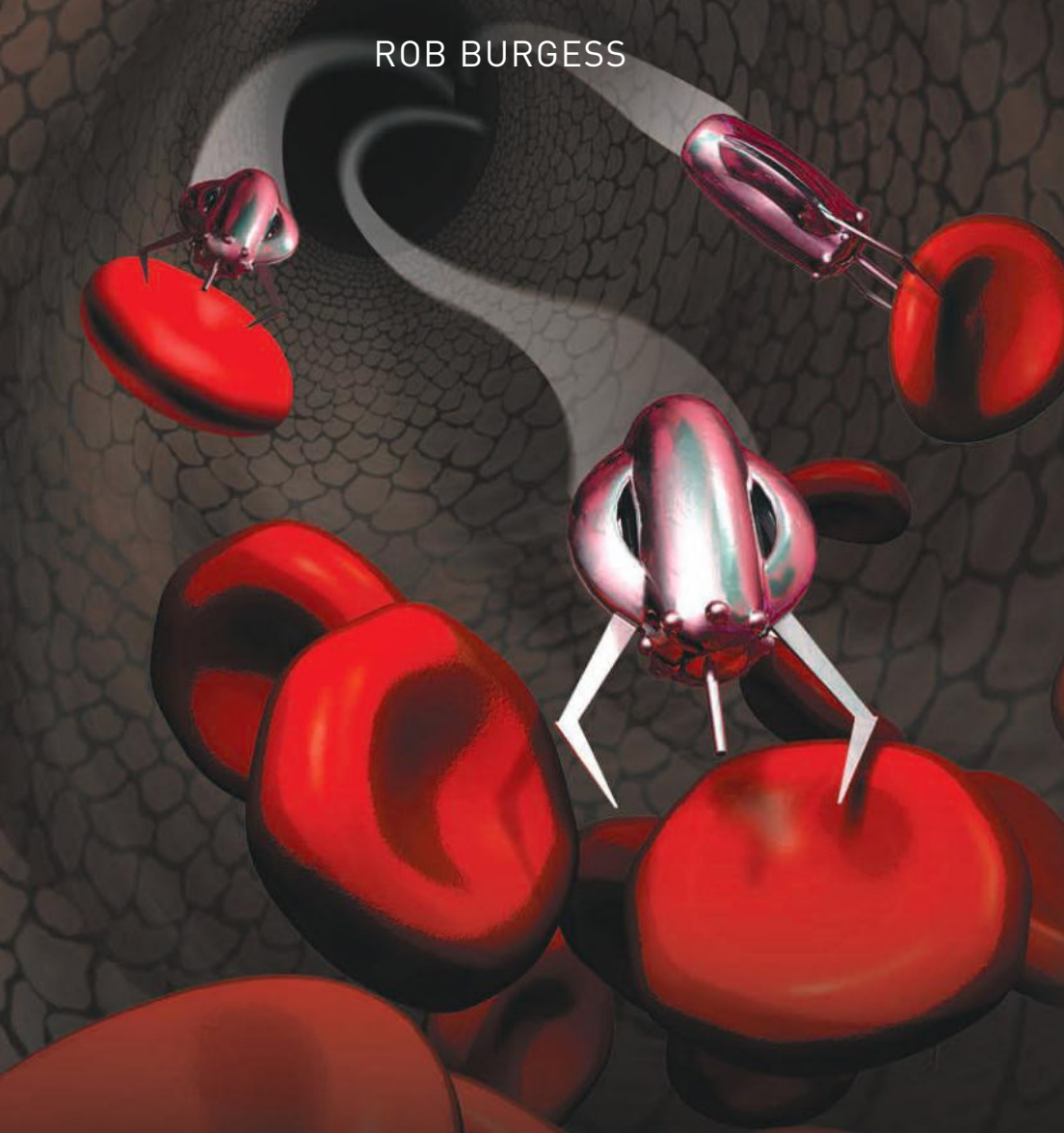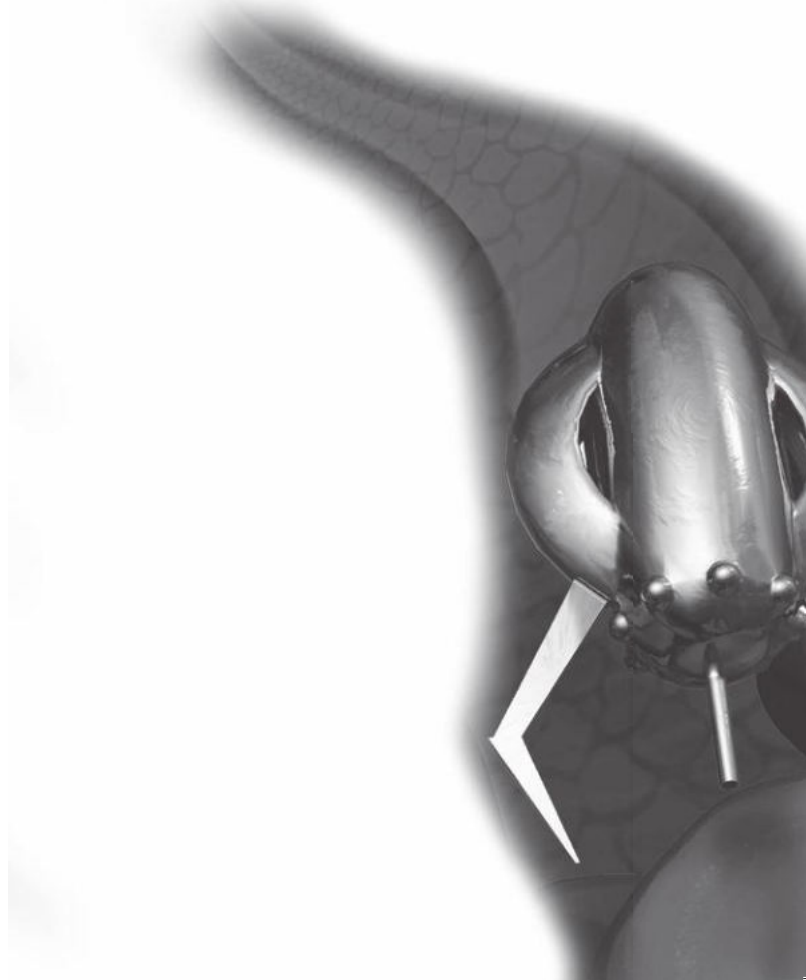ROB BURGESS

UNDERSTANDING
NANOMEDICINE

An Introductory Textbook

UNDERSTANDING
NANOMEDICINE

This page intentionally left blank

ROB BURGESS

UNDERSTANDING
NANOMEDICINE
An Introductory Textbook

To

my wife, Jane,
daughter, Zoie,
mother, Lola, and
father, Bob

This page intentionally left blank

Contents

Acknowledgment

I would like to sincerely thank all the scientists and doctors who allowed for the reprinted publication of their research and data in this book. It is your undying and unselfish pursuit of advances in nanomedicine that will transform diagnostics and therapeutics as we know it and inspire the next generation of nanomedical researchers. My hat is off to each and every one of you.

This page intentionally left blank

Preface to the Professor

As I researched the currently available textbooks covering the basic principles, applications and promise of nanotechnology as it applies to medicine, I noted a dearth of introductory material tailored specifically for students. While a number of comprehensive books exist outlining the promise of the nanosciences as they apply to medical applications, including most recent advances in medical research, these texts fail to properly introduce the student to nanoscience and nanotechnology as it applies first to biology and to potential therapeutics and diagnostics applications. Thus this text is devoted to the basic principles of nanotechnology, focusing on nanomaterials and nanoparticles, as with respect to the whole of nanoscience, these sectors hold the most promise for the future of medicine. It is tailored towards real-world applications of medical nanotechnologies with a heavy emphasis on specific examples from the existing literature available in this area. It is NOT (with the exception of Chapter 10) a compilation of thoughts and essays describing what may occur in the realm of nanomedicine in the distant future. The book is written at an introductory level to allow the student to have a firm grasp of the principles of nanotechnology first, followed by a discussion on the relationship between nanoscience and biology, and ending with the majority of the text outlining medical applications. As much of the content is not considered

to be central scientific dogma but rather exciting yet preliminary research, the text is often written in review format, giving full credit to researchers for their published findings and citing appropriate scientific articles. As the scientific discipline of medical nanoscience advances, it is anticipated that this text will mature into a more basic and fundamental description of the field as is the case for biology or chemistry.

I have organized the contents of this book to emphasize the basic principles of nanotechnology and how they might apply to the betterment of mankind through an improvement in human health. I point out that nanoscience, like all disciplines, is not an exact science, and that much of the material presented is based on hypothesis and backed up by experimental results. A great deal of emphasis is placed on the published experimental research and results of key leaders in the field. To accomplish this task, I have included:

1. A comprehensive description of the basic principles and definitions of nanoscience and nanotechnology.

2. A breakdown in the origins and chemical makeup of some of the most widely used nanomaterials and nanoparticles in medical research.

3. A concentrated focus on detailing the relationship between nanoscience and biology.

4. Descriptions of and principles behind the most high-profile nanotechnologies, nanomaterials and nanoparticles currently studied for applications in medicine.

5. An extensive review of the top five areas of therapeutic focus involving nanotechnology.

6. An entire section on *in vivo* targeting of nanoparticles utilizing cell type-specific ligands.

7. A breakdown of the principles behind the use of nanoparticles in thermal ablation therapy, emphasizing the most high-profile published examples.

8. An overview of the use of nanoparticles to deliver drugs *in vivo*

9. Descriptive explanations behind the principles and detail on the use of nanomaterials and nanoparticles as contrast agents in medical diagnostic applications.

10. A glimpse into the future of nanomedicine and what the student can expect to evolve regarding nanotechnology-based diagnostics and therapeutics, finishing with the intriguing concept of the "Singularity."

This book is organized to naturally transition from a basic understanding of the principles, including physics, behind, for example, nanoparticles and nanomaterials, to how these principles might be exploited and used to treat or at the very least efficiently diagnose human disease or anomalies. Each chapter introduces topics and vocabulary at a very basic level and transitions to more advanced coverage as the student's knowledge level matures.

Chapter 1 begins with an overview of the origins of nanoscience and nanotechnology and progresses to explain the physical principles behind nanostructures and nanotools. Although not related to medicine, industrial applications of both nanostructures and nanotools are cited as examples to give the student a firm understanding not only of the benefits of nanoscience but also about how the physics of nanotechnology can be exploited for gain. The chapter finishes with a shift in focus towards the relationship between nanoscience and biology, thus introducing the student to the major focus of this book.

In Chapter 2, I hope in on the basic potential for nanotechnology, centered around nanoparticles and nanomaterials, to impact therapeutics, specifically that in relation to cancer. A breakdown in the types of nanoparticles currently being explored for cancer treatment primarily via hyperthermia is presented, with specifics on different modes of action. In this section I describe the physics, principles and therapeutic concepts behind the use of nanoparticles, combined with external fields for thermal ablation. This is followed by a comprehensive breakdown of targeting nanoparticles to specific sites for tumor cell ablation outlining targeting agents and targeting moiety attachment. The chapter finishes with an overview of the use of nanoparticles for anticancer drug delivery, describing both locally and intravenously applied therapeutic platforms.

Chapter 3 focuses on nanotechnology-driven tissue engineering applications such as scaffolds for tissue repair. It begins with a breakdown of the most high-profile types of nanofibers used in scaffold development and details their compositions. This includes both natural and synthetic examples. Techniques for the synthesis of certain nanofiber types are

described, such as electrospinning, and the chapter concludes with real-world examples of nanofiber applications in tissue engineering, such as for bone and vasculature repair.

Chapter 4 covers the impact that nanotechnology is beginning to have on neuroscience and the treatment of neurodegenerative disease. Examples of neuronal/neural matrices based on nanomaterials are cited and described. This is followed by a special section on how nanomaterials might effectively address the age-old problem of therapeutic delivery across the blood-brain barrier (BBB). Specific examples of nanomaterial/nanoparticle strategies for BBB crossing are described and backed up by *in vivo* data from a number of researchers. Chapter 4 also cites examples of the neuroprotective effects of some nanoparticle systems such as those designed to be anti-oxidants and finishes with by describing some intriguing examples of combination nanoparticle/cell carrier strategies for applications in clinical neuroscience.

Surgery is perhaps the oldest form of medicine known to man and thus I have dedicated an entire chapter to nanotechnology's emerging impact on this field. Chapter 5 begins with a description of the need for new biocompatible biomedical implant coatings. This is followed by a description of several nanotechnology-based implant coatings currently under development, including, for example, those of nanostructured hydroxyapatite and metalloceramic origins. Surgery is addressed next with an explanation of the need to better minimize surgical damage and illustrations of nanotechnologies to address this issue such as nanopulses and next-generation nanocoatings for surgical instruments. Next the chapter addresses the need for better wound-healing technologies and outlines examples of how nanotechnology is already making significant inroads into this area with applications such as nanosutures, nanofiber-based bandages and antibiotic nanocoatings. Chapter 5 ends with a look at laser- and non-laser-based intracellular nanosurgery and how it is impacting basic biomedical research and may impact therapeutics in the future.

Chapter 6 tackles both the current potential and limitations of existing cell culture methods and how nanotechnology may provide new avenues for growing cells for research purposes as well as cell transplant therapeutics. A brief history of cell culture is given and the most popular cells for manipulation *in vitro* are described. The chapter's emphasis is on the development of new cell culture matrices that more effectively mimic

the natural *in vivo* environment. A comparison of 2D vs. 3D cell culture methods is made illustrating the advantages of 3D for both scale and *in vivo* mimicry. Examples of nanomaterial-based scaffolds for cell culture are cited, including those of both natural and synthetic origin. Techniques for the efficient cellularization of nanoscaffolds are also described and the chapter concludes with some unique applications of titanium and magnetic nanoparticle systems for cell culture.

Chapter 7 is therapeutically centric and focuses on the use of nanoparticles as drug delivery vehicles. The basic principles behind both active and passive drug delivery are outlined and this is followed by a thorough description of synthetic and natural nanomaterials currently under study as drug delivery platforms. Examples include the widely studied PLGA and PEG synthetic polymers along with some controversial delivery systems such as fullerenes. It concludes with a section listing and describing naturally occurring nanomaterials used or under study for drug delivery such as liposomes and gelatin.

Aside from therapeutic applications, diagnostics is clearly the area of medicine where nanotechnology holds the most promise. Chapter 8 is dedicated to nanotechnology-driven advancements in diagnostics that may allow for earlier and/or more efficient and sensitive detection of disease. The chapter begins with descriptions and illustrations of examples in *in vitro*-based nanodiagnostics such as nanobiochips and nanobiosensors. Nanolaser spectroscopy and nanoproteomics are also covered in this section. A detailed breakdown of the most widely studied nanotechnologies and methods for *in vivo* nanodiagnostics follows the *in vitro* section. Gold and magnetic nanoparticles acted upon by external fields for imagery are cited as examples, and intriguing research into the use of liposomes and micelles to deliver metal nanoparticles for *in vivo* diagnostics concludes the chapter.

In Chapter 9, I have chosen to focus on governmental influence on nanotechnology and, where possible, emphasize the effects it is beginning to have on the emerging field of nanomedicine. The chapter is broken down into two primary sections. The first illustrates government funding and promotion of advancements in nanotechnology. The second seeks to give the student a thorough understanding of government's attempts at regulating this rapidly maturing area of science. I have delineated the growing influence of major world governments on nanotechnology and have completed both sections with examples of globally and internationally

coordinated efforts at impacting nanotechnology in general and nanomedicine in particular.

The book concludes with a glimpse into the conceptual future of nanomedicine in Chapter 10. Here I take many of the more futuristic concepts and examples regarding medical applications and advancements of nanotechnology from leading nanoscientists and theoreticists around the world and describe them in enough detail to capture and peak the student's interest and imagination in what may lie ahead for the future of diagnosis, therapy and nanotechnology itself.

It should be noted that at the end of each chapter I have drafted a set of key terms in the form of a glossary. In choosing the terms I have attempted to drive home the most important points made within that chapter's text. In addition, I have also listed a review section of questions at the end of each chapter that are designed to provoke the student's intellect and grasp of the contents of that particular chapter. The questions are meant to be thought-provoking and many may be answered correctly in a number of different ways given the essay format. The answers to these questions can be found at www.understandingnano.org. It is up to the discretion of the professor whether or not to utilize these additions to each chapter, but I am convinced that if the glossary and review sections are properly studied the student will have a firm understanding of the most critical concepts from each chapter and section of this book.

As always, I am most certainly appreciative of comments and criticisms regarding the content and format of *Understanding Nanomedicine: An Introductory Textbook*. If you have input or suggestions pertaining to this book, I'd love to hear from you as your response will most certainly impact future editions.

Rob Burgess
www.understandingnano.org

Preface to the Student

As of the writing of this publication there is no introductory textbook available which sufficiently teaches the emerging concepts and principles behind nanotechnology and its potential enormous impact on the field of medicine. The science of nanotechnology is maturing at such a rapid pace that I feel the time is now to address its most promising area of application, and that is medicine. It is crucial for the future scientists, researchers and medical specialists of our time to have a strong grasp and understanding of both the potential for nanotechnology to revolutionize therapeutics and diagnostics, and the risks associated with these endeavors. I firmly believe that if you take the time to study and enjoy this introductory text you will not only appreciate the future impact that nanotechnology will have on man's health and well-being but also begin to form your own concepts and ideas on how to realize that impact.

With the exception of Chapter 10, I have based this composition of the concepts and examples presented in this book solely on hard facts and published data. I have not, for example, glossed over the possibility of nanoparticle toxicity but rather cited references to it where appropriate. The book is heavily focused on the use of nanoparticles as thermal ablation agents or drug delivery vehicles, as these areas are perhaps the largest areas of focus for nanotechnology with respect to medicine today. In addition,

as much of the content is not considered to be central scientific dogma but rather exciting yet preliminary research, the text is often written in review format, describing profound data and research and giving full credit to scientists and medical doctors for their published findings by citing appropriate scientific articles.

The chapters are organized largely as self-contained in subject material, beginning with non-medical definitions and descriptions of nanotechnology and transitioning to biology and putative uses of nanotechnology in medicine. The material transitions from the fundamentals of nanoscience to applications of those fundamentals and physical properties for the betterment of medicine and medical research. Each chapter is followed by a glossary of key terms and a set of review questions. I strongly urge you to study these in order to gain a thorough understanding of that particular chapter's material.

It is also recommended that the assigned material be read and thoroughly reviewed prior to the corresponding lecture. In addition, I suggest that you review the key terms at the end of each chapter and make an attempt at answering the review questions prior to the material being covered either in class or during study sessions. The answers to these questions can be found at www.understandingnano.org. This will allow you to have a basic grasp of the principles and subjects presented or discussed and make the lecture series more interesting and enjoyable. Also, take thorough notes in class and recopy those notes, preferably on the same day to re-emphasize the material. You will retain it longer and have less difficulty for recall during exams. If you are so inclined it is also recommended that you reread the text covered by lecture after class to aid understanding and retention.

Finally, I am always seeking comments, including both praise and criticism, regarding my manuscripts and publications. I cannot obtain more legitimate and valuable feedback than from the students for which this book was written. If you have ideas or suggestions for how I might make future editions of this book more useful, please contact me.

Rob Burgess
www.understandingnano.org

Reviewers

The author and publisher would like to express their sincere appreciation to a number of scientists and researchers who have taken considerable time and effort to assist with the development of this book. Nanotechnology is a diverse and wide-ranging scientific discipline, and we owe a great deal to the specialists who reviewed this material:

Rockford K. Draper
Professor, Molecular & Cell Biology and Chemistry
University of Texas at Dallas
Richardson, Texas

Gareth Hughes
President and CEO
Medical Nanotechnologies, Inc.
Dallas, Texas

This page intentionally left blank

1

Fundamentals of Nanotechnology

"Human health has always been determined on the nanometer scale; this is where the structure and properties of the machines of life work in every one of the cells in every living thing. The practical impact of nanoscience on human health will be huge."

Richard E. Smalley, Ph.D. (1943–2005)
1996 Nobel Laureate

NANOTECHNOLOGY AND ITS ORIGINS

Nanotechnology can be defined as the study of the control of matter below sizes of 100 nanometers, often on a molecular or atomic scale. The term "nanoscience" is often utilized interchangeably with "nanotechnology." The prefix "nano" is an ancient Greek term meaning "dwarf." Efforts in

nanotechnology and nanoscience often center around the construction or application of molecularly, or perhaps even atomically, precise structures for use in various applications.

While concepts in nanoscience date back to as early as 500 B.C. when philosophers believed that matter consisted of indestructible components (now referred to as "atoms"), the modern day origination of nanotechnology, not including the use of that specific term, can be traced back as far as 1867 to a first mention of its concepts, without the use of the term nanotechnology, by the Scottish theoretical physicist and mathematician James Clerk Maxwell when he described **Maxwell's Demon**, a tiny entity capable of handling and manipulating individual atoms (Figure 1.1).
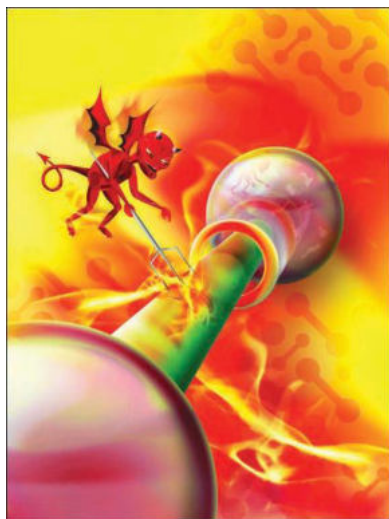
FIGURE 1.1 Artist's rendering of Maxwell's Demon controlling the function of a rotaxane molecule. (Image courtesy of Peter MacDonald, Edmonds, UK; reprinted with permission.)

In 1959 the theoretical observations of Dr. Richard Feynman were outlined in his seminal lecture titled "There's Plenty of Room at the Bottom" (see Focus Box 10.2 in Chapter 10). Feynman was a physicist who first proposed that it may someday be possible to precisely control the molecular or even atomic makeup of certain structures in a manner similar to what occurs in nature, for example as in the synthesis of a protein via translational mechanisms. His theory was based upon a top-down approach (described

later in this chapter) to achieve the desired scale of finished structure which is either molecularly or atomically precise in nature. In 1974 the term "nano-technology" was first introduced by Japanese physicist and Tokyo Science University Professor Norio Taniguchi and defined as "the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule." In general this early definition fits with the current pursuit of nanotechnology in areas such as materials science, instrumentation and even biotechnology. Three years later Dr. Tuomo Suntola (see Focus Box 1.1) and colleagues invented **atomic layer deposition (ALD)**, also known as **atomic layer epitaxy**, a process that allows for the efficient depositing of thin film layers of a thickness equal to that of a single atom. This process is still widely used today in the field of silicon-based microelectronics. It was in the early 1980s that nanotechnology got a technological boost with the invention of the **scanning tunneling microscope (STM)** (see below). This instrument set the stage for the manipulation of individual atoms and is still widely used today in the pursuit of atomically precise assembly. The evolution of **cluster science**, the study of small clusters of atoms no greater than $3*10^7$ in number, also played a significant role in the maturation of nanotechnology as it exists today. Combined, the existence of the STM and its application in cluster science resulted in the discovery of the fullerenes in 1985 and carbon nanotubes several years later, and has directly resulted in many of the basic advances in nanotechnology recently observed.

In addition to the advances in the fundamentals of science surrounding nanotechnology, it was Dr. K. Eric Drexler's tireless efforts in the 1980s at promoting its future impact and value in that peaked the

---

Focus Box 1.1 Tuomo Suntola and atomic layer deposition

In 1974 Dr. Tuomo Suntola of the Helsinki University of Technology invented atomic layer deposition based on self-limiting chemical reactions that allow for the precise control of film thickness deposition. In contrast to chemical vapor deposition, ALD is accomplished by pulsing chemical reactants followed by chemisorptions onto the substrate. This process is easy to initiate and doesn't place many restrictions on chemicals used thus opening the door to successful deposition of a wide variety of reactants. (Photo courtesy of the Physics Foundation Society; reprinted with permission.)
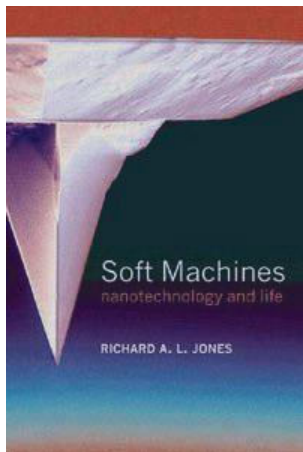
world's interest (see also Chapter 10 and Mechanical Nanocomputers). Through countless speeches and the publication of his book "Engines of Creation: The Coming Era of Nanotechnology" Dr. Drexler changed the face of materials, instrumentation and medical research as we know it today. Some twenty years later, in 2004, Richard Jones of the University of Sheffield published a book titled "Soft Machines" that allowed for an entertaining and thorough explanation of nanotechnology at the layman's level. It was this book that peaked the public's curiosity of this emerging field and began to tie together the fields of nanotechnology and biology. In it he suggests that nanoscale products, instrumentation or devices should be designed to mimic what occurs naturally in biology, i.e. as **biomimetics**, due to the precision and efficiency that has evolved in biological settings over time.

Nanotechnology can be broken down into three primary categories of research and applied science. These include:

(1) nanomaterials,
(2) nano-instrumentation and
(3) nanomedicine.

It is important to note that each classification or category is not mutually exclusive of the others and a great deal of overlap exists, for example, many types of nanomaterials are being used for or are under study for use in nanomedicine. Related to cluster science, the invention of the scanning tunneling microscope and the discovery of fullerenes as well as carbon nanotubes, there are certain developments in each of these fields which stand out as major advancements driving yet more interest and research in the field of nanotechnology. In the area of nanomaterials, the synthesis and study of semiconductor nanocrystals in 1985 and related quantum dots led to an explosion of research on nanoparticles of many different types. Each of these seminal advancements in nanotechnology has now begun to have an impact on the emerging field of nanomedicine.

There are two approaches to nanoscale manipulation: stochastic and deterministic. **Stochastic nanotechnology** refers to the manipulation

and handling of atoms and molecules in a chemical or bulk fashion. An example of stochastic processes used in nanotechnology includes ALD. **Deterministic nanotechnology** is defined as the handling of individual atoms and molecules. The literal pushing of atoms around on a substrate, such as that first accomplished by Eigler and Schweizer at IBM in 1989 using an scanning tunneling microscope in which the letters "IBM" were spelled out as Xenon atoms on a Nickel substrate, would be considered a deterministic process (see Figure 1.21 below). While either approach is sound conceptually, stochastic nanotechnology has made much more of an impact on material science and the semiconductor industry than deterministic, primarily due to scaling advantages.

THE BASICS OF THE NANOSCALE

The first observations and measurements at the nanometer scale were successfully made by Hungarian-born Richard Adolf Zsigmondy who won the Nobel Prize in Chemistry in 1925 for his research on colloids. In 1903 he invented the **ultramicroscope**, which is a system of illumination for viewing tiny particles (Figure 1.2). It is based on light scattering and not light reflection as is the case of a standard microscope.
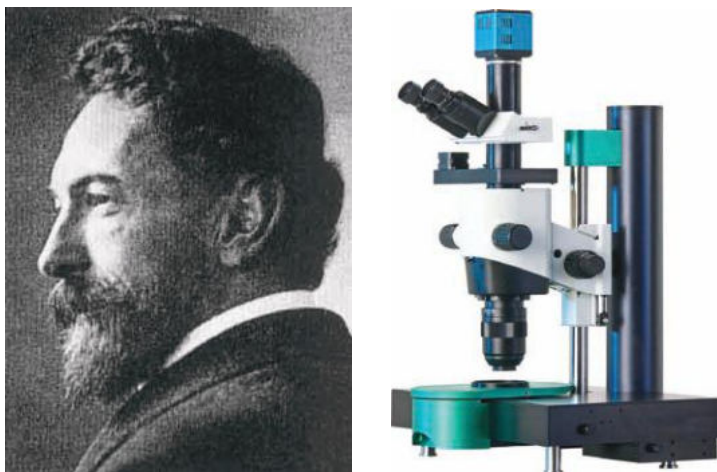
FIGURE 1.2 Richard Adolf Zsigmondy and a modern-day ultramicroscope. (Zsignomdy photo courtesy of Scientific-Web.com. Ultramicroscope photo courtesy of LAVison Biotec; reprinted with permission.)

He utilized the ultramicroscope for a vast number of studies over the years and in 1914 characterized gold sols and other nanomaterials and formulated the first classification system in the nanometer range that was based on nanoparticle size. This concept has since been expanded to include the **nanoscopic scale**, which is defined as the size at which the expected fluctuations of particle properties including motion and behavior can no longer be reduced to below a desirable threshold. It is the point at which a particle's basic physical properties change and are no longer governed by standard physical laws, but rather by surface area effects, or "quantum effects."

Nanotechnology research centers on the study of particles that can be classified as a certain number of nanometers in size. One **nanometer (nm)** is one billionth of a meter, or 10^{-9} meters. As a general rule of thumb, nanotechnology refers to technological applications or research conducted in the realm of a scale less than 100 nanometers (nm). One can grasp the concept of nanotechnological research by comparing the scale of known objects as in Figure 1.3.

Objects classified as falling within the nanoscale may also be conceptualized at the macro level. For example, the size of a marble as compared to the earth is roughly equivalent to a nanometer in relation to a meter. Another interesting example that allows for some grasp of the nanoscale relates to time. It has been calculated that a single whisker on a man's face grows about one nanometer during the time it takes him to raise the razor to his face during shaving.
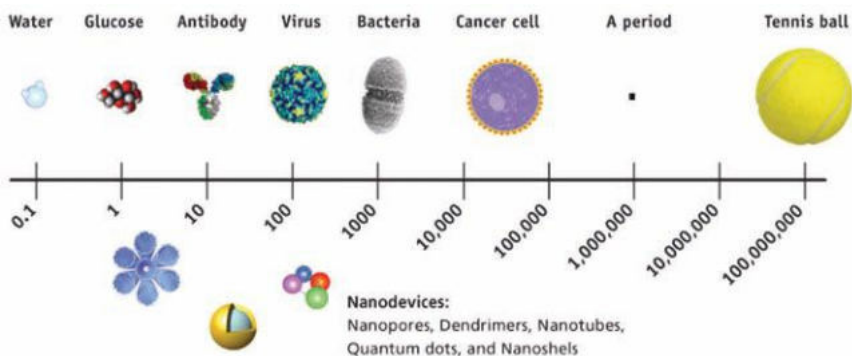
FIGURE 1.3 Nanoscale and examples of objects of different sizes. (Courtesy of U.S. Food and Drug Administration; reprinted with permission.)

NANOMATERIALS AND NANOPARTICLES

Nanomaterials are those which are defined as having unique physical properties derived from the inherent nanoscale dimensions of the material. These properties are the result of large surface area-to-volume ratios inherent at the nanoscale which drives novel quantum mechanical traits. A primary example is the change in the electronic properties of a material known as the **quantum size effect**, which is defined as unusual properties of extremely small crystals that arise from confinement of electrons to small regions of space in one, two, or three dimensions. The result of quantum effects often yields new mechanical and even catalytic properties. The term **nanoparticle** is often used interchangeably with nanomaterial but typically defines the individual molecules that make up the nanomaterial in bulk. It is the quantum properties of these individual particles which yields changes in the physical and/or catalytic properties of a material. Carbon nanotubes, for example, are considered nanoparticles individually yet components of a larger nanomaterial when properly organized by physical or chemical procedures. Improvements in stress vs. strain properties using carbon nanotubes and other nanoparticles in bulk dispersions are already making a significant impact across a number of industrial disciplines. Materials containing carbon nanotubes have been used in industrial and consumer applications to aid the physical properties of certain products. Bicycle frames, golf club shafts and sail boat masts, for example, have been supplemented with carbon nanotube-based materials to increase strength while decreasing the overall weight of the object (Figure 1.4).

Nanomaterials generally can be classified as falling into one of two categories: **fullerenes** or **inorganic nanoparticles**. The two sections below outlined the physical properties and differences between these two categories, citing specific examples of each.

Fullerenes

Fullerenes are characterized as cage-like hollow pentagonal or hexagonal molecules composed of carbon atoms. They are known as the third form or allotrope of carbon, after diamond and graphite and represent a separate class of carbon-based molecules. They typically exist in either spherical or tubular form (Figure 1.5). Carbon nanotubes fall into the nanomaterials fullerenes class and are of intense interest in the scientific

FIGURE 1.4 Examples of products containing carbon nanotubes. (Images courtesy of Zyvex Performance Materials; reprinted with permission.)

community due to their unique physical (strength) and electrical properties.

Fullerenes may be synthesized by several different methods including arc discharge, chemical vapor deposition or laser ablation. **Arc discharge** is the perhaps the most common and involves the passage of a high
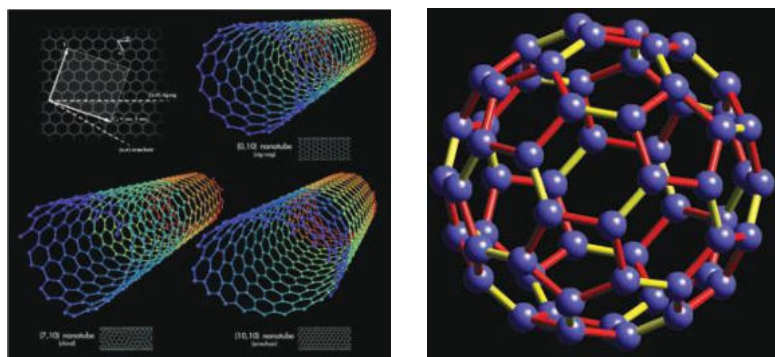
FIGURE 1.5 Two classes of fullerenes, buckyballs and single-walled carbon nanotubes. (Images courtesy of Kevin Taylor, the University of California at Davis and the Live Journal; reprinted with permission.)

electrical current between two graphite electrodes in an inert gaseous environment. Residues generated from the resulting plasma arc contain a variety of types of fullerenes. C_{60} fullerene, also known as "buckyballs," has also been successfully synthesized via organic chemical reactions (see text box). This molecule resembles a soccer ball and has a total of 12 pentagons and 20 hexagons. **Chemical vapor deposition (CVD)** is a chemically induced reaction in which two process gases and a carbon-containing gas are bled at high temperatures (700°C) into a reaction chamber containing a substrate layer of metal catalyst particles. CNT growth initiates at the metal catalyst sites as the carbon gas is broken down at the metal particle sites (Figure 1.6). CNTs grow as extensions of the metal particles and CNT diameter is directly proportional to catalyst particle size. For industrial-scale applications, CVD appears to be the most cost-efficient method of CNT synthesis, primarily due to the fact that CNTs may be grown on a substrate in bulk bypassing collection steps required by other methods.

FIGURE 1.6 Plasma-enhanced chemical vapor deposition synthesis of carbon nanotubes. (Image courtesy of Wikipedia; reprinted with permission.)
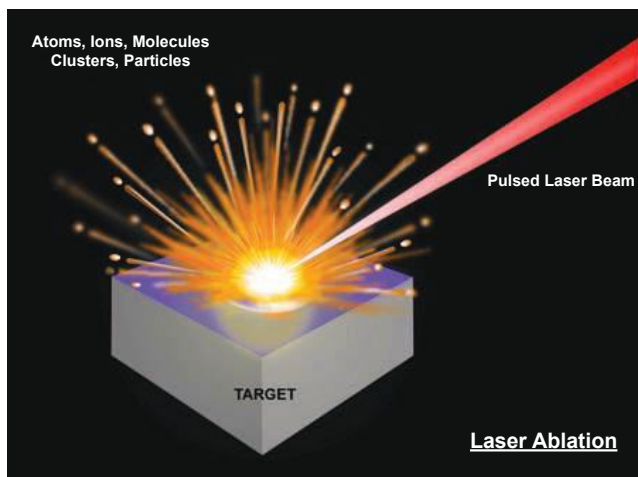
FIGURE 1.7 Diagrammatic illustration of the laser ablation method for the synthesis of fullerenes. (Image courtesy of Applied Spectra, Inc.; reprinted with permission.)

Laser ablation is another method for the synthesis of fullerenes in which a pulsed laser is directed at a graphite target, thus vaporizing the material at extremely high temperatures while an inert gas is slowly bled into the chamber (Figure 1.7).

Invented in 1995 by the late Professor Richard Smalley and colleagues at Rice University (see Focus Box 1.2), laser ablation allows

Focus Box 1.2 Richard Smalley, Robert Curl, Harold Kroto and the buckminster fullerene
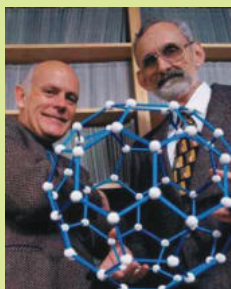
The late Dr. Richard Smalley (left) (1943–2005) and Dr. Robert Curl, professors at Rice University, along with Professor Harold Kroto at the University of Sussex discovered C_{60}, otherwise known as Buckminster Fullerene or "Buckyballs" in 1985. This seminal discovery revealed the most common naturally occurring fullerene and the smallest (1 nm in diameter) in which no two pentagons share an edge and lead to their receiving the 1996 Nobel Prize in Chemistry. (Photo courtesy of Rice University; reprinted with permission.)

for the very efficient generation of multi-walled carbon nanotubes (see description below). A modification of that procedure using metal catalysts such as cobalt and nickel and graphite composite later allowed for the synthesis of single-walled carbon nanotubes. While the yield is higher, typically 70% CNTs vs 30% by arc discharge, the method is more expensive. Due to unique intrinsic physical and electrical properties, carbon nanotubes have shown tremendous promise as agents for the destruction of cancer cells in the body and for other medical treatments. As this text is focused on medical applications of nanotechnology, a detailed description of carbon nanotube physical and chemical properties is thus warranted.

The origin and first discovery of carbon nanotubes (CNTs) is controversial and many reports describe their first characterization as occurring in 1991 by Sumio Iigima of NEC. Yet literature describing CNTs can be traced back to as early as 1952 when images of carbon tubes with an average diameter of 50 nm were published by Russian scientists L. V. Radushkevich and V. M. Lukyanovich. A variety of other reports between 1952 and 1991 also describe carbon tube-like structures with nanometer-scale diameters made by arc discharge and vapor growth techniques, yet the structures were not formally referred to as carbon nanotubes. These include a paper by French scientist A. Oberlin and Japanese scientist T. Koyama in 1976 depicting images of carbon nanoscale fibers made by a chemical vapor growth technique. In 1979 arc discharge methods were described to produce nanoscale carbon fibers by John Abrahamson at the University of Canterbury in New Zealand. In 1981 the first description of carbon nanotubes as rolled graphene sheets into multilayers was presented by Soviet scientists and six years later a patent was issued to the company Hyperion Catalysis for the synthesis of cylindrical discreet carbon nanofibrils.

As Abrahamson suggested, **carbon nanotubes** can be described as sheets of graphene rolled back upon themselves into cylindrical tubes. Unlike spherical fullerenes such as C_{60}, CNTs are composed only of hexagons. It is the intrinsic electrical properties of CNTs that result in electrostatic attraction between tubes and thus cause a clustering effect to make them insoluble. **Exfoliation** procedures are thus required for proper separation of CNTs in solution. Exfoliation may involve mechanical shearing, chemical modification or a combination of the two to reduce attractive forces. In fact, a simple process such as the application of ultrasound is

FIGURE 1.8 Photos of ultrasound equipment (left) and ultrasound exfoliated carbon nanotubes (right). (Images courtesy of Hielscher USA, Inc.; reprinted with permission.)

sometimes effective at the exfoliation of certain sample populations of carbon nanotubes (Figure 1.8).

CNTs exist either in single-wall (SWNT) or multi-wall (MWNT) formats depending upon the method and control of synthesis (see below). CNTs are uniquely strong, exhibiting on average 50–100 times the strength of steel yet they weigh much less. This makes them quite valuable for certain products and technologies requiring strong materials.

Single-Walled Carbon Nanotubes (SWNT)

A single-walled carbon nanotube can be thought of as a single sheet of graphene rolled back upon itself with seamless fusions at geometrically-corresponding carbon atoms. Multiple layers are not present in SWNTs but rather they are hollow. The diameter of a typical SWNT is around 1 nanometer yet its length can vary greatly and depends upon its mode of synthesis. The final structure of a SWNT is measured by what is known as the **chiral vector**, which is represented as two indices (n, m) unique to the chirality or achirality of the SWNT. Depending upon how the graphene sheet is rolled back upon itself, the CNT may or may not exhibit chirality. n and m represent the number of unit vectors in the graphene sheet in two directions, respectively. When n and m are equal, the achiral "armchair" formation is the result. When $m = 0$, an achiral "zigzag" shape is formed and when n and m are unique positive integers chirality is observed (Figures 1.9 and 1.10).
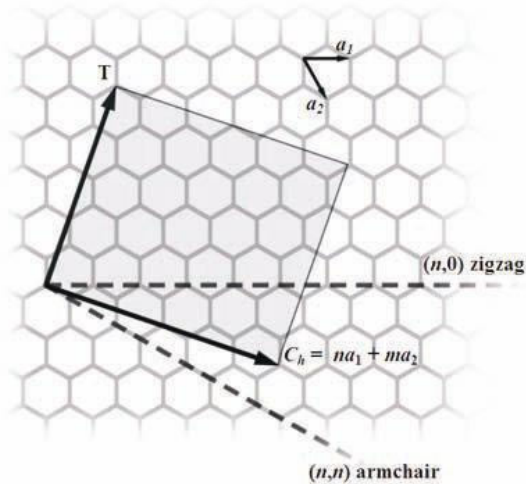
FIGURE 1.9 Chiral vector diagram of a graphene sheet describing its "rolling" into a carbon nanotube. T = CNT axis, a_1 and a_2 = graphene unit vectors, Ch = CNT vector. (Image courtesy of Wikipedia; reprinted with permission.)

Single-walled carbon nanotubes exhibit several features that distinguish them from multi-walled carbon nanotubes for practical applications. Their unique electrical properties, coupled with their small size, suggest that they may allow for future further miniaturization of microelectromechanical
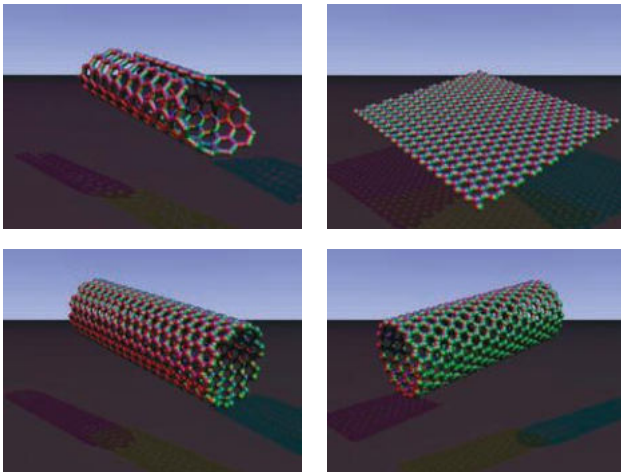
FIGURE 1.10 Graphene sheet, armchair (n,n), zigzag $(n,0)$, chiral (n,m). (Image courtesy of Wikipedia; reprinted with permission.)

devices currently used widely in the electronics industry. One example of this is a nanoscale SWNT electrical wire made possible by the tube's excellent electrical conductivity properties. In addition, SWNTs are much more uniform in molecular structure and their manufacturing can be more tightly controlled than that of MWNTs. These characteristics are of crucial importance from an FDA perspective if carbon nanotubes are ever to be utilized for the treatment of disease.

Multi-Walled Carbon Nanotubes (MWNT)

Multi-walled carbon nanotubes (MWNTs) resemble a graphene sheet rolled back upon itself several times over, thus producing a "chicken wire" type of molecular structure (Figure 1.11). This is known as the **Parchment model**. In the **Russian Doll** model, concentric circles of graphene sheets, the equivalent of SWNTs, are arranged within each other to produce a similar effect. In either case, the distance between carbon atoms from
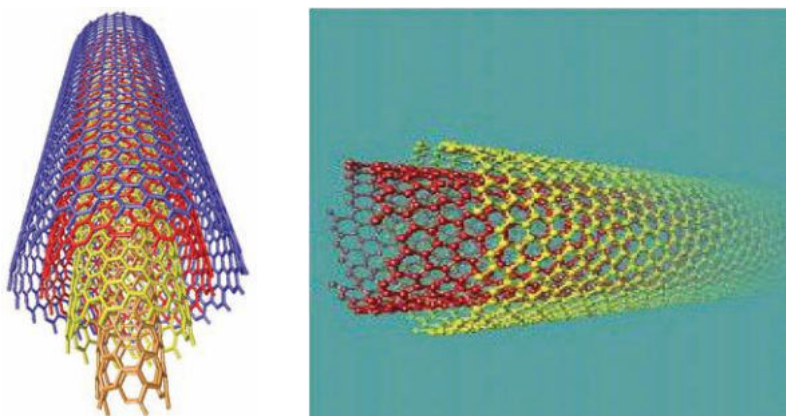
FIGURE 1.11. Diagrammatic examples of multi-walled carbon nanotubes. (Image courtesy of the University of Surrey Materials Institute and the Institute of Precision Engineering; reprinted with permission.)

an inner to outer layer is approximately 3.3 Angstroms. Multi-walled carbon nanotubes, especially double-walled (DWNTs), are much cheaper to produce than SWNTs, exhibit considerable resistance to chemical degradation and can be functionalized with the addition of new entities without breaking carbon=carbon double bonds which will often change the physical properties of the tubes. Functionalization of MWNTs allows for dispersion in a variety of solvents and very efficient as well as uniform attachment to host materials. These properties make MWNTs preferable over SWNTs for bulk materials production and use in composites to product a variety of products such as those in the sporting goods industry. Easton Sports and Zyvex Performance Materials have teamed up to produce and market a number of sporting goods containing functionalized MWNTs. These include both baseball bats and hockey sticks. The use of MWNT-containing composites to manufacture the shafts of these products reduces overall weight while increasing tensile strength and stiffness. This allows for a transfer of weight to the head of the bat or stick thereby increasing torque capacity and theoretically improving accuracy through stiffness.

While it is clear that both SWNTs and MWNTs exhibit unique mechanical properties, from a medical perspective it is the thermal and absorptive characteristics of SWNTs which warrant further discussion. The ability to absorb energy from external fields and convert that energy into heat for cell destruction is at the center of carbon nanotube applications in disease treatment and is known as thermal ablation. These absorptive and emotive properties of CNTs are discussed in detail below.

Thermal Properties of Carbon Nanotubes

All types of carbon nanotubes exhibit the property of **ballistic conduction**, which is defined as the unimpeded flow of particles carrying a charge or specific energy across long distances. The extreme small size of CNTs, which makes them resemble one-dimensional structures, drives size quantization effects and makes them ideal ballistic conductors. Conduction can involve either electrons or quantum vibration effects known as **phonons**. Thermal conductivity is thus almost unparalleled in comparison to other materials. It is estimated that due to ballistic conductivity properties CNTs have the ability to conduct up to up to 6000 $W \cdot m^{-1} \cdot K^{-1}$ at room temperature. Compare this to the well known and widely used thermal conductor copper, which typically transmits around 385 $W \cdot m^{-1} \cdot K^{-1}$. As carbon nanotubes are extremely stable even at temperatures up to 2800 °C under vacuum they

make ideal candidates for thermal conductivity applications. This thermal conduction efficiency also allows for the emission of heat generated by the absorption of applied external fields.

Absorptive Properties of Carbon Nanotubes

As mentioned above, carbon nanotubes can be considered one-dimensional structures due to their graphene sheet makeup. The optical properties of carbon nanotubes are based on this one-dimensional nature and involve unique electronic transitions that must be defined by a **density of states (DOS)**, which is the number of available states that an electron may occupy at each energy level. As one-dimensional structures, and in contrast to those defined by three dimensions, CNT DOS is not continuous but rather gradually descends and then ascends as sharp spikes known as **Van Hove Singularities**. Electronic transitions typically occur from $v_1 - c_1$ and $v_2 - c_2$ respectively although rare weak events from $v_1 - c_2$ may occur (Figure 1.12). This electronic state spiking phenomena is typical of one-dimensional materials and it is the structure of the carbon nanotube that drives the energy state between Van Hove Singularities and thus can be altered or tuned to a desired threshold for a particular application. One example of this is the illumination of carbon nanotubes dispersed in a polymer matrix with ultraviolet light.

Metallic SWNT

$v_1 \to c_1$ corresponds to the "first van Hove" optical transition

Semiconducting SWNT

$v_2 \to c_2$ corresponds to the "second van Hove" optical transition
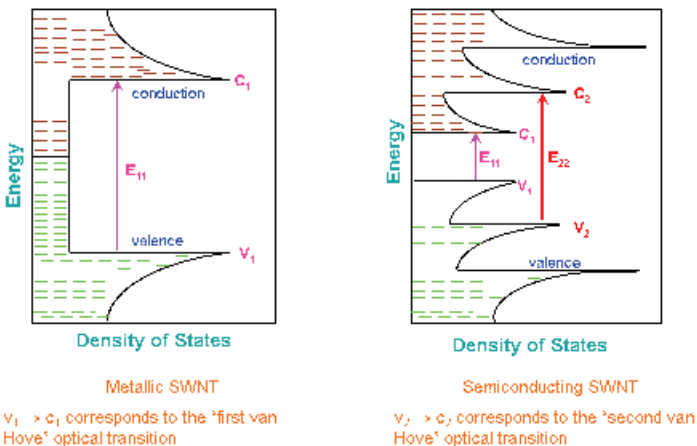
FIGURE 1.12 Graphs of density of states vs. Energy for metallic and semiconducting single-walled carbon nanotubes. (Image courtesy of Wikipedia; reprinted with permission.)

The absorption of photons by carbon nanotubes is the result of electronic transitions from valence to conduction within specific energy levels. These are sharp peaks in contrast to broad distributions for three-dimensional materials and can be used to identify the type of CNT and quality of a sample via UV/VIS spectroscopy. Given the physical characteristics of carbon nanotubes, most absorb light in the near infrared spectrum (800–2500 nm). Absorption of light in this wavelength range results in electronic excitation which is rapidly converted to thermal emission due to the excellent thermal conductivity properties of CNTs. A great deal of research has been conducted on exploiting this phenomenon as a way to thermally ablate cancer cells within the body yet the technique is hampered by the inefficient penetration of near IR light into the deep tissues of the body.

Recently, researchers have discovered the unique property of carbon nanotubes to absorb radiofrequency (RF) waves and correspondingly emit heat in a manner similar to that when exposed to near IR light. As RF waves are not impeded by biological materials, including tissues of the body (and even bone!), the possibility of deep tissue RF/carbon nanotube-mediated thermal ablation of cancer cells is now being explored. Referred to as **Kanzius RF Therapy**, this revolutionary medical technique may change the way cancer is treated (see Focus Box 1.3 and detailed description in

Focus Box 1.3 John Kanzius and RF-mediated cancer treatment

The late John Kanzius was a radio and TV engineer and serial inventor that developed what is now known as Kanzius RF Therapy for the treatment of cancer. The invention is based on the sensitivity of gold or carbon nanoparticles to radiofrequency wave exposure. Introduction of nanoparticles into tumors followed by exposure to RF waves results in the rapid emission of heat from the nanoparticles thereby thermally killing local cancer cells. Although still in experimental stages Kanzius RF Therapy shows much promise for the future treatment of cancer and possibly other diseases such as those of viral origin. (Photo courtesy of *Discover* Magazine; reprinted with permission.)

Chapter 2). Gold nanoparticles also exert a similar sensitivity to RF and have been studied in this respect (see also Chapter 2).

Perfluorocarbons

Perfluorocarbon (PFC) nanoparticles consist of carbon backbones surrounded by fluorine atoms. PFC molecules can be in cyclic or linear in shape and consist only of carbon and fluorine. PFCs have been in production for quite awhile and can be manufactured by three primary methods: direct reaction of fluorine with hydrocarbons, the **Fowler Process** or electrochemical fluorination (EFC). In the Fowler Process, a two-step procedure using cobalt-fluorine derivatives as reactants yields the final product, perfluorohexane, according to the below reactions:

$$2\ CoF_2 + F_2 \rightarrow 2\ CoF_3$$

$$C_6H_{14} + 28\ CoF_3 \rightarrow C_6F_{14} + 14\ HF + 28\ CoF_2$$
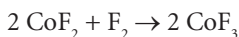
The PFC is typically bled off in the vapor to near 100% purity. In electrochemical fluorination a hydrogen fluoride reactant is used in an electrolytic reaction to yield a final product in one step as in the example reaction below, again for the production of perfluorohexane (Figure 1.13).

$$2\ N(C_6H_{13})_3 + 90\ HF \rightarrow 6\ C_6F_{14} + 2\ NF_3 + 45\ H_2$$
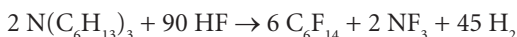
As will be discussed in detail in later sections, medical applications for PFCs include eye surgery, diagnostic imaging and cancer therapeutics.

Inorganic Nanoparticles

Nanoparticles, sometimes referred to as **nanocrystals**, may be composed of oxides, metals, semiconducting materials or even biological/naturally
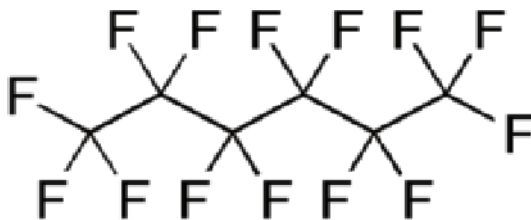
FIGURE 1.13 Molecular structure of perfluorohexane.
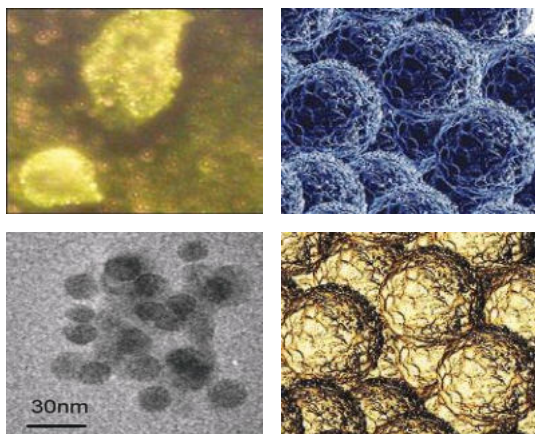
FIGURE 1.14 Gold nanoparticles, silver, iron, and CdSe nanoparticles (quantum dots). (Images courtesy of TopNews Health, NextScience.org, The University of Texas at Dallas and GreenTech Gazette; reprinted with permission.)

occurring species and often exhibit striking physical and chemical properties in manners similar to that for fullerenes. These inherent and unique properties often lend themselves to a diagnostic or therapeutic application and may include magnetic, light emission or thermal emission characteristics. In the majority of cases it is the high surface-to-volume ratio of nanoparticles that drives their unique physical, electrical and chemical properties.

The nomenclature often confuses fullerenes as nanoparticles yet they actually reside in a class of their own. PFCs, although organic, may fall into the classes of both nanomaterials and nanoparticles depending upon usage and final structure. Examples of truly inorganic metal nanoparticles include cadmium (Cd) (quantum dots), colloidal gold, silver, iron and platinum (Figure 1.14). Depending upon known toxicity, some of these types of nanoparticles may act as excellent diagnostic and therapeutic platforms. The history and properties of some of these have been expanded upon below.

Types of Biocompatible Inorganic Metal Nanoparticles
Gold Nanoshells
Invented in 1988 by Rice University researcher Naomi Halas (see Focus Box 1.4), gold **nanoshells** show extreme promise for the treatment of cancer

Focus Box 1.4 Naomi Halas and gold nanoshells

Naomi Halas is a Rice University-based researcher who knows no bounds. While working at IBM in 1987 she co-invented a dark pulse soliton and later gold nanoshells. It is the latter that shows extraordinary promise as a possible agent for the eradication of cancer cells in the body. She also is pursuing nanotechnological platforms for the detection of samples as small as a single molecule, and applications in energy and global health. She currently heads the Laboratory for Nanophotonics at Rice and is the recipient of the Cancer Innovator Award from the Congressionally Directed Medical Research Programs of the US Department of Defense. (Photos courtesy of KCTS9; reprinted with permission.)

via photothermal ablation. As spherical multilayered particles, nanoshells are similar in 3D geometry and composition to gold nanoparticles, are typically less than 100 nm in diameter and contain a dielectric (non-conducting) glass core internalized by a metallic (often gold) sphere or shell (Figure 1.15). Their optical properties are determined by the thickness and material composition of the core and shell, which can be tuned by

FIGURE 1.15 Diagrammatic illustration of a gold nanoshell. (Image courtesy of the U.S. National Institute for Science and Technology; reprinted with permission.)

modifying the synthetic process to alter the relative size of these layers. Nanoshells possess the property of **quantum plasma oscillation**, which is the collective oscillation of electrons. Such oscillation properties account for the tunability of nanoshells and vary depending on corresponding core and shell size. Custom synthesis of nanoshells allows for the optimization of light wavelength sensitivity within the visible and near infrared regions. It is the contact of light with appropriately tuned nanoshells that alters charge effects and thus results in light-to-heat (photothermal) conversion and the potential for hyperthermic ablation of unwanted cells or foreign material in the body. In addition, the optical properties of gold nanoshells make them ideal candidates for biomedical imaging and diagnostics. Yet these applications will require an enhancement of their relatively weak optical signal output.

Superparamagnetic Nanoparticles

A number of types of nanoparticles, including primarily iron, nickel and cobalt, exhibit magnetic properties that may be exploited for therapeutic or diagnostic benefit. In the case of ferro- and ferrimagnetic nanomaterials, the basic magnetic structure alters due to physical constraints when particle size is reduced below roughly 100 nm in diameter. At these sizes a single domain state is created from the larger macroscopic magnetic domain structure. This results in a fundamental change in the way magnetization reversal occurs and has yielded a new class of magnetic materials with unique magnetic properties. It was in 1949 that theoretical physicists Edmund Clifton Stoner and Erich Peter Wohlfarth of the University of Leeds were the first to characterize this new class of magnetic nanomaterials (actually nanoparticles) and the physical mechanism behind their novel magnetic properties.

One physical aspect of this new class of magnetic nanoparticles is the ability to control a property known as **coercivity**, defined as the intensity of a magnetic field to drive the magnetization of a particular material to zero after saturation. This is valuable in the electronics and chip information storage industry and has resulted in the development of a wide range of metallic and oxide magnetic nanoparticles in the diameter range of 4 nm–100 nm. Iron nanoparticles display magnetic properties due to a phenomenon known as **superparamagnetism**, defined as random flipping of the magnetization direction due to the influence of temperature (Figure 1.16). Superparamagnetism has applications in the biomedical

FIGURE 1.16 Magnetic iron oxide nanoparticles, 10 nm in diameter, spiked due to the application of a magnetic field. (Image courtesy of Science Photo; reprinted with permission.)

areas of imaging (contrast agents for MRI), biological separation (cell, nucleic acid, proteins, etc.) therapeutics (thermal ablation, drug delivery) and intracellular delivery (magnetofection).

Silver Nanoparticles

Silver nanoparticles may be manufactured by several different methods including physical vapor deposition, wet chemistry or least commonly ion implantation. **Wet chemistry synthesis** of silver nanoparticles involves the application of a reducing agent to reduce a silver salt such as silver nitrate in the presence of a stabilizing agent, such as bovine serum albumin (BSA), which stabilizes the high surface energy created on the nanoparticles as a byproduct of the reduction process. The use and application of silver nanoparticles is mostly limited to the medical industry due to the general acceptance of silver as safe for biomedical applications, although they have

FIGURE 1.17 Examples of surgical instruments coated with silver nanoparticles. (Image courtesy of Allianz; reprinted with permission.)

been used to coat ion battery cathodes as well as more recently on the surface of household appliances. Perhaps the two most intriguing medical applications of silver nanoparticles include as a component of bone cement and in the fighting of bacterial infections. They have also been used in the manufacture of surgical instruments and even surgical masks (Figure 1.17).

Other Types of Biocompatible Nanoparticles

This section explores a series of biocompatible nanoparticles that do not fall into the classes of fullerenes or metals and may be organic or inorganic in nature. Emphasis is place on examples which have been vigorously pursued for various medical applications.

Dendrimers

Dendrimers are synthetic polymers exhibiting branched-like configurations that achieve structural perfection. Depending upon their size they may be considered micro- or macromolecules, yet as they are often coupled to nanoparticles in medical research applications dendrimers warrant discussion here. It was in 1978 that Fritz Vogtle at the University of Bonn in Germany first synthesized dendrimers and this was soon followed by researchers at Allied Corporation (1981), Dow Chemical (1983) and Newkome (1985). All of these groups executed a method known as

divergent synthesis in which dendrimer molecules are synthesized from the internal core outward. Dendritic macromolecules were successfully constructed in 1990 by Cornell researchers in an outward-in approach with pre-synthesized dendrons attached to the core as the final step. This process is known as **convergent synthesis**. In either divergent or convergent synthesis, an active site is required for repeated reactions to build the branched chains and this site must be protected between synthetic events. As a result it is difficult if not impossible to synthesize large quantities of dendrimers thus limiting their use to certain applications that require microscale amounts. More recently a number of groups have employed a process known as **click chemistry**, which describes the chemical joining of small units together to quickly and reliably produce substances and materials, to produce polyphenylene dendrimers.

As polymeric molecules, the structure of dendrimers has a considerable impact on both their chemical and physical properties, which can best be described by comparing them to linear polymers. For example, in solution linear polymers tend to behave as flexible coils while dendrimers form a densely packed sphere. Dendrimers have a much lower viscosity than linear polymers and the availability of many side chains aids in solubility. This is dependent upon the presence of hydrophilic outer side chains which may allow for the solubilization of a hydrophobic counterpart, such as a drug, hidden within the dendrimer core, a process known as **encapsulation**. Depending on peripheral side chain identity, drugs may also be attached to the outer fringes of a dendrimer macromolecule for delivery. These side chains may also be utilized for binding to a specific target such as a cell or virus using targeting moieties (Figure 1.18).

Micelles and Liposomes

In 1913 the University of Bristol's William McBain proposed the theory that the high electrical conductivity of sodium palmitate solutions can only be the result of the presence of "colloidal ions," a detergent-like substance that spontaneously forms spherical clusters, now referred to as micelles. **Micelles** are three-dimensional spherical accumulations of surfactant molecules evenly dispersed in a liquid (a colloidal mixture). They often range in diameters below 100 nm and other shapes may exist in addition to spheres and include simple bilayers and cylinders, with shape depending upon the inherent properties of the surfactant
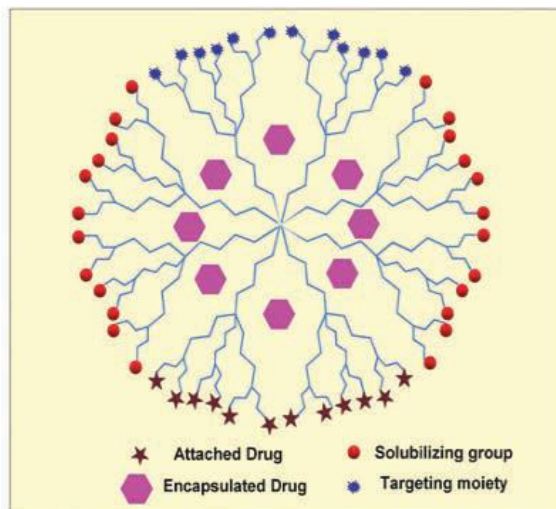
FIGURE 1.18 Diagrammatic illustration of a dendrimer/multiple drug complex. (Image courtesy of NanoPharmaceuticals; reprinted with permission.)

molecules as well as the external environment. pH, ionic concentrations and temperature, for example, have been known to play roles in micellular shape. In an aqueous environment (solution) micelles have surfactant molecules oriented with a hydrophilic head outside of the sphere protecting an internal hydrophobic tail and corresponding core. Due to the polymorphic nature of the surfactant molecules micelles tend to form spontaneously in solution.

Micellular structure, an outer hydrophilic shell surrounding a hydrophobic core, is the basic concept behind the phospholipid bilayers membrane of eukaryotic cells. It is often contemplated that the origins of the cell began via spontaneous formation of micellular bilayers and spheres millions of years ago. Yet phospholipid bilayers actually contain two layers of lipid molecular oriented towards one another in an inverted fashion. This allows for protection of the cytoplasmic hydrophilic environment from the external aqueous environment. **Liposomes** are derivatives of micelles which contain dual opposing layers and most closely resemble the structure of the cell membrane. Given their unique structure and ability to sequester molecules from solution coupled with cell membrane compatibility, micelles and liposomes have been studied extensively in the medical arena of drug delivery (Figure 1.19).
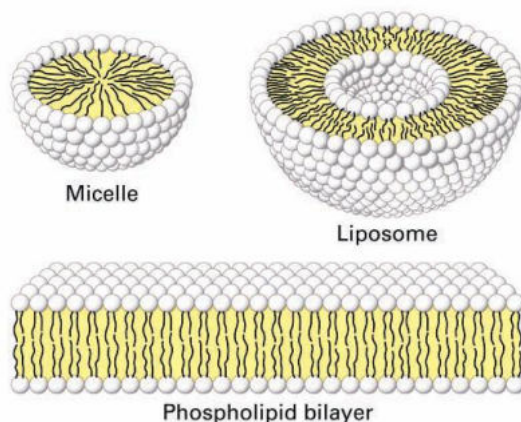
FIGURE 1.19 Diagrammatic illustrations of a micelle, liposome and phospholipid bilayers. (Image courtesy of The University of Miami, Department of Biology; reprinted with permission.)

The formation of micelles, while influenced by environmental factors like pH, is also dependent upon a certain energy threshold which must be overcome by the number of monomers presentation in solution. Known as the **critical micelle concentration** (CMC), it is the concentration of surfactant monomers above which spontaneous micelle formation occurs. In general, as the concentration of surfactant molecules increases in solution the surface tension between surfactant and solution decreases to a point where micelle formation begins to occur. This phenomenon can be represented as:

$C = CMC$, $(d^3F/dC_t^3) = 0$
$F = \mathbf{a}[\text{micelle}] + \mathbf{b}[\text{monomer}]$: function of surfactant solution
C_t: total concentration

where **a** and **b** are proportional constants and depend upon a range of properties of the entire solution or mixture.

The second most important factor in micelle formation is the temperature of the system. Micelles tend to form more easily at higher temperatures, and the **Krafft temperature** is defined as the minimum temperature of a system at which spontaneous micelle formation occurs. Below this temperature monomer surfactant molecules remain as monomers and in crystalline form, even in an aqueous environment.

NANOTOOLS

In the broadest sense, **nanotools** are defined as instruments that allow for the observation, fabrication or manipulation of materials and particles with nanometer precision. Tools of this nature may be on the order of large scale laboratory equipment such as **scanning tunneling** or atomic force microscopes, or as small as a carbon nanotube probe. Below are described some key tools that have changed the face of nanotechnological research as we know it today.

Scanning Tunneling Microscope

The **scanning tunneling microscope** (STM) is an instrument developed by IBM scientists Gerd Binnig and Heinrich Rohrer in the early 1980s that allows for viewing the surface of a wide variety of substances at the atomic level (see Focus Box 1.5). STM images are the result of applying an electrical current to probe the density of electronic states, which correspond to the density of the material being probed. Typical resolutions resulting from STM microscopy fall in the range of 0.1 nm lateral and 0.01 nm deep. An STM may be used either under a vacuum or in air/gas ambients. Temperature ranges for successful application of STM microscopy fall

Focus Box 1.5 Binnig and Rohrer's scanning tunneling microscope

In 1981 IBM scientists Drs. Gerd Binnig (left) and Henrich Rohrer were awarded the Nobel Prize in Physics for their invention of the scanning tunneling microscope. The STM allowed, for the first time, the ability to form an image of single atoms on a metal or semiconducting surface by scanning a needle-shaped probe over the surface of the sample at a height of only a few atomic diameters and then processing the resulting current fluctuations into an image. (Photos courtesy of Technisch Universitat Darmstadt and Tainano.com; reprinted with permission.)
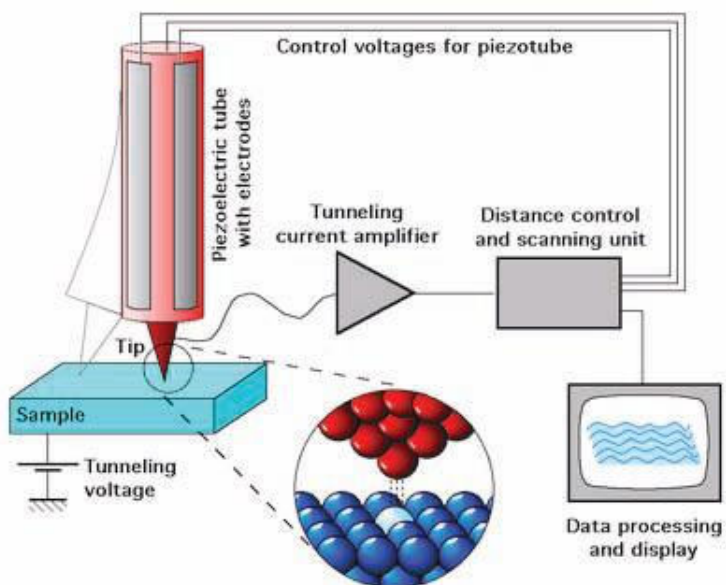
FIGURE 1.20 Schematic of a scanning tunneling microscope. (Image courtesy of Absolute Astronomy; reprinted with permission.)

anywhere from zero degrees Kelvin to several hundred degrees Celsius. The tunneling aspect of STM, also known as **quantum tunneling**, results from electrons traveling across the space between a probe and a semiconducting material surface. It is the variations in current due to differing electron densities as the probe passes over the material that result in an image when converted by software.

Figure 1.20 is a diagrammatic illustration of the components of a typical STM, which include circuitry for applying an electrical current, piezoelectric-controlled x, y, z platform, scanning tip, distance control and scanning unit, current amplifier and computer. The physical properties of the tip are crucial to clear resolution and depend upon the nature of the material and the tip's radius of curvature with the sharper the tip the sharper the image. Tip composition includes those made of platinum-iridium, tungsten and gold. Tips are made by either mechanical or electrochemical etching methods and those that end at a single atom are most ideal, hence carbon nanotubes have recently been used in this context as well as for atomic force microscopy (Figure 1.23).

The process of STM probing involves scanning the probe at a specific distance over a material or sample in an optimal pressure and temperature-based environment suited for that particular sample. Fluctuations in current due to changes in the density of states are processed as raw data and result in an image of the material's surface. Given the precise piezoelectric-based movement capabilities of scanning tunneling microscopy it has also been utilized to directly manipulate the surface of a material by using the probing tip to literally "push" materials at the level of a single atom. As mentioned above, this was first evidenced by researchers at IBM who used an STM tip to move Xenon atoms adhered to a nickel substrate into an arrangement spelling the letters "IBM." The same STM tip was then utilized to image the resulting change in atomic structure. These studies have since been expanded upon by other researchers in nanotechnology including Professor Saw-Wai Hla at Ohio University who created the letters "OU" for Ohio University with individual silver atoms on an Ag(111) surface (Figure 1.21). These advances in atom manipulation may be the first examples of the long-sought "bottom-up" approach to atomically precise manufacturing (see below).

Atomic Force Microscope

A close cousin of the scanning tunneling microscope, the **atomic force microscope** (AFM) was invented by Gerd Binnig, Calvin Quate of Stanford University and Christoph Gerber of the University of Basel in 1986. As is similar to that for an STM, AFM imaging utilizes the precision of piezoelectric positioners. Imaging, however, is accomplished not through **quantum tunneling** but rather by "feeling" the surface of the sample with
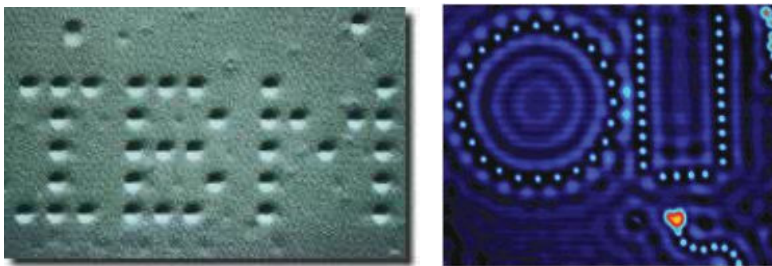
FIGURE 1.21 The letters "IBM" and "OU" spelled out through individual atom manipulation using an STM. (Images courtesy of Foresight Institute and Ohio University Nanospintronics and Nanomagnetics; reprinted with permission.)

a probe tip. Over twenty years later it is still perhaps the most widely used instrument for imaging at the nanoscale.

The basic components of an AFM include a silicon-based cantilever and attached probe similar to that for an STM, an electronically controlled scanning base, laser, corresponding photodiode detector and computer for image conversion (Figure 1.22). It is generally understood that the sharper the probe the higher the resolution achieved, and thus researchers are constantly seeking next-generation probe tips such as carbon nanotubes (Figure 1.23). The theory behind AFM imaging is straightforward and is based on **Hooke's Law** of elasticity which states that the extension of a spring is in direct proportion to the load added to it. In the case of an AFM, the cantilever is the spring and changes in spring load translate to changes in surface topography. Many forces can be measured via AFM including mechanical, van der Waals and electrostatic. Cantilever deflection during the scanning of a sample is typically monitored using a laser focused on the end of the cantilever. Reflection of the laser to a photodiode array allows for collection of a dataset which is converted into an image.

Commercial Nanotools: The nProber™

Moore's Law was introduced in 1965 by Gordon Moore, a founder of Intel, when he made the observation that the number of transistors that can be inexpensively placed on an integrated circuit doubles
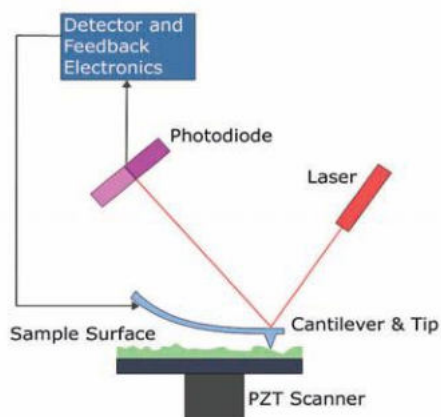
FIGURE 1.22 Photo and schematic of an atomic force microscope. (Images courtesy of Wikipedia; reprinted with permission.)
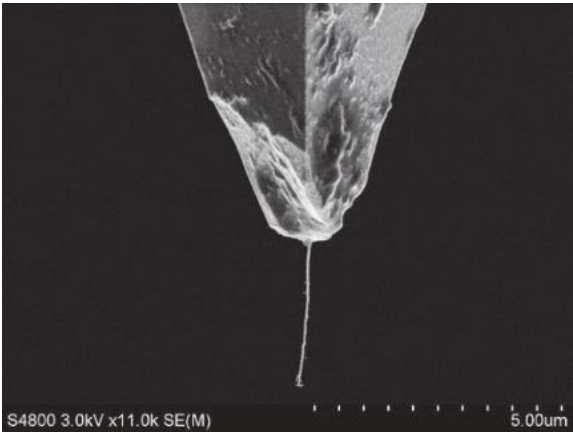
FIGURE 1.23 Carbon nanotube AFM probing tip. (Image courtesy of David W. G. Voyle, NanoTsunami; reprinted with permission.)

approximately every two years, a phenomenon that has resulted in node size scaling into well below the 100 nm range (Figure 1.24).

As this trend has continued uninterrupted over the last 40 years, electronics chip manufacturers have required more sophisticated and precise instrumentation for **failure analysis**, the process of collecting and analyzing data to determine the cause of integrated circuitry failure
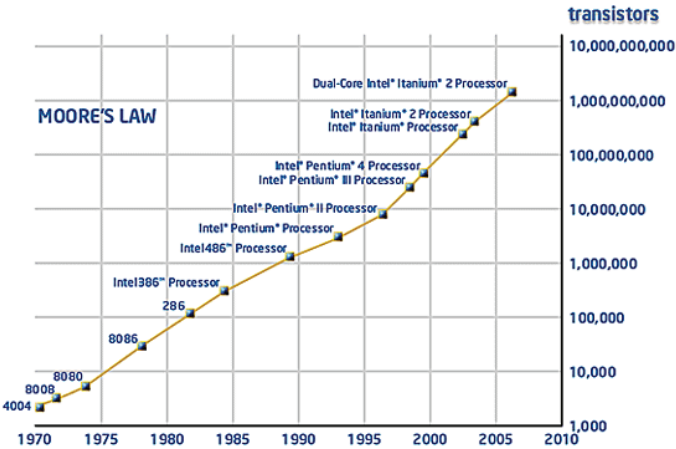
FIGURE 1.24 Graphical representation of Moore's Law. (Image courtesy of Rodney Van Meter, Keio University; reprinted with permission.)

FIGURE 1.25 Zyvex Instrument's nProber. (Left) The instrumentation setup including scanning tunneling microscope, probing equipment and computer data analysis hardware. (Right) Eight positioners precisely land probes on nanosized nodes of an integrated circuit chip. (Photos courtesy of Zyvex Instruments; reprinted with permission.)

and how to prevent it. In chip-based failure analysis **IV (current-voltage) curves** are generated to provide data on chip electronic integrity. This is accomplished by landing probes on nodes and passing an electrical current from one node to another. Due to ever decreasing chip node size and inversely increasing node number per chip more precise instrumentation is needed to effectively perform failure analysis. Companies such as Santa Barbara, California-based Multiprobe, Inc. and Richardson, Texas-based Zyvex Corporation have developed an instrument, referred to as the nProber™, that allow for nanoprecise manipulation of probes at levels now approaching 30 nm node size. With eight positioners to allow the simultaneous landing of eight individual probes it allows researchers an increase in the characterization of IC curves during microchip analysis. The coupled manipulators are precise enough to allow for routine probing below 100 nm (Figure 1.25). It will be interesting to see the limits to which this instrumentation can provide reliable IV curves as node size continues to decrease. It will be even more interesting to see how long Moore's Law remains in effect below the next transformational technology to replace the integrated circuit is invented.

CURRENT MANUFACTURING RESEARCH

During the past decade a great deal of interest and effort has been directed at striving for the ability to manufacture a material or object with atomic

or, at the very minimum, molecular precision. There are a number of reasons why such precise manufacturing is desirable over current methods including the complete elimination of waste and cost savings due to parallel assembly. The terms "top-down approach" and "bottom-up approach" were first introduced by the Foresight Institute, a California-based nonprofit organization and think tank founded by Dr. Eric Drexler, in 1989 to distinguish between the two primary methods to accomplish this level of precise manufacturing. Both approaches to atomically precise manufacturing are described below.

Top-Down Approach

A **top-down approach** to manufacturing involves the use of microfabrication machinery to externally control the atomically or molecularly precise synthesis of a desired material. An example of top-down manufacturing is optical photolithography, which is the process of etching away at a substrate to produce a desired, precise geometric pattern using light and photo-resistant chemicals. A number of different types of optical photolithographic systems have been developed and are used in research and industry worldwide (Figure 1.26).

In layman's broad terms, a top-down approach to manufacturing suggests the manufacture of materials and instrumentation which can be used for the manufacture of smaller and more precise materials and instrumentation, thus cycling down to a desired end product which may be molecularly or atomically precise. Some like to think of it as robots that build smaller robots that build smaller robots … and so on!

Bottom-Up Approach

Bottom-up approaches to nanoscale synthesis are focused on the assembly of a material, object or device from individual components. Said assembly often, but not always, involves the concept of self-assembly, whereby a molecule, for example, can wrap upon itself based on inherent chemical and physical properties to form a 2-dimensional or 3-dimensional shape (Figure 1.27 and discussed in more detail below). In addition, positional assembly, relying on the same properties, might also be accomplished between separate molecules. A great deal of research in this area focuses on the exploitation of nucleic acid bonding properties for the generation of self-assembled 2D structures (see discussion below on DNA Nanotechnology).

FIGURE 1.26 Micrascan II DUV photolithographic system at Albany University in New York. (Image courtesy of Albany University; reprinted with permission.)
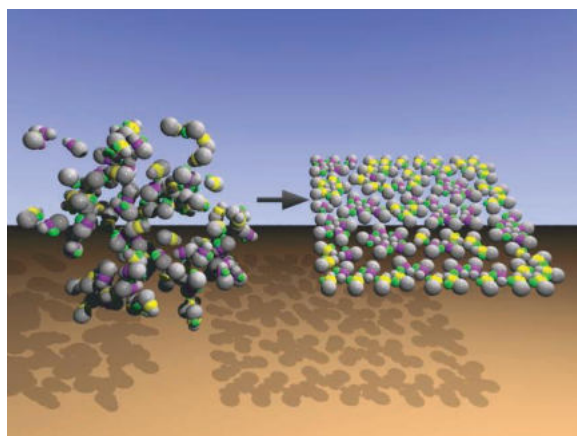
FIGURE 1.27 Molecular self-assembly. (Image courtesy of University of Victoria, Department of Chemistry; reprinted with permission.)

Atomically Precise Manufacturing

Atomically precise manufacturing (APM) is defined as the ability to manufacture materials and structures at the atomic (or at least molecular) scale with controlled precision. It is a fusion of disciplines including chemistry and engineering. The ultimate goals of APM are to completely eliminate waste and streamline manufacturing capabilities through a process called **parallel assembly**, in which objects are manufactured along many pathways simultaneously. Future large-scale commercial applications of APM are thought to bring enormous benefits to society in such areas as the economy, healthcare and defense. The challenge of achieving APM is currently being attacked through applications of both the top-down and bottom-up approaches. As discussed above, top-down approaches include the development of micro- and nano-manipulator machines that could theoretically build smaller versions of the same machines and so on until precise manufacturing at the atomic or molecular level has been achieved. Bottom-up approaches involve the "pushing" of individual atoms using, for example, STM or AFM-based approaches to bond and assemble a unique structure, one atom at a time (Figure 1.28). While it is clear that each method has a long way to go in terms of achieving atomically precise manufacturing of a structure, the end result could impact manufacturing at a level not seen since the Industrial Revolution.
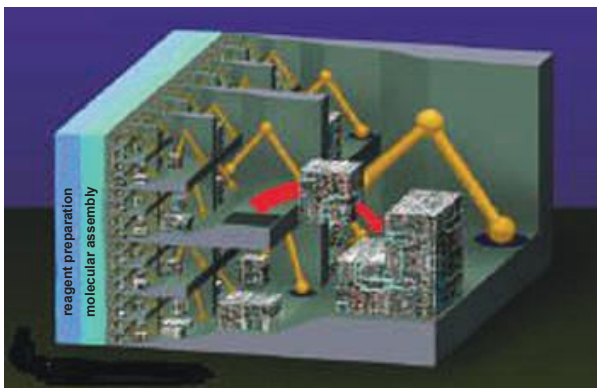
FIGURE 1.28 Diagrammatic illustration of a convergent bottom-up approach to atomically precise manufacturing. (Image courtesy of K. Eric Drexler, Nanosystems, 1992; reprinted with permission.)

BIONANOTECHNOLOGY

The terms bionanotechnology and nanobiotechnology are often used interchangeably to describe the same scientific discipline, which represents a merging of the fields of biology and nanotechnology. **Nanobiotechnology** is defined as the development and use of nanotechnological devices for use in biotechnology. One example is the application of nanoparticles to treat or manage disease. This is an intense research field and is covered extensively throughout this book. Conversely, **bionanotechnology** refers to the use of biomolecules for applications in nanotechnology. Examples of this include the exploitation of nucleic acid physical properties in self-assembling research as described below. Bionanotechnology is focused on the nanotechnoligcal makeup and properties of living organisms and how best to apply the corresponding principles at the nanoscale. A related discipline is **bionanoscience**, which is a field of research focusing on the nanoscale physical and chemical properties of naturally occurring biological (or at least biomicking) structures and materials.

Nucleic Acid Nanotechnology

Nanotechnological research which seeks to utilize the inherent inter- and intra-molecular recognition properties of nucleic acids to build structures is known as **nucleic acid nanotechnology**. While this can be accomplished using either DNA or RNA as base components, the vast majority of research in this area has been on DNA applications due to its stability and ease of synthesis in comparison to RNA. Progress in this field represents some of the first advances in the control of molecularly precise manufacturing. It is based on the inherent **Watson-Crick base-pairing** properties critical to and required for the formation of the DNA double helix. This phenomenon was named after James Watson and the late Francis Crick, two University of Cambridge researchers who discovered the structure of DNA in 1953, for which they were awarded the 1962 Nobel Prize in Physiology or Medicine along with researcher Maurice Wilkins.

DNA is an unbranched polymer consisting of four subunits known as nucleotides. These include adenine (A), cytosine (C), guanine (G), and thymine (T). It can exist in varying lengths, is typically around half a nanometer (20 angstroms) in width and is designated as having a five prime (5′) end and a three prime (3′) end, named after the positioning of phosphate and hydroxyl groups at the termini. Linkage between
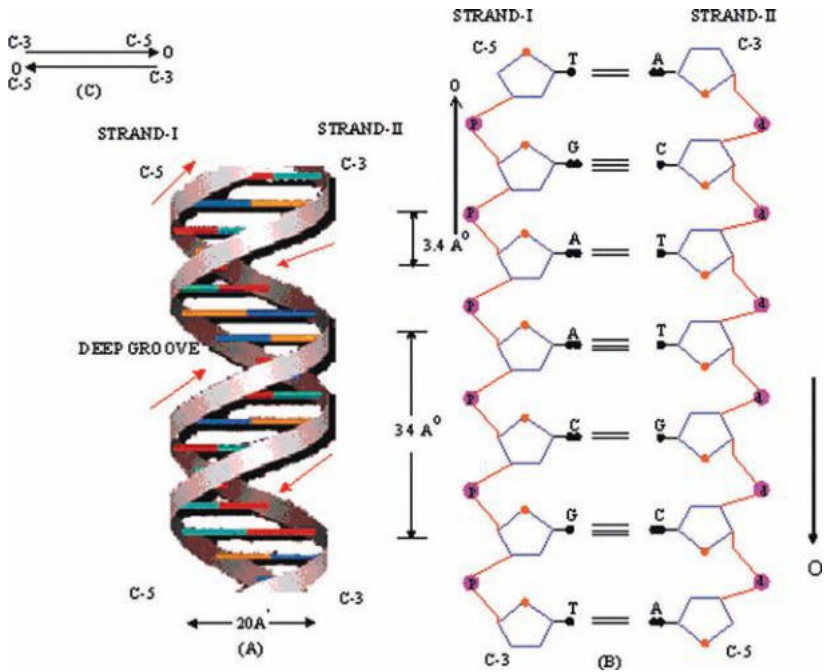
FIGURE 1.29 Diagrammatic illustration of a DNA double helix and flattened image emphasizing Watson-Crick base-pairing. (Image courtesy of Pink Monkey; reprinted with permission.)

these nucleotides within a single strand of DNA occurs via strong **phosphodiester bonds**. A DNA double helix is formed through hydrogen bonding between compatible base pairs on each strand. Watson-Crick base pairing thus occurs between A and T and between G and C. Purines, A and G, always form bonds with pyrimidines, T and C. G/C bonding is significantly stronger due to three hydrogen bonds vs. two for A/C base-pairing (Figure 1.29).

The unique 5′ to 3′ phosphodiester bonding and Watson-Crick base-pairing properties of double-stranded DNA result in the formation of a precise, rigid molecule that is stable at room temperature in a variety of solvents. Often referred to as "building blocks," the individual nucleotides and their bonding characteristics provide an excellent mechanism for which to assemble two-dimensional and even three-dimensional nucleic acid-based structures. A number of groups, most notably that led by Paul Rothemund at the California Institute of Technology, have studied this phenomenon. Dr. Rothemund and colleagues have generated

Focus Box 1.6 Nadrian Seeman and DNA-based molecular self-assembly

The pioneering professor Paul Rothemund at California Institute of Technology has focused much of his career on macromolecular design and topology with a particular emphasis on DNA-based self-assembly. Dr. Rothemund's group has developed computer software that enables the optimization of nucleic acid sequences to form desired 2D or 3D structures. The researchers have synthesized the nucleic acid sequences suggested by the calculations, and they do self-assemble in the predicted manner. The ultimate goal of Dr. Rothemund's research is to use DNA as a scaffold for the assembly of larger precise entities such as biological macromolecules or electronic circuitry. (Photos courtesy of Paul Rothemund; reprinted with permission.)

"**DNA origami**" by folding long single-stranded DNA into structures and filling in as needed with short oligonucleotides, all based on Watson-Crick base-pairing properties (Figure 1.30). Studies by Rothemund and others will no doubt open the door to nucleic acid-based bottom-up molecularly precise manufacturing and may allow for the creation of real-world practical nanoscale machinery and working devices (e.g., molecular motors) that will have an impact on virtually every aspect of our lives.

Nanotechnology's Potential Impact on Medicine

Nanomedicine can be defined as the medical application of nanotechnology. It is an all-encompassing term that captures the essence of research, diagnostic and therapeutic applications involving nanotechnology that span a wide range of medical disciplines. Strategies for employing nanotechnology in medicine have been contemplated and pursued to manage, treat or even cure virtually every type of ailment known to man. Examples include the application of nanomaterials science to treat burns, nanoelectronic circuitry to enhance neuronal signaling and communication in the brain, and nanoparticle-mediated thermal ablation of cancer cells just to name a few. Although the potential to impact virtually any area of
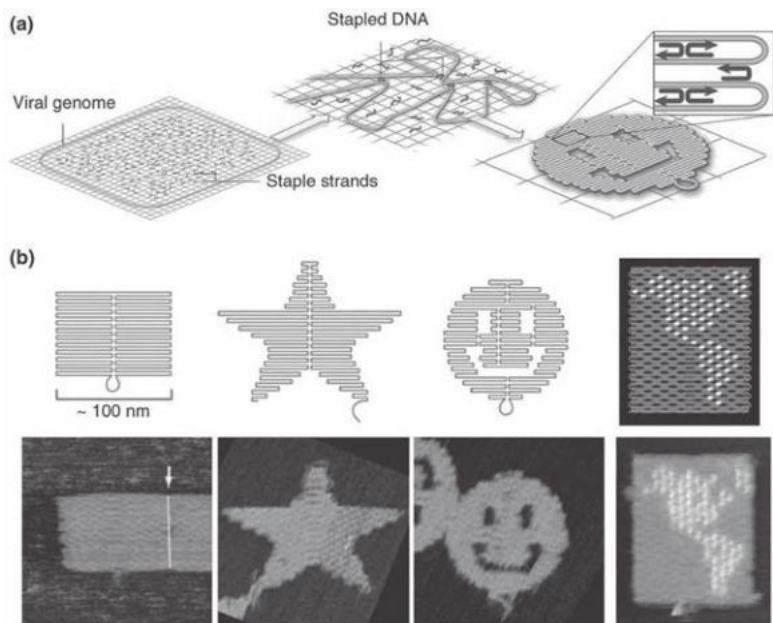
FIGURE 1.30 DNA origami created by Dr. Paul Rothemund at the California Institute of Technology. (Courtesy of Rothemund, 2006; reprinted with permission.)

medicine exists, nanomedicine can be broken down into specific major medical disciplines as outlined in Table 1.1.

Typically over the last ten years or so various nanotechnological platforms that may play a role in future medical applications have converged upon a specialization towards diagnosis, therapy or sometimes, as is the case for magnetic nanoparticles, both (Figure 1.31). The study of nanoscience and the optimization of technologies such as liposomes and carbon nanotubes have driven the design of methodologies that are tailored to address specific diseases and medical anomalies. Liposomes, for example, represent excellent nanoscale-based drug delivery vehicles and thus tend to be focused on delivery to certain cell types as in the treatment of cancer. Nanotemplates provide unique access to extremely high-throughput capabilities for rapidly defining the presence of a biomarker and thus lend themselves as excellent platforms for diagnostics applications.

Rapid progress is being made to drive these advances in nanomedicine, especially in the area of cancer treatment. The remainder of this book

Table 1.1 Medical disciplines and example nanotechnology platforms to address them

Discipline or Medical Focus	Example of Nanomedical Application
Cancer	Attach nanoparticle or nanomaterial to cancer cell and destroy it.
Tissue Engineering	Use nanomaterials as scaffolds to drive tissue growth and differentiation.
Clinical Neuroscience	Supplement the nervous system with nanoparticles or nanomaterials to enhance neuronal signaling and survivability.
Surgery	"Welding" of tissues using nanoparticle substrates.
Stem Cell Culture Matrices	Use nanomaterials as scaffolds to grow and differentiate stem cells into tissues for transplant.
Diagnostics	Nanoparticle-based contrast agents.

breaks down each of these concepts and the basic principles behind how nanomedicine and the corresponding strategies developed may positively affect specific areas of medicine. While still in its infancy when compared to other medical technologies, nanomedicine is undoubtedly here to stay and will have an enormous impact on the future health and well-being of man.
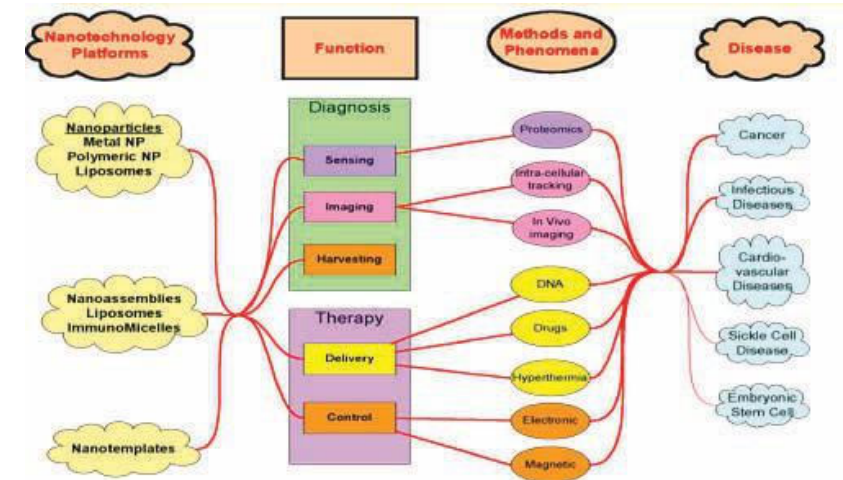
FIGURE 1.31 Concepts in nanotechnology-driven medicine.

CHAPTER SUMMARY

Nanotechnology and Its Origins

1. Early concepts in nanotechnology date back to 500 B.C.
2. Richard Feynman was the first modern-day scientist to propose the scientific field of nanotechnology.
3. Technologies such as atomic layer deposition and scanning tunneling microscopy have made considerable impacts on the advancement of nanotechnology.
4. K. Eric Drexler was pivotal in promoting the future impact of nanotechnology.
5. The three primary categories of nanotechnological research are nanomaterials, nano-instrumentation and nanomedicine.
6. The two approaches to nanoscale manipulation are stochastic and deterministic in nature.

The Basics of the Nanoscale

1. Richard Adolf Zsigmondy formulated the first nanometer range classification system.
2. One nanometer (nm) is one billionth of a meter, or 10^{-9} meters.

Nanomaterials and Nanoparticles

1. Nanomaterials exhibit a quantum size effect.
2. Fullerenes are the third allotrope of carbon and exhibit unique physicochemical properties.
3. Fullerenes may be synthesized by arc discharge, chemical vapor deposition or laser ablation.
4. Richard Smalley, Robert Curl and Harold Kroto received the Nobel Prize for their discovery of the buckyballs.
5. Carbon nanotubes have unique intrinsic electrical properties that result in electrostatic attraction of one another.
6. Carbon nanotubes exist in single-wall or multi-wall formats and are uniquely strong.
7. The structure of a single-walled carbon nanotube is measured as a chiral vector.
8. Multi-walled carbon nanotubes exist in the form of Parchment or Russian Doll models.
9. Carbon nanotubes are highly thermally conductive and efficiently absorb certain external fields such as RF waves and near IR light.

10. Kanzius RF Therapy is based on gold nanoparticle or carbon nanotube sensitivity to RF waves.
11. Perfluorocarbons (PFCs) are carbon backbones surrounded by fluorine atoms and can be synthesized by direct reaction of fluorine with hydrocarbons, the Fowler Process or EFC.
12. Gold nanoshells are comprised of a dielectric glass core surrounded by a gold sphere or shell and have unique tunable optical properties.
13. Superparamagnetic nanoparticles such as iron oxide have shown much promise in diagnostic imaging applications.
14. Silver nanoparticles may be manufactured by physical vapor deposition, wet chemistry or ion implantation and are accepted as safe for *in vivo* medical applications.
15. Dendrimers may be synthesized by divergent synthesis, convergent synthesis or click chemistry and are ideal for carrying drug payloads to disease sites within the body.
16. Micelles and liposomes are both hydrophilic and hydrophobic in nature and also act as efficient platforms for drug delivery.

Nanotools

1. The scanning tunneling microscope (STM) allows for imaging resolution at the atomic level and has been instrumental in advancing the field of nanotechnology.
2. The atomic force microscope (AFM) is a cantilever-based imaging instrument that yields 3d images based on a phenomenon known as Hooke's Law.
3. Zyvex Instruments' nProber was developed to address the phenomenon known as Moore's Law for IC failure analysis applications.

Current Manufacturing Research

1. The two primary methods of nanoscale synthesis are the top-down and bottom-up approaches.
2. Atomically precise manufacturing (APM) marries the disciplines of chemistry and engineering and is pursued for purposes of eliminating waste and streamlining manufacturing procedures.

Bionanotechnology

1. Watson-Crick base pairing properties of nucleic acids may be manipulated to promote DNA or RNA self-assembly.

2. Nadrian Seeman's work on DNA-based self assembly has resulted in computer algorithms which can formulate the design of nucleic acids that self-assemble into 2D and 3D structures.
3. Nanotechnology is now beginning to make a significant impact on many medical disciplines in imaging, diagnosis and therapeutics.

KEY TERMS

- Nanotechnology
- Nano
- Maxwell's Demon
- Atomic Layer Deposition
- Scanning Tunneling Microscope
- Cluster Science
- Biomimetic
- Stochastic Nanotechnology
- Deterministic Nanotechnology
- Ultramicroscope
- Nanoscopic Scale
- Nanometer
- Nanomaterial
- Quantum Size Effect
- Nanoparticle
- Fullerene
- Arc Discharge
- Chemical Vapor Deposition (CVD)
- Laser Ablation
- Carbon Nanotube
- Exfoliation
- Chiral Vector
- Parchment Model
- Russian Doll Model
- Coercivity
- Superparamagnetism
- Wet Chemistry Synthesis
- Dendrimers
- Divergent Synthesis
- Convergent Synthesis
- Click Chemistry
- Encapsulation
- Micelle
- Liposome
- Critical Micelle Concentration (CMC)
- Krafft Temperature
- Nanotool
- Scanning Tunneling Microscope (STM)
- Quantum Tunneling
- Atomic Force Microscope (AFM)
- Hooke's Law
- Moore's Law
- nProber
- Failure Analysis
- IV Curve
- Top-Down Approach
- Optical Lithography

- Thermal Ablation
- Ballistic Conduction
- Phonon
- Density of States (DOS)
- Van Hove Singularities
- Kanzius RF Therapy
- Perfluorocarbon (PFC)
- Fowler Process
- Nanoparticle
- Nanocrystal
- Nanoshell
- Quantum Plasma Oscillation
- Phosphodiester Bond

- Bottom-Up Approach
- Self-Assembly
- Atomically Precise Manufacturing (APM)
- Parallel Assembly
- Nanobiotechnology
- Bionanotechnology
- Bionanoscience
- Nucleic Acid Nanotechnology
- Watson-Crick Base-Pairing
- Phosphodiester Bond
- DNA Origami
- Nanomedicine

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. Describe the nanoscale.
2. List the major types of nanoparticles and nanomaterials currently studied in nanoscience.
3. Compare and contrast divergent vs. convergent dendrimer synthesis.
4. Define and write the equation for critical micelle concentration (CMC).
5. Describe the basic principles behind an atomic force microscope.
6. What is Moore's Law?
7. Compare and contrast top-down vs bottom-up approaches to nanotechnology research.
8. What are the principles behind Watson-Crick base-pairing?
9. Describe the theory behind molecular self-assembly concepts and cite and example of a structure formed by this technology.
10. What are the major areas of medical focus for nanotechnology?

2

Nanoparticles and Hyperthermic Cancer Therapeutics

In this chapter, the use of nanoparticles as tools for the treatment and management of cancer is examined. Two delivery platforms are described: the localized, non-specific application of nanoparticles and the use of targeting moieties to direct nanoparticles to cancerous cells within the body. Each of these platforms is based on taking advantage of nanoparticle sensitivity to external fields described in Chapter 1. The use of nanoparticles for targeted cancer drug delivery will be discussed in Chapter 7.

NANOPARTICLES AND THERMAL ABLATION

Mechanisms of Action

This section describes the mechanistic action of how thermal energy is generated by nanoparticles acting upon by external fields. Particular

attention is paid to both the use of near infrared light and radiofrequency waves as the sources for external energy as these have shown the most promise from a safety perspective.

Metals and Light

Metals, especially gold, are unique in their abilities to interact with external fields such as electromagnetic waves including light, radiofrequency and even x-rays. When the wave is of the appropriate wavelength or frequency, the metal particles exhibit an effect known as **surface plasmon resonance (SPR)**, which is defined as the oscillation of free electrons along a particle's surface upon exposure to an external field. A **surface plasmon** is a free electromagnetic wave and it will travel in a particular direction parallel to the metal and external interface. In order to yield a surface plasmon resonant effect, the increase in the magnitude of oscillations must intensify electron scattering effects and convert the external field into heat. It is well known that gold and silver are excellent conductors of surface plasmon resonance, yet gold is much safer when introduced into the body (Figure 2.1). Gold nanoshells are similar to gold nanoparticles in their ability to exhibit SPR, yet they are also tunable, based on size and composition, to different wavelengths of light thus making their utility much more valuable for medical applications.

Metals and Radiofrequency Waves

Light is not the only external field that can drive metal nanoparticles to release energy in the form of heat. Radiofrequency (RF) waves have also been shown to promote thermal emission from metal nanoparticles, most notably gold (Figure 2.2). The mechanism of action is thought to be based on the agitation of the metal ions as they follow an alternating current fluctuating in different directions. The agitation results in ion vibrations and ultimately heat release. Pioneering research by the late John Kanzius in this area has significantly advanced this field and could potential transform the way certain types of cancer are treated. His visionary efforts also include the application of RF and carbon nanotubes for cancer therapeutics (see Focus Box 1.4 in Chapter 1).
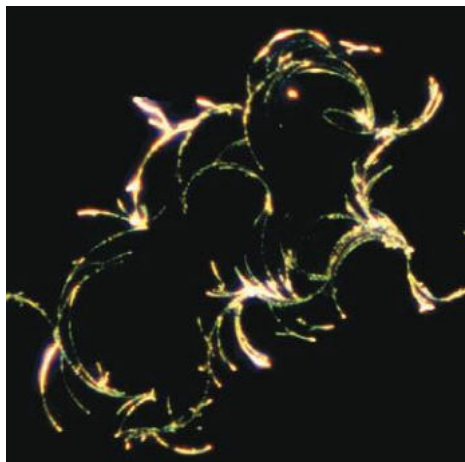
FIGURE 2.1 Gold nanoparticles exhibiting surface plasmon resonance (SPR). This dark-field image was formed by accumulations of colloidal gold nanoparticles (40 nm diameter) on a coverslip. The green color comes from the resonance Rayleigh scattering of single gold nanoparticles due to surface plasmon resonance excitation. Because the wavelength of the surface plasmon resonance associated with the nanoparticles is dependent on particle size, shape, and local dielectric environment, the dark-field image exhibits different colors. (Image courtesy of Image Technology Group; reprinted with permission.)

Carbon Nanotubes and Radiofrequency Waves

Carbon nanotubes have been well documented to act as tiny antennas for radiofrequency waves. The absorption of RF by CNTs properly solubilized by molecules such as Kentera™ generates a large amount of thermal energy which can be utilized in a biological setting for therapeutic initiatives.

The mechanism of action behind CNT heating in the presence of an RF field has been under much scrutiny given the physics behind the phenomenon. How is it that carbon nanotubes can convert radiofrequency waves into thermal emission when the properties of the system just don't add up? For example, at a frequency of 13.56 MHz (the medically-approved frequency for use of RF) the wavelength of the RF field is around 22 meters. This greatly exceeds the average carbon nanotube length by a ratio of about 1 um of RF to 300 nm of nanotube. Thus the two entities shouldn't be compatible from an absorption perspective and resonance

(a)

(b)
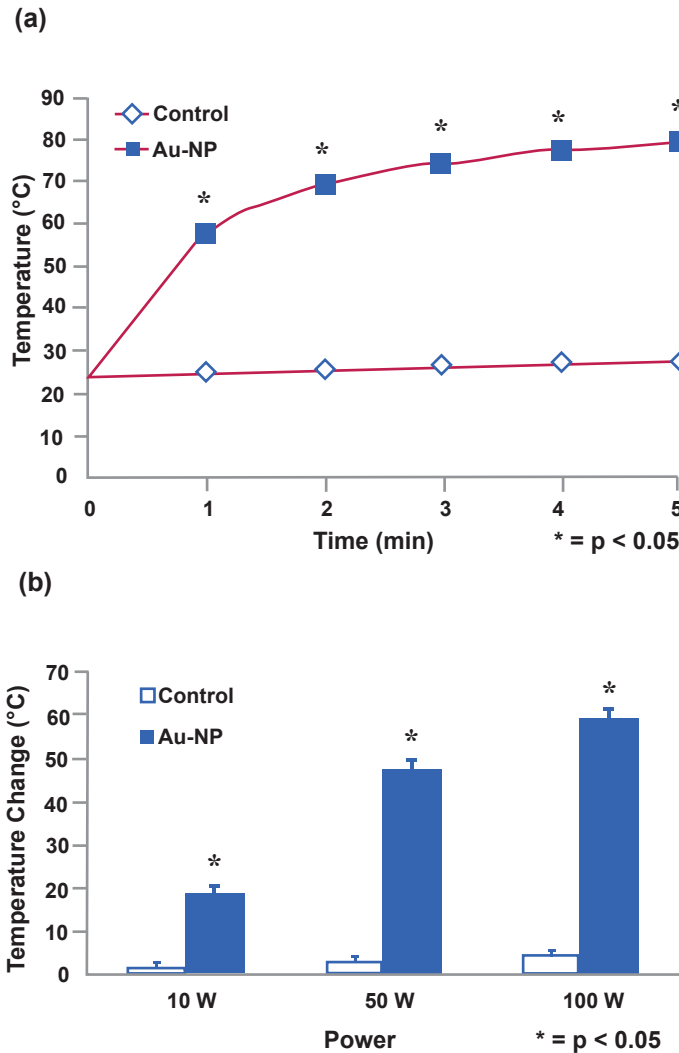
FIGURE 2.2 Au-NP solutions are heated selectively and efficiently in RF field. (A) Solutions of Au-NP (squares) or water (diamonds) were exposed to the RF field at 50 W and temperatures were measured over time. Results reported are averages of three separate experiments. (B) Solutions of Au-NP (black) or water (white) were exposed to the RF field at 10, 50, or 100 W and temperatures were measured after 3 minutes. Results reported are the averages of three separate experiments. (Images courtesy of Cardinal *et al.*, 2008; reprinted with permission.)

shouldn't be observed. It has been speculated, though not proven, that observed thermal emission may be based on the resistive conductivity of the carbon nanotubes coupled with their high aspect ratios of length/ surface. This aspect ratio ranges from 300 to 1000 for a typical carbon nanotube. Thus, for CNTs measuring an average of 1 micron in length, a peak RF field strength of 15 kV/m might drive thermal emission. An alternative explanation is that in a biological environment CNTs may self-assemble to create larger antennae up to microns in length which would be much more efficient at RF wave absorption and thus corresponding thermal emission.

The killing of cancer cells and desired elimination of tumors within the body using heat has been a focus in the area of cancer therapeutics for many years. It has been demonstrated that applying a thermal field locally to cancerous tissue will result in modest cell death and some reduction in tumor size. In addition, more recent studies have suggested that the localized heating of tumors and corresponding cancer cells makes them more sensitive and susceptible to drug and chemotherapeutic treatments. The use of nanoparticles which emit heat when exposed to external fields takes this basic concept a step further, and literally places nanosized "antennas" which allow for the generation of a thermal field deep within a tumor. In the case of radiofrequency and carbon nanotube-mediated cancer cell thermal ablation, carbon nanotubes are introduced into the tumor and the tumor (along with introduced CNTs), is subsequently exposed to radiofrequency waves at an approved frequency for medical applications (13.56 MHz) for a defined period of time, usually several minutes. Passage of RF waves from transmitter to receiver through the tumor results in a rapid increase in temperature as a result of CNT RF wave absorption (Figure 2.3).

As described above, this may also be accomplished using CNTs or gold nanoshells and near infrared (near IR) light exposure at a wavelength around 800 nm. For near IR applications no external receiver is required. The mechanism of action for carbon nanotubes exposure to near IR is described below.

Carbon Nanotubes and Light

The mechanism of action for thermal emission by carbon nanotubes exposed to near IR light is a great deal more straightforward than for that pertaining to the use of RF. Both single-walled and multi-walled CNTs
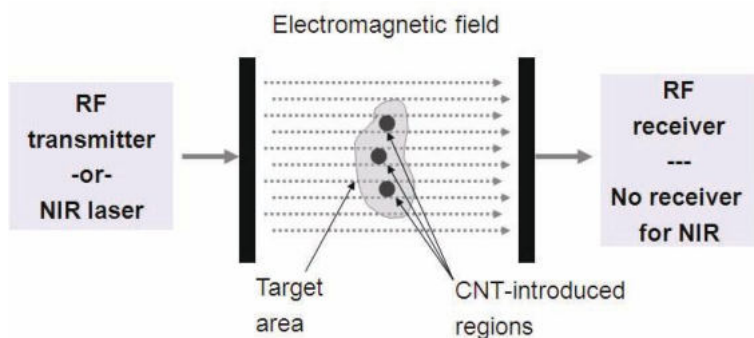
FIGURE 2.3 Diagrammatic illustration of the mechanism of action behind nanoparticle-mediated thermal ablation of cancer cells. (Image courtesy of Gareth A. Hughes, Ph.D.; reprinted with permission.)

efficiently absorb near IR light in the wavelength range of 700–1100 nm, yet it is interesting that MWNTs behave and dipole antennae that absorb a broad spectrum of light while SWNTs have a much more specific absorption spectra. A closer look at SWNTs suggests that as chirality properties are altered the absorption spectra is as well and optimal absorption may occur in the presence of light of wavelengths as long as 1250 nm. In the case of either MWNTs or SWNTs the absorption of light results in optical stimulation of the CNTs to produce vibration and subsequent thermal energy emission.

Carbon Nanotubes, Heat and Cancer Therapeutics

Hyperthermia is defined as temperatures above 40°C, at which point living biological entities such as cells begin to die. Irreversible damage to cells rapidly occurs at temperatures above 45°C. The use and application of carbon nanotubes, both for hyperthermia-medicated cancer cell ablation and drug delivery as discussed in Chapter 7, is dependent upon effective solubilization of CNTs in an aqueous/biological environment. Both single- and multi-walled carbon nanotubes possess intrinsic **van der Waals** intertube **attractive properties** which are defined as relatively weak attractive forces between molecules other than those due to covalent bonds or ionic bonding. Between two molecules van der Waals attractive forces are negligible, but between trillions it can result in considerable insolubility of the nanoparticles and clumping in solvents such as water.
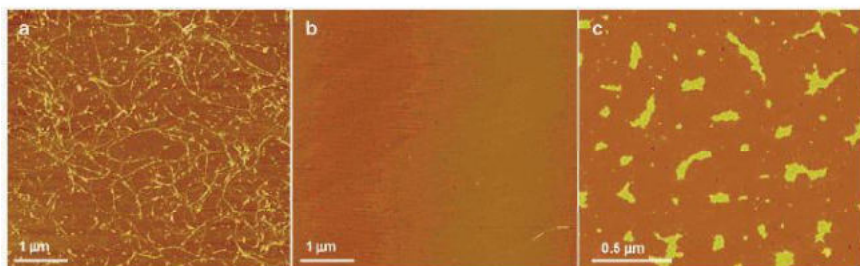
FIGURE 2.4 AFM image of nano-1/SWNT dispersion compared to SDS/SWNT and nano-1 control samples. (a) Nano-1/SWNT dispersion revealing many long SWNTs. (b) SDS/SWNT sample exhibiting minimal dispersion of SWNTs. (c) Nano-1 control sample lacking SWNTs. (Images courtesy of Zorbas *et al.*, 2004; reprinted with permission.)

This must be addressed to generate CNT solutions containing individual water-soluble tubes for their effective use *in vivo*. Gregg Dieckmann, Inga Musselman, Rocky Draper and colleagues at the Nanotech Institute of the University of Texas at Dallas have developed a peptide-wrapping system for the effective dispersion, solubilization and functionalization of carbon nanotubes of biological applications. The system employs a 29-amino acid peptide made by solid phase peptide synthesis procedures, referred to as **nano-1**, which has been designed to form an amphiphilic a-helix. Nano-1 has apolar residues which occupy one face of the helix, binding to the CNT and reducing van der Waals attractive forces between CNTs. Polar residues on the opposite face are predicted to interact with polar water molecules in solution thus increasing solubility. A mixture of this peptide with SWNTs was sonicated and subsequently ultracentrifuged, with the fraction containing the peptide-wrapped carbon nanotube fraction isolated from the sample. Atomic force microscopy was used to characterize the peptide-wrapped CNTs (Zorbas *et al.*, 2004 and Figure 2.4).

Other molecules have been designed for the dispersion of carbon nanotubes in a variety of host solvents such as Kentera (Case Study 2.1).

Superparamagnetic Nanoparticles, Magnetism and Mechanism of Action

Ferro- and ferrimagnetic nanoparticles have unique properties that lend themselves as ideal agents for thermal emission in the ablation of cancer

---

Case Study 2.1: Localized Injection and Use of Carbon Nanotubes to Ablate Cancer Cells

Chris Gannon and others at the University of Texas M.D. Anderson Cancer Center in Houston have developed reagents and procedures for the *in vivo* ablation of cancer cells using a combination of solubilized single-walled carbon nanotubes and radiofrequency waves based on the concept of Kanzius RF Therapy (see Focus Box 1.4). In creating an animal model for human liver cancer, Gannon's group injected the human hepatic VX2 cancer cell line into the flanks of adult New Zealand white rabbits and subsequently transferred sections of these tumors into the liver parenchyma of rabbits. Working with researchers at the University of Texas at Dallas and the nanotechnology company Zyvex Corporation, Gannon's group used RF waves and SWCNTs non-covalently functionalized with the polymer Kentera, which allows for dispersion and solubilization in an aqueous or physiological environment, to thermally ablate these tumor *in vivo*. Intratumoral injection of Kentera-conjugated SWCNTs followed by exposure to RF at 600W for 2 minutes resulted in complete thermal necrosis of the tumor tissue. Other than expected minimal thermal injury to tissues surrounding the tumors, no additional toxicity or organ damage was observed (Gannon *et al.*, 2007).

---

cells. As discussed in Chapter 1, these particles exhibit a phenomenon known as **superparamagnetism** in which a flipping of the magnetization direction is influenced by temperature. How is this magnetic effect converted into heat? Ferro- and ferrimagnetic nanoparticles exhibit a property known as **magnetic hysteresis**, in which atomic dipoles become aligned with the magnetic field, no matter the direction. Upon exposure of the nanoparticles to a positive, then negative, then positive magnetic field a hysteresis loop is created and this loop is dissipated by the nanoparticles in the form of heat. The power dissipated in this process is referred to as the **Specific Absorption Rate (SAR)** and can be represented by the following equation:

$$\text{SAR} = Af$$

where A is the area of the hysteresis loop and f is the frequency of the magnetic field.

A is expressed in Joules/gram of nanoparticle and is referred to as the "specific loss" of the material. *A* is a very complex component of the SAR and in the case of magnetic nanoparticles depends on a multitude of factors including the magnetic frequency of the magnetic field, their overall volumetric concentration, their magnetiocristalline anisotropy *K* and the temperature of the system.

As earlier in the chapter, in all the systems described above the temperature of biological environment in general and the cancer cells in particular must reach around 45°C for efficient thermally induced necrosis to occur. Alternatively, a temperature of 42°C may allow for more effective targeted drug or chemotherapeutic treatment to occur as cells tend to be more permeable when heated to temperatures in this range.

Treatable Types of Cancer

The use of nanoparticles in combination with external fields such as radiofrequency waves or light may be effective for the treatment of a variety of cancers, but one common property regarding the integrity and type of cancer is obvious: the tumors must be solid and the cancer cells non-circulating. Leukemia, for example, a circulating cancer of the white blood cells, is currently not amenable to this form of cancer treatment. There are two primary reasons for this. In order to yield an appropriate level of temperature increase a high concentration of "antennae" nanoparticles must be locally present. A spreading of nanoparticles throughout the body, whether individually attached to cancer cells or not, will not yield the desired increase in temperature under current research conditions (this may change in the near future with improvements in the technology). Secondly, the process of placing a patient with nanoparticles present throughout the body under exposure to RF waves or light could result in thermal ablation of healthy tissue and is thus too dangerous to be contemplated. While highly precise and efficient targeting of nanoparticles to specific cancer cells is a major goal of today's research in this area, it will be awhile before these platforms may be considered for treating cancers such as leukemia. Thus cancers theoretically treatable using nanoparticles and external fields for thermal ablation are solid in composition and include, but are not limited to:

- Bone
- Brain
- Breast
- Cervical
- Colorectal
- Head and Neck
- Liver
- Lung
- Melanoma
- Ovarian
- Pancreatic
- Prostate
- Testicular

NON-SPECIFIC, LOCALIZED USE OF NANOPARTICLES FOR TUMOR ABLATION

Initial studies on the use of nanoparticles coupled with external fields for therapeutic purposes, particularly in the area of cancer treatment, were directed at localized introduction or non-specific accumulation within the cancerous tissues of the body. Localized introduction involves the injection or permeabilization of nanoparticles directly into the tumor, with the goal of producing a nanoparticle concentration high enough that application of an external field such as light or radiofrequency waves will yield the desired effect of cellular thermal ablation with minimal damage to surrounding tissues. These initial studies paved the way for more sophisticated research focused on targeting nanoparticles to particular cell types in order to decrease exposure of surrounding healthy tissues to the cell killing effects of thermal ablation (discussed in more detail in the next section). Below are two case studies highlighting some of the more high-profile research on non-specific applications of nanoparticles to eradicate cancer.

TARGETING NANOPARTICLES TO SPECIFIC SITES FOR TUMOR ABLATION

One of the hallmarks of cancer is the uniqueness of the cells forming the tumor or driving the metastatic fate of cancerous tissue. These cells often exhibit characteristics that other cells within the body don't, the most obvious of which is the unchecked ability to divide. Uncontrolled cell division often directly or indirectly impacts the morphological and molecular makeup of cells and may result in the presence of unique molecular and biochemical markers that can be exploited to single out cancer cells within the body. Other molecular and biochemical signatures unique to cancer cells may themselves be the driving force behind unchecked cell division.

Focus Box 2.1 Ellen Vitetta and targeting cancer cells

Dr. Ellen Vitetta, Director of the Cancer Immunobiology Center and Professor of Microbiology and Immunology at the University of Texas Southwestern Medical Center in Dallas, Texas, is a pioneer in the area of targeting cancer cells with drugs and has now turned her attention to targeting carbon nanotubes for purposes of thermal ablation. Her group has recently targeted SWNTs to Daudi cancer cells *in vitro* using monoclonal antibodies which bind to receptors expressed on these cells and ablated these cells via exposure to near IR light. She is now translating these findings in animal models of cancer. (Photo courtesy of UT Southwestern; reprinted with permission.)

If the cancer cell-specific biomarker is a cell surface receptor it may allow for the homing or targeting of a particular therapeutic agent to sites on the outer surface of the cell membrane. A great deal of research has been focused in this area prior to the advent of potential nanotechnology-based therapeutics endeavors. As a result, a number of cell surface receptors have been defined as being either entirely unique to a specific type of cancer or as overexpressed on the cell surface to a level at which utilizing them as homing sites in targeting strategies is pursued. Table 2.1 outlines some

Table 2.1 Examples of well characterized cancer cell surface markers over-expressed in certain types of cancer	
Cell Surface Marker	**Cancer(s) Exhibiting Overexpression**
Her2-Neu	Stage IV Breast
Integrins	Numerous Cancers
TGF beta Receptor	Colon
EGF Receptor	Lung, Head and Neck
VEGF	Colorectal
CD Markers	Numerous Cancers

Table 2.2 Classification of cancer cell targeting platforms

Targeting Agent	Mode of Action	Cancer Application	Known Example(s)
Monoclonal Antibody	Cell Surface or Internalized	Breast, Colorectal, Head and Neck, Lymphoma	Herceptin, Erbitux, Avastin, Rituximab
Small Molecule	Cell Surface, Intracellular	Lung	Tarceva, Iressa
Aptamer	Cell Surface, Intracellular	Renal, Lung	AS1411 (in dev.)
Peptide	Cell Surface, Intracellular	Breast, Thyroid	RGD peptide (in dev.)

of the more high profile cell surface markers that have been exploited for targeting drugs and immunotoxins as therapeutics.

Targeting Agents

The targeting of cell surface receptors which are uniquely expressed on cancer cells provides a very powerful mechanism for homing a therapeutic or imaging platform in on an undesirable cell type while theoretically excluding other healthy cell populations within the body. There are a number of different types of targeting agents which bind specifically to particular cell surface receptors. Many of these have been utilized as the therapeutic platforms themselves with no attached payload. Some of the most widely studied and currently used in a clinical setting include monoclonal antibodies, small molecules, peptides and even aptamers. Examples are listed in Table 2.2.

Monoclonal Antibodies

Unlike polyclonal antibodies, **monoclonal antibodies** are identical antibodies due to the fact that they are produced by one type of immune cell. Using current hybridoma (mouse/human hybrid cells) technology originally developed by Georges Kohler, Cesar Milstein and Neils Jerne (see Focus Box 2.2), monoclonal antibodies can be produced which

<div style="background-color:#d9e89a;">

Focus Box 2.2 Georges Kohler and discovering monoclonal antibodies
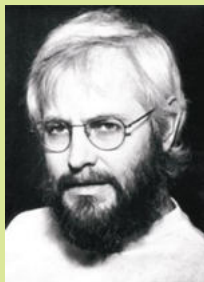
German biologist Dr. Georges Kohler, along with Argentine scientist Cesar Milstein and Danish researcher Niels K. Jerne, won the 1984 Nobel Prize for Physiology or Medicine for their 1975 research on the immune system and discovery of technologies and procedures for monoclonal antibody production. In their research they created a fusion between a myeloma cell line, which had lost its ability to secrete antibodies, and a healthy antibody-secreting B cell line. Selection for the successfully producing cells resulted in a highly controllable system for monoclonal antibody production. (Photo courtesy of the Dana Foundation; reprinted with permission.)

</div>

bind tightly to virtually any material or **antigen**, which is defined as a substance that prompts the generation of antibodies which specifically bind to it. Antigens typically consist of proteins or polysaccharides. **Epitopes** are antigenic determinants present on an antigen through which actual binding occurs. When applied as a therapeutic a particular monoclonal antibody is referred to as "MAb." Monoclonal antibodies are far superior to polyclonal antibodies with respect to their controlled manufacturing procedures and their reproducible affinity for specific target antigens.

Antibody-antigen binding can be quantified by what is known as the **binding affinity**, which is defined as the strength of an antibody's binding to its antigen epitopes. All interactions between antibody and epitopes contribute to the value of the binding affinity. These are always non-covalent, transient interactions that can be influenced by a variety of environment factors including temperature, pH and ionic concentrations. Binding affinity is thus represented by an **association constant K** according to the following equation:

$$K = \frac{[Ab-Lg]}{[Ab][Lg]}$$

Case Study 2.2: The Enhanced Permeability Retention Effect and Gold Nanoshells for Cancer Therapeutics

In efforts at developing new technologies for the thermal ablation of cancer cells, Jennifer West and colleagues in the Department of Bioengineering at Rice University have taken advantage of a phenomenon known as the **enhanced permeability and retention effect (EPR)** (for detailed description see Chapter 7), in which a preferential accumulation of certain sizes of molecules occurs in tumor tissues. This is due to newly formed but abnormal vasculature consisting of wide fenestrations at the tumorigenic site which leads to preferential size-based permeabilization. West and colleagues created a murine animal model via subcutaneous injection of colon carcinoma cells in immuno-compromised mice. After the tumor had reached a certain size polyethylene glycol (PEG)-coated gold nanoshells were injected intravenously (via the tail vein) into the mice and allowed to accumulate in tumorigenic tissue via EPR for approximately six hours. Exposure of the tumors to near infrared light with a diode laser tuned to 808 nanometers for 3 minutes resulted in the ablation of all visible tumors, with the mice appearing tumor free greater than 90 days post-treatment (Figure 2.5). Described by West *et al.* as a non-invasive cancer treatment procedure, this is one of the first examples of successful *in vivo* photothermal ablation of cancer cells using a combination of nanoparticles with an external field (O'Neal *et al.*, 2004).
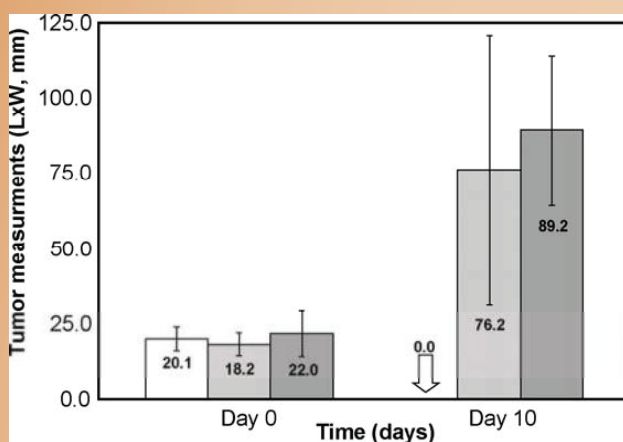
FIGURE 2.5 Mean tumor size at day zero and ten following treatment for experiment (white), sham (light grey) and control (grey) tumor populations. (O'Neal *et al.*, 2004; reprinted with permission.)

where the terms in brackets represent the concentration of

- antibody-ligand complexes [Ab–Lg],
- unbound antibody [Ab], and
- unbound ("free") ligand [Lg].

The association constant K can also be represented more conveniently according to the **Scatchard equation**

$$K = \frac{r}{(n-r)(c)}$$

where

r = the ratio of the concentration of bound ligand to the concentration of antibody molecules placed in the system,

n = the number of ligand binding sites on the antibody molecule (i.e., its valence), and

c = the concentration of unbound ("free") ligand.

A typical strong interaction between antibody and antigen through binding epitopes is represented by values of K around $1-3*10^{12}$/mole as is evidenced by Hahnel and Twaddle's thorough analysis on the interaction between estrogen and its receptor in various breast cancer cell lines (Table 2.3).

Monoclonal antibodies used for therapeutic purposes may bind to a variety of antigens present on the surface of tumor and cancer cells. Examples of this are listed in Table 2.4. They may also be utilized to deliver a number of different types of payloads to destroy the targeted cells in question including radioactive ligands, cytokines, toxins, liposomes containing drugs and specific killer cell types. This is depicted in Figure 2.6. These **immunoconjugates**, which are defined as antibodies linked to a second molecule such as a toxin, radioisotope or label, may act at the cell surface as killing agents or be internalized to release payloads intracellularly.

Small Molecules

Small molecules are defined as low molecular weight organic molecules that are not considered to be polymers. They are usually designed to interact tightly with biological materials including nucleic acids, proteins or polysaccharides and to cause a change or effect upon the target or cell presenting the target. At a maximum size of around 800 Daltons, most

Table 2.3. Association constants and number of binding sites for estradiol receptors of hormone-dependent carcinomas

Carcinoma	Association Constant ($\times 10^{12}$/mole)	No. of binding sites ($\times 10^{14}$ mole/mg)
BAG	1.797	15.1
BOT	1.520	5.7
CAP	2.331	8.0
CHA	2.495	5.2
DEN	2.348	15.2
DYM	3.051	5.7
FER	1.630	4.5
FIN	0.953	7.6
FLE	8.860	30.3
GEB	2.083	5.9
GOO	1.811	10.4
GUY	8.839	4.8
HAN	7.778	10.8
HIC	4.717	6.4
HLL	2.106	44.0
HNA	0.692	45.0
HOL	2.264	55.0
HYA	0.289	19.5
ITZ	0.749	62.8
MED	3.695	3.8
MIL	1.260	13.6
MIT	2.891	27.3
MOF	2.003	56.7
MRA	3.698	7.0
PAP	2.974	10.6
PAR	3.738	7.4

Table 2.3. (*Continued*)

Carcinoma	Association Constant ($\times 10^{12}$/mole)	No. of binding sites ($\times 10^{14}$ mole/mg)
PIO	1.053	6.8
PRA	5.338	21.8
ROG	1.196	12.9
SCR	2.915	4.5
SHE	6.675	32.4
SMA	1.794	5.7
TUL	11.018	12.5
WAN	1.475	15.6
WIL	3.819	5.8
WIM	1.229	46.3
WOO	2.466	14.9
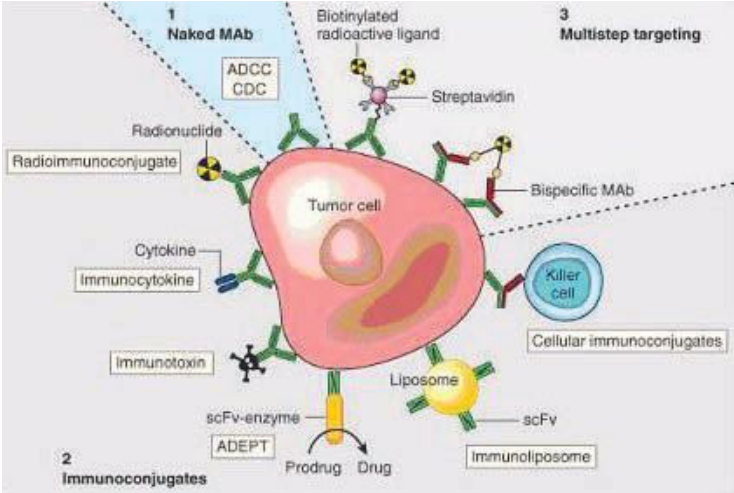
FIGURE 2.6 Diagrammatic illustration of different types of binding sites for monoclonal antibodies present on a typical cancer cell. (Image courtesy of Wikipedia; reprinted with permission.)

Table 2.4. Monoclonal antibodies approved by the FDA for cancer therapy

Generic (Trade mark)	Target antigen	Isotype	Species	Payload	Mechanism of action	Antitumor therapeutic activity	FDA year of approving
Rituximab (Rituxan™)	CD20	IgGI k	Chimeric	–	Induction of apoptosis, ADCC, CDC, chemosensitization	Low grade B-cell NHL	1997
Trastuzumab (Herceptin™)	Her-2/neu	IgGI k	Humanized	–	ADCC, chemosenzitation, CCA, inhibition of angiogenesis	Her-2 overexpressed metastatic breast cancer	1998
Alemtuzumab (Campath-IH™)	CD52	IgGI k	Humanized	–	ADCC, CDC	B-cell CLL	2001
Cetuximab (Erbitux™)	EGFR (Her-I)	IgGI k	Chimeric	–	inhibition of angiogenesis, chemosensitization and radiosensitization, CCA, ADCC	Metastatic colorectal cancer, head and neck cancers	2004
Bevacizumab (Avastin™)	VEGF	IgGI k	Humanized	–	Inhibition of angiogenesis	Colorectal cancer	2004
Panitumumab (Vectibix™)	EGFR	IgG2 k	Human	–	Inhibition of cell growth, induction of apoptosis, decreased proinflamatory cytokines and VEGF production	Metastatic colorectal cancer	2006
Gemtuzumab ozogamicin (Mylotarg™)	CD33	IgG4 k	Humanized	Calicheamicin	Double-stranded DNA breaks and cellular death induced by payload after intracellular hydrolysis	CD33+ relapsed AML	2000
Ibritumomab tiuxetan (Zevalin™)	CD20	IgGI k	Murine	90-yttrium	Cellular death induced by β-emitter, induction of apoptosis, ADCC, CDC	Low grade or follicular, relapsed or refractory, CD20+ B-cell NHLs; Rituximab-refractory follicular NHL	2002
Tositumomab (Bexxar™)	CD20	IgG2a λ	Murine	131-iodine	Cellular death induced by γ-emitter, induction of appoptosis, ADCC, CDC	Relapsed CD20+ B-cell NHL; Rituximab-refractory NHL	2003
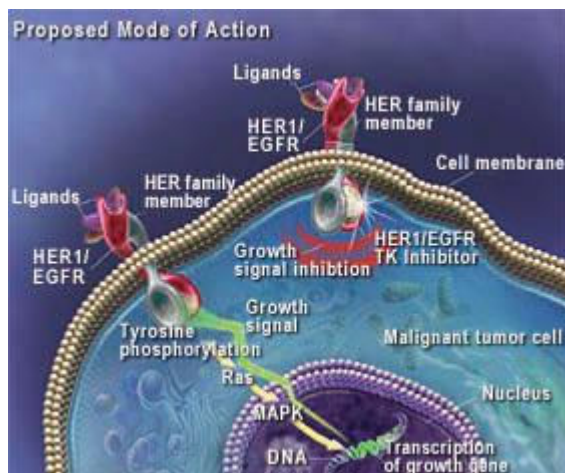
FIGURE 2.7 Illustration of the proposed mechanism of action for the small molecule drug Tarceva. (Image courtesy of Genentech, Inc.; reprinted with permission.)

small molecules can rapidly diffuse across cell membranes to effect the intended action intracellularly. They act upon targeted cells in a number of ways as either agonists or antagonists of cell function and viability. These primarily include an alteration of cell signaling to yield a therapeutic benefit. Most drugs fall into the small molecule category. Tarceva (Erlotinib hydrochloride) and Iressa (Gefitinib) are excellent examples of targeted small molecule platforms that attack lung cancer cells through interaction with the epidermal growth factor receptor (EGFR) which is preferentially expressed on the surface of these cells (Figure 2.7).

Both Tarceva and Iressa bind the intracellular ATP binding site of the tyrosine kinase domain and inhibit its function of autophosphorylation thus causing apoptosis of the cells through inhibition of the anti-apoptotic Ras signaling cascade. The specific intracellular binding capacity of these small molecule drugs may also be optimal for targeting a thermal ablation platform such as a carbon nanotube into the cell's cytoplasm.

Aptamers

Aptamers are defined as oligonucleic acid or peptide molecules that bind to a specific target molecule. Most research on aptamers has occurred

in nucleic acid aptamer development. Aptamers in this respect are often identified from large random sequence pools by desired target binding specificity. The selection technology, referred to as **SELEX** (<u>s</u>ystematic <u>e</u>volution of <u>l</u>igands by <u>ex</u>ponential enrichment) allows for very rapid *in vitro* selection of aptamers that tightly bind anything from proteins to small molecules to even other nucleic acids. This process has been recently automated by Andrew Ellington and colleagues at the University of Texas-Austin in collaboration with Somalogic, Inc. As a result, the selection process has been shortened to three days from the typical six week experiment. In addition, aptamers can be combined with **ribozymes**, nucleic acid-based enzymes, to be cleaved and thus activated upon binding to a target ligand. They can also be combined with other targeting moieties such as peptides for additional specificity (Figure 2.8).

Perhaps the most high profile aptamer therapeutic currently under development is known as AS1411 and has been designed to target nucleolin receptors on the surface of renal and acute myeloid leukemia (AML) cancer cells. AS1411 was originally developed as a potential cancer therapeutic by Professor Paula J. Bates of the University of Louisville in Kentucky. Upon binding, nucleolin receptors internalize the aptamer subsequently resulting in an activation of apoptosis. The
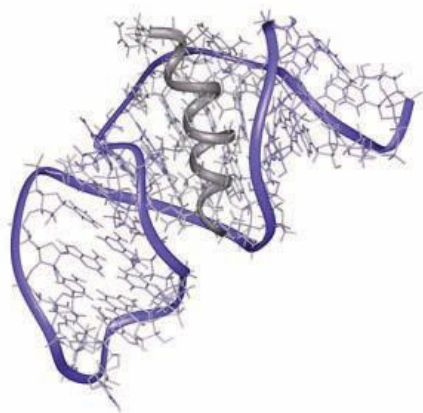
FIGURE 2.8 RNA Aptamer coupled with the HIV Rev response element RRE binding peptide. (Image courtesy of Institute for Cellular and Molecular Biology, The University of Texas at Austin; reprinted with permission.)

design and clinical trial research on AS1411 is being spearheaded by London-based Antisoma.

Peptides

Peptides are short oligomers synthesized from the joining of α-amino acids via peptide bonds, often by a process known as **Solid-Phase Peptide Synthesis (SPPS)**, which is a method for the synthesis of peptides using a deprotection and washing procedure (see Focus Box 2.3). The lengths of peptides vary greatly, but in general they are much shorter than proteins also known as polypeptides which typically consist of hundreds of amino acids linked by peptide bonds. Peptides are often designed to mimic a particular ligand's binding affinity for a receptor or other cellular target of interest. This allows for the efficient use of a small portion of the ligand for binding and thus targeting a specific receptor and cell type. The **RGD** (arginine-glycine-aspartic acid) peptide, for example, was derived from the active binding site of many extracellular matrix proteins for which integrin receptors bind to allow for the attachment of cells to the ECM (Figure 2.9). As integrin receptors are overexpressed in a number of different types of cancer cells, the RGD peptide may act as an excellent targeting moiety for chemotherapeutic, drug or even nanoparticle delivery.

Focus Box 2.3 Robert Merrifield and solid-phase peptide synthesis

The late Robert Merrifield (1921–2006) was a pioneer in the art of peptide synthesis. In 1963, as a researcher at the Rockefeller Institute for Medical Research, Dr. Merrifield championed the development of Solid-Phase Peptide Synthesis (SPPS) which utilizes repeated cycles of amino acid coupling followed by deprotection and washes resulting in an efficient synthesis procedure due to the removal of excess reagents from the system. The technique is routinely used today at the industrial scale and has changed little over the past 50 years. In 1984 he Nobel Prize in Chemistry for these studies. (Photo courtesy of Michigan State University; reprinted with permission.)
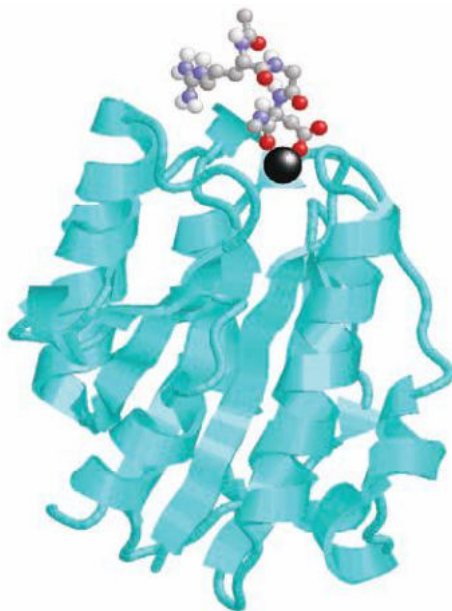
FIGURE 2.9 Diagrammatic illustration of the docking of an RGD peptide to the Mg-dependent active site of the human 3 integrin receptor. The peptide is illustrated in the molecular structure at the top of the figure.

Each of the above, MAbs, small molecules, aptamers and peptides provide an invaluable resource for targeting nanoparticles to unwanted biological material such as cancer cells, bacteria or viruses present within the body. As discussed, targeting may occur on the surface of cells or intracellularly, even driving nanoparticles into the nucleus via, for example, binding to nuclear hormone receptors. These targeting platforms will no doubt play an integral role in the development of a wide range of targeted nanoparticle-based therapeutics.

Targeting Moiety Attachment

Covalent Attachment

Covalent attachment of targeting agents to nanoparticles involves the creation of permanent bonds between the agent and the nanoparticle. A **covalent bond** is a type of chemical bond in which atoms shares electron

pairs with one another or with additional covalent bonds. This sharing can occur between sigma or pi orbital electrons. It is one of the strongest types of bonds known and is considered permanent when referring to the attachment of a targeting agent with a nanoparticle. Covalent bonding does not require that the two atoms involved in the bond are of the same identities, only that they share similar **electronegativities**, which is defined as the ability of an atom to attract electrons in the formation of a covalent bond. This attraction force must be similar between atoms sharing a covalent bond. Therefore, a covalent bond between two atoms doesn't necessarily require that they are equal in identity but that they have similar electronegative properties. In addition, it should be noted that covalent bond strength depends not only on the sharing of electrons but also the angular relationship of the atoms involved in and adjacent to the bond.

While there are a number of methods for covalently attaching targeting agents to carbon nanotubes, covalent bonding is one of the most preferred due to the tight, relatively permanent interaction. In an elegant study, David Scheinberg's group at Memorial Sloan Kettering Cancer Research Center in New York has developed a reproducible organic synthesis methodology that allows for the efficient permanent covalent attachment of antibodies to single-walled CNTs. Specifically, his team functionalized the side walls of water-soluble SWNTs through a multistep synthesis procedure that allowed for the covalent attachment of antibodies, radio-metal ion chelates and fluorescent probes to the tubes. These reagents were subsequently analyzed *in vitro* and *in vivo* in a murine lymphoma animal model and shown to efficient target and thus recruit the binding of SWCNTs to tumor cells in each system.

The **reaction flow** is as follows:

1. Shortening and purification of SWCNTs by oxidative acid digestion
2. Sidewall functionalization of 1 with amino groups
3. Modification of amino groups with bifunctional chelate
4. Derivatization of remaining free amine groups
5. Introduction of reactive sulfhydryl groups and conjugation of thiolated antibody
6. Preparation of non-targeting radiolabeled control
7. Preparation of radiolabeled targeting SWCNT

Non-covalent Attachment

Targeting moieties may also be attached to nanoparticles via **non-covalent bonding**, which is considerably weaker and can often result in the dissociation of the targeting agent from the nanoparticle if the bonding strategy is not optimal for the desired environment. In addition, nanoparticles may be attached to other nanoparticles (or fullerenes) via non-covalent bonding and thus enhance a particular diagnostic or therapeutic application through superior optical, electronic and absorptive properties. Case Study 2.3 illustrates this for gold nanoparticle attachment to carbon nanotubes.

One of the strongest known non-covalent bonds is that between the tetrameric protein avidin, discovered in egg whites, and biotin, otherwise known as vitamin B7. The **dissociation constant, Kd**, defined as the propensity for two bound objects to reversibly dissociate from one another, is an excellent mathematical representation of this tight bond. *Kd* is the reciprocal measurement of the association constant *K*, presented earlier. It is represented as:

$$Kd = \frac{[\text{Ab}][\text{Lg}]}{[\text{AbLg}]}$$

where the terms in brackets represent the concentration of

- unbound antibody [Ab], and
- unbound ("free") ligand [Lg].

For avidin and biotin has been measured to be around 10^{-15} M. This makes the interaction between avidin and biotin one of the strongest non-covalent associations known. Case Study 2.4 is an example of taking advantage of this extremely tight albeit non-covalent binding interaction between the biotin and avidin to allow for the efficient attachment of a targeting moiety to CNTs.

Non-covalent attachment of targeting moieties is the preferred method with respect to therapeutic applications of carbon nanotubes for a number of reasons. First, covalent modification of carbon nanotubes introduces changes in the inherent structure of the tubes which in many cases affects their ability to absorb energy from external fields thus decreasing the amount of absorbed energy available for conversion into heat during resonance. This decrease in CNT thermal emission will no doubt have a detrimental impact on cellular ablation efficiency. In addition, the introduction of

Case Study 2.3: Non-covalent Interaction between Gold Nanoparticles and Multiwalled Carbon Nanotubes via an Intermediatory

In a study on nanoparticle interactions, researchers at the Center for Nanotechnology at Chung Yuan Christian University in Taiwan have developed an elegant method for the non-covalent attachment of gold nanoparticles (Au-NPs) to multi-walled carbon nanotubes via the use of the intermediary bridging agent orthomercaptoaniline. The specific goal was to anchor gold nanoparticles to the sidewalls and ends of CNTs through a phenomenon known as **pi electron stacking** (also known as pi-pi interactions), which is defined as a stacked arrangement of often aromatic molecules due to interatomic interactions between pi electrons. One functional domain of orthomercaptoaniline was bound to the surface of the CNTs via pi electron stacking in a manner that allowed for the preservation of the inherent CNT electronic characteristics. The second functional domain allowed for linkage of Au-NPs via a thiol linker, again, through pi electron stacking. The finished product thus contained MWNTs non-covalently linked to Au-NPs via two instances of pi electron stacking, made possible by an aromatic bridge molecule (Yeh, *et al.*, 2009).
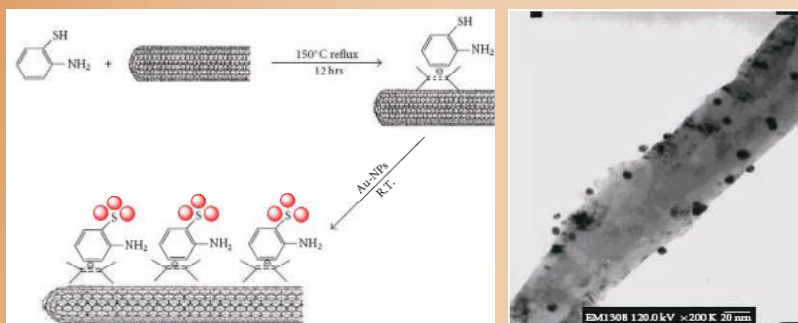
FIGURE 2.10 Diagrammatic illustration (left) of the procedure for Au-NP attachment to MWNTs and TEM image (right) of the final Au-MWCNT-thiol functionalized complex. (Yeh, *et al.*, 2009; reprinted with permission.)

Case Study 2.4: Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes

Ellen Vittetta and colleagues at the University of Texas Southwestern Medical Center in Dallas, Texas (see Focus Box 2.1) have designed an avidin-biotin system for the flexible non-covalent attachment of a variety of targeting and monitoring moieties to carbon nanotubes. In this study biotinylated polar lipids were utilized to prepare stable SWCNT dispersions soluble in water that were subsequently attached to one of several neutralite avidin-derivatized MAbs. These antibodies were directed against the cancer cell marker CD22, which is known to be upregulated in Daudi lymphoma cells. Following specific attachment of the antiCD22-SWCNT complexes to cells *in vitro* exposure to near infrared light resulted in considerable cellular ablation which was dependent upon bound MAb-CNT complexes. These studies demonstrate the development of a unique platform for efficient non-covalent attachment of targeting antibodies to carbon nanotubes that allows for specific targeting of the complexes and corresponding cellular ablation when exposed to NIR light (Chakravarty, *et al*., 2008).
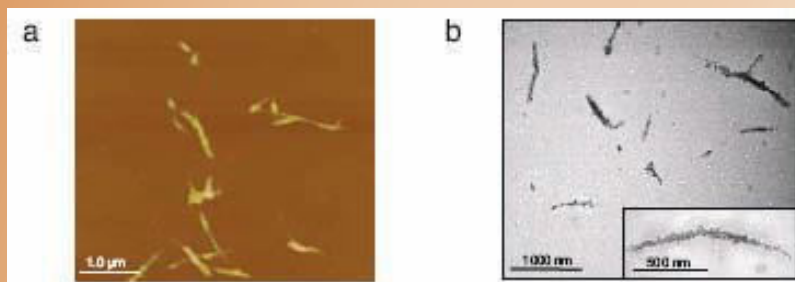
FIGURE 2.11 Water-soluble CNTs functionalized with biotinylated polar lipids. (a) AFM image of B-CNTs shows CNTs coated by the biotinylated polar lipid, DSPE-PEG-biotin. (b) TEM images of individual B-CNTs show uniform coverage of biotin after immunodetection with gold-labeled anti-biotin. (*Inset*) Higher magnification of a B-CNT coated with gold-labeled antibiotin. (Chakravarty, *et al.*, 2008; reprinted with permission.)

permanent changes to the molecular structure of CNTs creates therapeutic platforms that in some instances have unknown molecular structures. This is an unfavorable situation from a Food and Drug Administration (FDA) approval perspective as the changes may actually increase their toxicity in certain respects.

IN VIVO ANTICANCER PLATFORM DELIVERY

The delivery of nanoparticles to site of disease may be accomplished by either of two primary methods: localized injection at the disease site or introduction into the blood stream. Each of these options along with their advantages and disadvantages is discussed below.

Localized Injection

Localized injection of nanoparticles involves their direct introduction as a suspension at the site of disease. The primary goal of localized injection is to quickly introduce a high enough concentration of the therapeutic agent at the disease site, such as, for example, within a deep tissue tumor, that will allow for efficient killing of unwanted cells or material without the risk of damage to surrounding tissues (Figure 2.12). In reality this is a difficult endeavor, and studies have shown that in almost all cases some damage to surrounding tissues occurs when localized injection procedures are implemented. A major advantage to localized injection includes no need for cellular targeting and binding to occur thus simplifying the therapeutic design and speeding up the process of treatment. The disadvantage is spreading of the non-targeted therapeutic to other areas of the body which could cause damage itself or certainly in combination with exposure of these areas to an external field resulting in unwanted cellular thermal ablation.

Intravenous Injection

Intravenous injection allows for the use of the patient's circulatory system to direct the therapeutic complex to the site of disease. It is almost always implemented with nanoparticles complexed to targeting agents as targeting moieties are needed to seek out and allow for specific binding of nanoparticles to target cells or material. One exception to this, discussed earlier in this chapter, is reliance upon the enhanced permeability retention
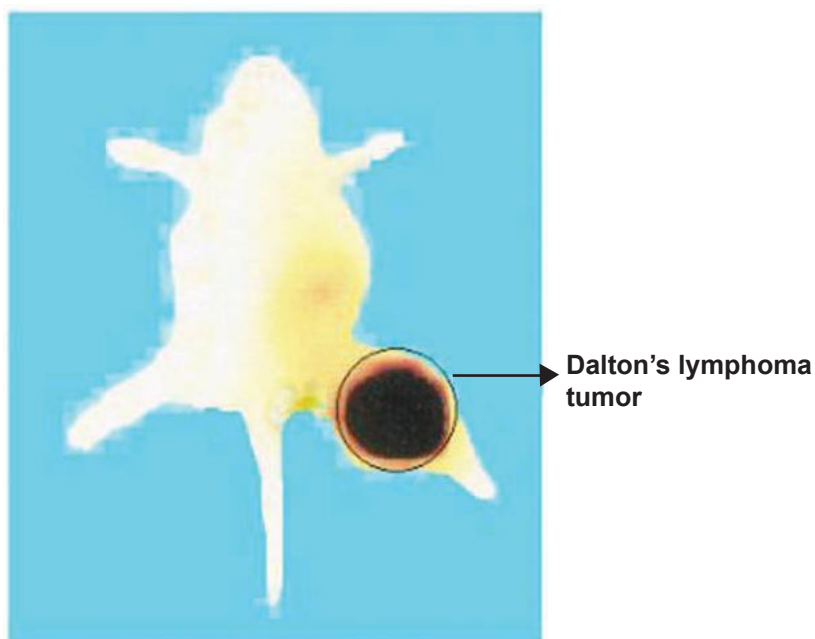
FIGURE 2.12 Intratumoral, localized injection of nanoparticles. Image   is of 99mTc-ETPL nanoparticles injected directly into a lymphoma tumor demonstrating prolonged tumor retention of nanoparticles but also a spreading of the nanoparticles into other areas of the specimen. The black and yellow portion in the figure indicates radio-labeled complex. (Image courtesy of ALASBIMN Journal; reprinted with permission.)

(EPR) effect, in which a preferential accumulation of certain sizes of molecules occurs in tumor tissues. The primary advantage to intravenous injection of nanoparticles is ease of administration with no guesswork involved in the depth and therapeutic concentration needed for localized injection. Intravenous injection, however, runs the risk of widespread nanoparticle distribution outside of the disease site, perhaps within organs which could be damaged by nanoparticle presence or a combination of the nanoparticle and external field resulting in unwanted thermal ablation of cells. Intravenous injection, therefore, is dependent on such factors as cellular targeting efficiencies, concentration administered, localized external field administration (if applicable) and timing between injection and therapeutic application if an external field is used.

CHAPTER SUMMARY

Nanoparticles and Thermal Ablation

1. Localized injection and targeted delivery are the two primary delivery platforms for nanoparticle-based cancer therapy.
2. Certain metals exhibit surface plasmon resonance (SPR), which may be exploited for thermal ablation of cancer cells.
3. Light, radiofrequency waves and magnetism are the three primary external fields used in metal SPR induction to create heat.
4. Solubilized carbon nanotubes efficiently absorb RF waves and near IR light which is converted into thermal energy.
5. Carbon nanotubes may be solubilized by a variety of methods including through the use of molecules such as Kentera and peptide-wrapping technologies.
6. The killing of cancer cells is a central focus of nanoparticle-mediated hyperthermia research.
7. Ferro- and ferri-magnetic nanoparticles undergo magnetic hysteresis in the presence of an alternating magnetic field thus producing heat.
8. Most types of cancer treatable by nanoparticle-based hyperthermia are solid in composition.

Non-specific, Localized Use of Nanoparticles for Tumor Ablation

1. Localized tumor treatment using nanoparticle-based hyperthermia requires high concentrations of the particles within the tumor site.
2. Initial studies on locally injected nanoparticles have paved the way for efforts directed at targeted nanoparticle delivery to specific sites of tumorigenesis and cancer cells.

Targeting Nanoparticles to Specific Sites for Tumor Ablation

1. Cancer cells express unique receptors that may be used as beacons for nanoparticle attachment.
2. Numerous naturally occurring and man-made targeting moieties exist for homing nanoparticles to cancer cells in the body including monoclonal antibodies, small molecules, aptamers and peptides.
3. The enhanced permeability and retention effect (EPR) is an indirect targeting phenomenon that allows for preferential accumulation of nanoparticles in tumors.

4. Monoclonal antibodies are widely used in cancer therapy and be used to efficiently target cancer cells to deliver nanoparticles, drugs, radioactive ligands, cytokines or toxins for cancer cell destruction.
5. Most cancer cell-specific drugs are considered to be small molecules and may act directly as the therapeutic or be used to deliver other payloads such as nanoparticles.
6. SELEX technology allows for the rapid and accurate identification of aptamers that bind to a specific target.
7. Peptides are synthesized through Solid-Phase Peptide Synthesis (SPPS) and most often designed to mimic a ligand's binding affinity for a particular target.
8. Targeting moieties may be attached to payloads by covalent or non-covalent bonding, often via an intermediary molecule.

In vivo Anticancer Platform Delivery

1. Either localized intratumoral or intravenous injection of anticancer platforms (drugs, toxins, cytokines, nanoparticles, etc.) may be implemented for delivery to cancer cells.
2. Localized injection is used to quickly achieve high concentrations of the therapeutic within the tumor.
3. Intravenous injection is implemented for either active or passive (EPR) targeting of cancer cells.

KEY TERMS

- Surface Plasmon Resonance (SPR)
- Surface Plasmon
- Hyperthermia
- Van der Waals Attractive Forces
- Nano-1
- Superparamagnetism
- Magnetic Hysteresis
- Specific Absorption Rate (SAR)
- Enhanced Permeability and Retention Effect (EPR)
- Monoclonal Antibody
- Antigen
- Epitope
- Binding Affinity
- Association Constant K
- Scatchard Equation
- Immunoconjugates
- Small Molecule
- Aptamer
- SELEX
- Ribozyme

- Peptides
- Solid-Phase Peptide Synthesis
- RGD Peptide
- Covalent Bond
- Electronegativity

- Non-Covalent Bond
- Pi Electron Stacking
- Dissociation Constant, *Kd*
- Localized Injection
- Intravenous Injection

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. Define Surface Plasmon Resonance and describe the mechanism by which thermal ablation is accomplished by metal nanoparticles and light.
2. Describe the mechanism by which carbon nanotubes combined with RF waves allow for thermal ablation.
3. Define Specific Absorption Rate and write the equation representing it.
4. List some examples of cancer types treatable with nanoparticles combined with external fields.
5. Explain the Enhance Permeability Retention effect and how it is being used to treat cancer.
6. What are some examples of well-characterized surface markers for cancer cell targeting and in what cancers are they overexpressed?
7. List the four main types of targeting agents and give one example of each.
8. Who is Georges Kohler and what did he discover?
9. Compare and contrast association constant, *K*, and dissociation constant *Kd*.
10. Compare and contrast covalent vs non-covalent attachment of targeting agents to nanoparticles.
11. Define pi electron stacking.
12. Describe the two types of nanoparticle injection procedures and give examples of nanoparticles used for each.

This page intentionally left blank

3

Nanofiber-Based Scaffolds and Tissue Engineering

The goal of tissue regeneration has long been a central focus of the medical research community and the recent efforts at pursuing stem cell-based therapeutics emphasizes this point. The repair of damaged tissues and cells via cell/tissue transplantation, excluding organ transplants, has been an emerging medical discipline exhibiting only modestly positive results over the last decade. **Autologous replacement therapy,** which is defined as the transplant of cells or tissues from one part of the body to another in the same individual, is often met with limited success due to such factors as morbidity at the site of injury and limited living engineered materials available for transplant. These factors, coupled with the lack of a stable biocompatible matrix for which to grow and thereby graft transplant materials at the host site, has led to a considerable

effort at identifying new bases for two- and three-dimensional support scaffolds that can provide much needed support and stability required during autograft procedures. Recent research on nanofibers has suggested that they may possess some of the critical properties required for scaffold-based support of cells and tissues in transplantation therapy. Nanofibers, for example, have an extremely high surface area to volume ratio. This, combined with an often microporous structure, strongly favors cell attachment, growth and proliferation, migration and in some cases differentiation. This chapter describes the current state of research and development on nanofiber scaffold-based tissue engineering. For a more comprehensive look at basic nanoscale cell culture platforms see Chapter 6.

COMPOSITION AND TYPES OF NANOFIBERS

Nanofibers are defined as fibers with diameters on the order of 100 nm or less. They can range from 50 nm to 100 mm or more in length and can be composed of synthetic or naturally occurring polymers or even pure carbon. Nanofibers of **polymeric origin**, those which contain and are organized into repeating structural units, include those synthesized from the following materials.

- Chitosan—Natural
- Collagen—Natural
- Gelatin—Natural
- Hyaluronic Acid—Natural
- Silk Fibroin—Natural
- Protein—Natural
- Poly(lactic acid) (PLA)—Synthetic
- Polyurethane (PU)—Synthetic
- Poly(ethylene-co-E-caprolactone) (PLLA-CL)—Synthetic
- Poly(caprolactone) (PCL)—Synthetic
- Poly(lactic-co-glycolic acid) (PLGA)—Synthetic
- PLGA-poly(ethylene glycol) (PLGA-PEG)—Synthetic
- Poly(ethylene terephthalate) (PET)—Synthetic

Natural Polymeric Nanofibers

Nanofibers based on natural polymeric materials such as collagen provide the obvious advantages of being quite similar, almost identical to the macromolecular substances present within the human body. Thus these materials are more readily acceptable within the *in vivo* biological environment than a foreign substance such as a carbon nanofiber. This yields the increased probability that the nanofiber scaffold and all associated materials will be successfully incorporated into the host and minimizes chances for immune rejection or toxicity. As listed above, examples of these include collagen, gelatin, protein, hyaluronic acid, chitosan, elastin and silk. Some of the more high-profile natural polymeric nanofibers are discussed below.

Collagen

Collagen is by far the most popular and well characterized of all natural biomaterials. Collagen is the fibrous constituent of cartilage, tendons, bone and other connective tissue present within the body. Nanofibers fabricated from collagen have been demonstrated to exhibit high compatibility with cells such as myoblasts which form muscle and chondrocytes which form bone (Figure 3.1).
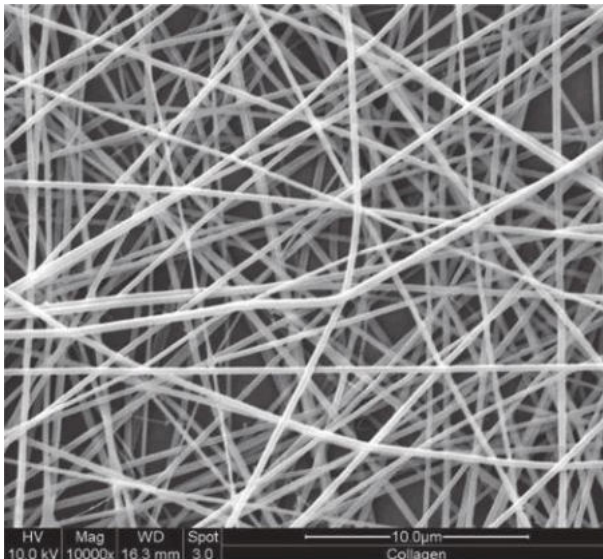
FIGURE 3.1 SEM image of collagen nanofibers. (Courtesy of IOP Science; reprinted with permission.)

Case Study 3.1: Chitosan nanofibers and bone cell growth

Scientists at the University of Washington in Seattle are making considerable progress in the development of nanotechnology-based cartilage repair scaffold platforms. The goal of these studies is to mimic the extracellular environment of cartilage in terms of microstructure and chemical composition and research on chitosan-based nanofiber scaffolds has been particularly promising. Miqin Zhang and colleagues have developed both chitosan and alginate-based nanofiber scaffolds that appear to recapitulate many of the properties of the *in vivo* microenvironment of cartilage. *In vitro* studies suggest that both osteoblasts and chondrocytes exhibit enhanced attachment to these nanofiber scaffolds compared to those of other classes. Upon attachment, the cells retain characteristic morphology and viability, critical parameters for bone tissue engineering applications (Figure 3.2).
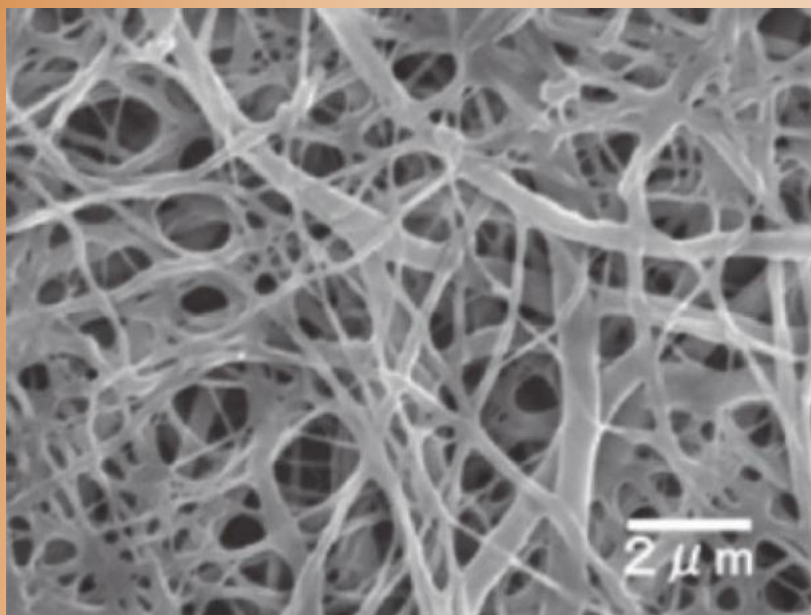
FIGURE 3.2 SEM images of electrospun chitosan-based nanofibers. (Courtesy of National Institute for Materials Science; reprinted with permission.)

Scaffolds built from collagen nanofibers and correctly cross-linked yield considerable strength and elasticity as well as other superior mechanical properties. Electrospun collagen nanofibers have now been used extensively for cell culture applications and are under intense study as scaffolds for tissue regeneration. In addition, it has been demonstrated that by blending type I collagen with other polymers such as poly(ethylene oxide) (PEO) a significant increase in the mechanical strength properties of the scaffold was observed. This opens the door for possible use of blended collagen-based scaffolds in a variety of tissue engineering therapeutic applications.

Chitosan

Chitosan is derived from chitin, which is a tough, protective semitransparent nitrogen-containing polysaccharide that is the primary component in the exoskeletons of arthropods as well as in certain fungal cell walls. Chitosan is a deacetylated derivative of chitin and has been used successfully to produce nanofibers of various sizes. Chitosan nanofibers have also been blended with PEO and these were shown to possess considerable structural integrity in water.

Hyaluronic Acid (HA)

Hyaluronic acid (HA) is a gel-like polysaccharide primarily located in the extracellular matrix of the synovial fluid of movable joints as well as in the eye humors and in connective tissue. It can be produced in nanofiber form by a process called electrospinning (described below) (Figure 3.3), yet as the application of electrospinning does not produce reliable and consistent HA nanofibers, Chu Um and colleagues at the State University of New York-Stony Brook developed the technique of **electro-blowing**, which combines electrospinning with air flow to yield the desired HA nanofiber properties. In addition, a group of South Korean researchers led by Tae Gwan Park at the Korea Advanced Institute of Science and Technology generated a three-dimensional macroporous HA nanofiber scaffold by electrospinning combined with a salt leaching process and has suggested its use in a variety of tissue regenerative medical applications (Figure 3.4). For example, under his direction chondrocytes were grown on HA water-swellable scaffolds and it was observed that adhesion as well as viability increased in direct proportion to HA content. Each of
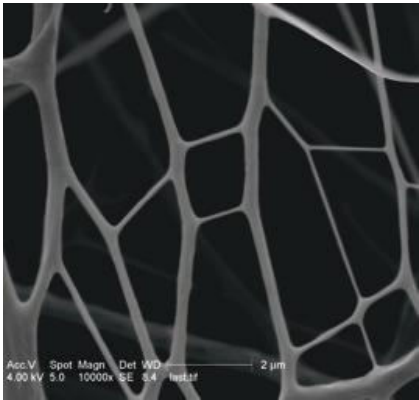
FIGURE 3.3 SEM image of hyaluronic acid nanofibers. (Courtesy of Caroline Schauer, Drexel University; reprinted with permission.)

these HA nanofiber synthesis techniques promises to open doors for the use of HA-based nanofibers in therapeutics.

Gelatin

Another naturally occurring biopolymer that has received much attention as a basis for nanofiber production is gelatin. **Gelatin** is a protein manufactured from the partial hydrolysis of collagen. In this process
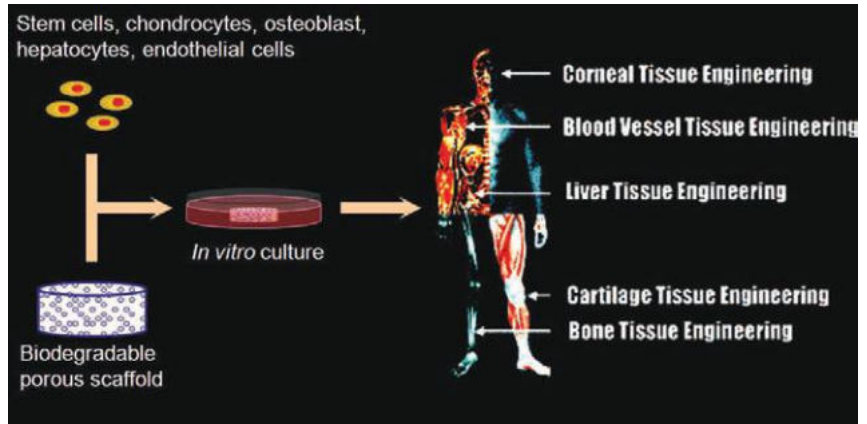
FIGURE 3.4 Diagrammatic flowchart of the use of a hyaluronic acid nanofiber scaffold for tissue regenerative therapeutics. (Courtesy of Tae Gwan Park, Korea Advanced Institute of Science and Technology; reprinted with permission.)

the bonds present within collagen that connect individual strands are broken down and rearrangement is allowed to occur, resulting in a gel-like consistency. The form that gelatin takes depends a great deal upon temperature. At higher temperatures gelatin is a liquid and upon cooling forms a solid. Temperature also affects the mechanical properties of gelatin with viscosity in water increasing at lower temperatures in general. In the presence of water gelatin forms a colloidal gel. **Colloids** are defined as a chemical mixture in which one substance is evenly dispersed throughout another. Researchers in the division of Bioengineering at the National University of Singapore have created gelatin/PCL biocomposite nanofibers using electrospinning techniques and demonstrated that scaffolds composed of these fibers have improved mechanical strength and water dispersion than either gelatin or PCL nanofibers alone. In addition, the researchers observed that bone and stromal cell attachment, division and extensive human fetal osteoblasts (hFOB) cell migration in the presence of these scaffolds were superior to other more conventional scaffolding systems. Other studies have suggested gelatin-based nanofiber scaffolds have promise for neural tissue and wound healing applications.

Silk Fibroin

In the production of silk, silkworms produce a unique protein known as fibroin. **Fibroin** is composed of anti-parallel layers of **beta sheets**, which is a secondary structure in certain proteins consisting of amino acid strands connected by five or more hydrogen bonds. The primary structure of fibroin consists of repetitive units of the following amino acid sequence:

$$(Gly\text{-}Ser\text{-}Gly\text{-}Ala\text{-}Gly)n$$

It is the repetitive natural of this sequence combined with the beta sheet secondary structure that gives fibroin significant tensile strength (stronger than Kevlar) while retaining considerable elasticity. Interestingly, spider dragline silk also contains fibroin. Nanofiber blends have been constructed using fibroin protein of silkworm or spider origin as the primary component along with various other reagents to improve biocompatibility. These include P(LLA-CL), collagen, chitin and even silver nanoparticles. Most of the research focus in this area has been on
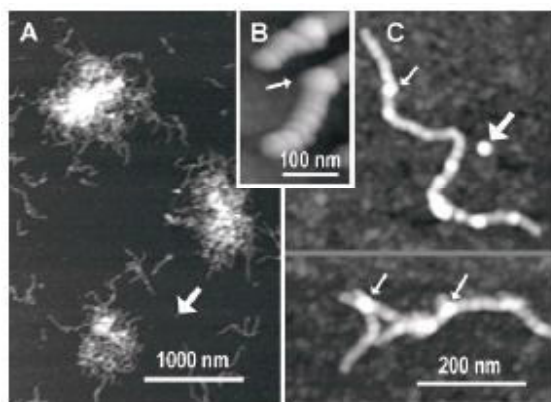
FIGURE 3.5 Silk nanofibers formed from pS(4+1) silk protein deposited on mica. AFM height images. (A) pS(4+1) silk protein is present primarily as aggregates of nanofibers. (B and C) Close-up AFM images of the pS(4+1) nanofibers show segmented substructure. Fat arrows indicate isolated blobs that are predicted to be segments of nanofibers, based on their sizes. Thin arrows indicate bulges, which often occur at branch points on nanofibers and may be due to nanofibers overlapping. (Oroudjev *et al.*, 2002; reprinted with permission.)

wound healing. When tested *in vitro* numerous cell types were successfully cultured using these scaffolds including keratinocytes and fibroblasts. H.G. Hansma and colleagues in the Department of Physics, University of California at Santa Barbara have comprehensively characterized the properties of spider silk fibroin nanofibers by atomic force microscopy and other methods and demonstrated that these nanofibers have a segmented substructure that differs considerably from the substructures of other proteins and gives it its unusual tensile strength and unique three-dimensional composition (Oroudjev *et al.*, 2002 and Figure 3.5). As Case Study 3.2 illustrates, three-dimensional silk fibroin nanofiber scaffolds have now been developed and utilized in a variety of *in vitro* and *in vivo* applications.

Human Protein

One concern that should be noted that in the cases of collagen, chitosan and gelatin is the lack of biocompatibility between these nanofiber scaffolds, which are composed of animal materials and bi-products, and humans. Immuno-rejection is a major issue in this respect and will need

Case Study 3.2: Silk fibroin nanofibers and self-assembly

Researchers in the Laboratory of Molecular Self-Assembly at MIT led by Dr. Shuguang Zhang have created silk fibroin nanofiber scaffolds through self-assembly protocols. These matrices have now been applied in several three-dimensional cell culture studies as well as in tissue engineering applications, including cell culture and even the repair of brain damage in rates (see Figure 3.6 for details).
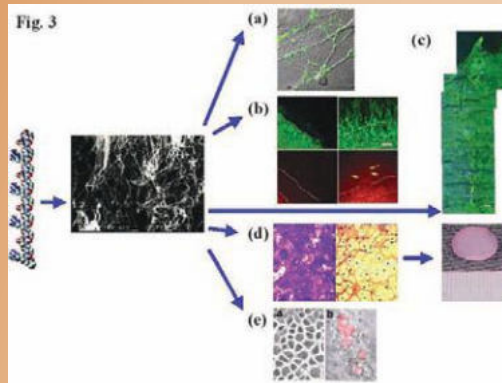
FIGURE 3.6 Self-assembling peptides form a three-dimensional scaffold woven from nanofibers. (a) Rat hippocampal neurons form active nerve connections, each green dot represents a single synapse. (b) Neural cells from rat hippocampal tissue slide migrate on the three-dimensional peptide scaffold. Cells on the polymer membrane (left) and on the peptide scaffold (right) are shown. Both glia cells (green) and neural progenitors (red) migrate into the three-dimensional peptide scaffold. (c) Brain damage repair in a hamster. The peptide scaffold was injected into the optical nerve area of brain that was first severed with a knife. The cut was sealed by the migrating cells after two days. A great number of neurons form synapses. (d) Chondrocytes from young and adult bovine encapsulated in the peptide scaffold. These cells not only produce a large amount of glycosaminoglycans (purple) and type II collagen (yellow), characteristic materials found in the cartilage, but also a cartilage-like tissue *in vitro*. (e) Adult rat liver progenitor cells encapsulated in the peptide scaffold. The cells on the two-dimensional dish did not produce cytochrome P450 type enzymes (left-panel). However, cells in three-dimensional scaffolds exhibited cytochrome P450 activity (right panel). (Courtesy of the Laboratory of Molecular Self-Assembly, Massachusetts Institute of Technology; reprinted with permission.)

to be addressed if these materials are to be used for *in vivo* therapeutics endeavors. Researchers have begun to address the issue of immunorejection by eliminating animal components altogether. The human protein **tropoelastin**, for example, has been of particular interested in this area for its biocompatible but also elastic properties. Tropoelastin is a 70 kDa water-soluble protein composed of multiple monomers covalently linked to one another to form the three-dimensional protein known as elastin. Tropoelastin can be synthesized in synthetic form and has been used in topical skin care and for wound healing. As demonstrated by researchers at Drexel University led by Peter Lelkes, electrospun tropoelastin nanofibers differ significantly from those of collagen origin, i.e. they are thicker and exhibit a ribbon-like shape as opposed to the round shape of collagen nanofibers. (Li *et al.*, and Figure 3.7). The Drexel team demonstrated the resulting scaffold's use in the three-dimensional culture and propagation of human embryonic palatal mesenchymal (HEPM) cells and compared it to their nanofiber scaffolds such as those of gelatin origin (Figure 3.8). In the manufacturing process they optimized solute concentrations and polymer delivery rate to control the diameter length of the nanofibers.

In the regenerative therapeutic areas of bone, tendon and cartilage tissue engineering the physical properties of scaffolds used are critical to long-term effectiveness. They must be able to withstand repeated and considerable internal and external forces to provide a realistic therapeutic benefit. The resulting scaffolds developed by Lelkes and colleagues were thus characterized for **stress**, a measurement of the amount of force exerted per unit area of a surface within a deformable body on which internal forces act, according to the following equation:

$$\textbf{Stress}\ (\text{gm/tex}) = \frac{\text{Force (gm)}}{\text{Area density (gm/m}^2) \times \text{width (mm)}}$$

where gm is the weight and m is the length of the tested strip of the specimen. **Strain**, which is defined as a geometrical measure of deformation representing the relative displacement between particles in the material body, was calculated by dividing the displacement of the scaffold by gauge length in millimeters. Stress vs strain curves allowed for the direct comparison of these properties in the form of comprehensive tensile strength for each polymeric nanofibrous scaffold (Figure 3.9).
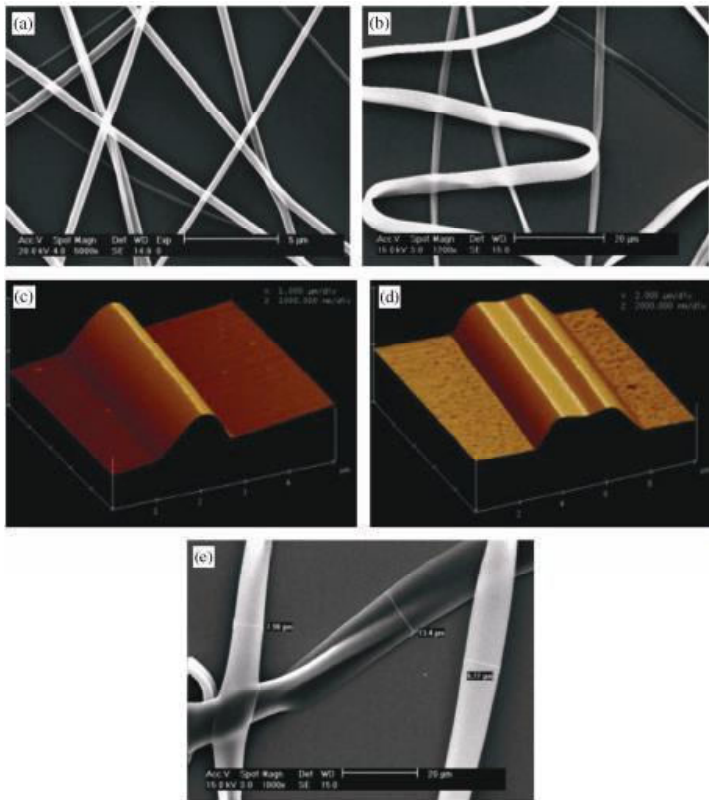
FIGURE 3.7 Comparison of electrospun collagen and tropoelastin fibers by SEM and AFM micrographs. (a) SEM micrograph of collagen fibers, magnification is 5000×. (b) SEM micrograph of tropoelastin fibers, magnification is 1200×. (c) AFM image of collagen fiber, showing the round shape of the fiber. (d) AFM image of tropoelastin, showing the ribbon shape of the fiber. (e) SEM micrograph of tropoelastin fibers, magnification is 1000×, showing the wider fibers at higher delivery rate. (Courtesy of Li *et al.*, 2005; reprinted with permission.)

Synthetic Polymeric Nanofibers

An extremely wide variety of synthetic polymers have been used to successfully synthesize nanofibers that have subsequently been studied for their use an application in numerous therapeutic areas such as those listed in Table 3.1. Much research has been focused on synthetic polymeric nanofibers due to the wide range of choices for base polymers as well
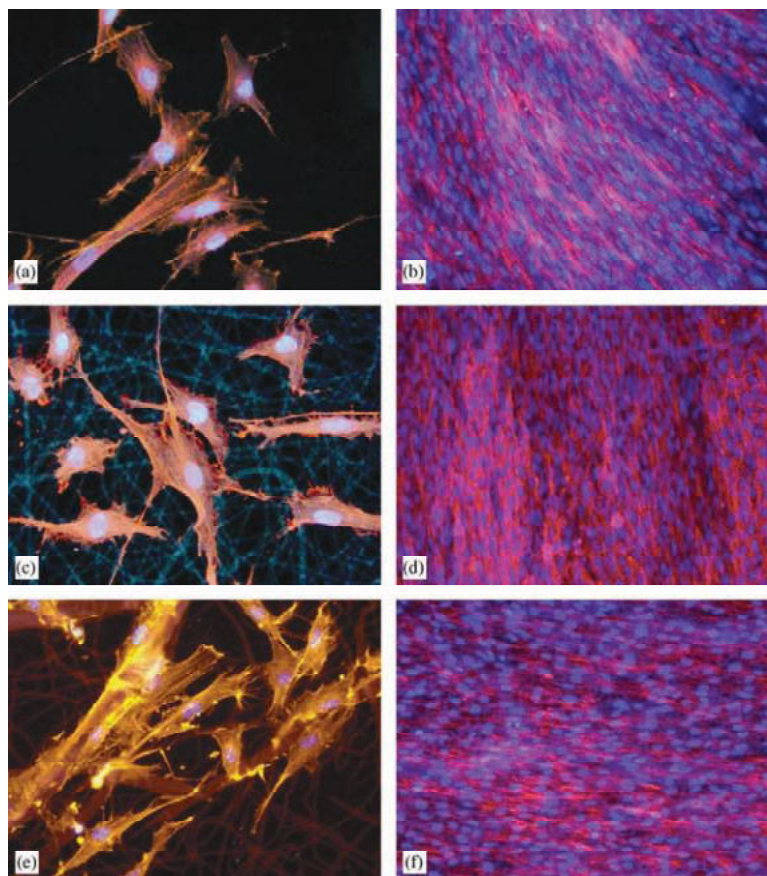
FIGURE 3.8 Morphology of HEPM cells on protein fiber matrices. Staining for nuclei-bisbenzimide (blue), actin cytoskeleton-phalloidin (red), fiber auto-fluorescence. HEPM cells attach, spread and form oriented monolayers on protein fiber matrices with typical fibroblastoid morphology. For details see Methods. (a, b) TCPS; (c, d) Gelatin; (e, f) Elastin; (a, c, e) HEPM after 48 h in culture (original magnification 400×); (b, d, f) HEPM monolayer at confluence after 72 h in culture (original magnification 200×). (Courtesy of Li *et al.*, 2005; reprinted with permission.)
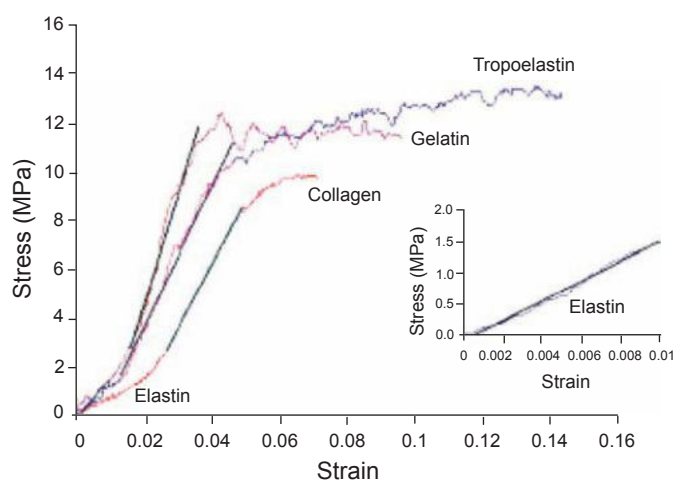
FIGURE 3.9 Microtensile test of electrospun natural polymeric fibers and morphology of HEPM cells on gelatin protein fiber matrices. Compared are tensile strengths of 8% gelatin, 10% collagen, 20% tropoelastin, and 20% elastin fibers. (Courtesy of M. Li *et al.*, 2005; reprinted with permission.)

as the use of the electrospinning process (described below) for efficient production of large quantities of nanofibers.

Poly(lactic-co-glycolic acid) (PLGA)

Poly(lactic-co-glycolic acid) (PLGA) is one of the most commonly used synthetic materials in the fabrication of nanofibers and a great deal

Table 3.1 Synthetic polymer-based nanofibers and their uses	
Polymer Nanofiber	**Use**
PLA	Blood vessel tissue engineering
PET	Blood vessel tissue engineering
PCL	Neural and cartilage tissue engineering
PLLA-CL	Biomimetic extracellular matrix for smooth muscle and endothelial cells
PLGA-PEG	Matrix for DNA delivery
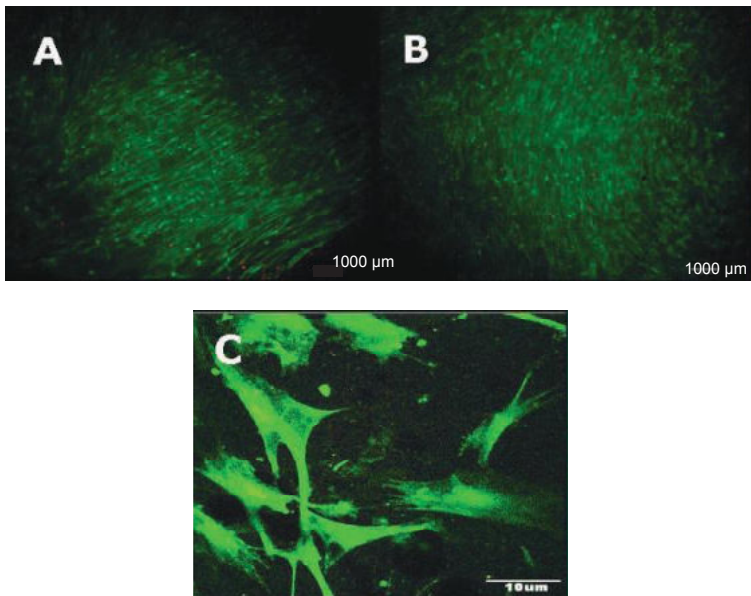
FIGURE 3.10 Fluorescence microscopy of cells grown on a PLAGA nanofiber matrix. Cell survival and morphology of hSF seeded on nonwoven electrospun PLAGA fiber matrices was determined by viability/cytotoxicity assay. Live cells appear as fluorescent green colour and dead cells as fluorescent red. Cell survival was determined on (A) 14 days, (B) 28 days and (C) 14 days at higher magnification. Cells maintained a normal morphology and survived on all the scaffolds. Similar results were also observed on the time points of 7 and 28 days (data not shown). (Courtesy of Kumber *et al.*, 2009; reprinted with permission.)

of research has focused on the use of PLGA-based nanofiber systems therapeutic applications. It is a copolymer that has been used in a variety of FDA-approved therapeutic devices owing to its biodegradability and biocompatibility. Cato Laurencin and colleagues at the University of Virginia developed a bio-resorbable PLGA-based nanofiber system for wound healing and drug delivery. In effect the nanofiber scaffold acts as a biodegradable gauze that also delivers antibiotics to infected areas (Figure 3.10).

Stavros Thomopoulos in the Department of Orthopedics at Washington University in St. Louis, Missouri has focused much of his research on the insertion point of tendon to bone and methods for healing localized injury here. Specifically, his group has engineered a PLGA-based nanofiber mat
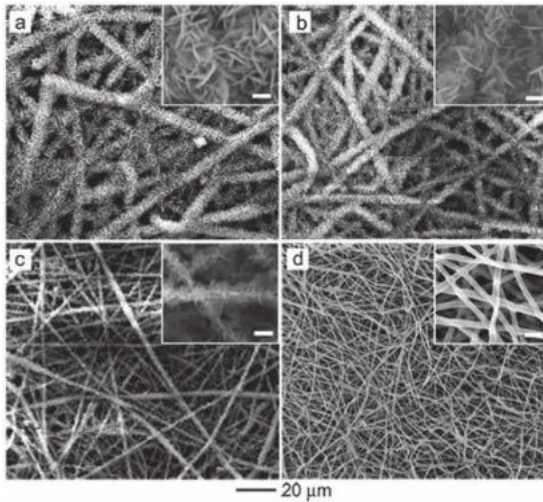
FIGURE 3.11 SEM images of calcium phosphate coatings on a nonwoven mat of PLGA nanofibers. The images were taken from different regions along the scaffold. The scale bars in the insets are 2 μm. (Courtesy of Stavros Thomopoulos, Washington University, St. Louis; reprinted with permission.)

coated with calcium phosphate in a concentration-dependent gradient (Figure 3.11). This was shown to be an effective system in influencing preosteoblast cells to migrate and differentiate in a murine animal model.

Synthetic Non-polymeric Nanofibers

Carbon Nanofibers

Carbon nanofibers are by far the most widely studied non-polymeric nanofiber for use in biomedical implants and, to a lesser degree, in tissue engineering. Carbon nanofibers have also been explored for their use in dental and orthopedic implants given the strength and durability of these materials. Discovered in 1889, **carbon nanofibers** are cylindrical nanostructures with graphene layers arranged as stacked cones, cups or plates (see Focus Box 3.1). The advantage to carbon nanofiber usage for regenerative medicine applications or in implants is the precision with which they are synthesized. They are produced from the catalytic decomposition of hydrocarbon gases or carbon monoxide over selected metal particles. The synthesis procedure most often utilized is catalytic plasma-enhanced chemical-vapor deposition (C-PECVD) (CVD is discussed in Chapter 1

Focus Box 3.1 T.V. Hughes, C.R. Chambers and the discovery of the carbon nanofiber
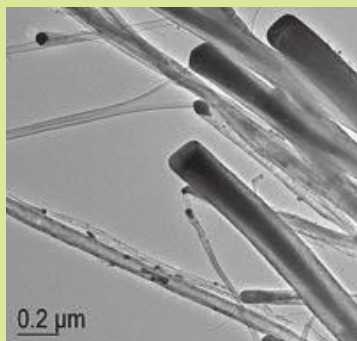
In 1889 scientists Drs. T.V. Hughes and C.R. Chambers were awarded a U.S. patent (#405,480) for a procedure promoting the growth of "carbon filaments," later confirmed as carbon nanofibers, from "swamp gas" (primarily methane) using a metallic crucible to drive the process. Unwittingly, the metal components of the crucible actually catalyzed the reaction! Carbon nanofibers have been used in a variety of industries for such applications as field electron emission sources, composite materials, oil spill remediation and now tissue engineering. (Photo courtesy of Wikipedia.org; reprinted with permission.)

with respect to carbon nanotube synthesis). Thomas Webster and colleagues in the Department of Biomedical Engineering at Brown University compared the functions of osteoblasts on carbon fibers of nano-sized diameters to those with diameters above 100 nm. They demonstrated increased proliferation with decreasing nanofiber diameter suggesting the smaller the fiber the better the scaffold. Brown University researchers have
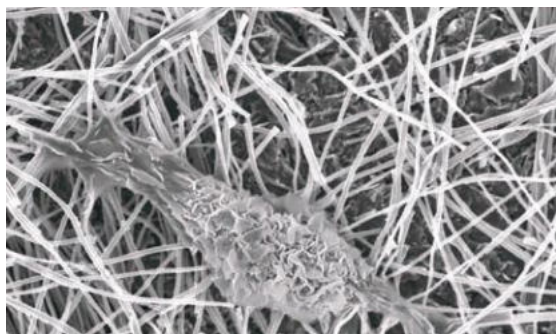
FIGURE 3.12 SEM image of a murine macrophage cultured on an open mesh of carbon nanofibers. (Courtesy of Laboratory for Environmental and Health Nanoscience, Brown University; reprinted with permission.)

also cultured macrophages successfully on carbon nanofiber scaffolds as illustrated in Figure 3.12.

TECHNIQUES FOR THE SYNTHESIS OF NANOFIBERS

A number of methodologies exist for the synthesis of a variety of different nanofiber types, yet with respect to carbon nanofibers many of these involve toxic metal catalysts and chemicals which exclude the final products from use as *in vivo* therapeutics. These will thus not be discussed here but instead focus will be placed on the three most popular nanofiber synthesis technologies which include electrospinning, self-assembly and phase separation.

Electrospinning

Electrospinning, defined as the use of an electrical charge to draw extremely fine fibers from a liquid, is perhaps the method of choice for the synthesis of nanofibers of a variety of different compositions but is certainly favored when using polymeric biomaterials as substrates. The technique offers the opportunity to tightly control the thickness and final composition of the nanofibers. It also affords the ability to control

Focus Box 3.2 William Gilbert and electrospraying

The late William Gilbert (1544–1603) was an English physician and natural philosopher who was regarded by some as the father of electrical engineering and magnetism. In the late 1500s Gilbert observed the ejection of water droplets from a source cone formed when brought into the proximity of an electrically charged piece of amber. This was the first documented case of electrospraying which was later refined into electrospinning, now a widely used technique for the synthesis of a variety of nanofibers. (Photo courtesy of BBC; reprinted with permission.)

porosity size in nanofiber meshes. The concept of electrospinning/ electrospraying has been around since the 1500s when William Gilbert, an English physician and natural philosopher (see Focus Box 3.2), observed a droplet of water form a cone shape when brought near a charged piece of amber, droplets were noted to be ejected from the cone tip. This was the first observation of **electrospraying**, which is the dispersion of a liquid into an aerosol using electricity as the catalyst. Electrospinning is an extension of this process whereby fibers rather than an aerosol are created. In the electrospinning process, characteristics are shared between both electrospraying and more conventional dry fiber spinning techniques. The procedure of electrospinning does not require complex chemical reactions or high temperatures which makes it an ideal methodology for fiber synthesis from a variety of starting materials, most notably biopolymers. The main components of the system are a polymer solution, polymer jet and high voltage supply (Figures 3.13 and 3.14).

The process involves the application of high voltage to a liquid droplet of the desired material, which subsequently becomes charged. The three-dimensional nature of the droplet is stretched due to electrostatic repulsion counteracting surface tension and a stream of liquid emerges
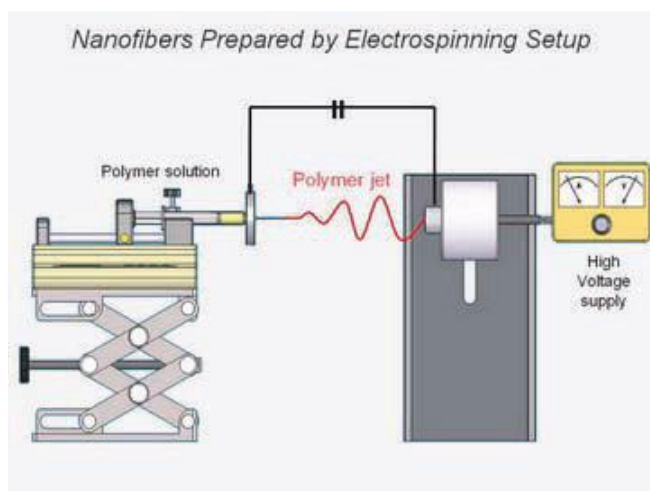
FIGURE 3.13 Diagrammatic illustration of electrospinning apparatus. (Courtesy of Wankei Wan, Western University; reprinted with permission.)
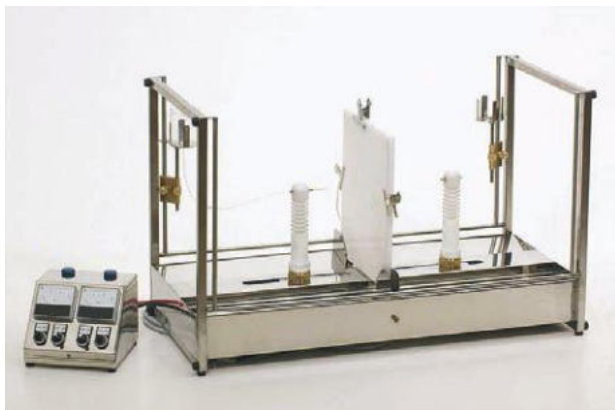
FIGURE 3.14. Electrospinning machine. (Courtesy of New Zealand Institute for Crop and Food Research; reprinted with permission.)

from the droplet surface. The exact point of this emergence is known as the **Taylor cone**. In order for fiber formation to occur, the molecular cohesion of the liquid must be at a critical level or the end result will not be fiber spinning but rather electrospray. If this is the case, a charged polymer jet of liquid is formed which begins to dry out in flight due to elongation and solvent evaporation, resulting in a change of current flow and migration of the charge to the fiber surface. Electrostatic repulsion causes a whipping effect on the fibers which are finally collected on a stationary or rotating grounded metallic collector.

Self-Assembly

Cells interact with their external environment through cell surface receptors that often specifically recognize and bind to extracellular matrix (ECM) proteins such as collagen and fibronectin. These interactions promote migration, cell division and in some cases differentiation to generate mature lineages in a tissue-specific manner. A recapitulation of the ECM environment for tissue engineering purposes is therefore thought to be advantageous for cell function as it pertains to therapeutic applications. While a number of researchers have spent much effort at attempting to recreate the ECM through the manufacture of various matrices often containing ECM-localized proteins, perhaps some of

the most promising advances have come in the form of non-covalently self-assembled peptide-rich nanofibers. **Self-assembly** is defined as the formation of an organized structure or pattern from pre-existing disorganized components as a result of specific local interactions and without external direction. In 1995 Matthew Tirrell and colleagues at the University of Minnesota developed a peptide-amphiphile (PA)-based system that allows for the self-assembly of thermally stable protein-based two- and three-dimensional architectures. An **amphiphile** is defined as a molecule containing a polar water-soluble head attached to a hydrophobic carbon tail and it is the amphiphilic nature of this system that promotes both self-assembly and biological compatibility. The polar peptide heads were derived from known collagen ligand sequences represented as:

Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro

This sequence is quite similar to the human alpha 1 (IV) 1263–1277 collagen ligand sequence. The hydrocarbon tail was originally a dialkyl chain later replaced and elongated by other researchers with a monoalkyl chain which resulted in significantly increased thermal stability. Both versions readily assembled into triple a triple helix three-dimensional formation at the liquid interface of aqueous solvents. These structures were later characterized for promotion of bioactivity and determined to be favorable for such phenomena as cell adhesion, migration and proliferation and it was postulated that this is due to the similarity between the PA structures and the natural ECM.

The preparation of nanofibers is the logical next step to achieve the desired structural dimensions of the system favorable for biocompatibility. This involves the reduction of cysteine residues present within the polar peptide heads to free thiol groups. These groups promote self-assembled cylindrical nanofiber formation below pH 4.0. This was accomplished by Samuel Stupp's group at Northwestern University in a seminal paper on PA-based nanofiber self-assembly. They used the standard reducing agent dithiotheritol (DTT) and followed by acidification to drive nanofiber self-assembly (Figure 3.15). The resulting fibers were roughly 5–8 nm in diameter and up to several microns in length. In addition, mineralization of hyaluronic acid (HA) crystals on the surface of these
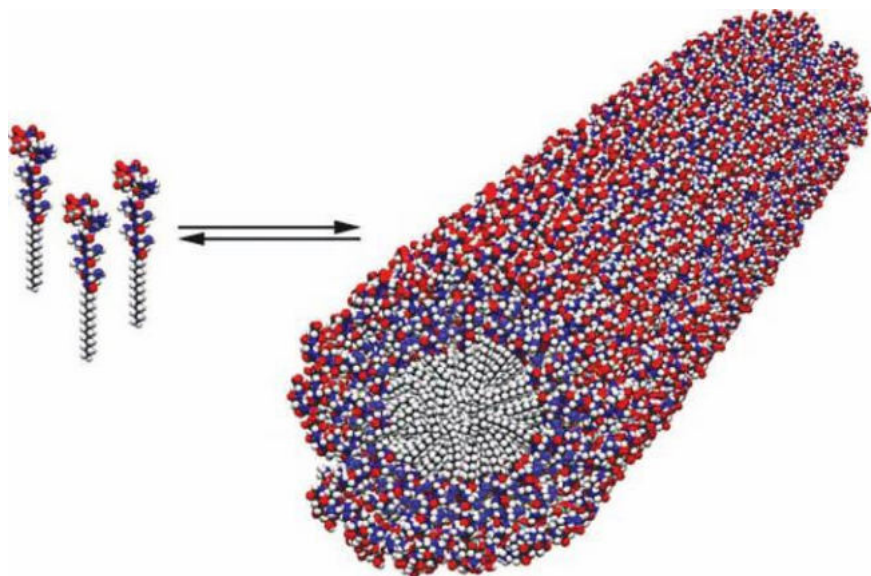
FIGURE 3.15 Diagrammatic illustration of a peptide amphiphile and its self-assembly into nanofibers. (Courtesy of Nanotope, Inc.; reprinted with permission.)

nanofibers was successfully accomplished with structure resembling that of actual bone tissue. Dr. Stupp's group went on to show that PA-based nanofibers coated with the neurite outgrowth-promoting epitope IKVAV resulted in the rapid differentiation of neural progenitor cells into neurons.

Other studies by the Northwestern researchers characterized the correlation between alkyl length and pH sensitivity and its effects on self-assembly. This led to the introduction of three unique methods for the formation of PA-based self-assembled nanofiber platforms: pH-controlled self-assembly, drying on surface self-assembly and divalent-ion-bonded self-assembly. Self-assembly was noted to be reversible and this reversibility was dependent upon pH (for an example see Figure 3.16). Reversible polymerization was also noted to improve nanofiber stability. Thus it is clear that the systems developed over the last 15 years involving self-assembled, reversible, pH dependent formation of nanofiber scaffolds is extremely versatile and has the potential to broadly impact scaffold-based tissue engineering.
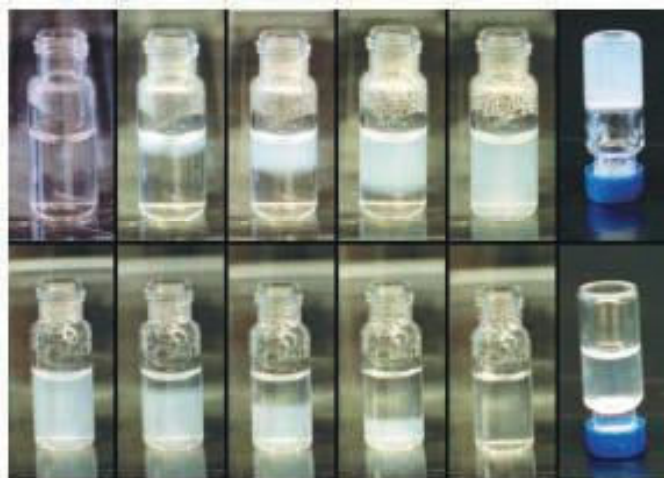
FIGURE 3.16 Time sequence of pH-controlled PA self-assembly and disassembly. (*Upper*) From left to right a peptide amphiphile dissolved in water at pH 8 is exposed to HCl vapor. As the acid diffused into the solution a gel phase is formed, which self-supports upon inversion (*Far Left*). (*Lower*) The same gel is treated with NH_4OH vapor, which increases the pH and disassembles the gel, returning it to a fully dissolved solution. (Courtesy of Hartgerink, *et al.*, 2002; reprinted with permission.)

Phase Separation

Phase separation is a technique by which nanofibrous foams are produced that are very similar in morphology to naturally occurring collagen present within the ECM. As is evidenced by the description below, the advantages of phase separation are its relative simplicity and the lack of equipment needed in comparison to either electrospinning or self-assembly. Developed in 1999 by researchers Peter Ma and Ruiyun Ma at the University of Michigan, thermally induced liquid-liquid phase separation involves five basic steps including:

1. Dissolution of the polymer material
2. Liquid-liquid phase separation
3. Polymer gelatinization (gelation)
4. Solvent extraction from the gel utilizing water
5. Freezing and freeze-drying under sealed vacuum

The **porosity,** ε, a critical parameter of nanofiber composition, is controlled by proper gelation procedures and its timing and duration must be optimized for both temperature and polymer concentration. It can be represented as:

$$\varepsilon = \frac{D_p - D_f}{D_p}$$

where D_f is the density of the fibrous matrix and D_p is the skeletal density of the polymer itself. D_p is calculated for the polymer as:

$$D_p = \frac{1}{\dfrac{1 - X_c}{D_a} + \dfrac{X_c}{D_c}}$$

where X_c is the degree of crystallinity of the polymer; D_a is the density of **amorphous** (lacking definite form) polymer and D_c is the density of 100% crystalline polymer. These formulas allow for a convenient numerical assignment to nanofiber matrix porosity.

The Michigan researchers refined **gelation**, which is defined as solidification by freezing, as well as cooling rates to prevent premature nanofiber network and unwanted platelet-like formations. Polymer concentration also plays a crucial role in general nanofiber properties, with a direct correlation between concentration and mechanical properties and an inverse correlation with porosity. Morphology in this system is primarily influenced by other factors such as the types of polymer and solvent used as well as temperature ranges applied during the process.

Porosity can also be controlled with the addition of porogens such as sugar and salt to create a macroporous three-dimensional scaffold of nanofibrous foam. Thus, in the end product, three levels of architecture exist including macroporosity controlled by porogen addition, interfiber distance controlled by polymer concentration and fiber diameter which is dictated by phase separation (Figure 3.17). If constructed properly nanofibrous foams generated by phase separation have been shown to promote cellular adhesion and protein adsorption, two parameters critical to cell viability and functionality.
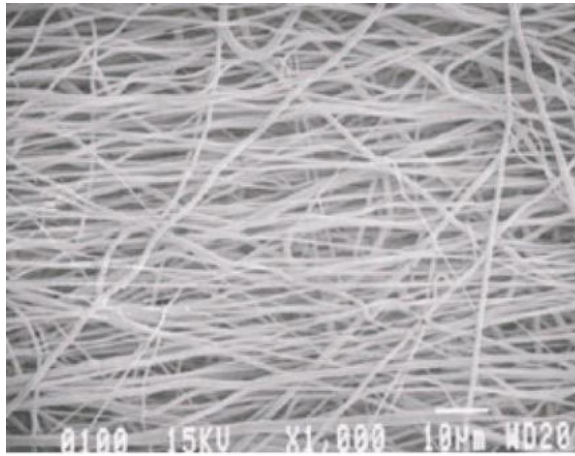
FIGURE 3.17 SEM micrographs of a PLLA fibrous matrix. (Courtesy of Polymer Journal; reprinted with permission.)

NANOFIBER APPLICATIONS IN TISSUE ENGINEERING

Numerous scaffolding systems have been developed and successfully used for a variety of tissue engineering applications, but it is only recently that nanoscale precision has been introduced into this endeavor for the production of nanofiber-based scaffolds that may yield superior performance and long-term integrity of the system. Several more high-profile physiological areas of study are described below. Nanofiber applications for neural tissue engineering has been reserved for a more detailed description in the next chapter.

Bone, Cartilage and Ligaments

The physical properties of nanofibers are crucial in determining their effectiveness as bone and cartilage tissue engineering platforms. Mechanical strength, pore size, porosity and the three-dimensional architecture of the final scaffold must adhere to strict minimum requirements given the stress and strain incurred by the skeletal system. It has been determined by a number of groups that nanofibrous scaffolds with a pore size in the range of 100–350 μm and porosity above 90% yield the most optimal results when assessing tissue growth and ultimately bone regeneration.
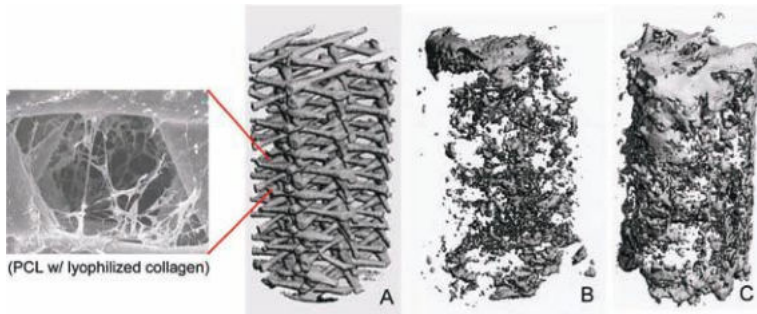
FIGURE 3.18 SEM and computerized images of mineralization. Seeding stem cells within PCL scaffolds coated with lyophilized collagen (image A with inset SEM) leads to new mineral formation by hMSCs (B) and hAFS cells (C) after dynamically culturing for 15 weeks in osteogenic media. (Courtesy of Robert E. Guldberg, Georgia Institute of Technology; reprinted with permission.)

Electrospun PCL-based scaffolds have received much attention in this area over the past decade. The application of these scaffolds in the context of stem cells provides a unique opportunity to create scaffolds containing desired terminally differentiated cell types derived from multipotent or possibly even pluripotent stem cells. For example, studies on mesenchymal stem cells (MSCs) derived from neonatal rat bone marrow have shown that these cells migrate deep within PCL nanoscaffolds and produce various ECM proteins *in vitro*. These studies were expanded in an *in vivo* rat model with the demonstration of both mineralization and type I collagen production (Yoshimoto *et al.*, 2003; Shin *et al.*, 2004). Researchers at Georgia Tech have taken these studies a step further and characterized mineralization of PCL nanoscaffolds by MSCs enhanced by coating with collagen (Figure 3.18).

Other studies have focused on the development and application of biodegradable nanofibrous scaffolds for therapeutic uses in which only a transient scaffold is needed to initiate inherent and autonomous tissue repair. For example, materials scientist Miqin Zhang and colleagues at the University of Washington in Seattle (also see Case Study 3.1) incorporated HA nanofibers into β-tricalcium phosphate (β-TCP) matrices via a combination of both gel casting and polymer sponge techniques. This resulted in increased mechanical strength of the system, as measured by compressive strength when compared to normal bone, yet still allowed for their decay over time. In addition, the researchers found that both the compressive strength and elastic modulus
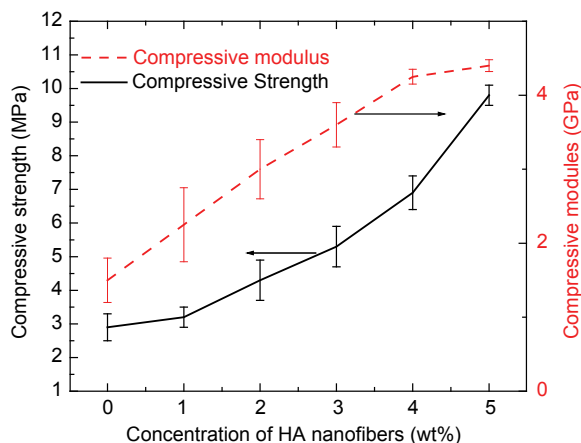
FIGURE 3.19 Compressive strength and modulus of porous nanocomposites scaffolds as function of concentration of HA nanofibers. (Courtesy of Ramay *et al.*, 2004; reprinted with permission.)

of the system increased with increasing concentrations of HA nanofibers (Ramay *et al.*, 2004 and Figure 3.19).

Cartilage is a stiff yet flexible connective tissue found throughout the human body and plays a major role in maintaining joint integrity. **Articular cartilage** is that which covers the surface of bones at the location of the joints. There is no vasculature within proximity of articular cartilage thus limiting access to sufficient numbers of chondrocytes or progenitor cells for repair, thus nanofibrous scaffolds which provide much needed reinforcement and tissue engineering possibilities would be of great advantage here. Shin and colleagues at Inje University in South Korea investigated the potential of a nanofiber-based poly(DL-lactide-co-glycolide) (PLGA) scaffold for cartilage reconstruction purposes. They demonstrated that, in addition to superior physical characteristics, extracellular matrix formation and cell proliferation in these scaffolds were superior to that of more conventional three-dimensional matrices (Shin *et al.*, 2006). Self-assembling peptide hydrogel nano-scaffolds have also been pursued for cartilage tissue engineering purposes. Seeding of the peptide KLDLKLDLKLDL with bovine chondrocytes followed by self-assembly into a hydrogel nano-scaffold yielded promising proliferation, ECM production and phenotype properties of the chondrocytes and it was observed that mechanical properties of the system increased over time
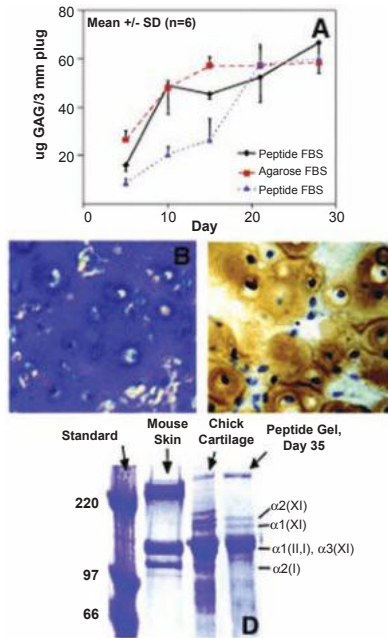
FIGURE 3.20 Matrix accumulation in chondrocyte-seeded peptide hydrogel. (A) Total GAG accumulation in cell-seeded peptide hydrogel cultured in FBS and ITS/FBS medium and in cell-seeded agarose. (B) Toluidine blue staining of chondrocyte-seeded peptide hydrogel, day 15. (C) Immunohistochemical staining for type II collagen in cell-seeded peptide hydrogel, day 15. Image width for *B* and *C* = 175 μm. (D) SDS/PAGE of collagens extracted from day 35 samples of chondrocyte-seeded peptide hydrogel. Standards: Chick cartilage for collagen II and XI banding pattern. Mouse skin identifies collagen I α-helix 2, indicative of collagen expression of a dedifferentiated, fibroblastic phenotype. (Courtesy of Kisiday *et al.*, 2002; reprinted with permission.)

suggesting matrix growth supported by the chondrocytes (Kisiday *et al.*, 2002 and Figure 3.20).

Ligaments are fibrous connective tissue that attaches bones to other bones and are primarily responsible for joint movement and stability. Ligament injuries, such as tears or stretching, are quite serious and difficult to repair or rehabilitate due to their limited tissue regeneration properties similar to that of skeletal muscle. In addition, if left unchecked, ligament injuries can result in the gradual degeneration of surrounding tissues. It is therefore imperative that, in addition to surgical methods, technologies be
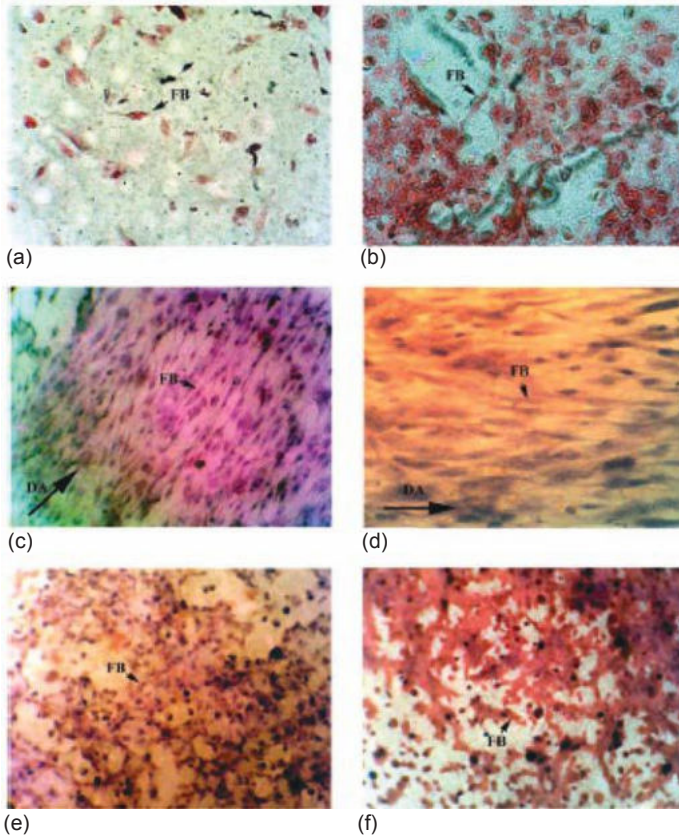
FIGURE 3.21 Histological observations of fibroblasts: on cast PU nanofibers. (a, b), on aligned PU nanofibers (c, d) and on randomly oriented PU nanofibers (e, f) after three and seven days of culture, respectively (FB: fibroblast; DA: direction of alignment). Spindle-shaped fibroblasts along the direction of fibers were observed in (c) and (d). (Courtesy of Lee *et al.*, 2005; reprinted with permission.)

developed for driving and enhancing ligament repair after injury. Aligned nanofibers have been intensely studied in this area. Researchers at Inje University in South Korea used the anterior cruciate ligament (ACL) as a model system and developed aligned PU nanofibers via electrospinning and seeded the scaffold with human ligament fibroblasts from the ACL. They discovered that the fibroblasts aligned themselves with the direction of the nanofibers and significantly more ECM was produced on aligned vs. non-aligned nanofiber scaffolds (Lee *et al.*, 2005 and Figure 3.21).

Skin

Skin is composed of the **dermis**, which is defined as the layer between the **epidermis** and subcutaneous tissue, and the epidermis, or outermost layer that is in contact with the environment. The epidermis consists of stratified squamous epithelium and **keratinocytes**, which serve to protect the body from outside threats. Keratinocytes constitute 95% of the epidermis and also serve to hold other intra-dermal cells such as lymphocytes within the epidermis. Skin injuries or wounds typically heal through the formation of epithelialized scar tissue from the epidermis and not as a result of true tissue regeneration. Said scar tissue, especially when formed in the absence of dermis due to severe burns for example, lacks appropriate strength and elasticity which results in pain and cosmetic issues. The dermis, in contrast, has a considerable capacity to regenerate. Thus the development of a system that allows for external wound or injury repair while simultaneously stimulating dermal tissue growth is at the forefront of research in this area. Christophe Egles and colleagues at Tufts University School of Medicine on Boston have developed a self-assembling peptide-based nanofiber scaffold that is coupled with epidermal growth factor (EGF) to stimulate epithelial growth (Schneider *et al.*, 2008 and Figure 3.22). In a human skin equivalent (HSE) tissue model they observed molecular self-assembly at the wound site to form unique three-dimensional structures and accelerated re-epithelialization in the presence of EGF roughly 5-fold over that in the absence of EGF.

Electrospinning has also been implemented for the development of nonwoven silk fibroin nanofibers for skin tissue engineering. Researchers at the Seoul National University College of Dentistry created electrospun silk fibroin nanofibers with high porosity and high surface area-to-volume ratio, a prerequisite for efficient cellular attachment, migration and growth. When coated with type I collagen, this scaffold was shown to promote keratinocyte and fibroblast adhesion and migration (Min *et al*. 2004). PU nanofiber membranes created by electrospinning methods have also been developed and determined to have superior oxygen permeability and regulated evaporative properties. These membranes thus were demonstrated to allow efflux of fluid from the wound yet prevented dehydration. In addition, the unique ultra-fine porosity of the system prevented foreign invasion and related infection of the wound (Khil *et al.*, 2003).
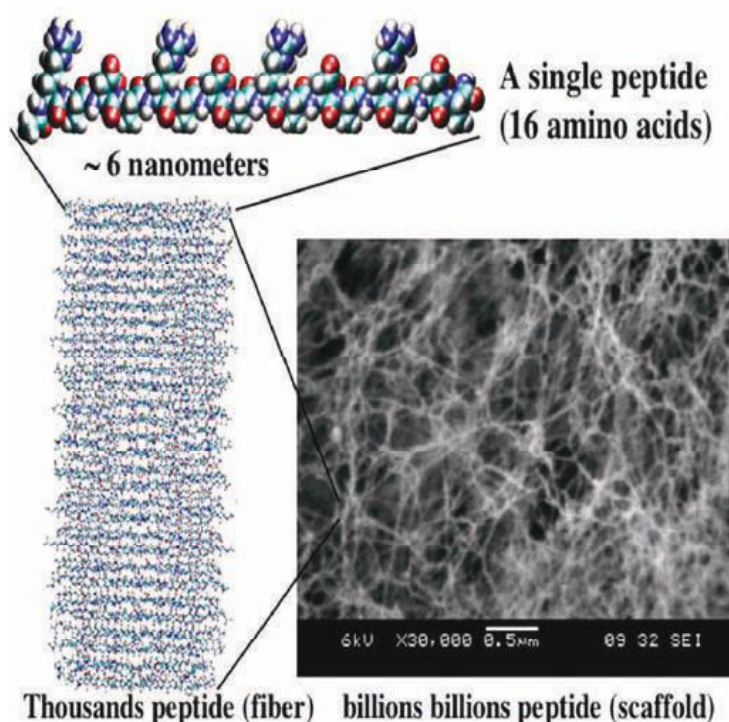
FIGURE 3.22 A designer self-assembling peptide nanofiber scaffold. A single peptide, approximately 6 nanometers, is shown. Thousands of peptides self-assemble to form a single nanofiber, trillions of peptides or billions of nanofibers form the scaffold that contains <99.5% water and 0.5% peptide materials. Positive and negative charges are labeled in blue and in red, respectively. (Courtesy of Schneider *et al.*, 2008; reprinted with permission.)

Vasculature

The **circulatory system** is an organ system designed to deliver nutrients, hormones and gases to cells within the body. In addition, it is crucial for removing waste and stabilizing body temperature as well as pH. It is accepted by most scientists that the circulatory system includes both the cardiovascular system, which distributes blood, and the lymphatic system, which distributes lymph. Damage to the vasculature of either of these systems will in most instances result in serious illness or death. Thus much attention has been focused on tissue regeneration repair technologies such as vascular grafts involving materials that are designed to have minimum impact with blood or lymph flow. Given the obvious advantages of nanoscale
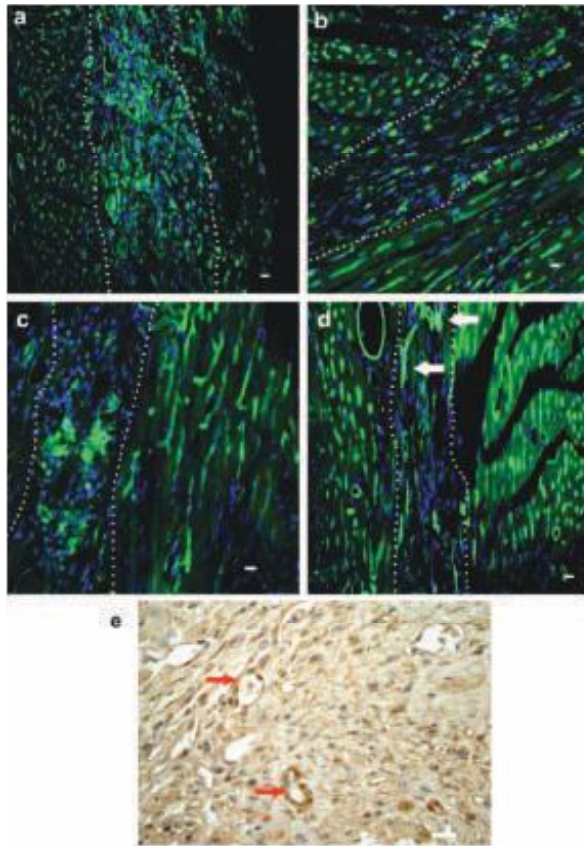
FIGURE 3.23 Endothelial cells spontaneously populate and organize in a cell-free peptide microenvironment. (a) Many cells (nuclei=blue, DAPI) within the peptide microenvironment (dotted lines as determined by light microscopy) stained positively with the endothelial cell marker isolectin-FITC (green) 7 days after injection. (b) Endothelial cells were still present within the microenvironment 14 days after injection and appeared to be elongated in shape. (c) Twenty-one days after injection, the endothelial cells appeared to be clustered within sections of the peptide microenvironment. (d) Distinct capillary-like structures (arrows) were seen within the microenvironment 28 days after injection. (e) Endothelial cell phenotype was confirmed by immunohistochemical staining for PECAM-1 (CD31, brown staining) within the peptide microenvironment at 28 days. Note the vessels within the gel highlighted by arrows that contain red blood cells. Bars represent 20 μm. (Courtesy of Davis *et al.*, 2005; reprinted with permission.)
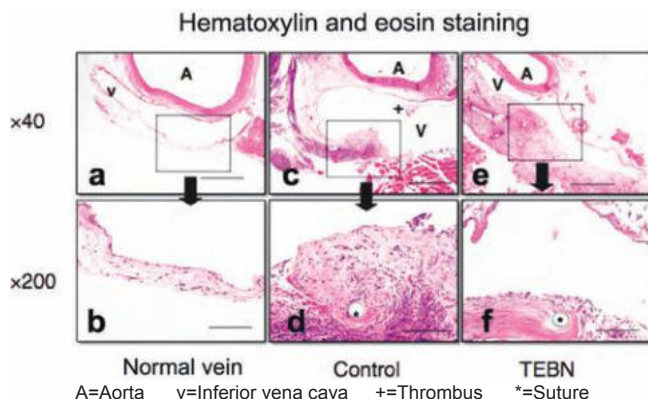
FIGURE 3.24 Histological findings in the anastomotic site at one week after operation. H&E staining of normal group (a) =40 (b) =200, control group (c) =40 (d) =200 and TEBN group (e) =40 (f) =200. The neointimal hyperplasia occurred at around the surgical suture (d). The neointimal hyperplasia is clearly decreased in TEBN group (f). Scale bar in (a), (c) and (e) is 500 mm and in (b), (d) and (f) is 100 mm. (Courtesy of Mutsuga *et al.*, 2009; reprinted with permission.)

materials with respect to porosity, elasticity, flexibility and stability recent efforts in the field have turned toward the use of nanomaterials, specifically nanofibers, for vasculature tissue engineering. Richard Lee and colleagues at Harvard Medical School have designed a self-assembling peptide system that promotes the survival of transplanted endothelial cells and enhances vascularization of the myocardium. They demonstrated that vascular smooth muscle cells are recruited to the microenvironment and appear to form vascular structures (Davis *et al.*, 2005 and Figure 3.23).

Nanofiber technology may also be used to prevent future vasculature collapse or obstruction post-surgery. Researchers in the Department of Cardiac Surgery at Nagoya University in Japan have included drug elution in a biodegradable nanofiber to aid in the prevention of postoperative obstruction of the pulmonary vein. Specifically, the drug Tacrolimus was incorporated into a poly(L-lactide-co-glycolide) biodegradable nanofiber scaffold, referred to as a TEBN, and used to treat a rat model of venous stricture. It was shown that the system reduced hyperplasia and preserved endothelialization (Mutsuga *et al.*, 2009 and Figure 3.24).

Ex vivo applications of nanofibers and nanomaterials have also been explored as matrices for the expansion and differentiation of stem cells into desired populations for cell therapy purposes. **Angiogenesis**
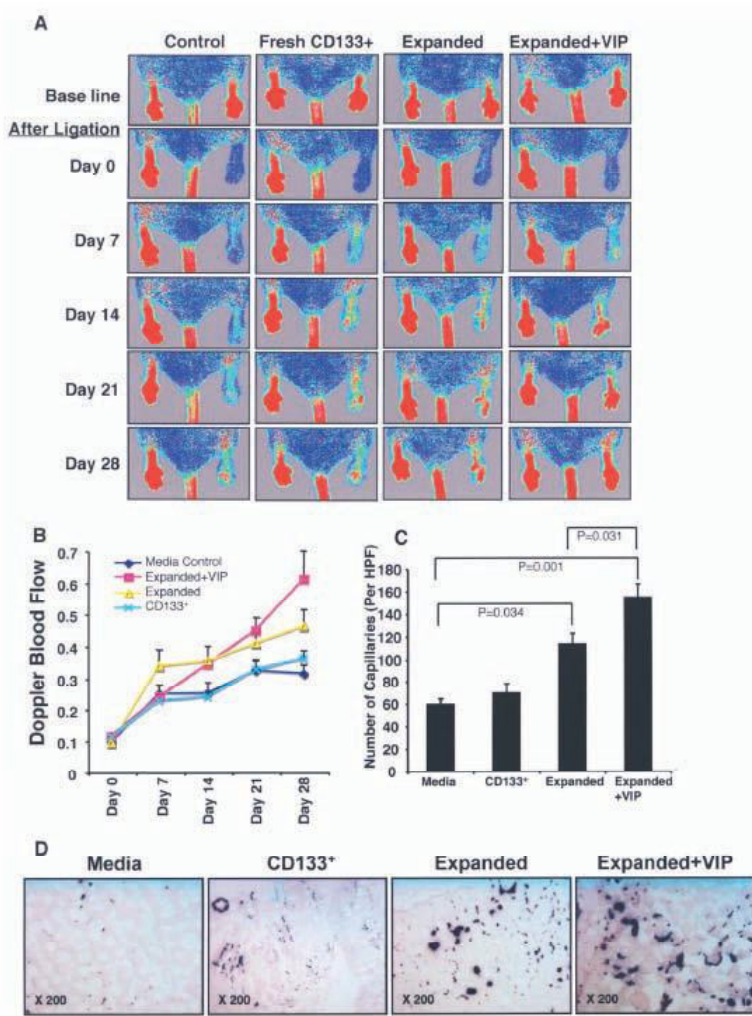
FIGURE 3.25 *In vivo* effect of manipulated stem cells in hind limb ischemic mouse model. Each group of ischemic mice was inoculated with stem cells or media as stated. (A) Doppler images for blood flow were taken for every 7 days, up to 28 days, and representative images are depicted. (B) Total images were analyzed and presented in a graphical format. Paired *t*-tests were performed for statistical evaluation. (C) Four sections of each sample were counted for alkaline phosphatase staining and five high-power fields (HPF) were counted for each section. Paired *t*-tests were performed to evaluate statistical significance. (Courtesy of Das *et al.*, 2009; reprinted with permission.)

is defined as a physiological process involving the growth of new blood vessels from existing ones. Pompili's group at Ohio State University created an *ex vivo* stem cell expansion system that allows for the expansion of human umbilical cord blood (UCB)-derived progenitor cells to enhance their angiogenic potential. The cells were expanded over 225 times in the system and expressed all the appropriate markers. Upon introduction into a mouse hind limb vascular injury model these cells were far more effective than non-nanofiber-cultured cells in blood flow restoration (Das *et al.*, 2009 and Figure 3.25).

NANOFIBER APPLICATIONS IN CONTROLLED DRUG DELIVERY

The controlled timing and release of drugs within a desired physiological setting not only allows for higher efficacy due to decreased saturation of the area but also promotes decreased toxicity through lower overall drug concentrations. Numerous polymeric materials have been used as delivery matrices yet many of these lacked the precision of a nanofiber-based three-dimensional environment that may allow for more controlled release of drugs resulting in yet higher efficacy and lower toxicity. Jo and colleagues in the Department of Pharmaceutics at the University of Mississippi created a self-assembling peptide amphiphile (PA) system comprising a cell-adhesive matrix metalloproteinase (MMP-2) embedded in a gel along with the anti-cancer drug cisplatin. MMP-2 cleaves the peptide GTAGLIGQRGDS. The final structure included self-assembled cisplatin-peptide nanofibers inside the gel and drug release was triggered by MMP-2 activity. Drug release was controlled by the concentration of the enzyme present within the system showing promise for both temporally and spatially controlled targeted cancer drug release (Kim *et al.*, 2009 and Figure 3.26).

Regulated protein release from polymer nanofiber scaffolds has also been studied as many peptides have been shown to have therapeutic effects. Gemeinhart's group in the Department of Biopharmaceutical Sciences at the University of Illinois examined the mechanism behind protein release from electrospun polymer nanofiber mats, specifically those of the model protein compounds bovine serum albumin (BSA) and anti-integrin antibody (AI). They determined that protein release was via **desorption**, defined as substance release from or through a surface. It was also observed
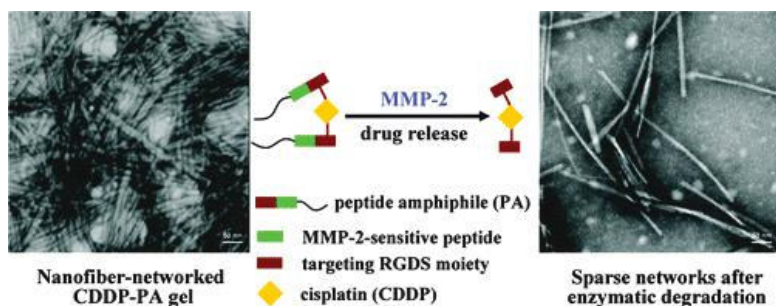
FIGURE 3.26 Enzyme-based controlled cisplatin release from a self-assembled peptide nanofiber scaffold. (Courtesy of Kim *et al.*, 2009; reprinted with permission.)

that the nanofibers did not decay at any appreciable rate (Gandhi *et al.*, 2009 and Figure 3.27).

Finally, biocompatible nanofiber/metal scaffolds have also been explored for controlled drug release capabilities. Researchers at the University of Arkansas prepared a biocompatible nanofiber scaffold on the surface of titanium foil via a one-step hydrothermal reaction. Adjustment of fabrication parameters such as reaction temperature, time and precursor concentration allowed for precise control of nanofiber length and diameter. The nanofibers were demonstrated to self-organize into macroporous scaffolds the pore size of which was ideal for controlled drug delivery via diffusion (Dong *et al.*, 2006 and Figure 3.28).
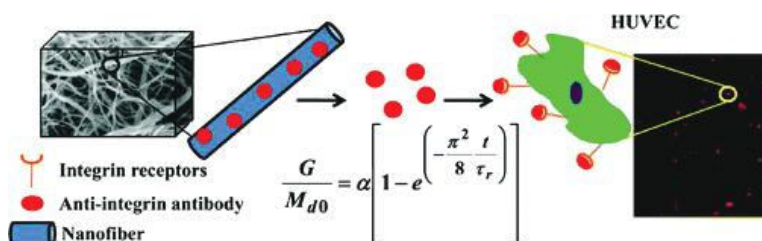
FIGURE 3.27 Flowchart of anti-integrin antibody release and binding to cells. Integrin receptors expressed on the surface of cells bind the anti-integrin antibody presented on the surface of nanofibers. (Courtesy of Gandhi *et al.*, 2009; reprinted with permission.)
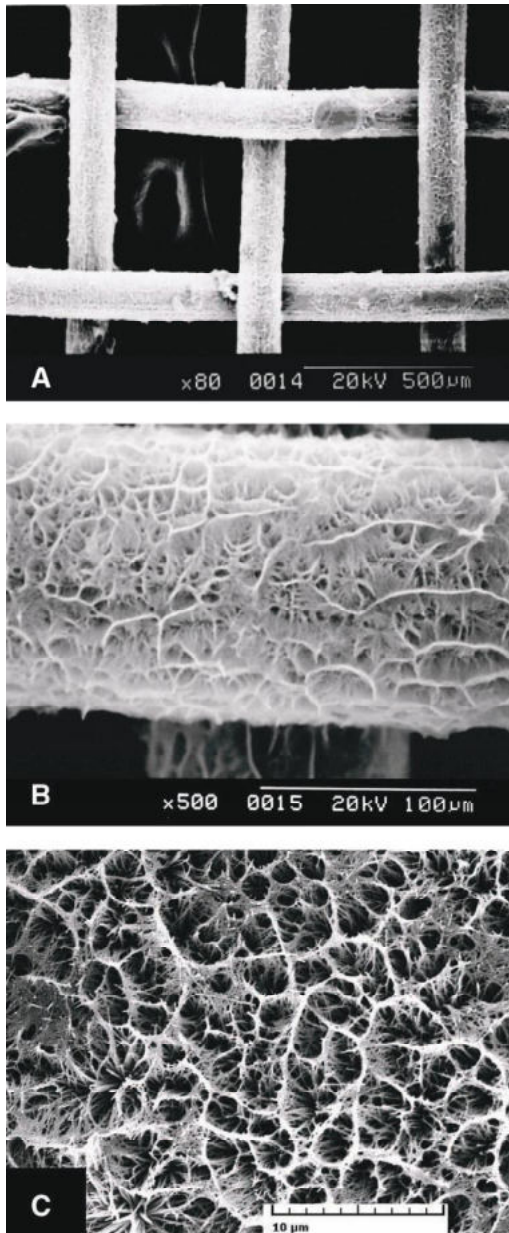
FIGURE 3.28 SEM image of array nanofibers on the Ti mesh. (A) A low magnification SEM photograph of Ti mesh. (B and C) High-magnification SEM photographs showing the 3D porous nanofibers on the Ti mesh. (Courtesy of Dong *et al.*, 2006; reprinted with permission.)

CHAPTER SUMMARY

Composition and Types of Nanofibers

1. Nanofibers are polymeric in origin, have diameters of 100 nm or less, are 50 nm to 100 mm or more in length and can be synthesized from a variety of materials.
2. Natural nanofibers provide the advantage of minimal toxicity and risk for immune rejection.
3. Collagen nanofibers are the most widely studied and have been used for cell culture and tissue engineering applications.
4. Chitosan, derived from the exosketetal protein chitin, has been used to produce nanofibers which promote bone cell growth.
5. Hyaluronic (HA) nanofibers can be produced by electrospinning or electro-blowing and have been demonstrated to enhance chondrocyte adhesion and viability.
6. Gelatin's physical form and mechanical properties are temperature dependent and it has been combined with PCL nanofibers to promote osteoblast growth.
7. Nanofiber blends containing fibroin have been developed and shown to act as excellent scaffolds for the culture of keratinocytes and fibroblasts.
8. Silk fibroin nanofibers may be synthesized by self-assembly.
9. Human tropoelastin protein may be used to create electrospun nanofibers.
10. Both stress and strain are two physical properties that must be taken into account when developing a scaffold for tissue engineering applications.
11. Synthetic polymeric nanofibers are popular for use in various areas of tissue engineering due to the wide range of base polymer choices and electrospinning ease of synthesis.
12. PLGA is a copolymer that has already been used in a variety of therapeutic devices due to its biodegradability and biocompatibility.
13. Carbon nanofibers represent an ideal platform for dental and orthopedic implants given their strength and durability.

Techniques for the Synthesis of Nanofibers

1. Electrospinning is the favored method of synthesis for polymeric nanofiber manufacture as it allows for tight control of final composition properties.
2. Electrospinning does not require complex chemical reactions or high temperatures.
3. Self-assembly is the method of choice for synthesizing nanofibers which recapitulate the ECM.
4. Peptide-amphiphile-based self-assembled nanofibers have been created and shown to promote neurite outgrowth and their self-assembly was shown to be reversible based on pH conditions.
5. Phase separation is a simple nanofiber synthesis process that doesn't require specialized equipment.

Nanofiber Applications in Tissue Engineering

1. There are strict requirements for mechanical strength, pore size, porosity and 3D architecture for nanofiber scaffolds used in tissue engineering.
2. Electrospun PCL-based scaffolds have been created which contain terminally differentiated cell types.
3. Some nanofiber scaffolds are biodegradable and disappear in the body over time.
4. Numerous cell types have been successfully cultured on both PLGA and PU electrospun nanofiber scaffolds.
5. Designer self-assembling nanofiber scaffolds have been used *in vivo* to promote wound healing via accelerated re-epithelialization and enhanced vascularization of the myocardium.

Nanofiber Applications in Controlled Drug Delivery

1. 3D peptide-based self-assembling nanofiber matrices have been used to deliver drugs in a spatially and temporally controlled manner.
2. The phenomenon of desorption has been exploited for the release of protein therapeutics from nanofiber mats.
3. Drug diffusion and release rates can be controlled by optimizing nanofiber scaffold pore size.

KEY TERMS

- Autologous Replacement Therapy
- Nanofiber
- Polymeric Nanofiber
- Collagen
- Chitosan
- Hyaluronic Acid
- Electro-Blowing
- Gelatin
- Colloids
- Fibroin
- Tropoelastin
- Stress
- Strain
- Poly(lactic-co-glycolic acid) (PLGA)
- Carbon Nanofiber
- Electrospinning
- Electrospraying
- Taylor Cone
- Self-Assembly
- Amphiphile
- Phase Separation
- Porosity, ε
- Amorphous
- Gelation
- Articular Cartilage
- Ligament
- Dermis
- Epidermis
- Keratinocyte
- Circulatory System
- Cardiovascular System
- Angiogenesis
- Desorption

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. List at least five types of building blocks for polymeric nanofibers.
2. How did researchers increase the mechanical strength of collagen containing nanofibers?
3. Describe the effect temperature has on gelatin.
4. What is the repetitive unit present in fibroin?
5. How did researchers address immunorejection issues in nanofiber scaffold development?
6. What is the formula for Stress?
7. List three synthetic polymer nanofiber types and describe the unique use of each.

8. What are the advantages of implementing electrospinning methods for nanofiber synthesis?
9. Compare and contrast electrospinning vs. electrospraying.
10. What are the five steps involved in thermally induced liquid-liquid phase separation?
11. Write the equation for porosity, ε.
12. In the system developed by University of Mississippi researchers, how does cisplatin get released from the PA scaffold?

4

Nanotechnology and Neuroscience

Neuroscience, which is defined as the field of study encompassing the various scientific disciplines dealing with the structure, development, function, chemistry, pharmacology, and pathology of the nervous system, is one of the largest areas of focus for clinical intervention to cure, or at the very least manage, neurological-based disorders. Neurological anomalies such as Parkinson's and Alzheimer's disease result from either the direct loss of neurons during neurodegeneration or a disruption in neurotransmitter-based intercellular signaling and communication affecting a person's cognitive and motors skills. The treatment of neurological disorders utilizing nanoparticles and nanomaterials has gained an intense focus over the last several years as doctors and researchers have realized the physical properties of these agents may aid in the prevention of neuronal cell loss or signaling disruption (see Table 4.1). The small size and resiliency of nanoparticles and nanomaterials in a biological setting are just a few of the ideal requirements for

Table 4.1 Theoretical applications of nanomaterials and nanoparticles in major areas of clinical neuroscience

Clinical Manifestation	Therapeutic Strategy	Nanomedical Platform(s)
Blood Brain Barrier Limitations	BBB Crossing Agents	Targeted Nanoparticles, Micelles, Liposomes, Dendrimers, Nanogels, Cells as Nanocarriers
Preventative CNS Care	Neuroprotection	Nanoparticle-based Oxides, Fullerenes

treating disorders of the nervous system. This chapter delineates two major areas of scientific advancement in medically relevant nanotechnology as they pertain to neuroscience, tackling neurodegeneration and crossing the blood-brain barrier.

NANOMATERIAL SCAFFOLDS AND NEUROREGENERATION

Neurodegenerative diseases have wreaked havoc on the human population for thousands of years. They are characterized as disorders in which brain and/or spinal cord cells are lost. Mature neurons do not regenerate, and replenishing them is dependent upon recently discovered neural stem cells. This process is inefficient at best and thus loss of mature neurons can lead to devastating permanent effects on cognition and even basic motor activity. Providing a biologically compatible scaffold or three-dimensional substrate upon which neural stem cells can propagate, migrate and differentiate is a central focus of the clinical neuroscience field. The most widely studied neurodegenerative diseases include:

- Adrenoleukodystrophy (ALD)
- Alcoholism
- Alexander's disease
- Alper's disease
- Alzheimer's disease
- Amyotrophic lateral sclerosis (Lou Gehrig's disease)
- Ataxia telangiectasia

- Batten disease (also known as Spielmeyer-Vogt-Sjögren-Batten disease)
- Bovine spongiform encephalopathy (BSE)
- Canavan disease
- Cockayne syndrome
- Corticobasal degeneration
- Creutzfeldt-Jakob disease
- Familial fatal insomnia
- Frontotemporal lobar degeneration
- Huntington's disease
- HIV-associated dementia
- Kennedy's disease
- Krabbe's disease
- Lewy body dementia
- Neuroborreliosis
- Machado-Joseph disease (Spinocerebellar ataxia type 3)
- MELAS—Mitochondrial Encephalopathy, Lactic Acidosis and Stroke
- Multiple System Atrophy
- Multiple sclerosis
- Narcolepsy
- Niemann Pick disease
- Parkinson's disease
- Pelizaeus-Merzbacher disease
- Pick's disease
- Primary lateral sclerosis
- Prion diseases
- Progressive Supranuclear Palsy
- Refsum's disease
- Sandhoff disease
- Schilder's disease
- Subacute combined degeneration of spinal cord secondary to Pernicious Anaemia
- Spielmeyer-Vogt-Sjögren-Batten disease (also known as Batten disease)
- Spinocerebellar ataxia (multiple types with varying characteristics)
- Spinal muscular atrophy
- Steele-Richardson-Olszewski disease
- Tabes dorsalis
- Toxic encephalopathy

In each of these cases, there exists a clear example of either cellular loss or the loss of proper signaling between neurons, most likely due to a blockage of neurotransmitter function. Parkinson's and Alzheimer's diseases are perhaps the most prominent and widely known of the diseases listed. They are both considered neurodegenerative disorders, yet the clinical manifestations of these diseases occur through different mechanisms. These represent excellent examples of disorders that may be treatable using nanotechnology-based approaches and thus warrant further consideration in detail.

Parkinson's Disease (PD) is a neurodegenerative disorder of the central nervous system that often impairs the individual's motor skills and speech. It is classified as a movement disorder and is characterized by tremors, **bradykinesia**, which is a slowing of physical movement, and widespread rigidity of the muscles. PD results from a loss of dopamine synthesis and related function, which is produced by the dopaminergic neurons of the brain. The causes of PD may be intrinsic, i.e. genetic, or extrinsic, i.e., exposure to a particular toxin or head trauma, but for the vast majority of individuals affected it is **idiopathic**, having no known cause. In any case it is the inherent loss of dopamine signaling that drives the physiological abnormalities and progression of the disease.

Alzheimer's Disease (AD) is currently known as the most common form of dementia and is to date incurable and terminal. Common symptoms include memory loss, confusion, irritability/aggression, mood swings and language breakdown. The pathophysiology of AD centers on the formation of extracellular plaques and tangles within the brain which are associated with neurodegeneration as well as an abundance of microglia and astrocytes. **Microglia** are resident macrophages of the brain and spinal cord and act as the first defense against foreign materials. **Astrocytes** are star-shaped glial cells that perform many functions including metabolic support of the endothelial cells lining the blood-brain barrier and providing nutrients to CNS tissue. The plaques themselves vary in both shape and size, averaging about 50 μm in diameter. They are primarily composed of beta-amyloid protein (Ab), which is a peptide of 39–43 amino acids cleaved from the amyloid precursor protein (APP). Mutations in the gene coding for APP have been associated with early onset AD and suggest abnormal cleavage drives Ab accumulation and plaque or tangle formation. The two-molecule tertiary form of the protein is thought to be the actual causative agent driving the progression of AD and is toxic to neuronal **synapses**,

which are junctions through which neurons signal to each other and to non-neuronal cells.

PD and AD share common characteristics with other neurodegenerative diseases, perhaps the most important of which is that the possible partial or full restoration of the neuronal signaling mechanisms crucial to proper functioning of the central nervous system could alleviate or at least manage the suffering involved. These and other neurodegenerative diseases could therefore be addressed with neuroregenerative therapeutic platforms. Indeed, this has been a major focus of neuroscientists and clinical neurologists for at least the past twenty years. Technologies that support the neurite and axonal (projections from the cell body of a neuron) growth may drive increased neuronal signaling and mitigate the overt symptoms of neurodegenerative disorders. Only recently has nanotechnology entered the scene as a basis behind neuroregeneration efforts. Nanometer-scale three-dimensional scaffolds (see also Chapter 3), for example, may provide a microenvironment conducive to neuronal survive and synapse formation. These materials often mimic some of the tubular structures found in nature such as rod-shaped bacteria or viruses, microtubules, axons and dendrites. In some cases they also can be custom synthesized to meet desired length-to-volume ratios and therefore be manufactured to precisely resemble structures in a neuronal microenvironment. Whether these materials would be used *in vivo* or as cell culture platforms outside of the body remains to be determined. Some promising materials studied as scaffold components include nanotubes and nanofibers and are discussed below.

Carbon Nanotube-Based Neuronal Matrices

Carbon nanotubes have long been thought to be an excellent source material for the development of neuronal matrices due to their electrical conductivity properties (see Chapter 1). It has been postulated by a number of groups that the passage of an electric current through a CNT-based matrix might stimulate neuronal signaling. Laura Ballerini's lab at the Center for Neuroscience at the University of Trieste in Italy developed a carbon nanotube grid that was demonstrated to promote both neuronal outgrowth formation and signaling. Specifically, dendrite elongation and cellular adhesion were supported along with a significant increase in network activity compared to controls (Lovat *et al.*, 2005 and Figure 4.1).
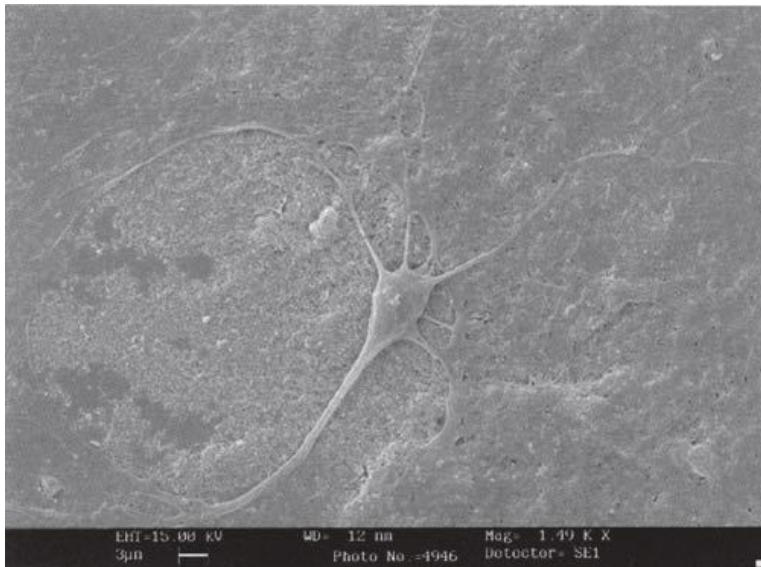
FIGURE 4.1 Neuron cultured on a carbon nanotube grid. (Courtesy of Lovat *et al.*, 2005; reprinted with permission.)

Ballerini's group also showed that carbon nanotube matrices improve the responsiveness of neurons, possibly by forming tight contacts with the cell membranes that favor electrical shortcuts between compartments of the neuron (Cellot *et al.*, 2009). Nicholas A. Kotov and colleagues in the Department of Chemical Engineering at the University of Michigan demonstrated the electrical stimulation of neuronal signaling utilizing a CNT-based matrix humanized with extracellular matrix protein. In this study, the researchers generated a laminin and single-walled carbon nanotube-based matrix as a thin film. Neural stem cells cultured on this film were shown to differentiate effectively into neurons and could be excited electrically to signal one another (Kam *et al.*, 2009 and Figure 4.2).

Vladimir Parpura's team at the Center for Nanoscale Science and Engineering, University of California-Riverside generated a carbon nanotube-based neuronal matrix that could be customized for systemic variation of matrix chemical properties through the attachment of different functional groups. This allowed for the manipulation of matrix charge and yielded the ability to control the outgrowth and branching of neuronal processes (Hu, *et al.*, 2004 and Figure 4.3).
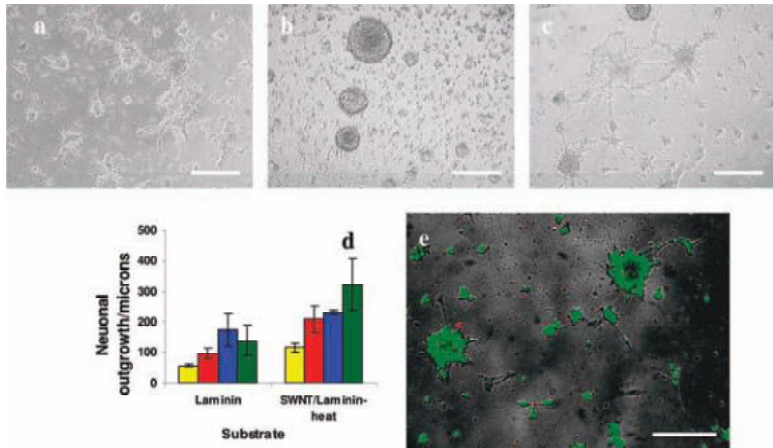
FIGURE 4.2 Micrograph assessing NSC cell adhesion and differentiation. 72 h after initial seeding on (a) laminin-coated glass slides and on 10 bilayers SWNT/laminin thin films that were (b) used as is or (c) heated at 300 °C for 10 min. (d) Distance of outgrowth from neurospheres after 24 h (yellow), 48 h (red), 72 h (blue), and 120 h (green) on laminin-coated slides and heat-treated SWNT/laminin film on slide. (e) Live-dead viability assay on seeded cells where live cells are stained green and dead cells are red. Scale bars are 200 μm. (Courtesy of Kam *et al.*, 2009; reprinted with permission.)
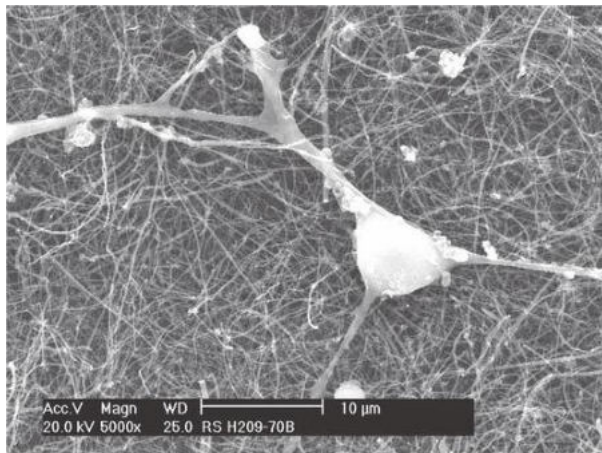
FIGURE 4.3 Scanning electron micrograph of a neuron cultured on a carbon nanotube matrix supplemented with specific functional groups to control outgrowth and branching. (Courtesy of Hu *et al.*, 2004; reprinted with permission.)

PC12 cells are neuronal precursor cells derived from a tumor originating in the rat adrenal medulla. When treated with nerve growth factor these cells readily differentiate into mature neurons. Barbara Nguyen-Vu and colleagues at Stanford University and NASA's Ames Research Center developed soft, free-standing vertically aligned carbon nanofiber (VACNF) arrays and demonstrated that they could be used as a multifunctional 3-D brush-like nanoengineered matrix. The carbon nanotubes were shown to bend in the presence of cells and speculated to alleviate micron-scale local mechanical cellular stress. In addition, the researchers postulated that the individual CNFs could act as probes to monitor cell mechanics as each is capable of responding to local forces. This makes it possible to characterize the local mechanical stress of specific regions of the cell body. The primary output of these probes can be measured as the **bending force, F**, which is defined as the force exerted upon a carbon nanofiber by interactions with a cell to result in its physical alteration. It is represented by the following equation:

$$F = \left(\frac{3EI}{L^3} \right) \delta$$

where E is Young's Modulus, I is the Moment of Inertia, L is the length of the CNF and δ is the lateral deflection of the tip from its original position. The Moment of Inertia, I is given by:

$$I = \pi(D_o^4 - D_i^4)/64$$

where D_o is the outer diameter and D_i is the inner diameter of the CNF. PC12 neurons were shown to form highly intricate neuronal networks in the VACNF arrays interspersed within the arrays and could be stimulated to signal electrically. Thus preliminary *in vitro* studies on carbon nanotube-based neuronal matrices are encouraging and it will be interesting to see what scientific results yield from CNT matrices implanted within the CNS *in vivo*.

The same researchers took these studies a step further and utilized VACNF arrays for the electrical stimulation of rat hippocampal brain slices. They demonstrated both excitatory post-synaptic potentials and somatic action potentials using this system and cited advantages over other stimulatory platforms which include lower electrode impedance, ability to

stimulate tissue through a biocompatible chloride flux and stable vertical alignment in liquid thus enabling access to spatially confined regions of neuronal cells.

Neuronal Nanotube Matrices from Other Materials

While carbon-based nanotubes are the most widely studied type of nanotube with respect to the development and application of neuronal cell culture matrices, other materials have also been described from which nanotubes can be produced and possibly used in neuronal cell culture. This includes DNA, proteins, synthetic polymers, glass and silicon. Techniques that drive nanotube formation from these materials include templating on porous filters such as alumina or silica, templating on electrospun nanofibers made of degradable polymers or the application of self-assembly as described above. These techniques yield the opportunity to synthesize a variety of nanotube designs from different starting materials. Thus the material identity and the final three-dimensional design can be tailored to meet the cell culture requirements of the particular cell type being studied. This is exemplified by David Martin and colleagues in the Regenerative Medicine Sciences Program at the University of Michigan, who generated a PEDOT nanotube/living neural tissue complex through direct polymerization which resulted in an electrically conductive network integrated within the living tissue. Electrical activity of the tissue was enhanced in this system. It should be noted, however, that **apoptosis**, or programmed cell death, of the cells increased in the presence of the polymer (Richardson-Burns *et al.*, 2007 and Figure 4.4). As the fabrication and study of non-carbon-based nanotube neuronal matrices matures it is clear that other platforms will make a significant impact on neuronal cell culture and regeneration.

NANOMATERIALS FOR CROSSING THE BLOOD/BRAIN BARRIER

The **blood-brain barrier (BBB)** is defined as a layer of tightly packed cells that make up the walls of brain capillaries and prevent substances in the blood from diffusing freely into the brain (Figure 4.5). It is the most restrictive barrier in the body and while it provides a critical neuroprotective property for the brain against foreign substance entry, it also creates a
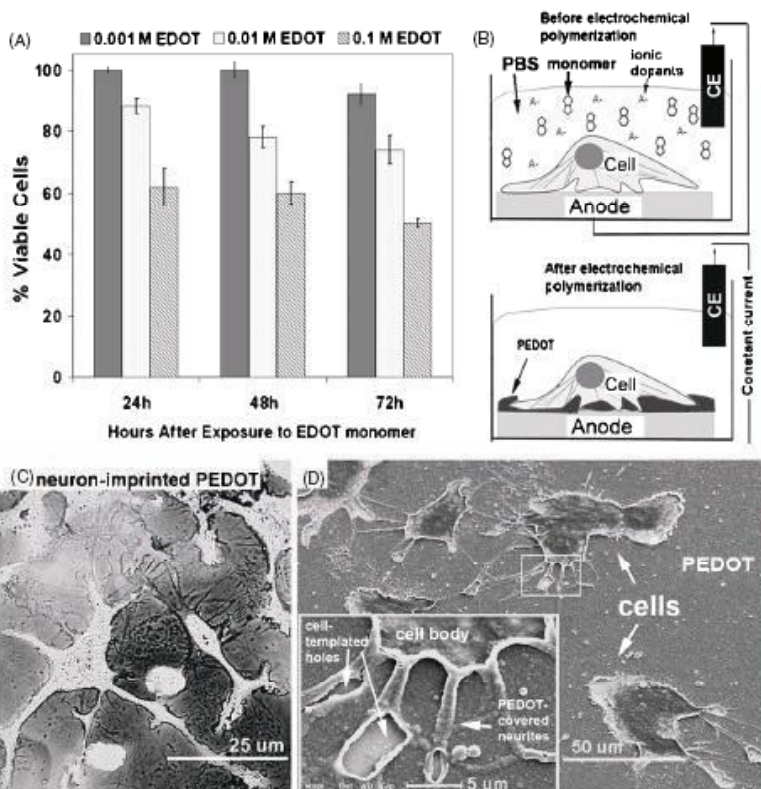
FIGURE 4.4 PEDOT can be polymerized in the presence of living neural cells resulting in a cell-templated, biomimetic conducting polymer matrix. (A) Time course of cell viability following exposure of SY5Y neural cells to increasing concentrations of EDOT monomer solution (0.001–0.1 M EDOT with 0.02 M PSS in PBS). Cell viability was determined by MTT assay using three independent cell samples for each time point and each concentration. (B) Diagram representing the process of electrochemical polymerization in the presence of living cells cultured on an electrode substrate. (C) Optical micrograph of PEDOT (dark substance) polymerized around live cells of mouse dissociated cortical neuronal cultures. The PEDOT intimately contacts the cell membranes revealing neurites, filopodia and nanoscale cellular processes. (D) Scanning electron micrograph (SEM) of PEDOT polymerized around neural cells reveals cell-shaped holes in the PEDOT matrix as well as cell-templated caves, tunnels and troughs. The nano and microscale fuzziness and roughness that is characteristic of PEDOT topology is also revealed by these SEM images. All cells were formaldehyde fixed prior to imaging and for SEM cells were also dehydrated with hexmethyldisilazane (HMDS). (Courtesy of Richardson-Burns *et al.*, 2007; reprinted with permission.)
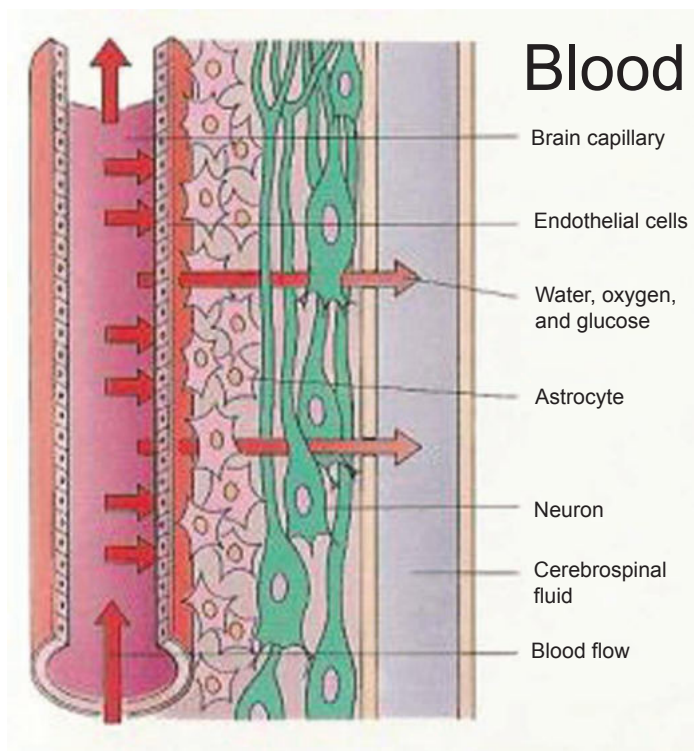
FIGURE 4.5 Schematic of the blood-brain barrier. (Courtesy of Gordon D. McHendry, Satori-5; reprinted with permission.)

problem for drug developers seeking to design brain-targeted therapeutics that can penetrate this barrier effectively.

The BBB prevents many small molecules and almost all macromolecules from entering the central nervous system and a critical component of it is **P-glycoprotein (P-gp)**, a membrane-associated protein expressed in the capillary endothelial cells. P-gp's role is to act as an efflux pump to prevent entry of foreign substances into the brain cavity and has been quite a "barrier," so to speak, for CNS drug developers. Current methodologies for drug delivery to the brain include temporary disruption of the BBB, invasive disrupture and the use of drug delivery vehicles specifically designed to cross into the brain cavity. It is the latter method, using drug delivery vehicles along vascular routes, that has been of much focus over the last ten years, especially with respect to

the development of novel nanomedicines. This is due to the fact that it is less invasive and potentially safer than the alternatives. Below some of the more high-profile nanotechnology-based platforms for drug delivery across the blood-brain barrier are discussed.

Micelles

As described in Chapter 1, micelles are a detergent-like substance that spontaneously forms spherical clusters. Researchers at the Institute of Bioengineering and Nanotechnology in Singapore have developed a micellular complex of core-shell nanoparticles formed by self-assembly of an amphiphilic peptide exhibiting strong antimicrobial properties against a wide range of bacteria, yeasts and fungi. These complexes were demonstrated to cross the BBB and suppress bacterial growth in the infected brains of rabbits.

Pluronic P85 is a di-functional block copolymer surfactant terminating in primary hydroxyl groups. It is a nonionic surfactant that is 100% active and has been shown to be relatively non-toxic (Figure 4.6). Pluronic block copolymers are under intense scrutiny for their potential to enhance peptide therapeutic delivery and efficacy. In general they have two hydrophilic PEG and one hydrophobic PPG blocks and have been shown to bind with the membranes of brain microvessel endothelial cells and to inhibit P-glycoprotein. Researchers at the University of Arizona have developed opioid peptide-containing micelles of P85 to enhance
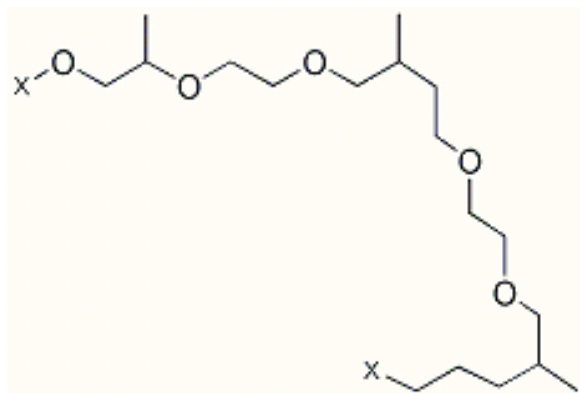
FIGURE 4.6 Pluronic P85 molecular structure. See text for details. (Courtesy of ChemicalBook.com; reprinted with permission.).

Focus Box 4.1 James William McBain and the discovery of micelles
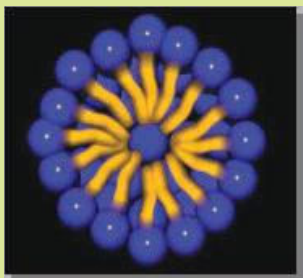
The discovery of micelles can be largely credited to the late Canadian colloidal chemist Dr. James William McBain (1882–1953). As early as 1913 at the University of Bristol in the UK he postulated that the high electrical conductivity of aqueous soaps such as sodium palmitate solutions could only be attributable to the existence of "colloidal ions," now known as micelles. In addition, he developed methods to lower the dew point of soap solutions in order to characterize their thermodynamic properties. He won the prestigious Royal Society of London's Davy Award for this research and discovery. (Photo courtesy of Envirosan Products; reprinted with permission.)

peptide **analgesia**, which is defined as a deadening or absence of the sense of pain without loss of consciousness. They showed a P85-specific inhibition of P-gp and enhanced cellular uptake of the opioids as well as morphine which resulted in significantly increased analgesia (Witt *et al.*, 2002).

Polyion complex (PIC) micelles allow for the incorporation of charged particles within their internal cores. They are formed through the reaction of hydrophilic block copolymers containing both ionic and non-ionic blocks with macromolecules of opposite charge like DNA or proteins. The final complex consists of a core containing polyion complexes of macromolecules and a copolymer ionic block surrounded by a nonionic shell. A number of groups have sought to exploit these properties for the *in vivo* delivery of charged macromolecules. At the National Cardiovascular Research Institute in Osaka, Japan, scientists developed polyion complex micelles containing plasmid DNA by self-assembly, yielding spherical nanoparticles with DNA in various forms such as supercoiled, circular or linear. Intravenous delivery revealed that incorporated DNA is able to persist as an intact molecule in the blood and could be delivered effectively to the liver as assayed by a fluorescent reporter gene (Harada-Shiba *et al.*, 2002 and Figure 4.7).
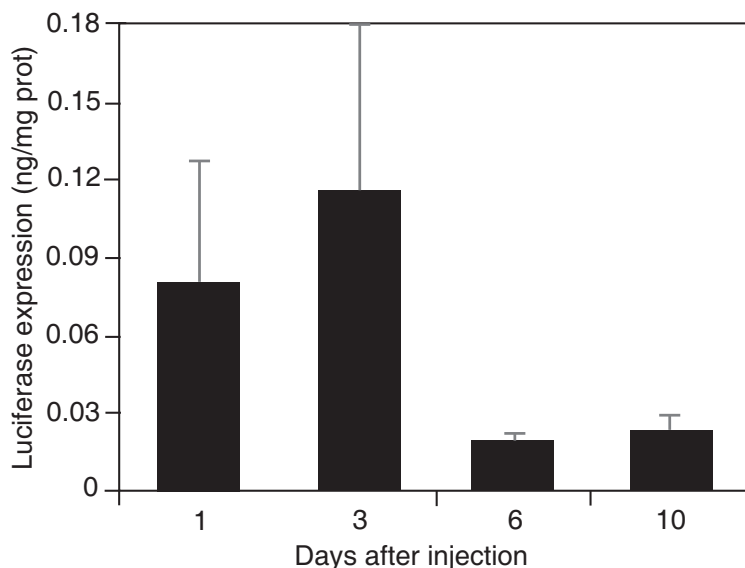
FIGURE 4.7. Time-dependent changes of gene expression in the liver after supramesenteric injection. PIC micelles prepared by mixing 50 µg of pGL3-control plasmid and PEG-PLL block copolymer at the charge ratio of 1:4 were injected. After the indicated time, the liver was homogenized and analyzed for luciferase activity. (Harada-Shiba *et al.*, 2002; reprinted with permission.)

While the BBB has yet to be addressed by polyion complex micellular platforms, their high loading capacity and the potential to release payload upon changes in environmental conditions puts them at the forefront of BBB drug delivery research.

Liposomes

Liposomes are one of the earliest nanomaterials developed for drug delivery purposes (defined in Chapter 1 and for a detailed discussion of drug delivery see Chapter 7). They are vesicles composed of lipid bilayers which protect an internal aqueous compartment that lends itself quite well as a hydrophilic drug-storage section. Lipophilic drugs may also be stored between the lipid bilayers and both can be released upon breakdown of the liposome. The **reticuloendothelial system (RES)**, which is a component of the immune system including monocytes and macrophages, is capable of clearing conventionally designed liposomes

from the circulation, yet decreasing liposomal size below 100 nm and coating them with polyethylene glycol (PEG) may extend circulation time. As of the writing of this book over 200 publications existed on the use of liposomes to target or cross the blood brain barrier for drug delivery purposes (see Case Study 4.1 for an example).

While Pardridge's group has chosen to focus primarily on the use of liposomes to deliver DNA-based constructs across the BBB, others have focused on drug delivery. Ulrich Bogdahn and colleagues in the Department of Neurology at the University of Regensburg in Germany have demonstrated the effectiveness of doxorubicin-containing pegylated liposomes for delivery of the drug across the BBB in glioma patients. **Doxorubicin** is a drug used in cancer therapy and works by intercalating within the DNA of cancer cells to prevent their survival through inhibition of macromolecular biosynthesis. It is particularly useful for the treatment of malignant **gliomas**, which are tumors in the brain or spine that arise from glial cells dividing out of control. In these studies, the authors noted long-term stabilization of glioma-based cancers in patients after treatment with liposomal doxorubicin, which has been used to treat other cancers unrelated to those of the brain such as Kaposi's sarcoma (Fabel *et al.*, 2001). Krys Bankiewicz and colleagues at the Brain Tumor Research Center at the University of California- San Francisco have been studying the coupling of liposome-mediated drug delivery across the BBB of rats with **convection-enhanced delivery (CED)**, which is the continuous injection under positive pressure of a fluid containing a therapeutic agent. CED was introduced by researchers from the U.S. National Institutes of Health in the early 1990s as a technique for the delivery of drugs that would otherwise not cross the blood-brain barrier by simple force of pressure. Using gadolinium- and fluorescence-based markers embedded within liposomes, they demonstrated magnetic resonance imaging (MRI)-based detection of marker delivery to sites of brain tumorigenesis. This was coupled with effective delivery of the drug Doxil to these same sites suggesting both diagnostic and therapeutic platforms integrated into one liposomal delivery platform.

Dendrimers

As discussed in Chapter 1, dendrimers are synthetic polymers exhibiting branched-like configurations that achieve structural perfection. They

Case Study 4.1: Noninvasive gene targeting to the brain

William Pardridge at the University of California in Los Angeles has played a critical role in the advancement of brain targeted gene therapy across the BBB via the use of liposomes. In this study, his group describes the efficient delivery of genetic constructs across the BBB of rats and throughout the tissues of the brain via non-invasive introduction of novel pegylated liposome plasmid carriers. Immuno-liposomes carrying a 7 kilobase expression vector coding for either luciferase or β-galactosidase were conjugated to a monoclonal antibody, OX26, which specifically binds to the BBB-expressed transferring receptor. As a neutral liposome (non-cationic), the vesicles were stable in the circulatory system. Brain-specific expression of the plasmid marker gene luciferase was observed to peak at roughly 48 hours post i.v. injection. β-galactosidase staining was further utilized to reveal in detail transgene activity throughout the brain including neurons, choroid plexus epithelium and brain microvasculature. These studies demonstrate the feasibility of using pegylated immunoliposomes to deliver gene constructs in plasmid form efficiently across the BBB via targeting strategies and open the door to potential gene therapeutic applications across the BBB (Shi *et al.*, 2000 and Figure 4.8).
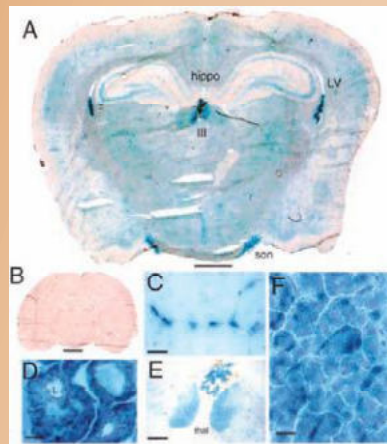
FIGURE 4.8 β-galactosidase histochemistry in brain and liver after liposome administration. (A–E) brain and (F) liver at 48 h after i.v. injection of the β-galactosidase gene packaged inside the OX26 pegylated immunoliposome (B–F). (B) The control brain. Magnification bars 5 1.5 mm (A), 2.2 mm (B), 57 mm (C), 23 mm (D), 230 mm (E), and 15 mm (F). (C) Punctate gene expression in intraparenchymal capillaries. (D) Gene expression in the epithelium of the choroid plexus. (E) The thalamic (thal) nuclei below the choroid plexus of the third ventricle, which is also visible in A. (F) Abundant gene expression in hepatocytes and localization of the β-galactosidase enzyme within the liver cell endoplasmic reticulum. (Shi *et al.* 2000; reprinted with permission.)

contain three specific organizational domains including: 1. The internal core to which branches are attached, 2. The outer shell of the branches surrounding this internal core and 3. The surface formed by branch termini which is **multi-valent**, having several sites of attachment, in nature. Dendrimers are typically smaller than a great deal of other nanomaterial types, especially with respect to those described in this section, ranging on average from 1.5 nm to 14.5 nm in diameter, and thus may make an ideal platform for crossing the BBB. It is during dendrimer synthesis that various solutes, such as those containing therapeutics, can be trapped within the interior cavities of the dendrimer. Degradation of the dendrimer and drug release depends upon the density of the dendrimer shell, and in some instances the density can be such that diffusion of solutes is inhibited even after harsh treatments such as extraction, heating or even sonication. Thus the synthesis of dendrimer-drug complexes must be tailored to meet desired diffusion requirements. Cationic dendrimers, for example, have been shown to exhibit toxicity and disrupt the tight junctions between cells. It was observed that these effects tended to increase directly with dendrimer generation and correspondence surface area. Dendrimer toxicity could be decreased with surface modification such as the addition of carboxylic groups, yet tight junctions were still disrupted with these modified dendrimer compositions (Kitchens *et al.*, 2005). Hemant Sarin and colleagues at the National Institute of Biomedical Imaging and Bioengineering in Bethesda, Maryland demonstrated the effective transvascular delivery of chemotherapeutic agent-containing dendrimers across the BBB and into gliomas. The researchers utilized polyamidoamine (PAMAM) dendrimers due to their small size even after successive generations of synthesis. They found that intravenously injected functionalized dendrimers less than 12 nm in diameter were able to traverse the pores of the blood-brain barrier of RG2 gliomas and demonstrated that those with long half lives accumulated in glioma cells (Sarin *et al.*, 2008 and Figure 4.9).

As described in the work of Sarin and colleagues mentioned above, one of the most widely studied types of dendrimers is **poly(amidoamine) (PAMAM)** due to its small size and binding affinities for a variety of different types of molecules. PAMAM dendrimers are the most common class of dendrimers and consist of an alkyl-diamine core and tertiary amine branches (Figure 4.10). They are available commercially in generations from G0 to at least G10 and can be synthesized with five different core
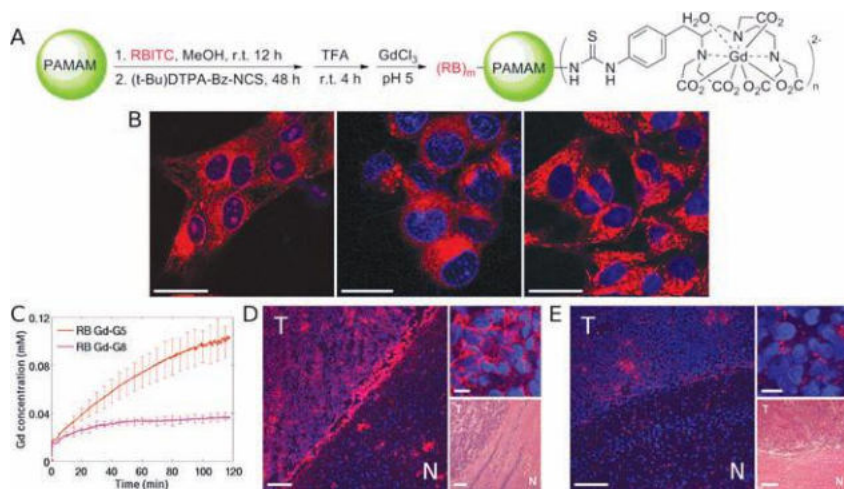
FIGURE 4.9 Fluorescence microscopy of glioma cell uptake of rhodamine B labeled Gd-dendrimer generations *in vivo* versus *ex vivo*. (A) Synthetic scheme for production of rhodamine B (RB) labeled Gd-polyamidoamine dendrimers. The naked polyamidoamine dendrimer is first reacted with rhodamine B and then with Gd-DTPA. (B) As shown by fluorescence microscopy *in vitro*, rhodamine B Gd-G2, rhodamine B Gd-G5, and rhodamine B Gd-G8 accumulate in glioma cells. Rhodamine B Gd-G2 dendrimers enter RG-2 glioma cells, and in some cases, the nucleus (left). Rhodamine B Gd-G5 dendrimers enter the cytoplasm of RG-2 glioma cells, but do not localize within the nucleus (middle). Rhodamine B Gd-G8 dendrimers enter RG-2 glioma cells *in vitro* (right). Shown are merged confocal images of blue fluorescence from DAPI-Vectashield nuclear (DNA) stain and red fluorescence from rhodamine B labeled Gd-dendrimers. Scale bars = 20 μm. (C) At 2 hours dynamic contrast-enhanced MRI shows substantial extravasation of rhodamine B Gd-G5 dendrimers and some extravasation of rhodamine B Gd-G8 dendrimers. Rhodamine B Gd-G5 n = 6, rhodamine B Gd-G8 n = 2. D) Low power fluorescence microscopy *ex vivo* of brain tumor and normal brain surrounding tumor shows that there is substantial accumulation of rhodamine B Gd-G5 dendrimers within tumor tissue (left, T = tumor, N = normal, scale bar = 100 μm). High power shows subcellular localization within malignant glioma cells (upper right, scale bar = 20 μm). Hematoxylin and Eosin stain of tumor and surrounding brain (lower right, scale bar = 100 μm). Tumor volume is 31 mm^3. E) Also shown by low power fluorescence microscopy *ex vivo* is some accumulation of rhodamine B Gd-G8 dendrimers within brain tumor tissue (left, T = tumor, N = normal, scale bar = 100 μm). High power confirms minimal subcellular localization within glioma cells (upper right, scale bar = 20 μm). Hematoxylin and Eosin stain of tumor and surrounding brain (lower right, scale bar = 100 μm). Tumor volume is 30 mm^3. (Courtesy of Sarin *et al.*, 2008; reprinted with permission.)
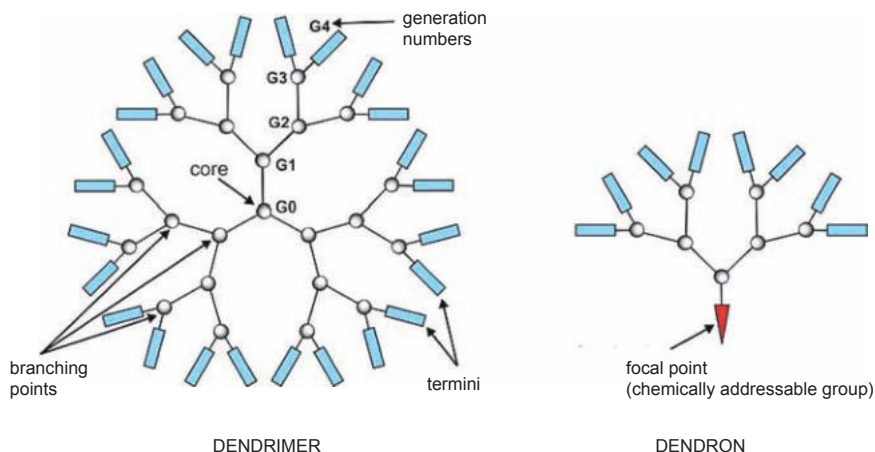
FIGURE 4.10 Structure of PAMAM dendrimer. (Courtesy of Wikipedia; reprinted with permission.)

types and ten functional surface groups. Most PAMAM dendrimers are supplied as solutions in methanol for improved long-term storage stability.

PAMAM dendrimers have been studied extensively primarily as an anticancer drug delivery platform to areas deep within sites of tumorigenesis. Details on this, citing other specific examples, are described and outlined in Chapter 7. Penetration of the BBB by PAMAM/DNA complexes have been demonstrated and confirmed by monitoring the expression of reporter genes within the brain. For example, researchers at Fudan University in Shanghai demonstrated the targeting of dendrimer complexes across the BBB and to sites within the brains of mice after intravenous delivery. Targeting was mediated using transferrin as the targeting agent and the transferrin receptor as the target, which is expressed on the surface of cells within the brain. **Transferrin** is a blood plasma protein involved in iron ion delivery to cells through transferrin receptor binding. Confirmation of gene delivery was by enzymatic assay of luciferase activity or observance of fluorescence.

Starburst® PAMAM dendrimers are a dendrimer subclass used in a wide variety of biomedical applications. They consist of multiple linear polymer arms attached to a central core and are structurally advantageous for attaching targeting, diagnostic or therapeutic moieties (Figure 4.11).
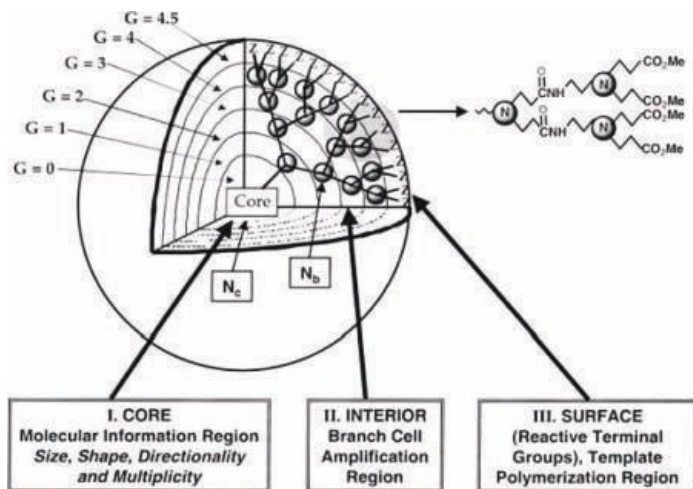
FIGURE 4.11 Three dimensional projection of Starburst dendrimer. Core-shell architecture for G = 4.5 PAMAM dendrimer with principal architectural components (I) core, (II) interior & (III) surface. (Courtesy of PharmaInfo.net; reprinted with permission.)

Starburst PAMAM/DNA complexes were shown to penetrate the BBB of mice after intramuscular injection and confirmed by efficient green fluorescent protein (GFP) reporter gene expression. In addition, the immunogenic ES312 compound also present in the dendrimer complex elicited an immune response significantly higher than from the introduction of DNA alone (Ding *et al.*, 2005).

Nanoparticles

Nanoparticles have also been studied extensively for their abilities to remain stable in the bloodstream and drive drug delivery across the BBB. The basic requirements for brain delivery are the same as for other nanomaterial platforms and include:

- Small size (<100 nm)
- Stability in the blood
- Avoidance of the RES
- Minimization of neutrophil activation, platelet aggregation and the activation of inflammatory responses.

Protein-based NPs such as those composed of human serum albumin (HSA) have shown promise in drug delivery across the blood brain barrier. Researchers at the Institute for Pharmaceutical Technology in Frankfurt, Germany have developed loperamide-containing HSA NPs chemically conjugated to **apolipoprotein E (ApoE)**. ApoE is a lipoprotein that binds specific receptors primarily expressed on the surface of liver and endothelial cells. Loperamide is a synthetic drug well known to not cross the BBB on its own due to P-glycoprotein activity and thus has no analgesic effects when administered. It was demonstrated that these NPs could efficiently cross the BBB and delivery loperamide to sites within the brain. The mechanism of action was suggested to be through ApoE interactions with lipoprotein receptors present in brain capillary endothelial cells (Michaelis *et al.*, 2006 and Figure 4.12).

Non-biodegradable NPs have also been studied for their potential to cross the BBB and act either as imaging agents themselves or delivery therapeutics within the brain. Jerome Engel's group at the Brain Research Institute/UCLA created iron oxide-based magnetic nanoparticles (MNPs) capable of crossing the blood–brain barrier (BBB) and of concentrating in the epileptogenic tissues of acute and chronic animal models of temporal lobe epilepsy. The MNPs consisted of **alpha methyl tryptophan (AMT)** covalently attached to an iron oxide-dextran conjugate. AMT has been used as a positron emission tomography (PET) ligand to identify epileptogenic tissues in several epilepsy conditions. The particles were shown to cross the
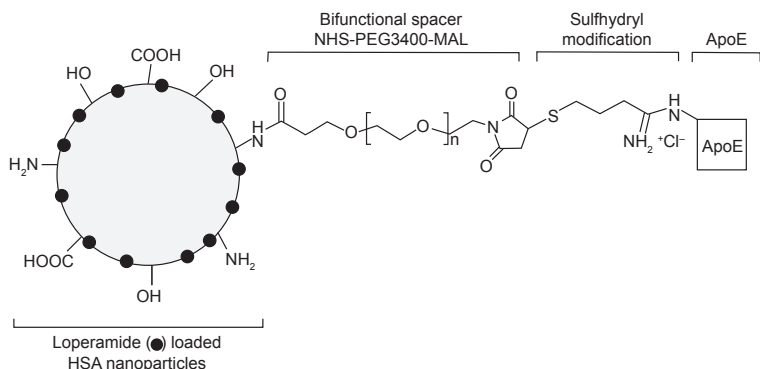
FIGURE 4.12 Schematic illustration of covalently apolipoprotein E-modified HSA nanoparticles. Representation not to scale. (Michaelis *et al.*, 2006; reprinted with permission.)

blood–brain barrier (BBB) and concentrate in epileptogenic tissues during the acute and chronic stages of a rat model of temporal lobe epilepsy, permitting their localization with standard MRI.

Polymer-based NPs have been pursued for their ability to drive drug delivery across the BBB primarily due to their biodegradable properties. As early as 1997 polymer-based NPs were shown to deliver analgesics as well as anti-cancer agents and anti-convulsants across the BBB. Jorge Kreuter's group at the Institute of Pharmaceutical Technology in Frankfurt, Germany developed polysorbate 80-coated polybutylcyanoacrylate (PBCA) NPs that effectively delivered loperamide across the BBB and produced analgesic effects in rodents after intravenous injection. **Polysorbate 80**, commercially known as Tween 80, is a nonionic nanoparticle surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid and is commonly used in the food industry thus confirming its safety. The data suggested that over-coating with polysorbate 80 induced brain capillary endothelial cell uptake of the NPs, followed by delivery of loperamide to the brain tissue. In addition, crossage of the BBB by polysorbate 80-coated PACA nanoparticles can be enhanced through the recruitment of apolipoproteins (Alyautdin *et al.*, 1997 and Figure 4.13).

Finally, a number of groups are now taking the next step and developing strategies for the delivery of specific therapeutic platforms across the BBB and deep within the brain tissue to treat some of the most debilitating disorders such as Alzheimer's disease (AD). Although a controversial finding, excessive levels of various metals have been identified post-mortem in Alzheimer's patients and suggested to exacerbate the disease. It is postulated that **oxidative stress**, which is defined as a condition of increased oxidant production in cells characterized by the release of free radicals and resulting in cellular degeneration. **Redox** refers to a chemical reaction between two substances in which one substance is oxidized and the other reduced. Oxidative stress plays a role in the development and progression of AD by promoting the accumulation of high levels of redox-active metals, particularly iron, contributing to this manifestation. Researchers are now creating NP delivery platforms that provide **metal chelators**, which are agents that bind metal ions and render them neutral and unavailable, deep within the brain, eventually allowing for metal removal from the brain via the circulatory system. In one particular study, University of
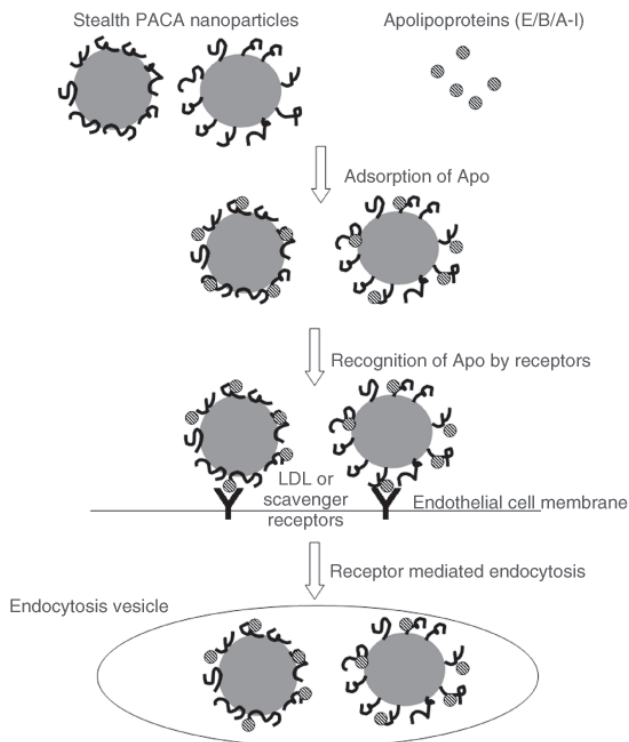
FIGURE 4.13 Diagrammatic illustration of PACA nanoparticle Apo-driven crossage of the blood-brain barrier. (Courtesy of Patrick Couvreur, CNRS; reprinted with permission.)

Utah researchers generated NPs covalently conjugated to iron chelators such as MAEHP and demonstrated efficient removal of iron from AD brain sections *in vitro* using this system (Liu, *et al.*, 2006, Figure 4.14 and for review see Liu *et al.*, 2009).

Nanogels

The term **nanogel** describes any mixture of nano-sized particles or fibers with a gel, typically one that is protein-based in the case of therapeutic or medicinal applications. Nanogels have been pursued for anti-cancer drug delivery directly into cancer cells. In addition, protein-based nanogels have been demonstrated to stop bleeding through protein matrix

A.   B.
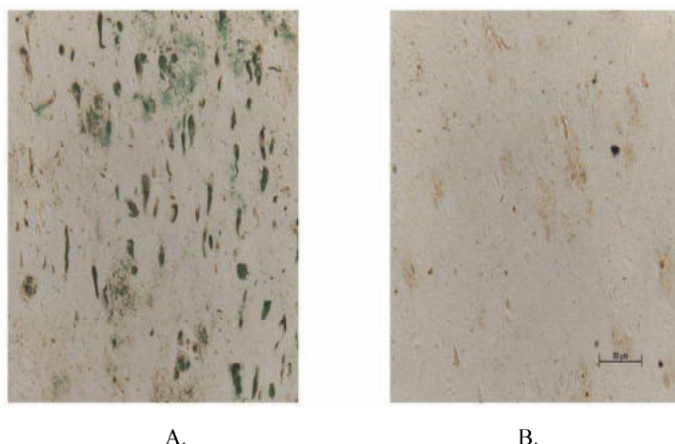
FIGURE 4.14 Lesion-associated chelatable iron in AD brain sections was depleted with iron chelator as evaluated by histochemistry. (A) Control and (B) MAEHP treated section. (Liu *et al.*, 2006; reprinted with permission.)

self-assembly after application at the site of injury. Alexander Kabanov at the Center for Drug Delivery and Nanomedicine at the University of Nebraska Medical Center in Omaha is perhaps the leading researcher in this area and has developed nanogels that allow for the delivery of DNA across the BBB (see Focus Box 4.2). Specifically, his group has developed a PEG-PEI-based nanogel that can bind and spontaneously encapsulate negatively charged oligonucleotides (Vinogradov, *et al.*, 2004 and Figure 4.15).

This resulted in a stable dispersion of solid nanoparticles in aqueous solution that averaged less than 100 nm in size. *In vitro* studies using bovine brain microvessel endothelial cells (BBMECs) suggested that this nanogel formulation will efficiently cross the BBB and deliver oligonucleotides intact within nanoparticles in the brain. Interestingly, Kabanov's group suggest that transport occurs via **transcytosis**, which is a mechanism for transcellular transport in which a cell encloses extracellular material in an invagination of the cell membrane to form a vesicle, then moves the vesicle across the cell to eject the material through the opposite cell membrane by the reverse process. This is further confirmed through a measurement of the

Focus Box 4.2 Alexander Kabanov and nanogels for DNA delivery

Alexander Kabanov, a professor at the University of Nebraska Medical Center, is perhaps the world's leading researcher in the use of nanogels to deliver nucleic acids as therapeutics. The main focus of his research is on the application of nanopolymers for gene delivery and he has refined and developed nanogel system for carrying oligonucleotides, DNA and siRNA as modes of gene therapy (see section on Nanogels). His unique platform draws on the ability of nanopolymers ionic chains to bind nucleic acids or proteins and has a load capacity higher than other drug carriers. (Photo courtesy of the University of Nebraska; reprinted with permission.)
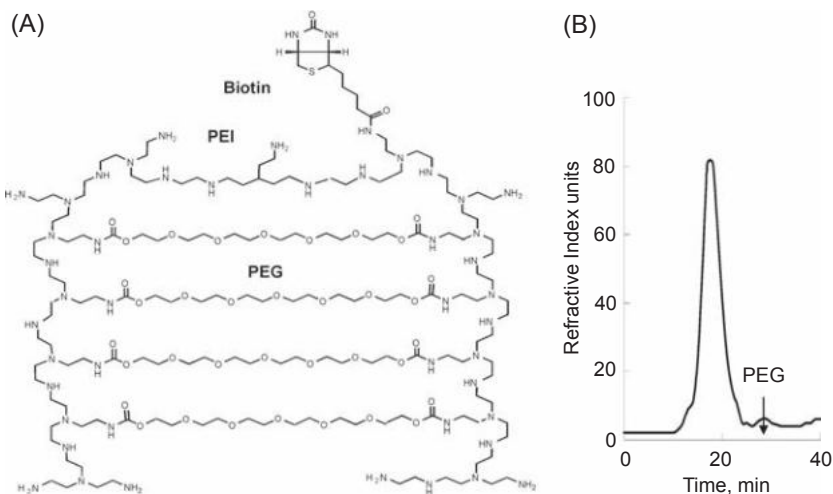
FIGURE 4.15 PEG-PEI nanogel and its characterization. (A) Schematic representing biotinylated nanogel network. (B) The chromatographic profile of GP-HPLC with refractive index (RI) detection of a biotinylated nanogel sample. (Courtesy of Vinogradov, *et al.*, 2004; reprinted with permission.)

apparent permeability of the cells with respect to the oligonucleotides-containing nanogels according to the below equation.

$$P_{app} = (V/AC_0) \times dC/dt \text{ (cm/s)}$$

where $V \times dC/dt$ is the steady-state rate of appearance of ODN at the basolateral side of the monolayers, C_0 is the initial ODN concentration at the apical side, and A is the surface area of the membrane. The authors demonstrated that nanogels containing insulin as a targeting agent were the most efficient at transport across BBMEC monolayers (Figure 4.16). Uptake in BBMEC monolayers was confirmed by fluorescence microscopy (Figure 4.17). The bovine *in vitro* studies were confirmed by biodistribution studies in the mouse (Vinogradov, *et al.*, 2004).
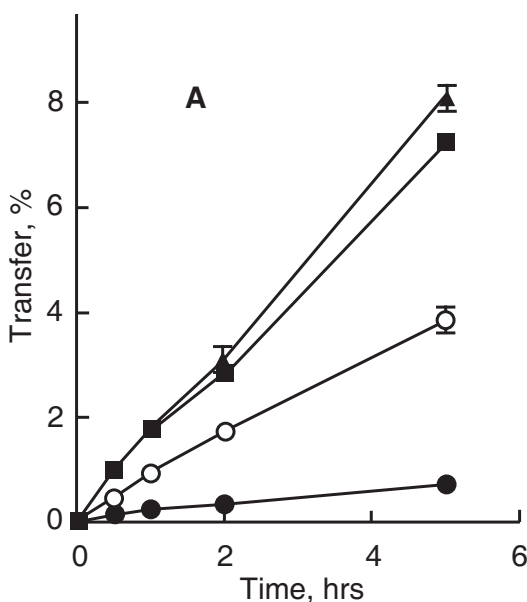
FIGURE 4.16 Transport of free ODN and ODN formulated with nanogel across BBMEC monolayers. The symbols correspond to free ODN (black circles); nanogel-ODN complex (open circles); transferrin-vectorized nanogel-ODN complex ODN (squares); insulin-vectorized nanogel-ODN complex (triangles). All nanogel-ODN complexes are prepared at N/P) 8. Data are means (SEM (*n*) 3). (Vinogradov *et al.*, 2004; reprinted with permission.)
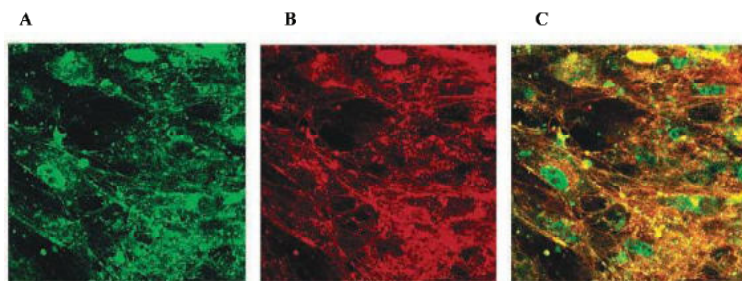
FIGURE 4.17 Confocal fluorescent microscopy of BBMEC monolayers after the incubation (2 h) with FITC-labeled ODN loaded into RITC-labeled nanogel. (A) FITC showing the cells. (B) RITC showing the nanogel. (C) The superposition of images A and B. Magnification X100. (Vinogradov *et al.*, 2004; reprinted with permission.)

NANOMATERIALS AND NEUROPROTECTION

The term "**neuroprotection**" refers to strategies and mechanisms for the prevention or reduction of neuronal apoptosis or degeneration. As described above regarding nanoparticles for crossing the blood brain barrier, it is clear that oxidative stress plays a considerable role in CNS neuropathology, often resulting from either programmed cell death or outright neuronal necrosis. Thus agents that minimize redox processes within the CNS show promise for the treatment or at least management of many CNS disorders. Below are some example nanoparticle platforms that have been studied for their anti-oxidative properties in this area.

Nanoparticle-Based Oxides (Anti-Oxidants)

Yttrium and cerium oxides (Y_2O_3 and CeO_2) are nanoparticles that are known to have anti-oxidative properties. Cerium oxide, for example, has a cubic structure and when prepared properly is characterized by monodisperse nanoparticles that are crystalline in nature (Figure 4.18).

Ceria itself tends to have a dual oxidation state, thus possessing oxygen vacancies or defects. This property and the related reaction chemistry can be represented by the below equation for cerium oxide.
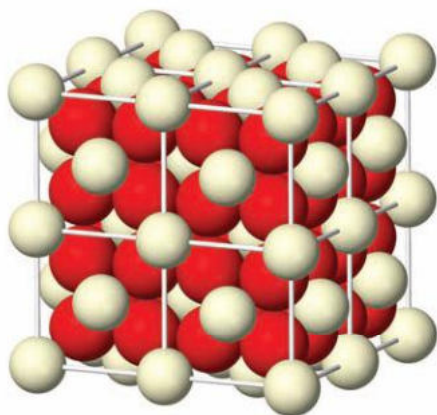
FIGURE 4.18 Illustration of the molecular three-dimensional nature of a cerium oxide nanoparticle. (Courtesy of Wikimedia; reprinted with permission.)

$$CeO_2 \underset{\text{oxidizing}}{\overset{\text{reducing}}{\longleftrightarrow}} CeO_{2-y} + \frac{y}{2}O_2$$
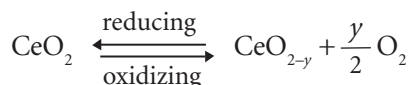
Dave Schubert and colleagues at the Salk Institute in La Jolla, California demonstrated that these nanoparticles could protect HT22 nerve cells *in vitro* from oxidative stress and that the neuroprotection observed is independent of nanoparticle size. Both nanoparticle types acted as effective anti-oxidants to limit the amount of reactive oxygen species present within the system required to kill the cells. **Reactive oxygen species (ROS)** are highly reactive oxygen free radicals that can damage cellular structures. Specifically, it was observed that ROS concentrations in untreated or glutamate treated HT22 cells were decreased in the presence of either nanoparticle type in a concentration-dependent manner (Schubert *et al.*, 2006 and Figure 4.19).

Fullerenes

Fullerenes typically have a composition and three-dimensional structure that are conducive to reactivity with and thus neutralization of oxygen free radicals. As described in Chapter 1, they consist of carbon atoms evenly
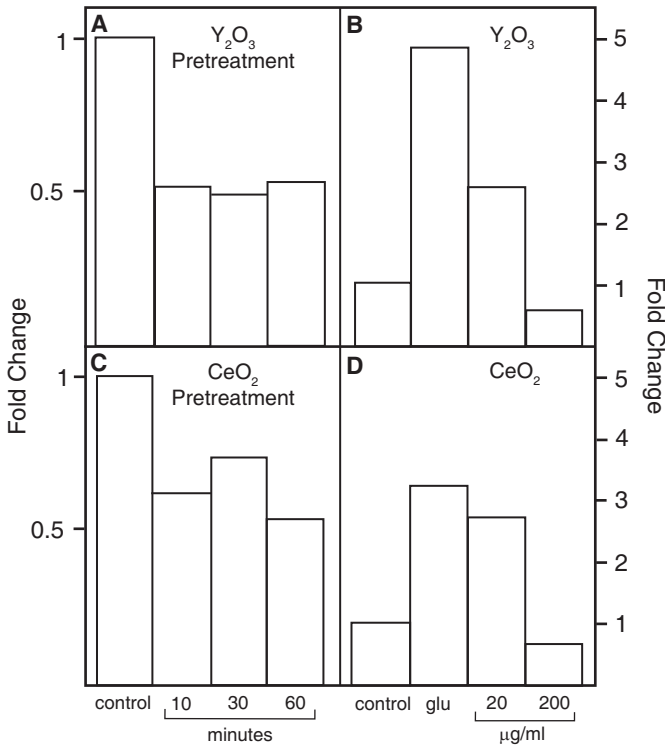
FIGURE 4.19 Y_2O_3 and CeO_2 nanoparticles are antioxidants. Cells were either exposed directly to Y_2O_3 (A) or CeO_2 (C) and ROS determined 10, 30, and 60 min later, or cells were exposed to glutamate for 8 h to generate endogenous ROS and then either 20 or 200 μg/mL of the nanoparticles added (B, D). The data are expressed as fold change in endogenous ROS in each experiment. The endogenous level of ROS was 14.35 (arbitrary units) in untreated cells and 67.66 units in cells treated with glutamate for 8 h. Each bar graph is the mean of 10,000 independent measurements, with an error of less than 2% between repeated measurements. (Schubert *et al.*, 2006; reprinted with permission.)

spaced and connected by double bonds. In solution, such as in a biological environment, this molecular structure lends itself well to reaction with oxygen free radicals. For example, Dennis Choi and colleagues in the Department of Neurology at Washington University School of Medicine in St. Louis were some of the first researchers to study the free radical scavenging properties of buckminsterfullerenes (C_{60}) as a neuroprotective mechanism. They confirmed anti-oxidant capabilities of buckminsterfullerenes on
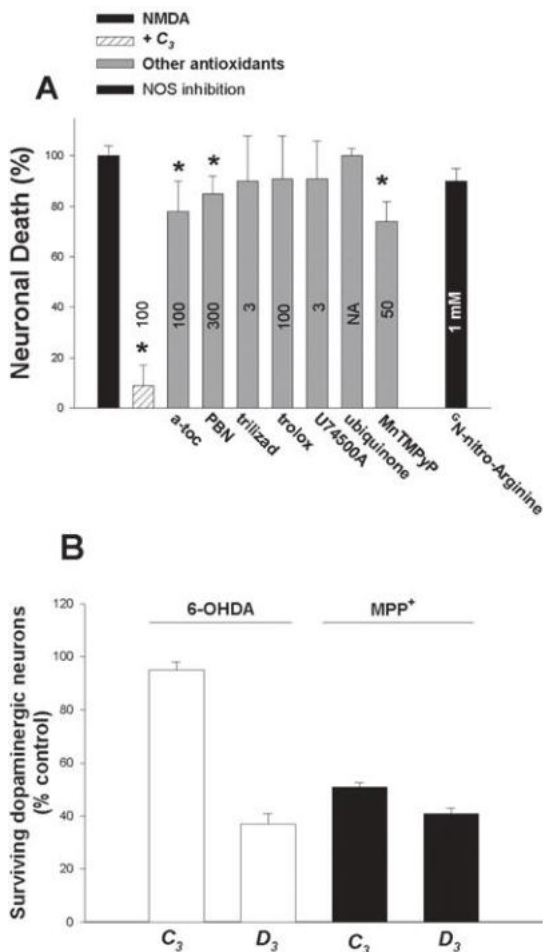
FIGURE 4.20 Neuroprotection by fullerenes. (A) Comparison of neuroprotection by various antioxidants and a NOS inhibitor versus the C_3 compound against NMDA toxicity. Cortical cultures were exposed to NMDA in the presence of the listed compounds. GN-Arginine was applied 4 h before application of NMDA, and re-applied during the NMDA exposure. Cell death was assessed by assaying LDH release and by examining the neurons by phase contrast microscopy. Concentrations on the bars reflect the most effective concentration of each drug, determined from dose response curves. (B) Carboxyfullerene isomers C_3 and D_3 differ in neuroprotection against 6-OHDA and MPP1 injury in cultured mesencephalic dopaminergic neurons. Cultures were exposed to 6-OHDA or MPP1 in the presence of C_3 or D_3. The percent of remaining dopaminergic neurons is shown. (Courtesy of Dugan *et al.*, 2001; reprinted with permission.)

cortical cell explant cultures *in vitro* and demonstrated a reduction in cell death as a result of reduced excitotoxicity. **Excitotoxicity** is the pathological process by which nerve cells are damaged due to over-excitation, more often a result of high amounts of the neurotransmitter glutamate. Serum-deprived apoptosis was also shown to be reduced by buckminsterfullerenes in this system (Dugan *et al.*, 1996). The same group later demonstrated that systemic administration of a C_3 carboxyfullerene isomer delayed motor deterioration and death in a mouse model of familial amyotrophic lateral sclerosis (FALS) and suggested that the mechanism may be through eliminating both superoxide anion and H_2O_2 via free radical scavenging (Dugan *et al.*, 2001 and Figure 4.20).

J.Y. Wu's group in the Department of Molecular Biosciences at the University of Kansas in Lawrence showed that **fullerenols**, which are defined as caged fullerene oxides, demonstrated inhibition of glutamate receptor activity, most likely through free radical scavenging, that resulted in lower calcium uptake by neurons and thus reduced glutamate-induced neurotoxicity via diminished intracellular calcium buildup. More recently, as Case Study 4.2 illustrates, some researchers have begun to combine the anti-oxidant effects of fullerenes with drugs to yield a two-pronged approach to neuroprotection.

CELLS AS NANOMATERIAL CARRIERS FOR CLINICAL NEUROSCIENCE

The majority of this text is focused in general on the extracellular positioning of nanomaterials/nanoparticles to exert some form of therapeutic or diagnostic effect. Yet it is important to consider that platforms involving the intracellular placement of nanomaterials and the use of cells as nanomaterial carriers in this sense are gaining attention from the scientific community. Immune cells such as macrophages, microglia and mononuclear phagocytes have been shown to ingest colloidal nanomaterials. These cells are capable of both delivery and release of their payloads to sites of tissue injury, infection or disease. Howard Gendelman's group at the University of Nebraska Medical Center in Omaha demonstrated this concept by using bone marrow macrophages (BMMs) to deliver the nanoparticle-formulated anti-retroviral agent indinavir to various organs and tissues in mice, with most accumulation occurring in the lungs, liver and spleen. A **macrophage** is a large white blood cell that ingests foreign particles and substrates via **phagocytosis** (cellular eating). Their size and carrier

Case Study 4.2: Reversal of axonal loss and disability in a mouse model of progressive multiple sclerosis

Howard Weiner and colleagues at the Center for Neurologic Diseases, Harvard Medical School addressed axonal degeneration *in vivo* using a combination therapeutic platform containing a water-soluble fullerene derivative (ABS-75) attached to an NMDA receptor antagonist. This therapeutic strategy combines both the anti-oxidant properties of the fullerene derivative with the anti-excitotoxic properties of the receptor antagonist. The goal was to block axonal damage and reduce disease progression in a mouse model for multiple sclerosis. They showed reduced disease progression associated with both reduced axonal loss and reduced demyelination in the spinal cord. In addition, the researchers confirmed that no memory loss had occurred in mice undergoing treatment suggesting that it does not affect cognition (Basso *et al.*, 2008 and Figure 4.21).
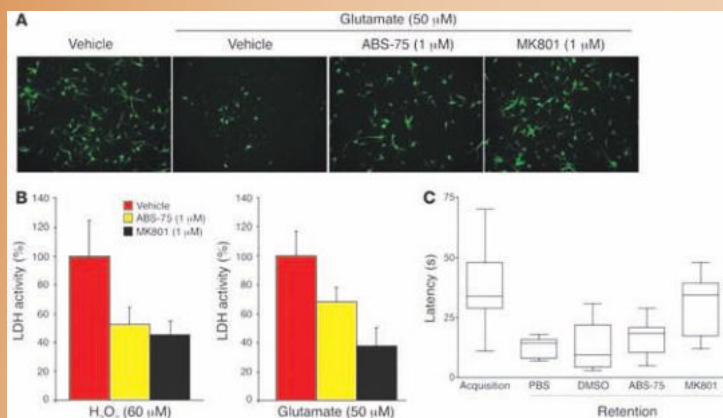
FIGURE 4.21 Fullerene ABS-75 protects neurons from oxidative injury and NMDA-dependent glutamate-induced injury but does not impair mouse memory function. Rat cortical neurons were challenged with either H_2O_2 or glutamate for 24 h in the absence or presence of ABS-75 or MK801. Glutamate- and H_2O_2-induced injuries were assessed by (**A**) MAP-2 staining and (**B**) LDH activity. The results showed reduced neuronal damage in fullerene ABS-75– and MK801-treated cultures. (**C**) Fullerene ABS-75 and MK801 effects on memory function were assessed on a plus maze apparatus. The results demonstrated that, unlike MK801, fullerene ABS-75 does not impair memory function. Original magnification, ×130. (Basso *et al.*, 2008; reprinted with permission.)

ability make them ideal vehicles for small molecule transport. Loading of BMMs with indinavir was accomplished by monolayer culture of the cells in the presence of the agent. To assess distribution, cells were labeled with [111]Indium for SPECT analysis.

Gendelman and Kabanov (discussed above under Nanogels) have since collaborated to develop a cell-mediated strategy for the delivery of a self-assembled PEG-PEI catalase complex. A **catalase** is a common enzyme found in nearly all living organisms which are exposed to oxygen. It typically functions to catalyze the decomposition of hydrogen peroxide, a harmful byproduct of normal metabolic processes, to oxygen and water. These "nanozymes" were around 60 nm to 100 nm in size, stable in pH and ionic strength, and retained antioxidant activities. BMM/nanozyme complexes were administered intravenously in mice and shown to deliver the catalase across the BBB into the brain. A significant presence of the catalase was also seen in various other organs (Batrakov, *et al.*, 2007 and Figure 4.22). Thus proof-of-principle now exists for the utilization of cell-nanomaterial hybrids in crossing the BBB.
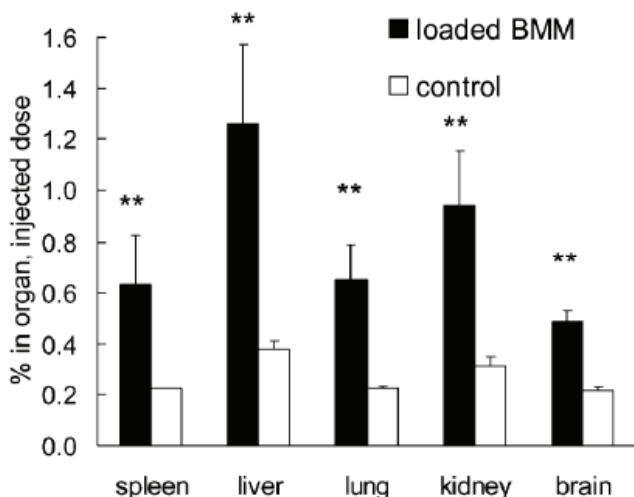
FIGURE 4.22 Biodistribution of ^{125}I-labeled nanozyme in MPTP-treated mice. Mice were injected with BMM loaded with catalase nanozyme or with nanozyme alone (control group). Twenty-four hours later mice were sacrificed and the amount of radioactivity was measured in various organs. Statistical significance of the BMM-loaded nanozyme transport compared to the nanozyme alone group is shown by asterisks: (**) $p < 0.005$. (Batrakova *et al.*, 2007; reprinted with permission.)

These same researchers have since expanded on this concept to utilize cells as delivery vehicles for other key molecules and factors that are well known to mitigate neurodegenerative diseases. These include anti-inflammatory growth factors and neurotrophic factors such as glial derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF).

CHAPTER SUMMARY

Nanomaterial Scaffolds and Neuroregeneration

1. Neurodegenerative diseases are characterized by loss of brain or spinal cord cells.
2. Nanoparticles and nanomaterials, due to their unique physical properties, may be used to treat neurological disorders.
3. Nanometer-scale scaffolds may mimic tubular neural structures such as axons and dendrites.
4. Carbon nanotube electrical conductivity properties have made them an excellent source of material for neuronal matrices.
5. Carbon nanotubes promote neuronal outgrowth and signaling.
6. CNT matrix charge can be manipulated through the attachment of different functional groups.
7. Vertically aligned CNTs may alleviate micron-scale mechanical stresses of cultured cells when used as a cell culture matrix.
8. Numerous materials may be used for neuronal cell culture applications including CNTs, DNA, proteins, synthetic polymers, glass and silicon.
9. Templating and self-assembly are the primary techniques for manufacturing nanotube-based cell culture platforms for neural or neuronal applications.
10. PEDOT-based nanotubes can be used to create a nanotube/living neural tissue complex with enhanced electrical activity.

Nanomaterials for Crossing the Blood/Brain Barrier

1. The blood/brain barrier (BBB) is the most restrictive barrier in the body primarily due to the presence of P-glycoprotein (P-gp).
2. Current methods for drug delivery across the BBB include its temporary disruption, more aggressive invasive disruption or the use of delivery vehicles.

3. Micellular complexes consisting of antimicrobial amphiphilic peptides have been shown to cross the BBB and suppress bacterial growth.

4. Pluronic P85 has been shown to inhibit P-gp and enhance the uptake of drugs across the BBB.

5. Polyion complex micelles allow for the incorporation of charged particles in their internal cores and possess characteristics which may make them ideal for delivery of therapeutics across the BBB.

6. Liposomes may incorporate drugs in their internal cores or between their lipid bilayers and have been extensively studied as delivery platforms across the BBB.

7. Convection-enhanced delivery may improve liposomal crossage of the BBB.

8. Dendrimers' small size may make them ideal for crossing the BBB and their multi-valency allows for efficient targeting moiety and therapeutics attachment.

9. PAMAM dendrimers are the most common class of dendrimers and can be synthesized with up to five different core types and ten functional groups.

10. Intravenously injected PAMAM dendrimers have been shown to efficiently cross the BBB of gliomas.

11. PAMAM dendrimers can deliver gene therapies across the BBB.

12. Requirements for nanoparticles as brain delivery vehicles include small size, stability in the blood, avoidance of the RES and minimization of inflammatory response.

13. Protein-based nanoparticles such as ApoE, which are biodegradable, can deliver drugs across the BBB.

14. Some non-biodegradable nanoparticles such as iron oxide meet the requirements for crossing the BBB and can be used to deliver therapeutics or as imaging agents themselves.

15. Polymer-based nanoparticles such as Tween 80 have been extensively studied as carriers of imaging agents and therapeutics into the brain due to their biodegradable and non-toxic qualities.

16. Excessive levels of metals in the brain may contribute to oxidative stress and ultimately the manifestation of Alzheimer's disease.

17. Nanoparticles are now being studied as delivery vehicles for metal chelators deep within the brain.

18. Nanogels have been studied as platforms for the treatment of cancer, wound healing and the delivery of gene therapies across the BBB.

Nanomaterials and Neuroprotection

1. Agents that minimize redox processes within the CNS show promise for the treatment or management of many CNS disorders.
2. Yttrium and cerium oxide are nanoparticles that exhibit anti-oxidative properties.
3. Fullerenes have a composition and 3D structure that are conducive to the neutralization of oxygen free radicals and have been shown to reduce excitotoxicity and thus the death of cortical cells.
4. Fullerenols have also been shown to be effective free radical scavengers and promote neuroprotection.

Cells as Nanomaterial Carriers for Clinical Neuroscience

1. Cells such as macrophages, microglia and phagocytes may be used as *in vivo* nanomaterial delivery vehicles.
2. A PEG/PEI catalase nanozyme complex was shown to be successfully delivered across the BBB by bone marrow macrophages.

KEY TERMS

- Neuroscience
- Neurodegenerative Disease
- Parkinson's Disease
- Bradykinesia
- Idiopathic
- Alzheimer's Disease
- Microglia
- Astrocyte
- Synapse
- PC12 Cell
- Bending Force, F
- Apoptosis
- Blood-Brain Barrier (BBB)
- P-glycoprotein (P-gp)
- Pluronic P85
- Analgesia
- Polyion Complex Micelle
- Reticuloendothelial System (RES)
- Doxorubicin
- Glioma
- Convection Enhanced Delivery (CED)
- Multi-valent
- Poly(amidoamine) (PAMAM)
- Methotrexate
- Cetuximab
- Kaplan-Meier Curve
- Transferrin
- Starburst® PAMAM dendrimers
- Apolipoprotein E (ApoE)
- Alpha Methyl Tryptophan (AMT)
- Polysorbate 80
- Oxidative Stress

- Redox
- Metal Chelators
- Nanogel
- Transcytosis
- Apparent Permeability
- Neuroprotection

- Reactive Oxygen Species
- Excitotoxicity
- Fullerenol
- Macrophage
- Phagocytosis
- Catalase

REVIEW QUESTIONS

(Answers to the review questions can be found at (**www.understandingnano. org.**)

1. List the three main areas of clinical neuroscience for which nanotechnology is making an impact and cite specific nanomedical platforms for each.
2. Compare and contrast Alzheimer's and Parkinson's diseases for both symptoms and proposed possible causes.
3. What are some of nanomaterials used to develop matrices for neuronal cell culture?
4. How does the blood brain barrier restrict foreign substance entry into the brain?
5. Describe how Pluronic P85 drives the delivery of drugs across the BBB.
6. Describe how polyion complex micelles are formed to contain charged macromolecules and cite an example of delivery into an organ.
7. What component of the immune system clears conventional liposomes from the circulation and how has nanotechnology addressed this roadblock to drug delivery?
8. Describe William Pardridge's research on the use of liposomes to deliver gene therapy platforms across the BBB and to the brain.
9. How does doxorubicin attack cancer cells and what type of cancer is it particularly useful in treating?
10. Outline the domains of dendrimer organization and explain how therapies are associated with them in the manufacturing process.
11. Why are PAMAM dendrimers so widely studied as drug delivery vehicles, especially across the BBB?
12. Give an example of a strategy for how researchers actively target nanoparticles for penetrating the BBB.

13. Describe the phenomenon of oxidative stress and give an example of how to mitigate its effects on Alzheimer's disease.
14. What is the equation for the apparent permeability of cells?
15. Write the redox reaction equation for cerium oxide and cite how Schubert's group used this nanoparticle for neuroprotection.
16. Define fullerenols and explain how fullerenols exhibit neuroprotective properties.
17. Cite an example of cells used as *in vivo* carriers for nanoparticles or enzymes.

5

Nanotechnology and Surgery

Surgery can be defined as "dealing with the treatment of injury, deformity or disease by both manual and instrumental means." It is well known that surgery is one of the oldest forms of medicine with archeological evidence of brain surgery dating back to 8000 B.C. Tools designed and utilized for surgical procedures made of bronze and steel have been discovered that date back to ancient Roman times and, in many cases, are remarkably similar to those used today. Yet improvements in the quality of surgical instrumentation (including implants), procedures and recovery have constantly been sought over the centuries. With the advent of nanotechnology, a new era in the field of surgery is about to emerge. This chapter outlines areas of surgery, including **intracellular nanosurgery,** defined as the manipulation of organelles within a cell by manual and instrumental means (see also "Chromallocytes" in Chapter 10), where nanotechnology has already begun to make a significant impact and sheds light on future nanotechnology-driven trends emerging in this field of medicine.

IMPLANT AND SURGICAL INSTRUMENT DESIGN

Nanocoatings for Implants

An **implant** is defined as an artificial object placed inside the body in order to replace, correct or repair a damaged bodily function. A variety of implants are in use today, examples of which include pins, rods and screws for bone repair and in some cases joint replacement. Other types of implants include electrical, such as artificial pacemakers, and biological, for example bio-materials surgically implanted to replace or repair damaged tissues and/or internal organs. Skin replacement and grafting of bio-materials on the surface of the body are also considered implants.

Implants are currently manufactured using four types of materials. These include metals or metal alloys, polymers, ceramics and composites. There are two primary factors relating to the biological environment that must be taken into account when designing an implant: biocompatibility and biomimicry. **Biocompatibility**, which is defined as the capability of co-existence with livings tissues without causing harm, is a crucial requirement in the design of medical implants. Current research in this area is focused on the production of implant coatings that provide both biocompatibility as well as longevity properties. In addition, researchers are seeking new implant coatings that may provide stronger adherence to surrounding tissues without the threat of toxicity. **Nanocoating**, which is the coating of a surface with a nanomaterial, of implants has been a major focus in the medical device industry over the past ten years. An example of nanocoating includes that of the Manchester, Connecticut company Inframat Corporation which specializes in the application of hydroxyapatite nanoparticles for coating biomedical implants (Figure 5.1).

Biomimicry, which is the process of utilizing the way nature produces something in order to create a manmade material, is crucial to the biological environment's acceptance of a foreign or unnatural entity such as an implant. Thus a great deal of effort has been focused on creating nanoscale materials which mimic or at least promote acceptance by the biological environment of a foreign material. The concept of biomimicry is discussed in detail throughout this chapter.

Nanomaterial Adsorption and Adhesion

Researchers have found that host response to nanomaterials is often quite different and in some cases more favorable to that of more conventional
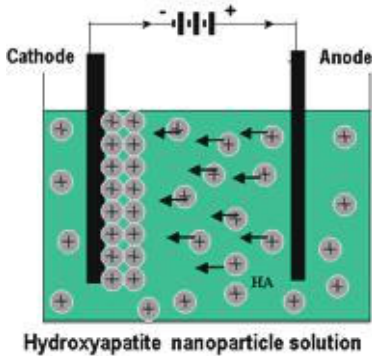
FIGURE 5.1 Inframat Corporation's hydroxyapatite nanocoating technology. (Upper Left) Diagrammatic illustration of procedure to coat implants with hydroxyapatite nanoparticles. (Bottom, left to right) Examples of implants coated with hydroxyapatite nanoparticles including dental, hip and knee implants, respectively. (Courtesy of Inframat Corporation; reprinted with permission.)

materials. Why would nanomaterials mimic the biological environment more accurately than more conventional materials? A key theme throughout this chapter pertains to the efficiency of **protein adsorption**, which is defined as the ability of a solid to attract and hold proteins on its surface. Efficient protein adsorption is critical for the recruitment and stable attachment of cells to a surface material. Tight cellular attachment to an implant's surface is crucial for its proper function in almost all cases. Cellular adhesive domains such as particular amino acid sequences of adsorbed binding proteins vitronectin, fibronectin and/or laminin mediate cellular attachment to a particular surface. Better access to these through enhanced nanomaterial properties may facilitate this binding. **Integrins**, for example, are dimeric cell surface receptors that mediate the attachment of a cell to the tissues surrounding it or the extracellular matrix. This is
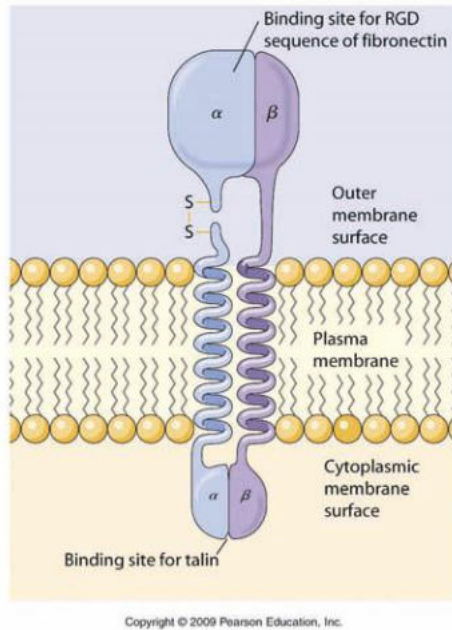
FIGURE 5.2 Diagrammatic illustration of the integrin receptor imbedded within a plasma membrane. (Courtesy of Memorial University of Newfoundland; reprinted with permission.).

through specific interaction with a well known amino acid sequence, RGD, present in fibronectin and other extracellular matrix proteins (Figure 5.2). **RGD** (Arg-Gly-Asp) is a peptide that is known to bind specifically to integrins and provide cellular attachment to the ECM.

Nanomaterials are well known to have unique surface energetics that may aid in the attraction and binding of cell surface receptor proteins, thus promoting cellular adhesion once a nanomaterial has been introduced into a biological environment such as into a patient. Nanomaterials have unique surface characteristics and energetics due to such properties as:

- Higher surface areas
- Higher surface roughness
- Increased surface defects (including grain boundaries)
- Altered electron distributions

Each of these as well as other inherent characteristics of nanomaterials will affect the efficiency of protein adsorption due to the fact that proteins are themselves nanoscale entities. The very chemical and three-dimensional nature of the nanomaterial surface may either promote or inhibit cellular adhesive domain and therefore cell binding and attachment. It has been postulated, and confirmed in some laboratories, that nanoscale features on the surface of certain nanomaterials can provide for more access to protein adsorption sites and thereby promote the adhesion of cells. The first study to reveal how proteins respond to different nanoscale features was conducted by graduate student D.C. Miller at Purdue University. In this study the nanomaterial PLGA was synthesized with varying sizes of exterior spherical bumps and tested for efficiency of interaction with the adhesive cell surface protein fibronectin. The study revealed, in general, that with decreasing sphere size came increase fibronectin adsorption (Liu *et al.*, 2006 and Figure 5.3).

Researchers are now pursuing the best nanomaterials and deposition methods for implant design. Edirisinghe's team at the University College London, for example, have elucidated a process for depositing bioactive nanoparticles in predetermined topographical geometries on both metallic and non-metallic surfaces. In this study they successfully deposited bio-compatible hydroxyapatite nanoparticles on both titanium and glass substrates electrolytically. Both two- and three-dimensional properties could be controlled in this process by varying mesh sizes/ geometries, spraying time and the electric field (Li *et al.*, 2008 and Figure 5.4).

The enormous potential impact of nanostructured materials and nanocoatings on the design and application of biomedical devices and, more specifically, implants warrants a detailed discussion of this discipline. There are three primary materials utilized for the generation of nanostructured coatings in the orthopedic and dental implant fields which include diamond, hydroxyapatite and metalloceramics. Table 5.1 summarizes the advantages and disadvantages of each with respect to their physical properties.

Nanostructured Diamond Coatings

It is no secret that diamond is the hardest known material. The **hardness** of a material refers to various properties in the solid phase that give it
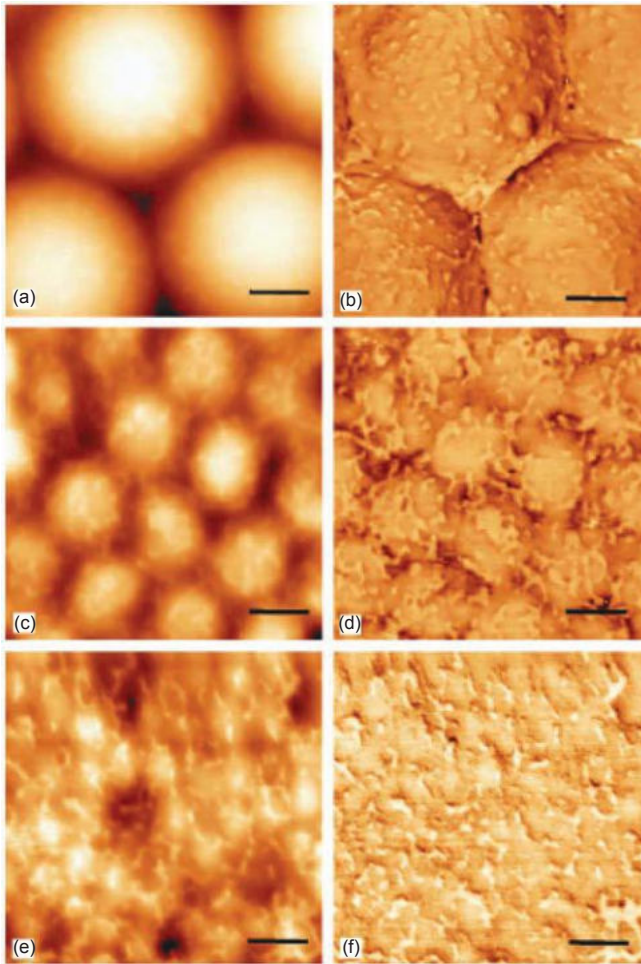
FIGURE 5.3 Representative AFM images of fibronectin (FN) adsorbed to PLGA surfaces. (a, b) 500 nm, (c, d) 200 nm and (e, f) 100 nm spherical bumps. Left columns (a, c, e) are height images and right column (b, d, f) are phase images. Additionally, (a) height and (b) phase images of FN adsorbed to 500 nm PLGA surfaces showed little to no interconnectivity between FN molecules; (c) height and (d) phase images of FN adsorbed to 200 nm PLGA surfaces showed a higher degree of interconnectivity between FN molecules; (e) height and (f) phase images of FN adsorbed to 100 nm PLGA surfaces showed well-spread FN molecules with a large amount of interconnectivity masking the underlying PLGA nanometer surface. Images are 750×750 nm. Scale bars are 150 nm. (Courtesy of Liu *et al.*, 2006; reprinted with permission.)
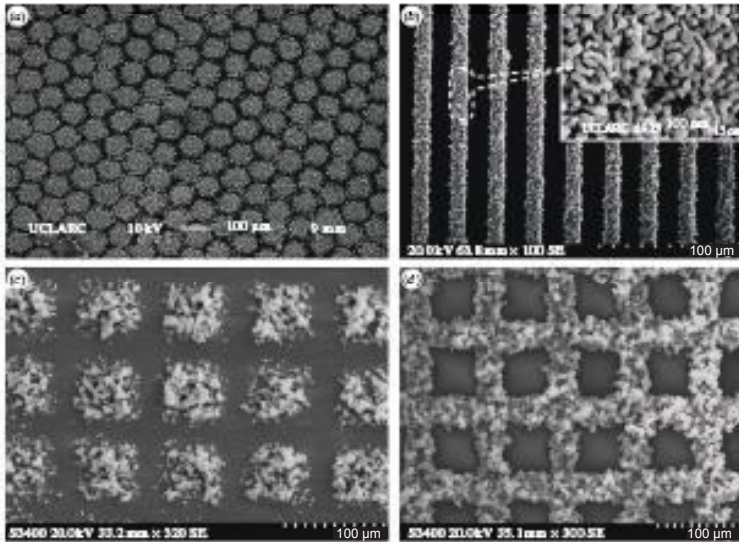
FIGURE 5.4 Scanning electron micrographs of nHA islands. (a) hexagonal nHA islands deposited on a glass substrate with the island diameter set at 50 mm, (b) nHA lines deposited on a titanium substrate with the width set at 50 mm, (c) square nHA islands deposited on a titanium substrate with the island diameter set at 50 mm and (d) nHA deposited on the gold template. (Courtesy of Li *et al.*, 2008; reprinted with permission.)

resistance to shape change when force is applied. It is typically reported in units of pressure, or **Pascals**, which is defined as one Newton per square meter (N/m^2 or $kg \cdot m^{-1} \cdot s^{-2}$). Other materials that are considered hard in their own right such as cubic boron nitride (c-BN) can reach, at best, about 50% of the hardness of diamond (47 GPa). As a result, carbon-based coatings such as diamond continue to dominate the super-hard materials industry. Recent advances in nanomaterials science have led to the development of nanostructured diamond coatings which exhibit unique combinations of hardness, toughness, low friction and corrosive resistivity. The most widely used deposition technique for producing hard carbon coatings is chemical vapor deposition, the basics of which were discuss in Chapter 1. Adjustments in the application of CVD allow for the customization of the resulting coating grain size (Catledge *et al.*, 2002 and Figure 5.5).

Due to its supreme biocompatibility, nanostructured diamond has been referred to as the "Biomaterial of the 21st Century." The two primary

Table 5.1 Advantages and disadvantages of the three primary types of nanostructured materials as they apply to orthopedic and dental implants (Catledge *et al.*, 2002; reprinted with permission)

Merits	Nanostructured Diamond	Nanostructured Hydroxyapatite	Nanostructured Metalloceramic
Advantages	• Combination of extreme hardness, low surface roughness and good fracture toughness • Good adhesion to titanium alloys • Good corrosion resistance • Easily tailored mechanical properties	• Promotes bone formation around implant • Increases osteoblasts adhesion, proliferation and mineralization	• Combination of hardness, very low surface roughness and extremely good adhesion • Good corrosion resistance • Can conform to shape of implant
Disadvantages	• Poor adhesion to cobalt, chrome and steel substrates • Conformal coatings not well developed yet	• Low toughness and flexural strength • Control of chemistry and microstructure remains a challenge	• Still in early stage of development and testing

advantages of using nanostructured diamond over other materials for the coating of biomedical implants is a reduction in abrasion and a resistance to protein absorption. Interactions of diamond nanocoatings with water, critical for biocompatibility, have been extensively studied in recent years. Researchers have now run simulations on nanostructured diamond coatings which suggest that a thin layer of ice could remain on
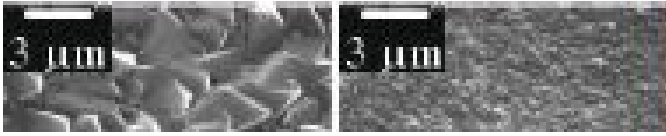
FIGURE 5.5 SEM images and Raman spectra of CVD diamond films grown with various mixtures. (a) H_2/CH_4 with a flow ratio of 500/88 and (b) $H_2/CH_4/N_2$ with a flow ratio of 500/88/8.8. Both films were grown with a chamber pressure of 125 Torr and an unconventionally high CH_4 fraction (15 vol %). The smooth nanostructured film in (b) results from the addition of nitrogen to the feed-gas mixture. (Courtesy of Catledge *et al.*, 2002; reprinted with permission.)

the coatings at temperatures well above body temperature. Maintenance of low-temperature and a short-range ordering of water molecules at the nanostructure interface are critical to keep abrasive effects low and allow for macromolecular adsorption to the interface. Diamond, unlike other nanostructured coatings, seems to meet this challenge as evidenced by research conducted by Efthimios Kaxiras in the Department of Physics at Harvard University. His group demonstrated a remarkable increase in the melting point of water on the surface of nanostructured diamond (Wissner-Gross *et al.*, 2007 and Figure 5.6).

Nanostructured Hydroxyapatite Coatings

Some of the most chemically stable and therefore widely used materials in biomedical applications are **ceramics**, which are defined as inorganic, non-metallic solids prepared by the actions of heat and subsequent cooling. They may be crystalline in structure or amorphous, in which there is no long range order of the positioning of the atoms as in, for example, glass. The chemical properties of ceramics can be controlled during synthesis in such a way as to custom tailor the strength, hardness, resistivity and thermal expansion of the material to meet the needs of the particular application being addressed. Customization of the ceramic surface as porous, for example, has been shown in countless studies to promote tissue growth as a preferred three-dimensional interface for cellular adhesion, migration and division. Given ceramics' high compressive strength, they have been routinely used in dental and orthopedic applications such as the development and use of crowns, bridges, false teeth and hip prostheses.
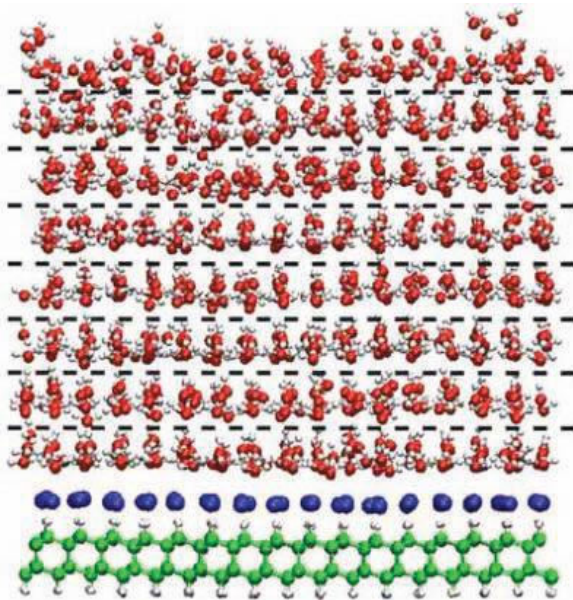
FIGURE 5.6 Diagrammatic illustration of a diamond/water interface. Water molecules (red and white) form orderly layers of ice on top of a layer of diamond (green) treated with sodium atoms (blue) in a simulation showing that such ice layers could persist well above body temperatures. Potentially, high temperature ice could make diamond coatings more suitable for implanted joints, heart valves, and other medical devices. (Courtesy of Alexander D. Wissner-Gross and Efthimios Kaxiras; reprinted with permission.)

With respect to the coating of biomedical implants, **hydroxyapatite (HA)** $[Ca_{10}(PO_4)_6(OH)_2]$ is the ceramic of choice (see also Figure 5.1). HA is a naturally occurring mineral and is the principle storage form of calcium and phosphorous in bone. It bears a striking resemblance to the mineralized structure of bone and has been shown to have high **osteoconductivity**, which is to promote bone formation proximal to an implant. Thin film deposition procedures adopted from the electronics industry and developed over the last ten years have now revealed the importance of nanostructured hydroxyapatite coatings. It is through these procedures that nanostructured HA coatings can now be made that mimic the surface and architectural features of bone. Nanometer-sized grains and a high volume fraction of grain boundaries can now be crafted from

HA, and these characteristics have been shown to increase osteoblasts adhesiveness, proliferation and mineralization. Other desirable effects of nanostructured HA implant coatings include high density, high abrasion resistance, durability and the ability to release calcium and phosphate ions which stimulate bone growth.

Ceramics such as HA are deposited on the surface of biomedical implants by a process known as **thermal spraying**, where a melted or heated material is literally sprayed onto a particular surface. Researchers at the Industrial Materials Institute, National Research Council in Canada have developed nanostructured HA coatings sprayed onto implants via the HVOF (high velocity oxy-fuel) process. HVOF generates higher **crystallinity** (lower degradation), which is the degree of structural order in a solid, and bond strength when compared to more conventional coatings, than other processes (Figure 5.7). The HVOF process uses high kinetic energy
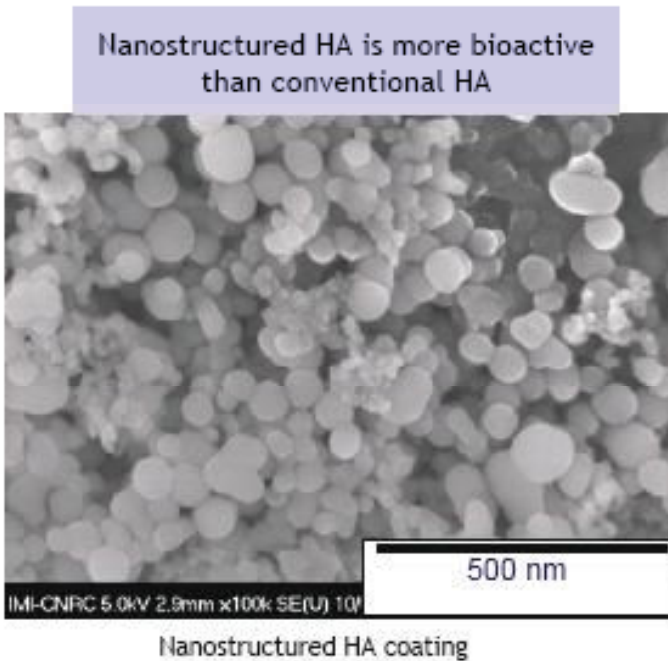
FIGURE 5.7 SEM image of a nanostructured hydroxyapatite coating. (Courtesy of the Industrial Materials Institute, National Research Council, Canada; reprinted with permission.)

and controlled thermal output to produce dense, low porosity coatings that exhibit considerable bond strengths, low oxide concentrations and extremely fine finishes.

Nanostructured Metalloceramic Coatings

In the orthopedic discipline of total joint replacements, long-term wear has been the number one issue affecting success rates. Over the past 30 years, metallic/plastic combinations such as polyethylene acetabular have been used yet wear has been cited as the critical weakness of devices made of this composite. As a result, researchers have begun evaluating alternative materials that address the issue of wear for total joint replacements. **Metalloceramics** are materials which contain both metallic and ceramic substances, the combination of which provides unique physical and structural properties. In this respect it has been discovered that combinations of certain ceramics and metals have superior wear characteristics compared to metal/plastic and other composites. Metallic materials are tough and **ductile** (capable of being hammered out as thin metals, malleable), while ceramic materials possess a combination of both covalent and ionic bonding. The issue here is a considerable adhesion problem when ceramic coatings are deposited on metallic substrates, primarily due to the difference in bonding types between the two materials. Thus researchers have now begun to use nanotechnology to develop metalloceramic materials which have a graded effect of metallic bonding at the substrate to covalent bonding at the coating surface. For example, researchers at the University of Alabama-Birmingham, in collaboration with Spire Corporation (Bedford, MA), have created a novel functionally graded nanocrystalline metalloceramic coating using a nanoprecise process known as **ion beam-assisted deposition (IBAD)**. IBAD is a materials engineering technique which combines ion implantation with simultaneous sputtering or another physical vapor deposition technique (Figure 5.8). It provides independent control of parameters such as ion energy, temperature and the rate of atomic species arrival during deposition. This level of control is invaluable for creating a gradual transition between a substrate nanomaterial and a deposited film.

The final nanomaterial has metallic inner layers and a hard ceramic outer coating. This type of material was designed specifically for Co-Cr-Mo components with a Cr/CrTi metallic layer at the interface which
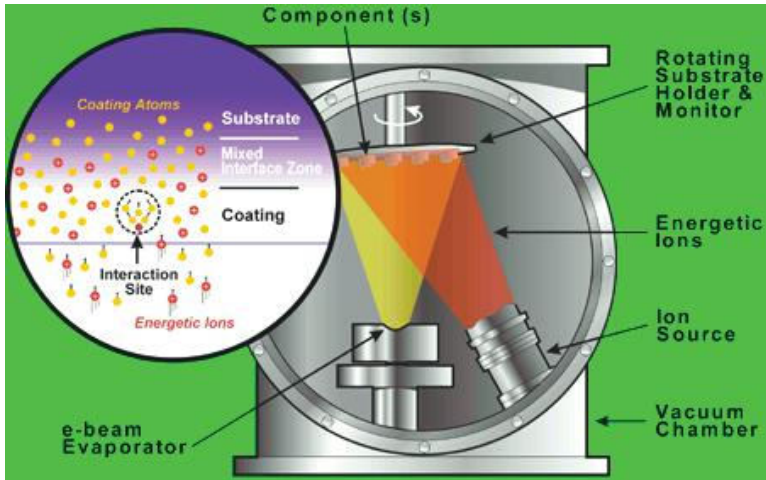
FIGURE 5.8 Schematic of ion beam-assisted deposition. The coating material is produced using a high-powered electron beam. Components to be coated are placed in a vapor of the coating material and individual atoms or molecules condense and stick to the surface. Highly energetic ions are simultaneously produced and directed at the component surface to complete the process. (Courtesy of Spire Biomedical Corporation; reprinted with permission.) Thus it is clear that nanostructured metalloceramics hold great promise.

was demonstrated to increase adhesion to the cobalt chrome substrate. Hardness was demonstrated to be much higher for coated vs. uncoated substrates (Figure 5.9).

In addition, the chromium metal layer increased adhesion between layers and increased the strength of the coating by serving as a barrier to crack formation and propagation. The advantages of this metalloceramic material over those more conventional are severalfold. A nanocrystalline structure with a grain size of around 10 nm provides an exceptional surface for cellular adsorption. The functionally graded layers in which bonding gradually changes from metallic to covalent enhance both the strength and toughness of the entire composite. Finally, the coating will have metallic adherence to other metals, but with significantly better wear resistance and a lower friction coefficient. Thus it is clear that nanostructured metalloceramics have great promise in the area of total joint replacement and will most likely impact other biomedical implants efforts as well.
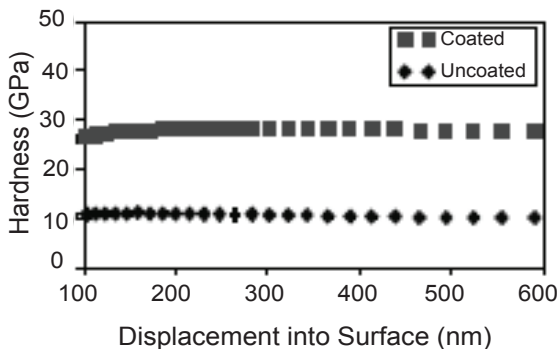
FIGURE 5.9 Nanoindentation hardness measurements of metalloceramic-coated and uncoated Co-Cr-Mo samples. Coated surfaces exhibit far greater hardness than those uncoated. (Courtesy of Catledge *et al.*, 2002; reprinted with permission.)

Nanopolymer Scaffolds

Nanostructuring is defined as the creation of nanosized physical properties throughout a material and can be accomplished by a number of methods including simply using nanoparticles themselves, e-beam evaporation, chemical etching, or lithography. The efficacy of bone growth on the resulting nanostructures depends on three primary properties including:

- **Topography**—affects cell colonization efficiency through attachment
- **Surface Chemistry**—affects both attachment and cell viability
- **Wettability**—generally more hydrophilic nanostructures work better in a biological environment

In addition to nanocoatings implemented to make implants biocompatible and increase biomimetic properties, nanostructuring of the bulk of the implant material has also been a major focus in recent years. **Nanopolymer scaffolds**, which are three-dimensional microstructures that are composed primarily of nanopolymers, have been developed that mimic the natural arrangement of minerals and are currently being used to create bone and tooth implants. In his research on bone formation in the presence of nanomaterials Dr. Thomas J. Webster and his team of scientists at Brown University's Division of Engineering and Orthopaedics have found that, in general, no matter what material chemistry is involved

FIGURE 5.10 Histology of rat calvaria (the skullcap) after implant of tantalum (Ta) scaffolds coated with either nanophase or conventional hydroxyapatite (HA). Red shows new bone infiltration which occurred in greater amounts on nano-HA-coated Ta than on either conventional HA-coated Ta or uncoated Ta. Left: Uncoated Ta scaffold; Middle: Conventional HA coated Ta scaffold; Right: Nano-HA coated TA scaffold (higher magnification). (Courtesy of Liu *et al.*, 2006; reprinted with permission.)

(ceramics, metals, polymers or composites) improvements in bone growth are seen when the material is nanostructured. Webster's group has focused much of their efforts on coated tantalum scaffolds (Liu *et al.*, 2006 and Figure 5.10). According to Webster's' group, the efficiency of bone regeneration is dependent upon implant material surface characteristics such as chemical composition and three-dimensional structure which primarily affect protein adsorption.

MINIMIZING SURGICAL DAMAGE

Nanopulses

In traditional, "open" surgery, it is necessary for the surgeon to cut through and thus damage healthy tissue in order to access the organ or area that requires surgical repair. No matter how minor the surgery, recovery times are often considerable after the performance of a traditional procedure. Thus doctors and researchers have been seeking ways to further minimize the invasive nature of surgery and have begun to focus on the use of electric fields in which **nanopulses**, defined as brief, nanosecond pulses of an external field, are directed at unwanted cells and/or tissues. For example, a team of researchers led by Karl Schoenbach of Old Dominion University and Stephen Beebe of Eastern Virginia Medical School are developing a method that could allow doctors to eliminate tumor cells present within the body, cell-by-cell through precise exposure to electric field nanopulses that result in a triggering of apoptosis (cellular suicide). The apoptotic

events are the result of caspase activation. **Caspases** are a family of cysteine protease enzymes which play crucial roles in the activation of apoptosis, in necrosis and inflammation. The technique involves exposure to gradients of megavolts per meter applied for nanoseconds at a time. The brevity of the pulses results in no damage to cell walls but wreaks havoc on intracellular organelles. This nanopulse system was demonstrated, through caspase activation, to kill tumor cells in tissue culture and slow tumor growth in mice, and represents a platform where no surgical intervention is needed (Nuccitelli *et al.*, 2006 and Figure 5.11).
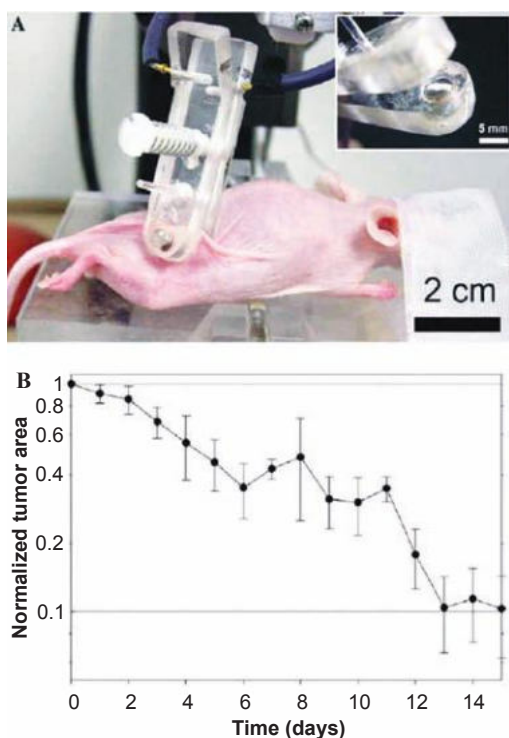
FIGURE 5.11 Nanosecond pulse tumor reduction. (A) Photograph of SKH-1 hairless mouse being treated with parallel plate electrode under isoflurane inhalation anesthesia. (Inset) Close-up of one of the plates of parallel plate electrode showing it recessed by 0.5 mm to allow a space for a conductive agar gel to be placed on it. (B) Mean change in normalized area of the transillumination image of six tumors from three mice treated with parallel plate electrodes using the same 4×100 pulse applications (3×100 on day 0 and 1×100 on day 4). Error bars indicate the SEM. (Courtesy of Nuccitelli *et al.*, 2006; reprinted with permission.)

Nanocoatings for Surgical Instruments

Surgical instrument design has always been in constant flux as surgeons continue to demand higher quality instrumentation that allows for minimal invasiveness and faster recovery rates. The combination of nanocrystalline materials, which have inherently desirable properties such as high strength and hardness, and amorphous metallic materials, which exhibit superior corrosion resistance, is of much focus in this area. A surgical instrument which possesses the properties of high corrosion resistivity along with significant wear protection is ideal, yet it is difficult to achieve high enough solidification rates to yield the desired properties for amorphous and nanocrystalline materials. Researchers at Praxair Surface Technologies in Indianapolis, Indiana have begun to address this by focusing on the use of detonation gun thermal spray technologies to marry these two materials as a coating on existing surgical instruments. The detonation gun thermal spray process involves the entrainment of powdered materials with the high velocity combustion products of a detonation wave as it propagates through a water-cooled barrel. A mixture of particles exits the barrel and impinges against a target substrate where the hot particles bond in overlaying platelets (Figure 5.12).

The researchers found that applying a mixture FeCrPC/nanoamorphous coating to numerous stainless steel surgical instruments (Figure 5.13) resulted in an almost three-fold increase in hardness and a significantly higher corrosion resistance.
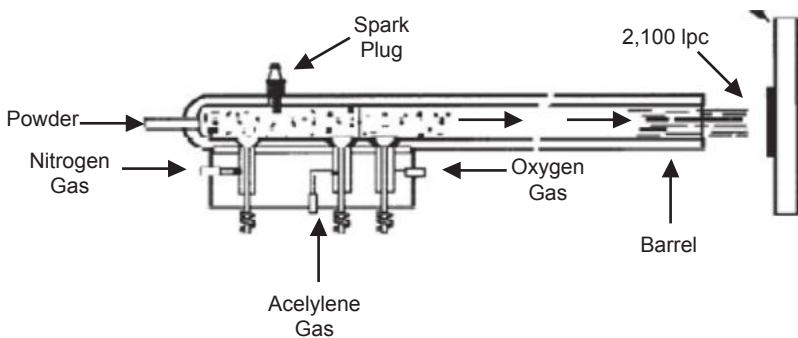
FIGURE 5.12 Schematic of the detonation gun process. (Courtesy of T. Shmyreva and J. Knapp, Praxair Surface Technologies and ASM International; reprinted with permission.)

FIGURE 5.13 Medical instruments with detonation coatings applied to working surfaces. (Courtesy of T. Shmyreva and J. Knapp, Praxair Surface Technologies and ASM International; reprinted with permission.)

POST-SURGICAL AND OTHER WOUND HEALING

It is clear that conventional surgery is quite traumatic on both the tissues being repaired and those surrounding the point of entry. Even the most precise surgical instrumentation is at best blunt in nature when viewed from the perspective of tissues and cells. Thus technologies which address this damage, either through minimization of it during the surgical procedure or during recovery are at the forefront of research in the medical field.

Point of Entry Repair

After any invasive surgical procedure it is advantageous to repair and stabilize the point of entry and underlying tissues to the greatest extent possible in order to promote rapid healing and minimization of scarring. For example, one of the oldest techniques for minimizing infection and the appearance of long-term scars is the use of sutures to close an open wound. A **suture** is a medical device that is employed to hold skin, internal organs and other tissues of the body together or in place following a laceration or surgical procedure. Sutures can be classified as either absorbable or non-absorbable. Absorbable sutures dissolve over time as the wound heals thus abrogating the need for removal. Non-absorbable must be removed after the majority of healing is complete, an often painful process. The most common types of sutures include nylon, polypropylene and vinyl, yet they can be fashioned from virtually any material that proves strong enough to maintain wound closure. Each

type of suture has advantages and disadvantages. Thinner sutures leave smaller holes in the skin yet lack the strength of thicker sutures, which tend to result in more visible scarring. Of importance to note is that, like the vast majority of foreign materials, most conventional sutures illicit some form of inflammatory response. Thus scientists and medical doctors have focused on addressing the weaknesses of conventional sutures. Some of this research has employed nanotechnology with specific examples described below.

Laser-Assisted Nanosutures

Case Study 5.1 below highlights the invention of **laser-assisted nanosuturing**, which is the application of a laser upon a material to create nanosized sutures. It is based on the use of light and chemistry to drive the bonding of tissues in order to promote healing and minimize scarring. It is a technological advance that will no doubt become common in the realm of post-surgical and laceration healing.

Nanofiber-Based Bandages

In addition to cutting-edge technological advances in the area of wound suturing, scientists and medical doctors are studying ways to increase the efficiency of healing through modifications in existing wound bandaging technology. Researchers Daniel Smith and Darrell Reneker at the University of Akron in Ohio created a urethane elastomer nanofiber-based bandage via electrospinning of nanofibers that is designed to allow for maximum oxygen penetration and exposure to a wound while still providing protection (Figure 5.14). The electrospinning process involves the addition of chemical precursors for nitric oxide (NO), which become encapsulated in the fibers as part of the manufacturing process. Upon activation by water, the bandage produces nitric oxide gas at concentrations regulated by the polymer identity. **Nitric oxide** gas is well-known to counter inflammatory responses and kill parasites and the high surface area to volume ratio of the nanofibers results in effective delivery of the medication. It accomplishes this by inducing DNA damage and degradation of iron-sulfur centers into iron ions and iron-nitrosyl compounds in bacteria.

Self-assembling peptide-based nanofibers have also been employed as wound dressings and confirmed to aid in the healing of burns in rodent models. Researchers in the Laboratory of Molecular Self-Assembly at

Case Study 5.1: Evaluation of photochemical tissue bonding for closure of skin incisions and excisions

(Photo courtesy of Harvard University)

Irene Kochevar and Robert Redmond, clinical researchers at the Wellman Center for Photomedicine at Massachusetts General Hospital in Boston, have developed a laser-based photochemical suturing technique that is showing much promise in pig animal laceration models. The procedure entails the use of a collagen fiber-based dye, **Rose Bengal** (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein), applied to the area to be sutured that is subsequently exposed to a potassium-titanyl-phosphate (KTP) laser. The laser activates fusion of the dye molecules/collagen fibers in the form of nanosutures. The advantages of this system are significant and include reduced inflammatory responses, reduced chances of post-suturing infection due to a lack of openings in the skin and less scarring compared to the use of traditional sutures.

MIT's Center for Biomedical Engineering have employed the RADA16-1 peptide, which consists of 16 alternating hydrophobic and hydrophilic amino acids and forms extremely stable β-**pleated sheets**. β-pleated sheets are a type of secondary protein structure consisting of strands of amino acids interconnected by five or more hydrogen bonds forming a twisted pleated sheet of protein that is stable under a variety of environmental conditions (Figure 5.15).

As discussed in Chapter 3, Christophe Egles' group in the Division of Cancer Biology and Tissue Engineering at Tufts University in Boston have applied RADA16-1 peptide-based β-pleated sheets to create nanofiber scaffolds loaded with Epidermal Growth Factor (EGF). These have now

FIGURE 5.14 Nanofiber wound dressing. (Left) Prior to application. (Right) Post application. (Courtesy of D. Smith and D. Reneker, University of Akron; reprinted with permission.)

been shown to accelerate wound healing in a bioengineered human skin equivalent (HSE) tissue model. The researchers demonstrated that the peptides underwent molecular self-assembly to form unique three-dimensional structures at the wound site, stably covering it and
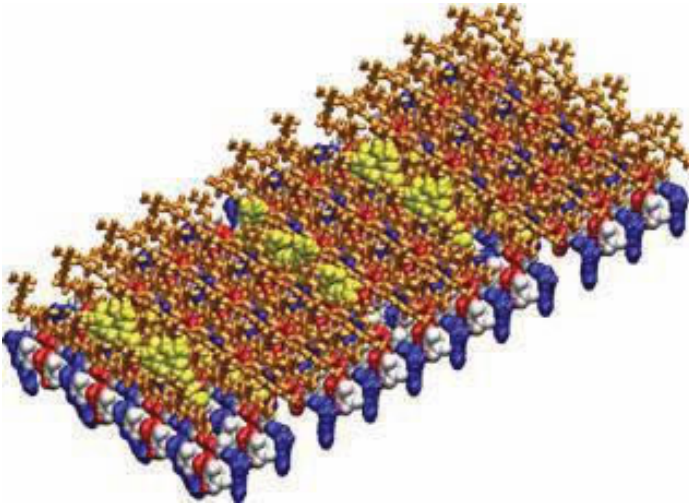
FIGURE 5.15 Molecular illustration of a RADA16-1 peptide β-pleated sheet. (Courtesy of the Laboratory of Molecular Self-Assembly at MIT; reprinted with permission.)
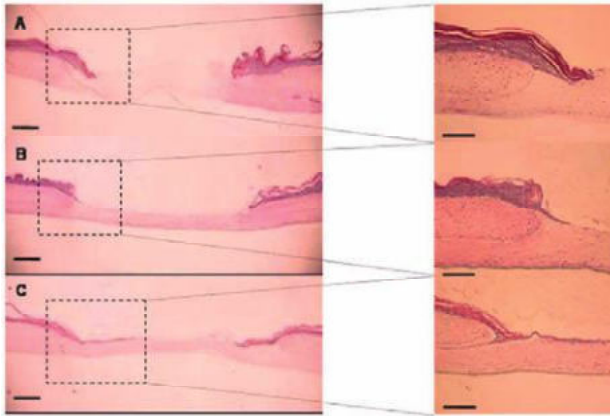
FIGURE 5.16 RADA16-1 peptide nanoscaffolds promotes wound healing. (A, B, C) Hematoxylin and eosin staining of the wound after 48 hours. (A) Control tissues where nothing is added on top of the wound. (B) Wound covered with a drop of RADA16-I. (C) Wound covered with a drop of peptide solution containing the growth factor EGF. The scale bar is 500 μm for wounds. For the inserts the scale bar is 100 μm. (Courtesy of Schneider *et al.*, 2008; reprinted with permission.)

releasing EGF which promoted re-epithelialization (Schneider *et al.*, 2008 and Figure 5.16).

Antisepsis

Sepsis, which is defined as an inflammatory state due to a known or suspected infection, is a major concern for surgeons following a surgical procedure and can wreak havoc on other types of open wounds such as lacerations. While many surgical procedures are routine and performed under relatively controlled conditions, the secondary risk of infection has plagued surgeons since the first operation was performed. In the United States alone over 2 million individuals acquire an infection post-surgery and 90,000 of these people die as a result. This number is much higher in third-world and developing nations due to a lack of basic hygienic conditions. Thus the development of new antiseptic technologies that reduce the possibility of post-surgical infection is a primary focus of the medical community. Two types of nanotechnological advancements that

could aid in this area, antibiotic nanocoatings and nanosilver, are described below.

Antibiotic Nanocoatings

Marek Urban and colleagues at the University of Southern Mississippi focus their research on the use of nanotechnology to deposit the antibiotic penicillin directly onto the surfaces of surgical instruments with the premise that the presence of penicillin will act as a real-time sterilization agent during and post-surgery. Thus this technology could also be classified as another example of a nanocoating, yet its purpose is not biocompatibility but rather anti-bacterial in nature. The researchers developed a nanotechnology-based method for modifying the surface of surgical instruments in a manner such that they were conducive to the adherence of penicillin molecules. By creating nanosized "spacer molecules" of varying lengths on the surface of the instruments penicillin was able to be efficiently attached. The polymer utilized for the study was polytetrafluoroethylene (ePTFE) commonly used in vascular graft prostheses, heart patches or staple prostheses. Modification of the polymer was accomplished by microwave plasma reactions in the presence of maleic anhydride (MA) which drove the creation of functional groups on the polymer surface. This reaction was followed by esterification reactions in the presence of polyethylene glycol (PEG) and the resulting surface utilized for the attachment of penicillin molecules (Aumsuwan *et al.*, 2007 and Figure 5.17). **Esterification** is defined as a chemical reaction resulting in at least one **ester product**, which is a compound produced by the reaction between an acid and an alcohol with the elimination of a molecule of water. The combination of reactions, in addition to providing attachment sites for penicillin, resulted in morphological surface changes that increased the area that could be in contact with bacteria. In addition, the spongy surface of the instrument mimicked a biological environment that promoted bacterial (*Staphylococcus aureus*) adhesion and thus its elimination due to the presence of penicillin molecules (Aumsuwan *et al.*, 2007 and Figure 5.18). The authors have suggested that this antibiotic-modified polymer surface could be coated on surgical instruments as well as implants to reduce bacterial infection rates in patients.
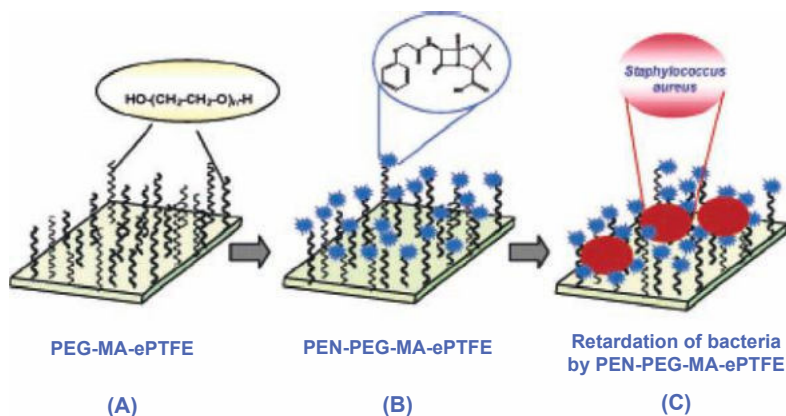
FIGURE 5.17 Representation of ePTFE surface modifications. (A) PEG-MA-ePTFE, (B) PEN-PEG-MA-ePTFE, and (C) retardation of bacteria by PEN-PEG-MA-ePTFE. (Courtesy of Aumsuwan *et al.*, 2007; reprinted with permission.)
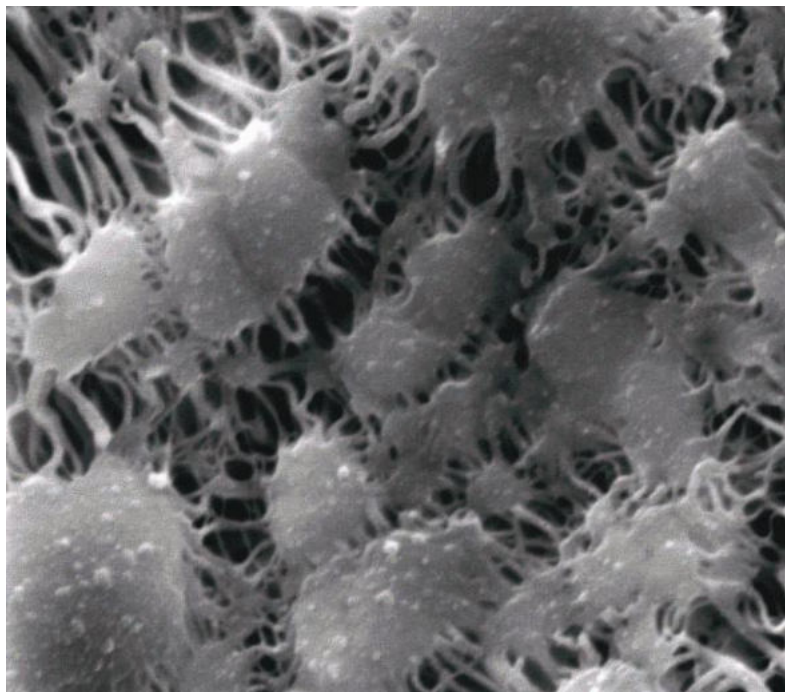
FIGURE 5.18 SEM image of a polymer surface designed for penicillin attachment and bacterial recruitment. (Courtesy of Aumsuwan *et al.*, 2007; reprinted with permission.)

Nanosilver

Nanoparticles have also been studied for their anti-infective properties. Metallic silver is known to be effective against infection from a wide range of bacteria and other microorganisms. It was introduced as an antimicrobial agent in the early 20th century and can be traced back to its first use in the 18th century, during which silver nitrate ($AgNO_3$) was used in the treatment of ulcers. Its use in this respect began to decline after the advent of antibiotics in the 1940s. Recently, however, topical use of silver to combat infection has gained popularity, particularly in the management of open wounds. This use has expanded to local applications post-surgery. The mechanism of silver toxicity on biological foreign invaders is based upon the bioactivity of silver ions (Ag^+) on enzymatic function. Silver ions bind various enzymes within a host bacterial, fungus or virally infected cell, inhibit their function and thus prevent such critical processes and oxygen uptake/metabolism or DNA synthesis (Figure 5.19).

Yet researchers have long sought ways to improve the efficiency of silver particles anti-infective properties and have thus turned their attention to silver nanoparticles. The extremely small size of nanoparticles, in the case of silver between 50 nm and 100 nm in diameter, results in a large surface area to volume ratio allowing them to more easily interact with pathogens. This has been shown to increase the overall efficiency of silver nanoparticle microbial killing. Researchers have estimated, for example, that one gram of silver nanoparticles yields enough antibacterial strength to effectively sterilize one hundred square meters of substrate material. A number of companies are now

Anti-microbial mechanism of silver ions.

As silver ions are taken into microbes, they react and bond to the cellular enzyme microbes. This inhibits enzyme activity and multiplication of microbes, thus extinguishing the microbes.
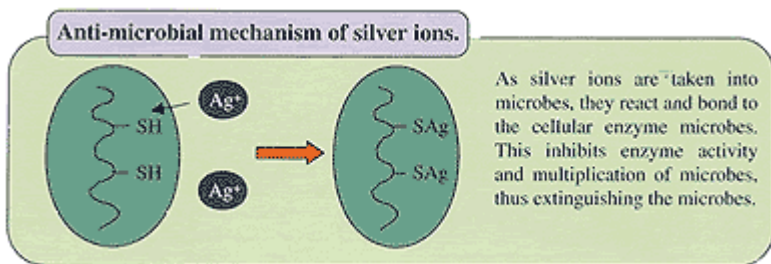
FIGURE 5.19 Silver anti-microbial mechanism of action. (Courtesy of Marubeni America Corporation; reprinted with permission.)

developing silver nanoparticles for wound dressing and post-surgical antiseptic applications. Through a process of ion bombardment of pure silver under vacuum followed by silver atom condensation on polyethylene substrates, Nucryst Pharmaceuticals, Inc., headquartered in Princeton, N.J., has created nanosilver-based wound dressings. Using this platform Dr. Robert Burrell's group discovered that nanocrystalline silver exhibited both antimicrobial and anti-inflammatory properties far superior to that of typical silver powders, although the mechanism of action is unknown (see Focus Box 5.1). The nanocrystalline silver enters the wound or post-surgical area through body fluids and can reportedly kill bacteria within thirty minutes after application and can last for several days depending upon the type of application. Researchers at Nucryst claim that the higher energy state of their nanosilver crystalline platform results in increased solubility thereby allowing better access to the site of injury. Other companies have chosen to couple silver and other nanoparticles to increase the concentration of silver atoms at a particular site of application. Nanosilva, LLC of Ocala, Florida has developed a silica-silver nanoparticle technology that aggressively dissociates molecular oxygen upon contact between O_2 and Ag nanoparticles. This results in considerable antimicrobial activity and has been applied for use in wound care, surgical equipment coating, implants, medical devices and prostheses (Figure 5.20).

Focus Box 5.1 Robert Burrell and nanosilver for wound care

Dr. Robert Burrell developed what is now believed to be the world's first commercial use of therapeutic nanotechnology (nanoAg). He began his research in 1991 after pondering the antimicrobial effects of silver and in 1995 made a major breakthrough with respect to the design of nanocrystalline silver wound dressings. At Westaim Inc. he developed Acticoat, a nanosilver-based wound dressing that speeds healing and reduces scar tissue formation. It is often used in burn units and is sold around the world by Smith and Nephew Plc. Dr. Burrell is currently Professor and Chair, Biomedical Engineering, CRC in Nanostructured Biomaterials, University of Alberta, Edmonton, Alberta. (Photo courtesy of the University of Alberta; reprinted with permission.)
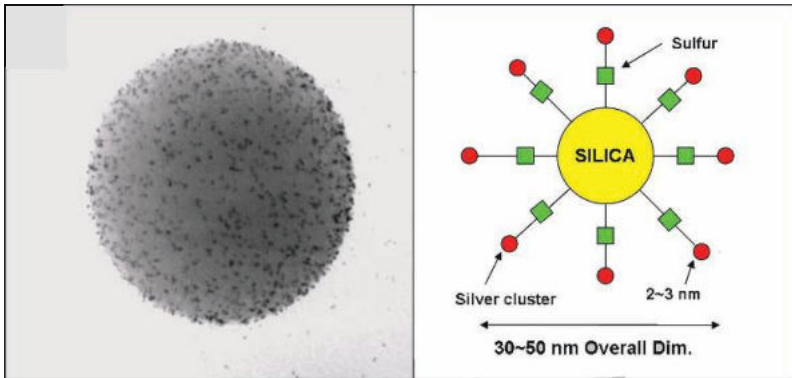
FIGURE 5.20 Silver-silica nanoparticles. (Left) transmission electron microscope image of a Nanosilva nanoparticle. (Right) Diagram of the complex nanoparticle. (Courtesy of Nanosilva, LLC; reprinted with permission.)

Silver nanoparticles may also be implemented as anti-viral therapies. In perhaps one of the most exciting studies regarding silver nanoparticles and potential anti-viral effects, a team of researchers at the University of Texas, Austin led by Miguel Jose Yacaman generated silver nanoparticles with an average diameter as small as 1 nm–10 nm using a mixing procedure with capping agents such as bovine serum albumin (BSA) to prevent large crystal synthesis and promote the formation of individual nanocrystals. The researchers demonstrated a size-dependent interaction with HIV-1 and suggested that the binding was due to preferential sulfur-bearing knobs of the **gp120 viral glycoprotein**, which is a glycoprotein expressed on the surface of HIV particles that plays a crucial role in viral binding to and entry into a cell. Intriguingly, silver nanoparticle binding to HIV-1 inhibited viral binding to host cells *in vitro*, most likely through a blockage of binding to host cells (Elechiguerra *et al*., 2005 and Figure 5.21).

INTRACELLULAR NANOSURGERY

The precise manipulation of eukaryotic cells at the level of the organelle, or **intracellular nanosurgery**, has been an important goal in the field of cell biology for a number of decades. The ability to control the biological function or alter the location of various intracellular structures such as mitochondria, vesicular traffic or perhaps even at the level of
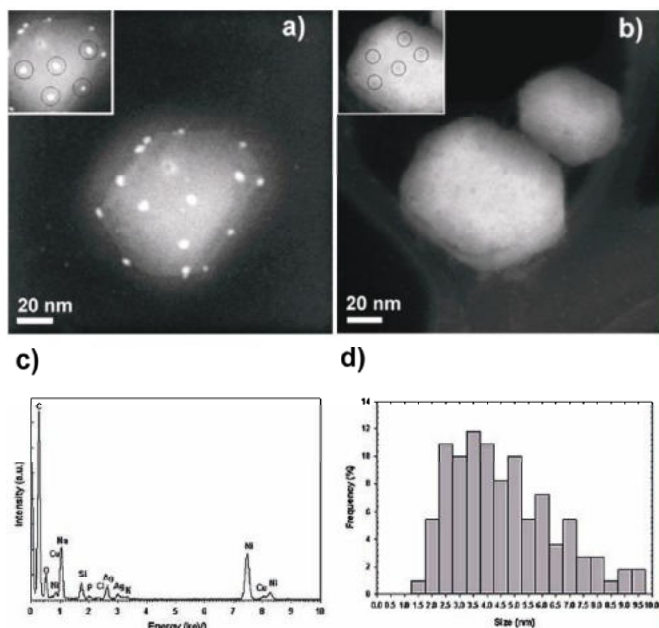
FIGURE 5.21 Silver nanoparticles and HIV-1. (a) HAADF image of an HIV-1 virus exposed to BSA-conjugated silver nanoparticles. Inset shows the regular spatial arrangement between groups of three nanoparticles. (b) HAADF image of HIV-1 viruses without silver nanoparticle treatment. Inset highlight the regular spatial arrangement observed on the surface of the untreated HIV-1 virus. (c) EDS analysis of image a) confirming the presence of Ag. The C signal comes from both the TEM grid and the virus, O, and P are from the virus, and Na, Cl, and K are present in the culture medium. Ni and Si come from the TEM grid, while Cu is attributed to the sample holder. (d) Composite size distribution of silver nanoparticles bound to the HIV-1 virus, derived from all tested preparations. (Courtesy of Elechiguerra *et al*., 2005; reprinted with permission.)

individual genes within the nucleus will drive a new era of research in cell biology (more detail on this futuristic concept is included in Chapter 10). Described below are some of the laser-based and non-laser-based techniques and instrumentation that have emerged in this area in recent years.

Laser-Based Nanosurgery

A **laser** (Light Amplification by Stimulated Emission of Radiation) employs a mechanism for emitting electromagnetic radiation, typically light via a

process of stimulated emission. The emitted light is usually a narrow beam with low divergence that can be adjusted and focused with optical lenses. It usually consists of **coherent light**, which is defined as light composed of in-step waves of identical phase and frequency. It is this coherence which gives laser light its intensity. In order to understand how laser energy might affect cells or tissues it is crucial to have a basic understanding of laser design and physical properties. A laser is composed of a **gain medium**, the source of optical gain, in an optical cavity which drives the gain, or increase in power of emitted light by transitioning the light from a higher to lower energy state. The optical cavity is primarily composed of two mirrors arranged to bounce light back and forth, each time passing it through the gain medium. As one of the mirrors, the **output coupler**, is partially transparent, the desired laser beam is strategically emitted through this mirror (Figure 5.22). Energy required for the amplification is known as **pumping** and is typically supplied as either light of a different wavelength or as an electrical current. The laser's final output of light may be either continuous wave (CW) or pulsed, with the pulsed version achieving much higher peak powers. It is the pulsed version which is most widely used in medical and biological applications due to the precise level of control and higher energy values achieved.

Focus Box 5.2 Theodore Maiman and the invention of the laser

Theodore "Ted" Maiman (1927–2007) was an American physicist who is credited with the invention of the first laser. Born in Los Angeles, California, he earned money during his teens repairing radios and other electrical appliances before pursuing his undergraduate degree at the University of Colorado and doctorate at Stanford. He developed the world's first functional prototype laser at Hughes Research Laboratories in Malibu, California on May 16, 1960 by shining a high-powered flash lamp on a rod made of ruby and coated with silver. Interestingly, the discovery was rejected as of little significance by the journal Physical Review Letters, but later accepted by the highly regarded journal Nature. (Photo courtesy of Bettman/Corbis; reprinted with permission.)
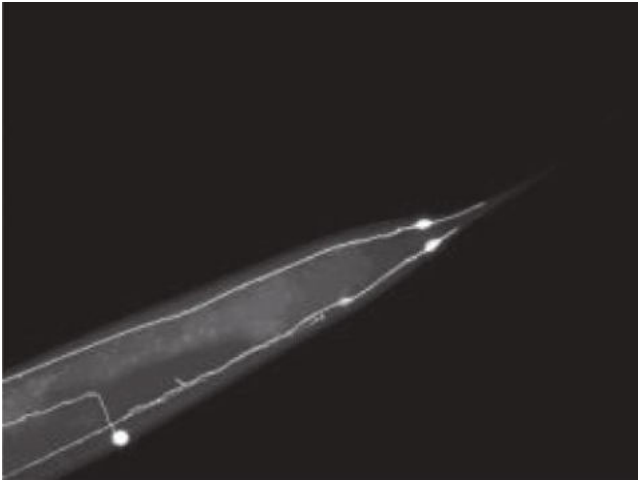
FIGURE 5.22 Laser surgery with living C. elegans. One worm is singled out in this image. (Courtesy of Craig Millman and Yanik Lab; reprinted with permission.)

Laser nanosurgery is defined as the use of ultrafast laser pulses to permit the precise ablation of cellular and subcellular structures. It may be applied in an *in vitro* or *in vivo* setting for basic research studies or therapeutic applications. A primary goal behind this precision is to minimize collateral damage and reduce the amount of compromised cellular viability. Recent advances in this technology have now allowed for the ability to dissect cells at the level of the intracellular organelles and thus characterize in real time the biological processes occurring within the cell's cytoplasm and nucleus. The use of laser nanosurgery to deliver exogenous materials to cells in developing embryos has also been demonstrated and is widely used in certain research fields such as for the production of genetically engineered mice (Kohli *et al.*, 2007 and Figure 5.23).

The two primary types of lasers utilized in laser-based nanosurgery include near-infrared (near IR) and pulsed ultraviolet (UV) lasers (Figure 5.24). Due to its ability to precisely control ablation effects, it is the latter that has received a great deal of attention to date with respect to nanoprecise ablation of subcellular structures.

Ultrafast laser nanosurgery is a process that uses very brief picoseconds and femtosecond laser pulses to ablate unwanted tissue. The primary advantage of ultrafast laser nanosurgery is that it allows for the
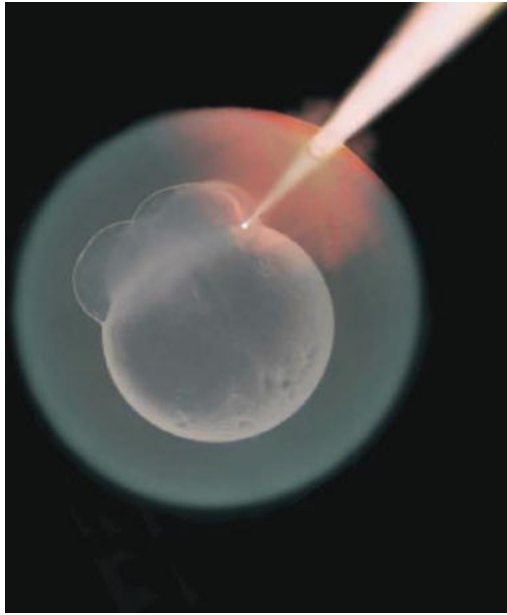
FIGURE 5.23 Laser surgery with living embryonic cells. Femtosecond laser pulses were focused beyond the chorion (the outer layer) and localized near or on the blastomere cells. With this method, the chorion remained intact. (Courtesy of Kohli *et al.*, 2007; reprinted with permission.)
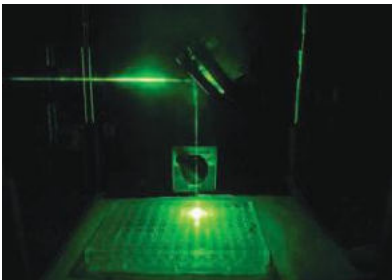
FIGURE 5.24 Examples of laser systems used in cell culture studies. (Left) Cells in a multi-well culture plate exposed to a near IR laser. (Right) Researcher exposing cells to UV laser pulses. (Left photo courtesy of Rod Rolle, the University of California, Santa Barbara; right photo courtesy Gwen Garden, the Center on Human Development and Disability at the University of Washington; reprinted with permission.)

evaporation of extremely small volumes of tissue with only very minimal heating or damage to the surrounding cells and tissues. The extent of tissue damage and ablation is dependent upon a number of factors including the laser's **pulse intensity**, defined as energy per area per unit time, the total number of pulses administered and the repetition rate of the laser. Interestingly, the wavelength of the laser appears to only have a slight affect on the efficiency of tissue or cellular ablation. The mechanism of action is based on the production of free electrons in tissues or cells after energy absorption through three primary processes which include multi-photon ionization, electron tunneling and cascade ionization. **Ionization** in this sense is defined as the formation of or separation of ions by heat. It is the density of the resulting free electrons which determines the tissue damage mechanism. Usually one of three different mechanisms of damage result in the desired effect and are outlined in Table 5.2 below.

Although an emerging technology, the use of ultrafast laser nanosurgery to study cells at the nanoscale is rapidly becoming routine in the field of cell biology. Eric Mazur, a Harvard University physicist, has adapted the use of laser in cell culture studies to manipulate cells with nanoprecision using femtosecond (one-quadrillionth of a second) bursts. Mazur and

Table 5.2 Damage mechanisms in ultrafast laser nanosurgery (Ben-Yakar *et al.*, 2009)

	Photochemical	Thermoelastic Stress Confinement	Plasma-mediated Ablation
Intensity Threshold	0.26×1012 W/cm^2	5.1×1012 W/cm^2	6.54×1012 W/cm^2
Mechanism of Damage	Free electrons participated in chemical reactions to form destructive reactive oxygen species (ROS) and lead to breaking of chemical bonds	Confinement of thermal stresses leads to formation of nanoscale transient bubbles	High pressure and high temperature plasma drives shock waves and cavitations
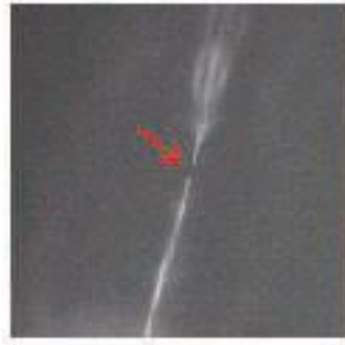
FIGURE 5.25 A single strand in the nerve cell of a tiny flatworm is cut using femtosecond nanopulses, removing the worm's sense of smell. (Courtesy of E. Masur and Harvard University; reprinted with permission.)

colleagues demonstrated that laser bursts delivered at nanosecond intervals could target and disrupt specific intracellular organelles such as a single mitochondrion within a mouse cell. His group used this system to sever a single nerve cell in a living flatworm, thus removing the worm's sense of smell (Figure 5.25).

Microfluidics is a scientific discipline that deals with the behavior, precise control and manipulation of fluids that are geometrically constrained to a small, sub-millimeter scale. Applications to study or manipulate biological systems using microfluidics approaches began at the cellular level and have now evolved to address more complex biological systems such as eggs, embryos and larvae. These systems have also yielded new opportunities for the close monitoring of changes in whole cell cultures in response to stimuli, including genome-wide changes in gene expression.

Microfluidics offers a number of advantages over other systems for monitoring biological samples that are in limiting amounts. These include small dimensions on the order of microns in size which provide both precise and rapid manipulation of samples and reagents which are often in limiting quantity; transparency of the materials making optical imaging possible; scalability due to the small nature of the system and affordability which is the result of cheap microfabrication techniques. Perhaps the most important property of microfluidic systems with respect to ultrafast laser nanosurgery is the ability to interface with robotic handling technologies that implement the use of microtiter plates. This

Focus Box 5.3 Adela Ben-Yakar and ultrafast laser nanosurgery microfluidics

Dr. Adela Ben-Yakar, an Associate Professor in the Department of Mechanical Engineering at the University of Texas, Austin has combined the fields of ultrafast laser nanosurgery and microfluidics to develop cutting-edge instrumental platforms for the study of nerve regeneration and the dissection of genome-wide changes in gene transcription as it relates to various biological processes. See *Case Study* 5.2 for details. Her research also spans the development of technologies and systems for laser endoscopy and laser micromachining. (Photo courtesy of the University of Texas at Austin; reprinted with permission.)

allows for the screening of large sample populations for phenotypic and possibly even genotypic changes pre- and post-treatment. It also allows for the trapping of bio-particles in a fluid environment. Researchers at the Microfluidics Laboratory of National Taiwan University, for example, have developed a fully integrated microfluidics device that allows for the non-invasive hydrodynamic trapping of bio-particles. In this device, suspended particles traveling above an active oscillator can be trapped without any solid contact. Trapping efficiency can be optimized by varying the flow rate of voltage applied. The device was used to enrich antibody concentration.

The combination of microfluidics with laser nanosurgical technologies has recently opened the door to high-throughput analysis and characterization of biological samples both before and after laser application. *Case Study 5.2* below highlights some of the recent advances in this area as they apply to the study of axonal regeneration in the nematode *C. elegans*. Systems like the one described allow for the opportunity to rapidly accumulate large datasets on *in vivo*-based manipulation of single cells. In this case, it was discovered that axonal regeneration post-neuroaxotomy occurs much faster than anticipated.

Case Study 5.2: Femtosecond laser nanoaxotomy lab-on-a-chip for in vivo nerve regeneration studies

The study of axonal regeneration in the nematode *C. elegans* requires precise **nanoaxotomy**, which is the process of surgically severing an axon at the nanoscale. Researchers in the Department of Mechanical Engineering at the University of Texas, Austin (see Focus Box 5.3) have developed a microfluidics device that enables the severing of axons using ultra-short laser pulses with high precision while simultaneously monitoring subsequent axonal regeneration activity. The unique features include an adjustable trap customizable for the size of the worms and system-integrated feeding modules that allow for the long-term follow-up study of axotomized worms post-nanosurgery. A two-layer microfluidic approach was used with top-layer pressurization after worm loading to immobilize each specimen. After neuroaxotomy the pressure is released and the worms are released into a feeding chamber for further study (Guo *et al*., 2008 and Figure 5.26).
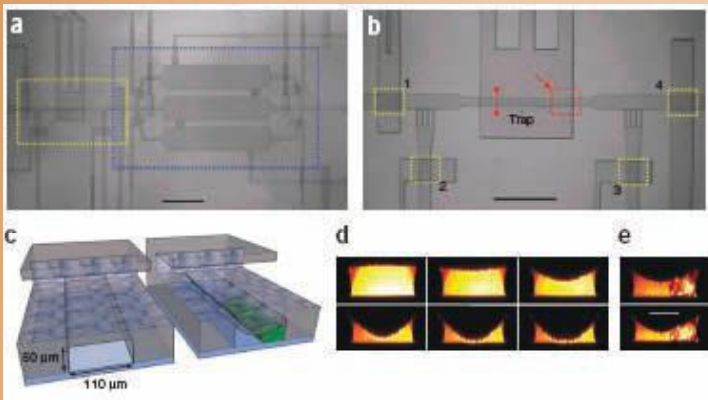
FIGURE 5.26 The nanoaxotomy lab-on-a-chip. (a) Overview of the chip with the trap system (yellow rectangle) and three recovery chambers (blue rectangle). (b) Magnified view of the trapping system (yellow rectangle in a). Valves 1–4 (yellow rectangles) respectively control inlet regulation, fine positioning of the worm (2 and 3) and gating to the recovery chambers. (c) Conceptual three-dimensional section renderings of the bilayer trap channels without and with an immobilized worm. (d) Two-photon images of cross-sectional profiles of the micro-channel in the trap area for increasing air pressures from 0 to 35, 70, 105, 140 and 175 kPa. (e) Cross-sectional two-photon images of a trapped worm at 105 and 140 kPa. Scale bars, 2 mm (a), 1 mm (b) and 50 mm (e). (Courtesy of Guo *et al*., 2008; reprinted with permission.)
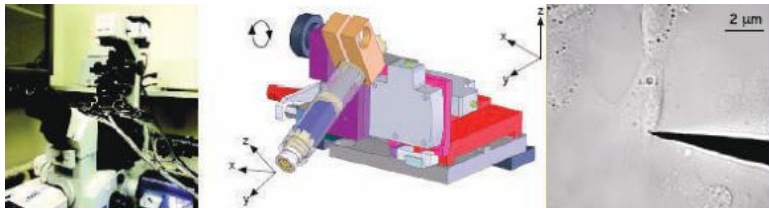
FIGURE 5.27 L200 nanomanipulation system and its application in nanosurgery. Installed on a Nikon Model TE2000 inverted microscope (left), drawing of coarse and fine positioners with axes depiction (middle) and probe insertion into a cytoplasmic sucrosome. (Courtesy of Meghana Honnatti and Gareth Hughes, Zyvex Labs; reprinted with permission.)

Non-Laser-Based Intracellular Nanosurgery

In addition to instrumental systems for applications in laser nanosurgery, there are a variety of other instrument-based platforms that enable the nanoprecise dissection or manipulation of cells at the level of organelles and which do not involve the use of lasers. Richardson, Texas-based Zyvex Labs, for example, developed the L200 system for nanoprecise manipulation of intracellular organelles. It provides the capability of positioning surgical tools, probes, electrodes or pipettes within close proximity of the cell. The resolution of nanomanipulation is extremely accurate, allowing researchers to probes various regions of the cell with nanoprecise accuracy (Figure 5.27). The development of

---

Focus Box 5.4 Babak Ziaie, "ferropaper" and micromotors

Researcher Babak Ziaie at Purdue University has developed an iron-based paper that might be used to create micromotors for the next generation of minimally invasive surgical instruments. A mixture of mineral oils and magnetic iron oxide nanoparticles impregnated on paper has resulted in a biologically-compatible micro-material that can be moved or caused to vibrate with a magnetic field. The invention could allow for the design of motors for micro-sized tweezers to manipulate individual cells or for flexible fingers to perform minimally invasive surgery. (Photo courtesy of Andrew Hancock, Purdue University; reprinted with permission.)

micromotors for the precise manipulation of micro- or nano-sized surgical instruments that are capable of performing surgery at the cellular level has also received much attention (see Focus Box 5.4)

Atomic force microscope (AFM) manipulation technology has also been successfully applied to cause intracellular organelle displacement or alteration. A team of researchers led by Chikashi Nakamura at the
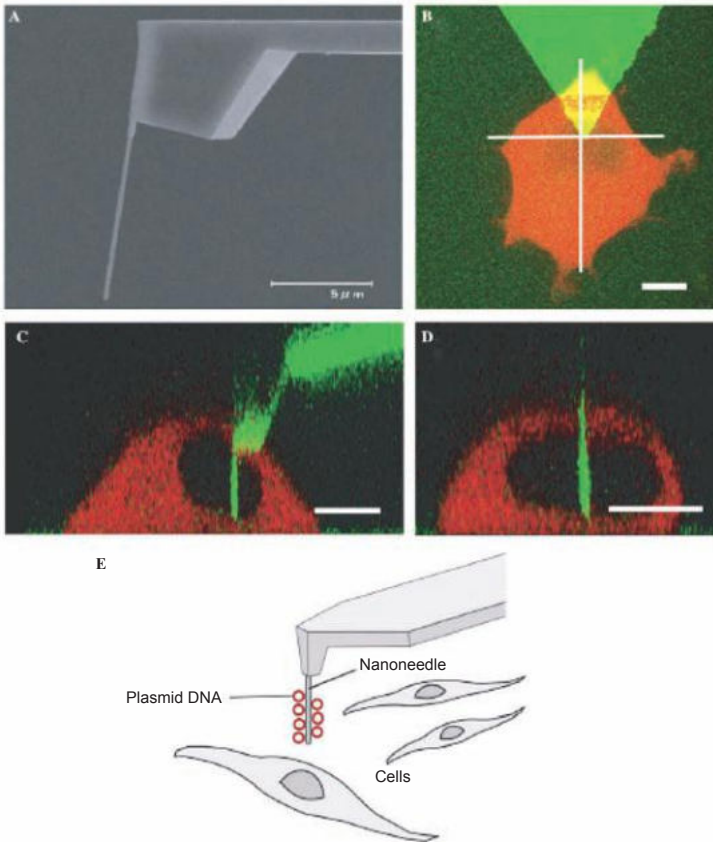
FIGURE 5.28 Fabricated nanoneedle inserted into a living cell. (A) An SEM image of the nanoneedle. (B) Stack of confocal slices of MCF-7 cells expressing DsRed2-NES excited at 543 nm and a FITC-labeled nanoneedle excited at 488 nm when the nanoneedle was inserted. (C) Cross-sectional image of green and red emissions processed from the confocal slices. (D) Vertical-sectional image of green and red emissions processed from the confocal slices. (E) Schematic representation of a single cell manipulation using AFM and a nanoneedle. Scale bars for all images indicate 5 microns. (Courtesy of Han *et al.*, 2005; reprinted with permission.)

Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology in Amagasaki, Japan have developed an AFM-based system that allows for the delivery of exogenous material into human cells over a long period without causing the death of the cells as would be the case for bulkier microinjection instrumentation. Specifically, a "**nanoneedle**" was etched from the tip of an AFM probe utilizing a focused ion beam (FIB) and sharpened to produce a variety of needs from 200 nm to 800 nm in diameter. These needles could be inserted directly into cells and utilized for the introduction of exogenous genetic material (plasmid DNA) attached to the sides of the needle (Han *et al.*, 2005 and Figure 5.28). It was found that needles etched to 400 nm and below in diameter yielded both superior long-term cell viability and exogenous gene expression compared to controls such as glass needles. No irreversible damage to the plasma membrane was observed, even after incubation of the cells with inserted nanoneedles for longer than one hour. This suggests a possible use of nanotechnology in the fields of cell biology and embryology, specifically with respect to improvements in microinjection survivability.

CHAPTER SUMMARY

Implant and Surgical Instrument Design

1. Implants are manufactured using metals, polymers, ceramics and composites and must adhere to strict biocompatibility and biomimicry guidelines.
2. Nanocoatings have been extensively studied for use in the implant industry due to their unique physical characteristics.
3. Proper protein adsorption to nanomaterial coatings is key to their effective use in implant design.
4. Integrin receptors on cell surfaces bind to ECM proteins such as fibronectin through recognition of the RGD amino acid sequence.
5. Nanomaterials have unique surface energetics that may aid in the binding of cell surface receptor proteins to nano-coated implants.
6. Electrolysis may be used to deposit nanoparticles on implant or surgical instrument surface.
7. The three primary nanomaterials used in coating implants are diamond, hydroxyapatite and metalloceramics.
8. Newly developed diamond nanocoatings exhibit unique combinations of hardness, toughness, low friction and corrosive resistivity.

9. Chemical vapor deposition is the most widely used technique for producing hard carbon nanocoatings.

10. The chemical properties of ceramics can be tightly controlled during synthesis to customize strength, hardness, resistivity and thermal expansion.

11. Hydroxyapatite (HA) is the preferred ceramic for biomedical implant coating as it is naturally occurring and mimics the mineralized structure of bone.

12. Nanometer-sized grains can now be crafted from HA which promote osteoconductivity.

13. Thermal spraying is the process of choice for coating ceramics onto the surface of biomedical implants.

14. Metalloceramics have unique physical and structural properties that promote superior wear characteristics.

15. Nanotechnology is now being used to create metalloceramic materials which have a graded effect of metallic bonding at the substrate to covalent bonding at the coating surface.

16. The efficiency of bone growth on nanostructures depends on topography, surface chemistry and wettability.

17. Nanopolymer scaffolds that mimic the natural arrangement of bone and tooth minerals have been designed to encompass the bulk of biomedical implant material.

Minimizing Surgical Damage

1. Traditional "open" surgery most often results in considerable recovery times and scarring.

2. Nanopulses trigger apoptosis and could in some cases replace traditional surgical removal of tumors.

3. Apoptosis triggered by nanopulses is the result of caspase activation.

4. Detonation gun thermal spray may be used to efficiently coat nanocrystalline/nanoamorphous materials on stainless steel surgical instruments.

Post-surgical and Other Wound Healing

1. The most common types of sutures are either absorbable or non-absorbable and include nylon, polypropylene and vinyl.

2. Laser-assisted nanosuturing (photochemical bonding) involves both the use of light and chemistry to drive the bonding of tissues with a fiber-based substrate.

3. Electrospun nanofiber bandages containing nitric oxide precursors allow for maximum oxygen penetration to a wound while still providing protection.

4. RADA16-1 self-assembling peptide nanofibers assemble into β-pleated sheets and accelerate wound healing by forming three-dimensional structures at the wound site.

5. Nanocoatings and nanosilver are the two primary nanotechnological advancements in the treatment or prevention of sepsis.

6. Spacer molecules on the surfaces of surgical instruments allow for the efficient attachment of penicillin.

7. Silver ions bind various enzymes within a host pathogen to inhibit their function and prevent processes necessary for survival.

8. The large surface area to volume ratio of silver nanoparticles allows them to more easily interact with pathogens resulting in an increased efficiency of microbial killing.

9. Silver nanoparticles are now being developed for post-surgical antiseptic and wound dressing applications.

10. Silver nanoparticles also exert antimicrobial properties by dissociating molecular oxygen.

11. The binding of silver nanoparticles to the HIV gp120 glycoprotein was shown to prevent virus/cell interactions.

Intracellular Nanosurgery

1. A primary goal of laser nanosurgery is to minimize collateral damage.

2. Laser nanosurgery is now widely used in research to deliver exogenous materials into cells.

3. Near infrared and pulsed ultraviolet are the two primary types of lasers used in laser-based nanosurgery.

4. Ultrafast laser nanosurgery allows for very minimal heating or damage to tissues surrounding the surgical site.

5. Ultrafast laser nanosurgery is based on the production of free electrons in tissues or cells after energy absorption.

6. A single mitochondrion in a mouse cell can be manipulated via ultrafast laser nanosurgery.

7. Advantages of microfluidics over other systems for characterizing biological samples include small dimensions, transparency, scalability and robotic interfacing capabilities.

8. Researchers are studying the development of micromotors which may be used for the next generation of minimally invasive surgical instruments.

9. Nanoneedles etched from the tips of atomic force microscopes have been shown to successfully deliver exogenous genetic material into cells.

KEY TERMS

- Surgery
- Intracellular Nanosurgery
- Implant
- Biocompatibility
- Nanocoating
- Biomimicry
- Protein Adsorption
- Integrin
- RGD Peptide
- Hardness
- Pascal
- Ceramic
- Amorphous
- Hydroxyapatite (HA)
- Osteoconductivity
- Thermal Spraying
- Crystallinity
- Metalloceramic
- Ductile
- Ion Beam-Assisted Deposition (IBAD)
- Nanostructuring
- Nanopolymer Scaffold
- Nanopulse
- Caspase

- Suture
- Laser-Assisted Nanosuturing
- Rose Bengal
  - Nitric Oxide
  - β-pleated Sheets
  - Sepsis
  - Esterification
  - Ester Product
- Gp120 Viral Glycoprotein
- Acticoat
- Intracellular Nanosurgery
- Laser
- Coherent Light
- Gain Medium
- Output Coupler
- Pumping (Laser)
- Laser Nanosurgery
- Ultrafast Laser Nanosurgery
- Pulse Intensity
- Ionization
- Microfluidics
- Nanoaxotomy
- Nanoneedle

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. List the types of implants that may be improved using nanotechnology.
2. How do nanocoatings improve the biocompatibility and biomimicry of implants?
3. Describe a mechanism by which cells interact with the ECM.
4. What are the unique surface properties of nanomaterials that make them affect protein adsorption efficiency?
5. List the three primary materials utilized for the generation of nanostructured coatings for orthopedic and dental implants and cite the advantages and disadvantages of each.
6. Why is diamond considered the "Biomaterial of the 21st Century"?
7. Why is hydroxyapatite the ceramic of choice for biomedical implants?
8. Describe how University of Alabama-Birmingham researchers created a functionally graded nanocrystalline metalloceramic coating.
9. List and describe the three primary properties of nanostructures that affect bone growth.
10. How do nanopulses trigger apoptosis?
11. Describe Kochevar and Redmond's nanosuturing technology.
12. How do nanofiber-based bandages increase wound healing efficiency over more conventional bandages?
13. What is the primary antimicrobial mechanism of action for nanosilver?
14. Describe the principle components of a laser and its mode of operation.
15. List the three primary types of ultrafast laser nanosurgery damage mechanisms.
16. Describe how University of Texas, Austin researchers combined ultrafast laser nanosurgery with microfluidics to study nerve regeneration.
17. List two examples of non-laser based intracellular nanosurgery and describe the instrumentation platforms used.

6

Nanomatrices for Cell Culture

Eukaryotic cell culture has been an indispensable and integral part of cell biology research for the past 100 years and has provided limitless opportunities to discern the molecular and biochemical properties of living cells. It is through cell culture that most of the pivotal studies and scientific breakthroughs in research on how cells function in the body have been made. This chapter focuses not on nanotechnology's impact on tissue engineering, which was covered in Chapter 3, but rather on the application of nanotechnology in *in vitro* cell culture and how it may provide new and unique avenues for propagating and differentiating a variety of cell types. Of particular emphasis and importance in this chapter is the concept of three-dimensional *in vitro* cell culture and how the physico-chemical characteristics and "nanogeography" of certain nanomaterials may drive a recapitulation of the true *in vivo* cellular environment.

A BRIEF HISTORY OF TISSUE (CELL) CULTURE

Tissue culture is defined as the growth of tissues and/or cells separate from an organism. The terms tissue culture and cell culture are today used interchangeably, although **cell culture** has been defined as the process by which cells are grown under controlled conditions in a laboratory setting. As early as 1882, scientists were attempting to culture tissues *ex vivo* (in an artificial environment outside a living organism). The first recorded undertaking was achieved by Sydney Ringer, a British clinician and pharmacologist who developed a saline solution, Ringer's solution, which allowed for the explanted culture of a beating frog heart. Interestingly, Ringer's Solution is still used today in many physiology laboratories. In 1885 a German zoologist, Wilhem Roux, cultured a portion of the medullary (neural) plate from a chicken embryo in saline successfully for several days, thus establishing the basics of tissue culture. In 1907 a Johns Hopkins University researcher, Ross Granville Harrison (see Focus Box 6.1), successfully cultured frog neural tube explants in a frog lymph **hanging drop tissue (cell) culture** (inverted suspension cell culture) system he had adapted from microbiologists studying bacteria. He watched the development of frog nerve fibers from the neurons

Focus Box 6.1 Ross Harrison and the invention of tissue culture

Born in Germantown, PA, Ross Granville Harrison (1870–1959) received his medical degree from Johns Hopkins Medical School in 1890 at the age of 19. After travel and work abroad he later returned to Johns Hopkins as an Associate Professor teaching anatomy and embryology until 1907. It was then that he devised a modified hanging drop tissue culture platform that allowed for the long-term culture of frog neuroblasts. For this work and his cumulative studies on nerve cell outgrowth ,which helped to form current understandings on nervous system function, he was considered as a Nobel Prize candidate. (Photo courtesy Wikipedia; reprinted with permission.)

FIGURE 6.1 Early cell culture laboratories (circa 1930) at Central Cancer Research Labs which would later become part of the National Cancer Institute. (Courtesy of NCI Visuals Online; reprinted with permission.)

in the explanted tissue. He had overcome the persistent problems of culture medium, observation limitations and contamination faced by other researchers to establish a relatively long-term culture system. His work was published in 1910 in the *Journal of Experimental Zoology* (Harrison, R., 1910). During the years immediately following this discovery tissue culture use and technology refinement blossomed and began to be used extensively in university and biotechnology research as well as industrial manufacturing (Figure 6.1). Considered a simple cell culture technique by today's standards, it opened the door to cell culture as a critical avenue for producing therapeutic monoclonal antibodies, vaccines and cell-produced drugs. In addition, the plethora of scientific data and information gleaned over the past 100 years regarding cellular mechanisms for growth, differentiation and plasticity have come from tissue culture studies. Notably, the hanging drop tissue culture technique is still used today to study, for example, embryonic stem cell differentiation through **embryoid body** (3D aggregates of embryonic stem cells) formation (Figure 6.2).
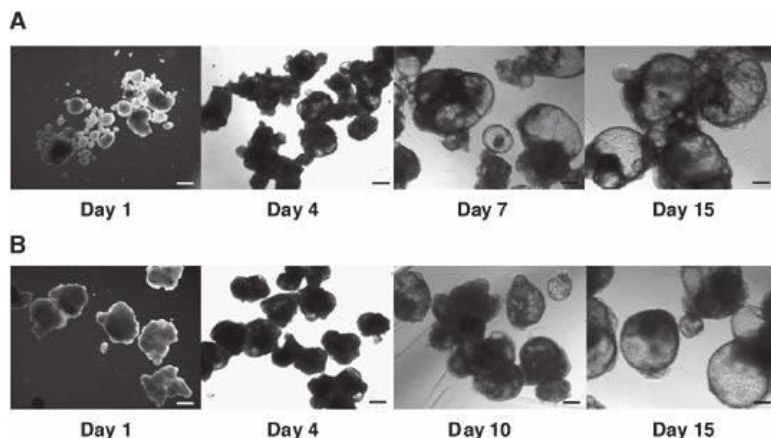
FIGURE 6.2 Mouse embryonic stem cell embryoid bodies formed by hanging drop tissue culture techniques. (Courtesy of Cerdan *et al.*, 2009; reprinted with permission.)

TYPES OF CELLS CULTURED

Since its inception over 100 years ago, cell culture has allowed for the establishment of literally thousands of different types of cell lines from virtually every tissue source. These include, but are not limited to:

- Cancer cells (see Figure 6.3)
- Fibroblasts
- Tumor cells
- Embryonic cells
- Embryonic stem cells
- Adult stem cells
- Adult immortalized cells
- Primary cells (direct explants)
- **Hybridomas** (hybrid cell lines of antibody-producing and myeloma B cells)

MANIPULATION OF CULTURED CELLS

General manipulation of cells in culture includes such routine procedures as media changes, passaging and **transfection** (the process of introducing nucleic acids into cells by non-viral methods) to achieve goals such as cell
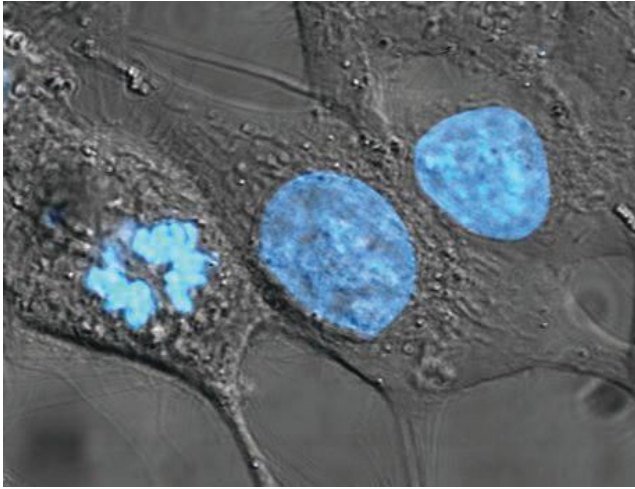
FIGURE 6.3 Bright field and fluorescence microscopy of Hela cells. One of the earliest human cell lines, descended from Henrietta Lacks, who died of the cancer that those cells originated from, the cultured HeLa cells shown here have been stained with Hoechst turning their nuclei blue. (Courtesy of Wikipedia; reprinted with permission.)

expansion, differentiation or genetic manipulation. In virtually every facet of cell culture the ultimate goal is to mimic the biological environment of a living organism in order to yield the most accurate data as to a cell's function during experimental manipulation. In order to create *in vitro* environments that are as *in vivo*-like as possible, several key issues must be addressed. Table 6.1 below lists some of the most critical.

Table 6.1 Issues and remedies in basic cell culture	
Issue	**Solution**
Nutrient Depletion	Change media frequently.
Accumulation of apoptotic and necrotic cells and debris	Changing of media removes unwanted dead cells and debris.
Negative effects of cell-cell contact	Frequent passaging of cells and low density plating.
Physical constraints of *in vitro* culture	Use of appropriate cell culture matrices.

As discussed in the next section, it is perhaps the dimensional physical constraints of 2D cell culture which have limited the true biomimicry of an *in vivo* environment.

2D OR NOT 2D?

All tissues and thus all cell types within the body are arranged in a three-dimensional (3D) environment. This architecture allows for unique cell-cell interactions and cell-ECM attachment. It also provides for the proper access to growth factors, extracellular ligands, nutrients and other cell signaling molecules inherent in the physiological system. Yet the vast majority of studies on cells in tissue culture have been undertaken in a two-dimensional (2D) format such as in a Petri dish, glass slide multi-well plate, etc. The disadvantages of 2D cell culture are numerous. For example, most cells within the body respond to gradients of signaling molecules. These molecular gradients drive countless cellular processes including cell determination and differentiation, organ development during embryogenesis, neural signaling and transmission, etc. Yet it is almost impossible to accurately recapitulate a 3D molecular signaling gradient in a 2D microenvironment. In addition, a 2D microenvironment alters cellular morphology to such an extent that its metabolism and overall transcriptional profile (genetic activity) is affected. The lack of an extracellular matrix and basement membrane significantly influences cellular metabolism and gene transcription as appropriate extracellular protein-protein signaling interactions are not made. The cellular morphological changes resulting from 2D culture also most likely result in preferential clustering of cell surface receptors in areas exposed to culture media thus further negatively influencing, for example, cell-cell communication. Conversely, the nanoscale features of the 3D *in vivo* environment allow for the proper orientation of cells and the control of cell spreading through limitations on surface area available for cell attachment. This all begs the question: How realistic is it to expect 2D cell culture to accurately mimic a 3D biological environment? Researchers now realize the importance and more accurately mimicking the 3D nature of the *in vivo* biological environment and have begun to place more attention on 3D cell culture applications (Figure 6.4).
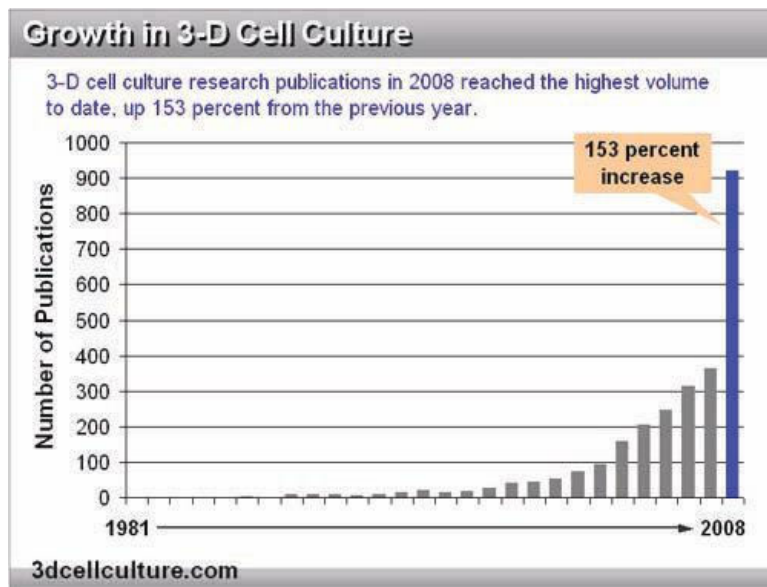
FIGURE 6.4 Increase in 3D cell culture-related publications as a function of time. (Courtesy of 3DCellCulture.com; reprinted with permission.)

3D CELL CULTURE AND THE ISSUES OF SCALE AND PURITY

In order to address the limitations of 2D cell culture a plethora of researchers spanning both academia and industry have developed 3D microfiber matrices that, in theory, more accurately mimic the *in vivo* biological environment. Synthetic versions of the 3D matrices are based on a variety of materials such as the polymers PLLA and PLGA. Yet in the majority of the cases the microfibers constituting the matrix average ~10–50 μm in diameter which is similar to the size of the majority of eukaryotic cells studied (~5–30 μm). As a result, upon cellular attachment to the microfibers of most conventional 3D cell culture matrices the cell is still considered to be in a 2D environment. In addition, the **micropores** (microsized gaps between fibers) separating microfibers in these systems often range in size from 10 μm to 200 μm across, similar to the size of a single cell (Figure 6.5). This is ~1000–10,000-fold greater than the size of the typical biomolecule such as a growth factor, for example. Biomolecular diffusion is the result and thus cells in this system often lack the access to
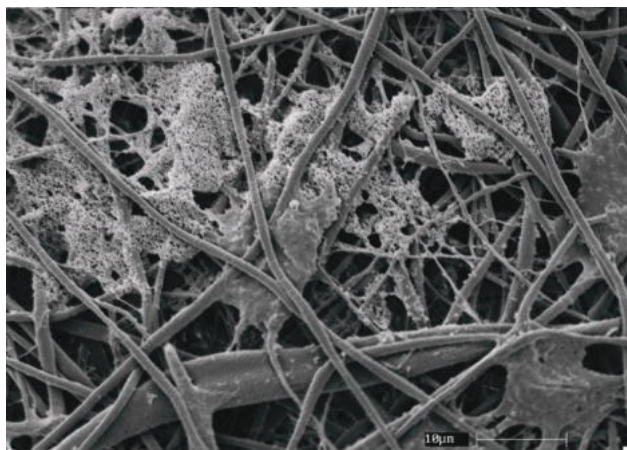
FIGURE 6.5. Osteoblast bone cells seeded on PHB/PLA 3D composite scaffolds modified fibers. Osteoblast cells attached under the PLA fiber on top of PHB granules. Note the relative size of the cells in comparison to the synthetic scaffold pore size. (Courtesy of Juana Mendenhall, Cornell University; reprinted with permission.)

nutrients and other biomolecules that would be easily accessible *in situ* (in a natural biological environment). In order to achieve an anatomically correct 3D microenvironment conducive to *in vivo*-like cell culture both the pore and fiber size of the 3D scaffold platform must be significantly smaller than the size of the cells being cultured thus mimicking the extracellular environment of a living organism.

To more effectively address the issue of scale in 3D cell culture, naturally occurring material substrates such as collagen, polyglycosaminoglycans and **Matrigel**™ (derived from **basement membrane**, which is the basic substrata for cellular structures throughout the body) have been used. While they do meet *in vivo*-like dimension requirements, each having a natural nanofiber consistency and origin, they frequently contain residual unwanted growth factors and impurities, making it difficult if not impossible to conduct controlled cell culture experiments that provide reproducible results. Thus the ideal 3D cell culture system should contain a fibrous matrix of appropriate scale coupled with a comprehensive validation of its purity, free from unwanted growth factors or biological contaminants. The remaining

sections illustrate a number of nanotechnology-based 3D cell culture platforms that address each of these critical requirements. It emphasizes nanotechnologies used for cell culture and not tissue engineering, which was addressed in Chapter 3.

SYNTHETIC NANOFIBER SCAFFOLDS

Polymer-Based

Polymer-based nanofiber scaffolds (see Table 3.1 in Chapter 3) have received much attention in the area of 3D cell culture, primarily due to the fact that they can be easily manufactured and architecture as well as purity can be precisely controlled. As discussed in Chapter 3, there are three primary methods for the synthesis of synthetic nanofiber-based 3D scaffolds: phase separation, electrospinning and self-assembly. When it comes to tissue/cell culture applications there are advantages and reasons for using one over the other. For example, phase separation allows for the precise control of pore architecture. Yet the most common method currently employed for nanofiber matrix manufacture is electrospinning. Electrospun nanofibers are produced through the application of a high electric field on a polymer solution, with a grounded region at some defined distance from the source. As charge accumulation overcomes the polymer solution surface tension, a spray of nanofibers is emitted (see Figure 3.15 in Chapter 3). Fiber whipping and elongation during solvent evaporation result in nanofibers with diameters ranging from 50 nm to several microns. This is in the same range as the diameter of collagen fibrils present within the extracellular matrix thus mimicking some 3D architectural aspects of the *in vivo* environment. Aside from these morphological advantages, the physical properties of electrospun nanofibers include high surface area to volume ratio, customizable pore size and high porosity needed for matrix penetration by cells. It is the combination of these properties that makes this system ideal for certain applications in 3D cell culture. Below are some examples of synthetic 3D polymer nanofiber matrices used in cell culture applications.

Ultra-Web®

Mouse ES cells have revolutionized the study of gene function through the systematic creation of knockout, or gene-deleted mice, yet their culture

is tedious and often requires the use of feeder cells as a support matrix. Sally Meiner's team in the Department of Pharmacology at Robert Wood Johnson Medical School in Piscataway, New Jersey focused on the use of the synthetic nanomatrix Ultra-Web® (Donaldson Co., Inc.) for the culture of mouse ES cells, specifically with respect to the maintenance of **pluripotency**, which is defined as a stem cell's ability to differentiate into many different lineages. **Ultra-Web** is a synthetic polyamide matrix that resembles the ECM/basement membrane through nanofibrillar 3D organization. Enhanced proliferation and self-renewal properties were observed for mouse ES cells grown on Ultra-Web in comparison to other tissue culture surfaces as measured by simple cell counting as well as characterization of expression levels for the pluripotency marker alkaline phosphatase (Nur-E-Kamal *et al.*, 2006 and Figure 6.6).
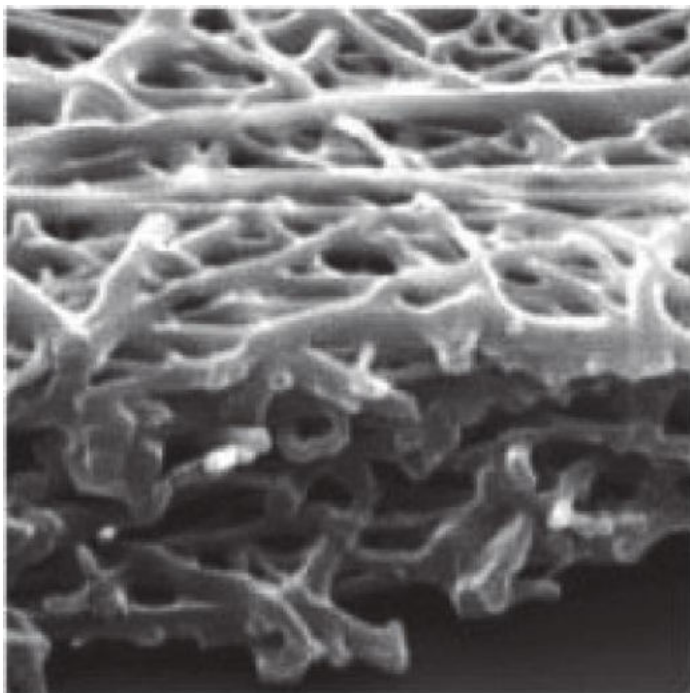
FIGURE 6.6 Ultra-Web. (Courtesy of Omnexus; reprinted with permission.)

PLLA-Based

Researchers in the Department of Orthopaedics and Rheumatology at the University of Marburg in Germany explored the effects of electrospun PLLA and PLLA/Col-1 synthetic nanofiber matrices on the culture of human tendon-derived fibroblasts. They observed a negative effect of PLLA alone on fibroblast growth which was not observed with the PLLA/Col-I (collagen I) blend. In fact, the blend activated the expression of the collagen I, III, X and decorin genes which are critical for correct tendon architecture (Theisen *et al.*, 2010 and Figure 6.7). These studies suggest the requirement for a collagen component in the 3D matrix for effective culture of this particular cell type.

Others have expanded on the use of PLLA-based nanoscaffolds to combine them with **nanospheres** (spherical particles having diameters of less than 100 nm) containing growth factors for the promotion of cell survival, propagation and differentiation. Peter Ma and colleagues in the Department of Biomedical Engineering at the University of Michigan in Ann Arbor have developed a system that utilizes PLLA nanoscaffolds combined with PLGA nanospheres seeded with recombinant human growth factor BMP-7 (bone morphogenetic protein 7). PLGA nanospheres
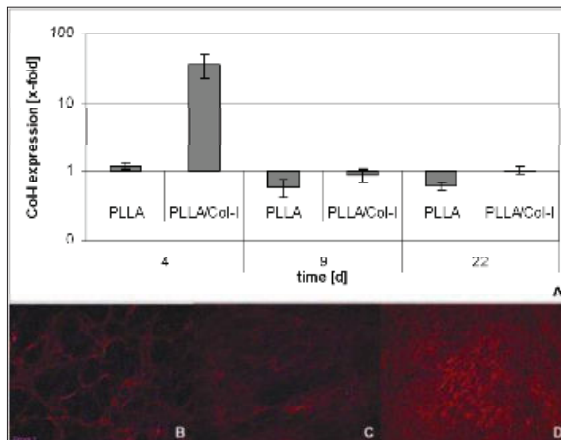
FIGURE 6.7 Influence of PLLA and PLLA/Col-I nanofibers on gene expression Col-I deposition of TDF's. (A) Time course of collagen-I gene expression of TDF's on nanofibers compared to cover slips control. (Lower) Immunofluorescence microphotographs of Col-I (red) deposition after 22 days of culture cover slip (B) control, (C) PLLA nanofibers and (D) PLLA/Col-I nanofibers. (Courtesy of Theisen *et al.*, 2010; reprinted with permission.)
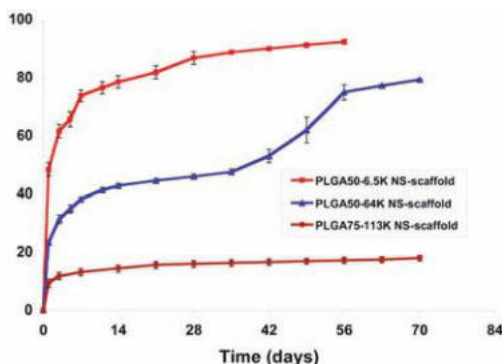
FIGURE 6.8 *In vitro* release kinetics of rhBMP-7 from nanospheres immobilized on nano-fibrous scaffolds. In 10 mM PBS with a BMP-7 loading of 200 ng/scaffold. Each data point represents a mean ± standard deviation ($n = 3$). (Wei *et al.*, 2008; reprinted with permission.)

coated with rhBMP-7 were seeded onto PLLA nanoscaffolds and the kinetics of growth factor release measured. It was observed that rhBMP-7 release could be temporally controlled by adjusting the chemical and degradation properties of the PLGA nanospheres (Wei *et al.*, 2008 and Figure 6.8). Controlled release of rhBMP-7 was shown to promote cell culture and induce bone formation.

Carbon Nanofiber-Based

Although generally considered for use in a two-dimensional environment, carbon nanofiber-based systems have been explored as cell culture matrices due primarily to their nanoscale physical composition. Thomas Webster's group in the Department of Physics at Purdue University in West Lafayette, Indiana (now at Brown University in Providence, Rhode Island) (other examples of Webster's research are discussed in Chapters 3 and 5) has studied carbon nanofibers placed on polymer substrates for their abilities to promote osteoblast culture. An imprinting method was used to place carbon nanofibers on a polycarbonate urethane (PCU) substrate. The system was then assessed for osteoblast adhesion and differentiation capacities. The results demonstrated selective adhesion and alignment of osteoblasts on carbon nanofibers coupled with enhanced calcium phosphate mineral deposition, an indication of differentiation, along linear patterns of carbon nanofibers (Khang *et al.*, 2006 and Figure 6.9).
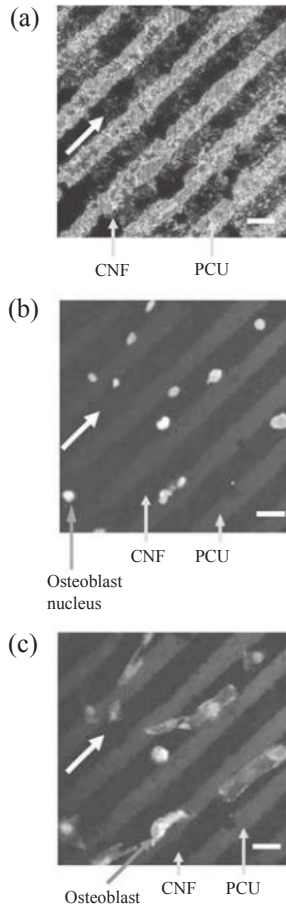
FIGURE 6.9 Fluorescence microscopy images of (a) carbon nanofiber (CNF) patterns on polycarbonate urethane (PCU), (b) selective osteoblast adhesion on CNF patterns on PCU, and (c) aligned osteoblast adhesion on CNF patterns on PCU. All bars = 20 μm; culture time = 2 days; all arrows show direction of CNF patterns.

NATURAL NANOFIBER SCAFFOLDS

Natural nanofiber scaffolds hold considerable promise for cell culture applications due primarily to biocompatibility, although ease of synthesis and processability also factor into their popularity in this

**Focus Box 6.2 Thomas Webster and nanophase material
cell culture**

As Director of the Nanomedicine Laboratories at Brown University, Thomas Webster has dedicated his career to the development of nanophase materials for biomedical implant applications. His group has evaluated various materials such as nanoscale ceramics, nanopolymers and carbon nanofibers for culturing cells from organ systems with the ultimate goal of using these systems in transplantation therapeutics. His lab has produced 25 patent applications, 8 books, 42 book chapters and over 300 scientific publications. (Photo courtesy of Brown University; reprinted with permission.)

area. Below are some examples of collagen- and silk fibroin-based matrices, two of the most popular natural nanofiber scaffold cell culture systems.

Collagen-Based

It is well known that the basement membrane is not smooth but rather covered with grooves, ridges, pits, pores and the fibrillary meshwork of the ECM. This 3D architecture is predominantly composed of intertwined collagen and elastin nanofibers. It is this network that not only provides tensile strength and mechanical rigidity to the system, but yields abundant binding sites for cell adhesion molecules. Thus, mimicking this system as a defined, reproducible cell culture platform may yield a much more accurate *in vitro* system for cell study than using synthetic nanomatrices. Collagen nanofiber matrices have received much attention recently due to their biocompatibility, ease of synthesis and the fact that they truly recapitulate a major component of the ECM. Researchers have studied 3D matrices composed of collagen nanofibers for the culture and *in vitro* manipulation of a variety of cell types. Case Study 6.1 below illustrates an example of this for the culture and osteogenic differentiation of mesenchymal stem cells.

Case Study 6.1: Growth of mesenchymal stem cells on electrospun type I collagen nanofibers

Oscar Lee and colleagues in the Department of Orthopedics and Traumatology at Taipei Veteran's General Hospital developed type I collagen nanofibers by electrospinning techniques and examined mesenchymal stem cell motility, morphology, growth, adhesion and osteogenic differentiation capabilities. Cells appeared to have a polygonal and flattened morphology and exhibited higher viability rates than on standard tissue culture plates. Differentiation was characterized via single cell RT-PCR of various marker genes to examine expression levels. Higher levels of the genes osteocalcin, osteonectin and osteopontin were observed at the single cell level on the nanofibers as compared to controls (Shih *et al.*, 2006 and Figure 6.10).
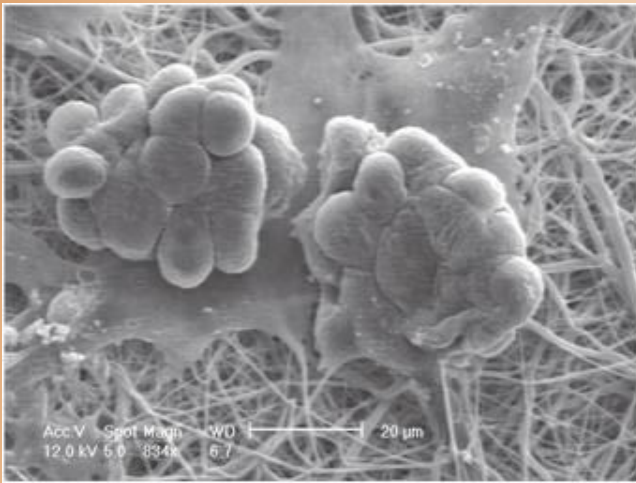
FIGURE 6.10 Scanning electron microscopy photomicrograph of cells on nanofibers. (Courtesy of Nanofiber Solutions; reprinted with permission.)

Silk Fibroin-Based

Silks offer excellent biocompatibility, controlled degradation properties and versatile processability, thus making them ideal platforms for cell culture matrices. The structure and application of silk fibroin nanofibers in tissue engineering is discussed in Chapter 3. This section illustrates some examples of silk fibroin as it applies to cell culture. For example, David Kaplan's group in the Department of Biomedical Engineering at Tufts University in Medford, Massachusetts has developed and utilized PEO-extracted and non-PEO-extracted electrospun silk fibroin nanofiber matrices for the routine culture of human aortic endothelial cells (HAECs) and human coronary artery smooth muscle cells (HCASMCs). The system promoted proliferation, alignment and elongation as well as differentiated marker gene expression (Zhang *et al.*, 2008 and Figure 6.11). In addition, HCASMCs were observed to form ECM as evidenced by collagen I production. It should be noted that the average diameter size of these matrices was around 370 nm, thus technically they are not classified as nanofiber scaffolds.
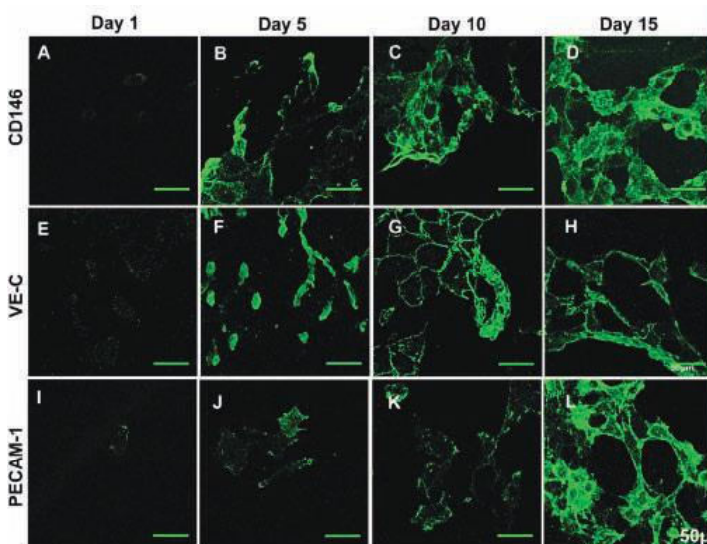
FIGURE 6.11 Immunocytochemistry staining of HAECs on electrospun silk fibroin scaffolds. Staining occurred on days 1, 4, 7 and 14 after initiation of cell culture. Scale bars are 50 μm. Abbreviation: VE-C, vascular endothelial cadherin. (Courtesy of Zhang *et al.*, 2008; reprinted with permission.)

Chitosan-Based

As discussed in Chapter 3, chitosan is a naturally occurring linear polysaccharide composed of randomly distributed glucosamines. It is produced by the deacetylation of chitin, a 3D structural component in the exoskeletons of crustaceans. Kam Leong's group in the Department of Biomedical Engineering at Duke University in Durham, North Carolina have taken the use of natural nanofiber scaffold systems a step further by developing a thermally responsive electrospun chitosan-based nanofiber system for the culture and differentiation of muscle cells. Hydroxybutyl chitosan (HBC), a modified form of chitosan, possesses the unique property of nanofiber degradation upon cooling to temperatures below the **lower critical solution temperature (LCST)**, which is defined as the critical temperature below which a mixture is miscible in all proportions. Human mesenchymal stem cells (hMSCs) were cultured in HBC nanomatrices for 3 days and their morphology characterized, both before and after cooling below the LCST to dissolve the matrix and leave a polymer-free cellular sheet. Specific attention was paid to nucleus structure and shape, as this is known to have wide implications on gene regulation. Differences in nuclear shape of hMSCs cultured in different systems were characterized using the following formula for **elliptivity, ε,** the measure of the elliptic nature of an object or shape:

$$\varepsilon = \sqrt{1 - \left(\frac{CD}{AB}\right)2}$$

where CD is defined as the length of the short axis and AB is the length of the long axis of the nuclear ellipse. The researchers observed considerable nuclear elliptivity and elongation in HBC culture vs. glass slide culture, an indication of cellular alignment, which was confirmed by broader analysis of cell morphology (Dang *et al.*, 2006 and Figure 6.12).

Biocomposites

Biocomposites are materials that contain natural fiber reinforcements. They may be mixtures of synthetic and natural or entirely natural components. A number of nanofiber-based biocomposites have been developed for use in tissue culture applications and it is the heterogeneous aspect of the systems which often provides and combines the advantages of using two or more
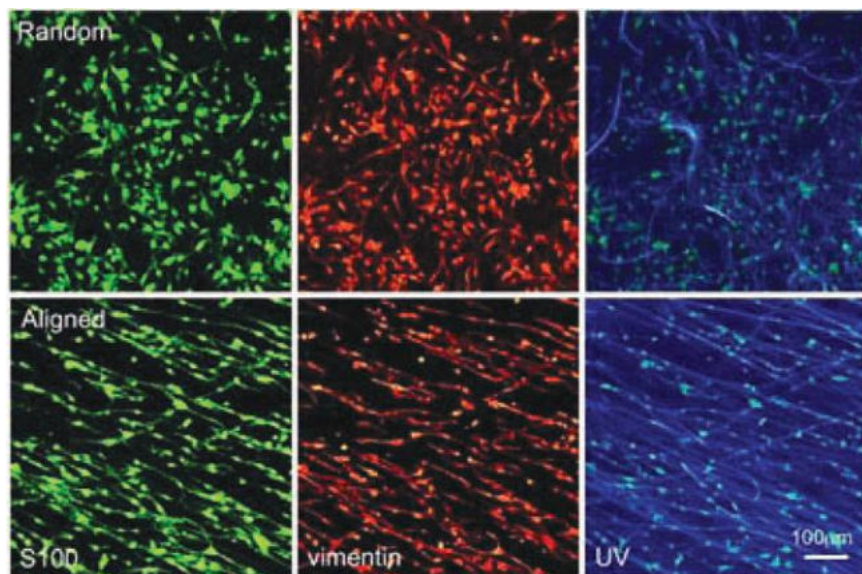
FIGURE 6.12 Cell sheet production and cellular morphology on random and aligned chitosan nanofibers. (Courtesy of Cosmo Bio; reprinted with permission.)

different matrix components. For example, Yoshihiro Ito and colleagues at the Kanagawa Academy of Science and Technology in Kawasaki, Japan have created a hydrophilic biocomposite nanofiber cell culture system composed of biodegradable and biocompatible poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and hydroxyapatite (HA). The surface of this biocomposite was demonstrated to promote adhesion of COS-7 cells in comparison to non-nanofibrous cast flat film controls suggesting the nanogeographic makeup of the system is the driving force behind cellular adhesion (Ito *et al.*, 2005). Yanzhong Zhang and colleagues at Dungua University and Shanghai and the National University of Singapore created a biocomposite composed of three naturally occurring nanofiber-based biopolymers. The matrix was created through doping of a dual electrospun hydroxyapatite/chitosan (Hap/CTS) nanofiber matrix with the bioactive component of collagen. Human fetal osteoblasts (hFOBs, discussed previously in Chapter 3) were cultured in this biocomposite and increased
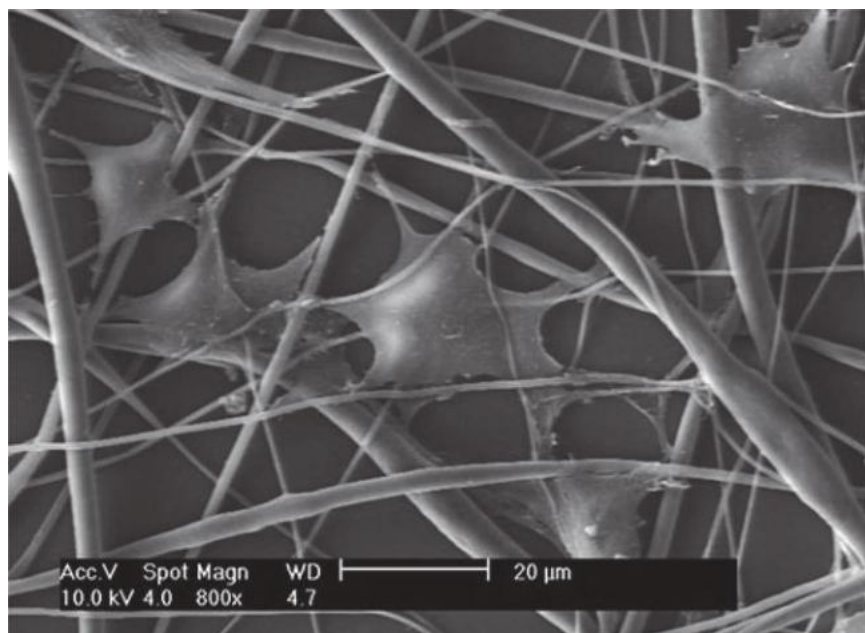
FIGURE 6.13 SEM Image of cells cultured on a collagen nanofiber matrix. (Courtesy of Nanofiber Solutions; reprinted with permission.)

proliferation, alkaline phosphatase expression and mineralization were observed compared to more conventional control matrices (Zhang *et al.*, 2010 and Figure 6.13).

Three-Dimensional Self-Assembling Peptides

Self-assembling peptides have been discussed elsewhere in this book, particularly with respect to tissue engineering platforms outlined in Chapter 3. The advantages of this system include their biological origin and thus biocompatibility, nanoscale and ease of assembly. Xiaojun Zhao's group in the Department for Biomedical Engineering at MIT describes a system for salt-facilitated self-assembly of designer peptide scaffolds. The RADA16-I, RADA 16-II, EAK16-I and EAK16-II systems have been shown to self-assemble spontaneously through the introduction of a peptide-containing aqueous solution into a salted solution (Zhang *et al.*, 2005 and Figure 6.14). Assembly can be controlled by varying the pH of the system
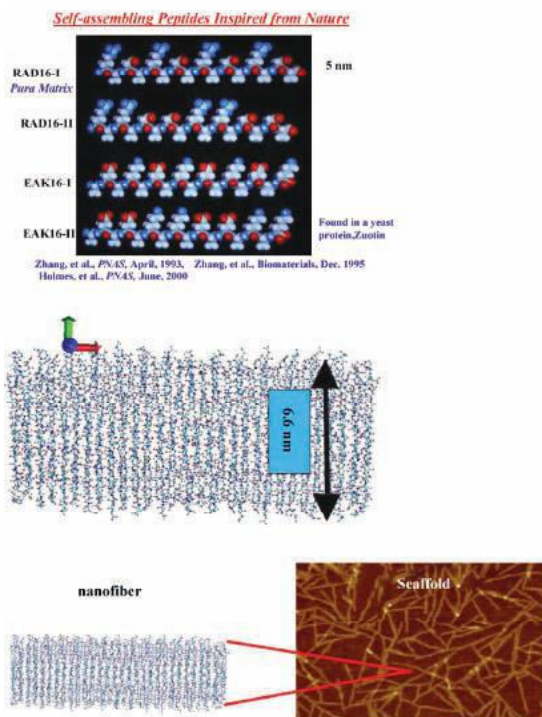
FIGURE 6.14 Molecular models of several self-assembling peptides. (Top) Molecular models of RADA16-I, RADA16-II, EAK16-I and EAK16-II. Each molecule is ~5 nm in length with eight alanines on one side and four negative and four positive charge amino acids in an alternating arrangement on the other side. EAK16-II is the first self-assembling peptide that was discovered from a yeast protein, zuotin. Blue = positively charged amine groups on lysine and arginine; red = negatively charged carboxylic acids on aspartic acids and glutamic acids. Light green = hydrophobic alanines. (Middle) Molecular model of hundreds self-assembling peptides form a well-ordered nanofiber with defined diameter that is determined by the length of the peptides. (Bottom) Thousands, millions and billions of self-assembling peptides form nanofibers that further form hydrogel, with great than 99% water content. (Courtesy of Zhang *et al.*, 2005; reprinted with permission.)

Table 6.2 Cell types successfully cultured in self-assembled peptide scaffolds

Mouse Fibroblasts	Bovine Calf and Adult Chondrocytes
Chicken embryo fibroblasts	Bovine endothelial cells
Chinese hamster ovary cells (CHO)	Rat adult liver progenitor cells
Rat pheochromocytoma cells	Rat cardiac myocytes
Rat neural stem cells	Rat hippocampal neural tissue
Mouse embryonic stem cells	Mouse adult neural stem cells
Mouse cerebellum granule cells	Mouse and rat hippocampal cells
Bovine osteoblasts	Hamster pancreas cells
Human cervical carcinoma cells	Human osteosarcoma cells
Human hepato-cellular carcinoma cells	Human neuroblastoma cells
Human embryonic kidney cells	Human foreskin fibroblasts
Human epidermal keratinocytes	Human neural stem cells

or by simply altering salt (NaCl or KCl) concentrations. Resulting scaffolds have nanofibers with an average diameter of ~10 nm and pore size between 5 and 200 nm.

The resulting scaffold structures created by Zhao's group were shown to support cellular attachment, survival and the induction of differentiation for a variety of mammalian primary and tissue culture cells (Table 6.2). In addition, the unique structures of these nanoscaffolds allow for some striking physical properties of the system, including stability across a broad range of temperatures and pH ranges.

Shuguang Zhang's team at MIT's Center for Biomedical Engineering has compared custom RADA16-I-based nanofiber matrices with respect to facilitating osteoblasts proliferation, differentiation and migration. They demonstrated the design, synthesis and analysis of several self-assembling peptide nanofiber systems specifically for the culture of osteoblasts, each of which presented different osteogenic growth factors to the 3D environment of the cells. In this study the researchers coupled osteogenic growth peptide
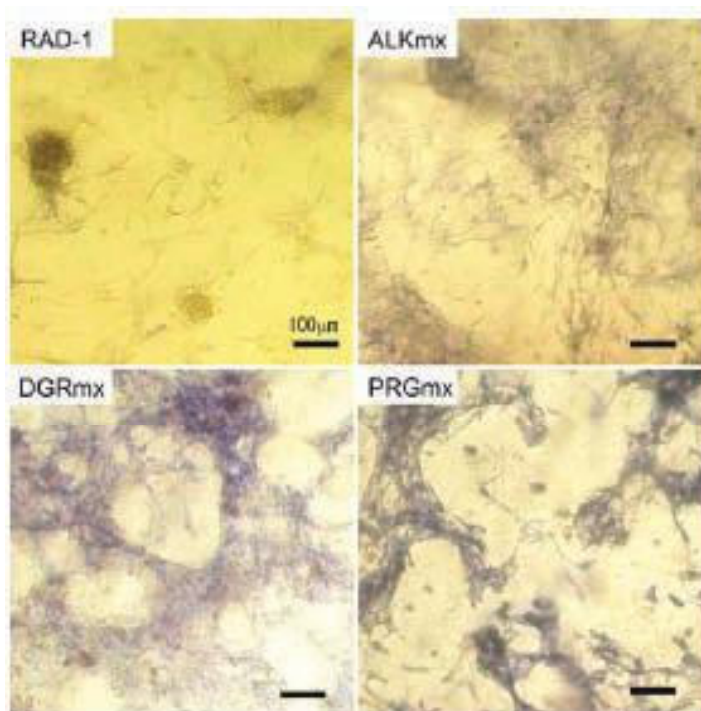
FIGURE 6.15 Alkaline phosphatase stained osteoblasts after culturing on different RADA16-I hydrogels. The bar represents 100 μm. (RAD-I) RADA16 1% (w/v). (ALKmx) ALK 1% (w/v)+RADA16. (DGRmx) DGR 1% (w/v)+RAD. (PRGmx) PRG 1% (w/v)+RADA16 (all mixture ratios are 1:1). The bluish color intensity correlates with the high alkaline phosphatase activity. RADA16 shows low cell adhesion to the hydrogel and the cells are aggregated. The cell attachment increases in DGR and PRG scaffolds were considered as a result of RGD cell attachment sequence. ALP, DGR and PRG showed higher ALP activities compared to RADA16-I, especially staining intensity of PRG. (Courtesy of Horii *et al.*, 2007; reprinted with permission.)

ALK, bone-cell secreted signal peptide, osteopontin cell adhesion motif DGR and the RGD peptide binding sequence PRG. They assessed mouse MC3T3-E1 cell proliferation rates, alkaline phosphatase activity and osteocalcin secretion, both bone-specific markers, and revealed an increase in each of these parameters compared to control systems (Horii *et al.*, 2007 and Figure 6.15). Interestingly, the PRG-based system promoted cellular migration into the 3D nanoscaffold.

CELLULARIZING NANOFIBER SCAFFOLDS

Cellularization is defined as the infiltration of cells into a particular environment. While numerous nanofiber-based cell culture scaffolds have now been developed, providing proper cell deposition within these matrices can be problematic. Successful and thorough population of the 3D matrix with cells is critical to their growth and manipulation as well as to the production of reproducible experimental data. It is true that cells, in most cases, will readily divide and migrate across the surface of a matrix, yet their ability to migrate within the innerspace of a 3D nanofiber matrix is limited. This is due primarily to the dense packing of the nanofibers and thus small pore size. Cells that have clumped in certain areas of the matrix or failed to properly infiltrate it will not yield valuable or reproducible scientific results.

Optimizing Nanoscaffold Architecture for Cellularization

A number of methods have now been developed to address cellularizing 3D nanoscaffolds. One strategy employs the interweaving of different nanofiber sizes to promote cellular infiltration. Researchers in the Department of Bioengineering at Rice University in Houston, Texas have created a layered nanofiber-microfiber mesh in which the inclusion of larger fibers interrupts normally dense nanofiber packing, thus allowing cells to fully colonize the 3D scaffold. In this study layered scaffolds of poly(epsilon-caprolactone) microfibers and nanofibers were generated by electrospinning the layers for different lengths of time (Pham *et al.*, 2006 and Figure 6.16). Rat marrow stromal cell infiltration was measured under both static and flow perfusion culture and shown to increase with decreasing nanofiber layer thickness.

Yet as the purpose of nanoscaffold use is to more accurately mimic the biological environment's nanogeography, the inclusion of microfibers in most cases is undesirable. Researchers in the Department of Materials Science and Engineering at Ohio State University in Columbus, Ohio led by Dr. John Lannutti have developed a combination electrospinning and salt leaching techniques to increase nanoscaffold pore size. By manipulating the Taylor Cone aspect of the electrospinning process (discussed in Chapter 3) a uniform distribution of salt particles within the nanofiber scaffold was produced thus resulting in deliberate, engineered delaminations and increased pore size (Nam, *et al.*, 2007 and Figure 6.17).
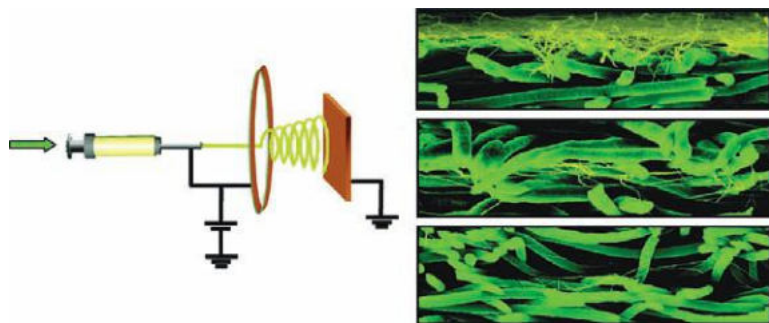
FIGURE 6.16 Schematic representation of the electrospinning setup. The flow rate (Q), distance (d), applied voltage (V), needle gauge (n), and polymer solution properties could all be varied to affect the properties of the resulting fibers. (Pham *et al.*, 2006; reprinted with permission.)

This same group has implemented laser ablation techniques for the introduction of "grooves" within nanofiber matrices to allow for avenues of cellularization internal to the matrix. Specifically, Lannutti's group performed laser ablation on a PCL and polyethylene terephthalate nanofiber mesh using a scanned femtosecond laser. Groove size and depth
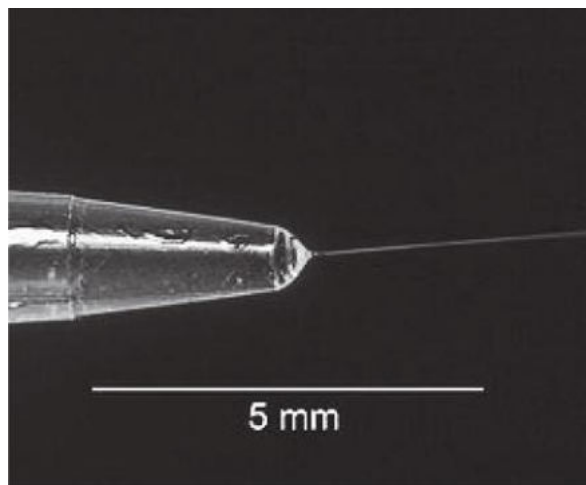
FIGURE 6.17 Photograph of a meniscus of polyvinyl alcohol in aqueous solution showing a fibre being electrospun from a Taylor cone. (Courtesy of Wikipedia; reprinted with permission.)
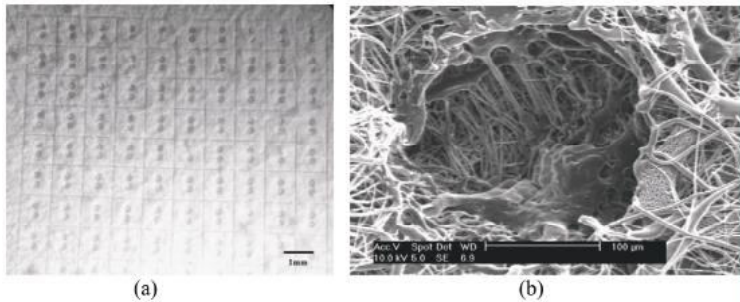
FIGURE 6.18 Laser ablated nanofiber mesh. (a) Matrix of microscale structures ablated in PCL nanofiber mesh and (b) magnified view of a laser-machined cavity in PCL nanofiber mesh (right) used for cell culture. (Courtesy of Choi *et al.*, 2007; reprinted with permission.)

could be adjusted by optimizing focus spot size, pulse energy and scanning speed (Choi *et al.*, 2007 and Figure 6.18).

Other techniques for increasing nanofiber scaffold pore size include the induction of ice crystals to provide solid collusions around which nanofibers are formed as well as the incorporation of a sacrificial nanofiber population that is removed prior to cell seeding. Robert Mauck and colleagues in the McKay Orthopedic Research Laboratory, University of Pennsylvania in Philadelphia used two separate spinnerets to co-electrospin a dual composite fiber-aligned nanoscaffold composed of PCL and PEO nanofibers. By adjusting the fabrication parameter a full range of sacrificial PEO nanofiber properties were produced. The researchers demonstrated that increases in the starting sacrificial fraction of nanofibers improved mesenchymal cell infiltration and distribution (Baker *et al.*, 2008, Figure 6.19 and see Case Study 6.2 below).

Cell Seeding within the Nanomatrix

It is clear that even when scaffolds possess the proper architecture to promote cellularization, scaffolds that are seeded on the surface with cells will eventually contain gradients of these cells, typically with higher concentrations on the surface and lower concentrations internal to the matrix. Although not convenient for most general and routine cell culture applications, the most common and direct method to overcome
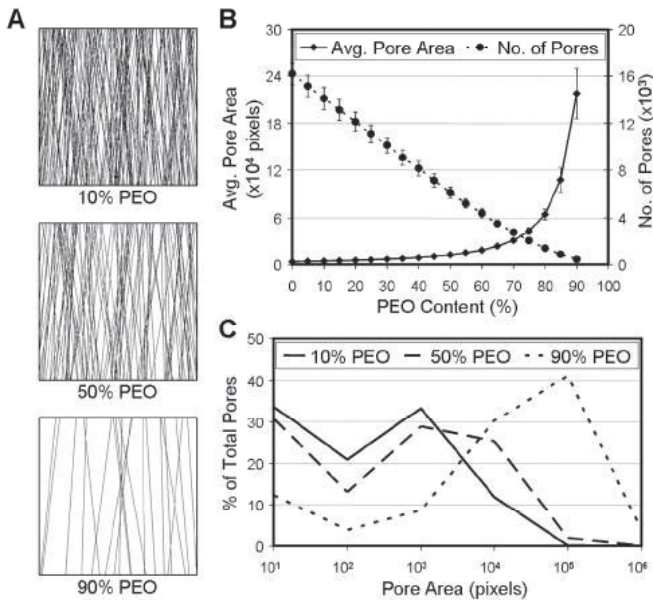
FIGURE 6.19 A simple model of composite scaffolds with increasing sacrificial fiber removal. A reduction in pore number, but an increase in average pore size is demonstrated. (A) Example composite scaffold layers representing scaffolds with 10, 50, and 90% sacrificial PEO fibers. (B) Increasing the PEO fiber fraction decreases the total number of pores (•) while increasing the average pore area (♦). (C) Pore area distribution shifts towards higher pore sizes with increasing PEO content. Data were averaged from 20 model iterations, each with a randomly generated fiber population. (Courtesy of Baker *et al.*, 2008; reprinted with permission.)

this phenomenon is to seed the cells on the scaffolds during synthesis. This methodology would be most likely used, for example, for tissue engineering applications. Electrospraying is the most common technique for accomplishing this. Mauck's group at U. Penn (discussed above) has also studied this area and was successful at cell deposition within a nanomatrix by electrospraying mesenchymal stem cells (MSCs) in gelatin with PCL electrospun nanofibers.

Researchers in the Department of Mechanical Engineering at University College London successfully electrospun porcine vascular and rabbit aorta smooth muscle cells into nanofiber matrices as highly concentrated cellular suspensions and found a long-term maintenance

Case Study 6.2: The potential to improve cell infiltration in composite fiber-aligned electrospun scaffolds by selective removal of sacrificial layers

Robert Mauck's group in the McKay Orthopedic Research Laboratory at the University of Pennsylvania in Philadelphia devised a strategy for co-electrospinning two separate fiber types followed by selective removal of one. In this study, poly(e-caprolactone) (PCL), which is a slow-degrading polyester, and the water-soluble polymer poly(ethylene oxide) (PEO) polymer were co-spun from two separate spinnerets to form a dual-polymer composite fiber-aligned scaffold. PCL served as the structural element and PEO, due to its water-solubility, represented the sacrificial fibers. PEO could be removed at will upon hydration and the sacrificial fraction of the matrix could be customized to yield different architectures (Baker *et al*., 2008 and Figure 6.20. Also see Figure 6.19 above).
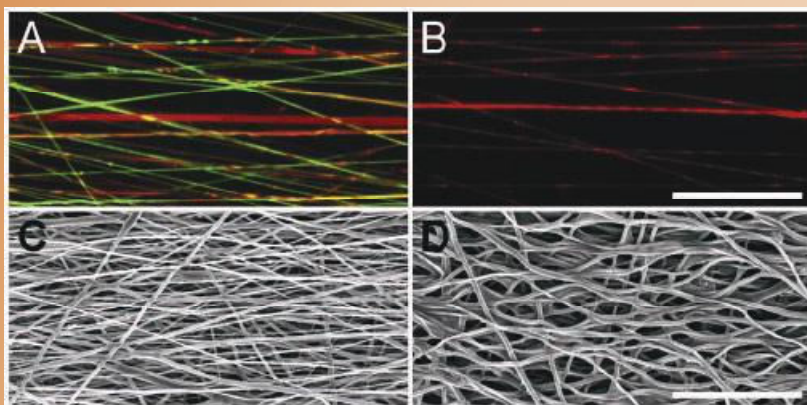
FIGURE 6.20 Composite fibrous scaffolds formed with individual fibers of distinct polymer composition. Removal of one sacrificial fiber population increases scaffold porosity. (A) Fluorescently labeled PCL (red) and PEO (green) fibers showed pronounced alignment and interspersion. (B) Submersion of scaffolds in an aqueous solution removed the PEO component while the PCL fibers remained intact. SEM images of as-spun (C) and post-submersion (D) composite scaffolds reveal increases in pore size with the removal of sacrificial PEO fibers. Scale bars: 50 μm. (Courtesy of Baker *et al*., 2008; reprinted with permission.)
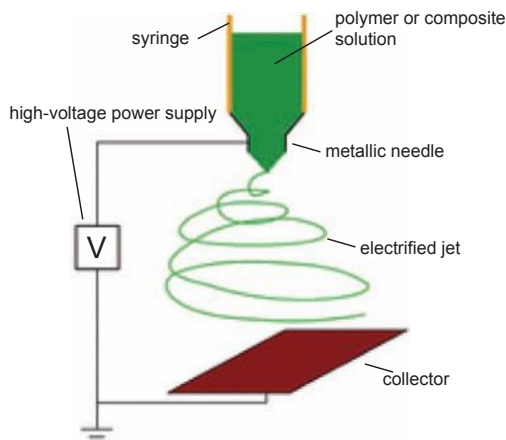
FIGURE 6.21 Diagram of an electrospinning setup. (Courtesy of Wikipedia.org; reprinted with permission.)

of cell viability. The electrospinning device employed a coaxial needle arrangement enabling the flow of a highly concentrated cell suspension in the presence of medical-grade polydimethylsiloxane (Jayasinghe *et al.*, 2007 and Figure 6.21).

These are all exciting techniques for cellularizing 3D nanofiber scaffolds, yet parameters such as solvent lethality, long-term cellular viability and contamination will need to be taken into account before they are routinely employed in tissue culture applications.

OTHER NANOTECHNOLOGY-BASED CELL CULTURE SYSTEMS

Titanium-Based Systems

Titanium nanoparticle-based systems have recently been explored, primarily in the area of 2D platforms, for the culture and propagation of stem cells. The primary advantage for using titanium nanoparticles as cell culture platforms is the ability to customize the matrix topography and roughness through adjustments in the thin film synthesis process known as **layer-by-layer (LbL) assembly**. LbL is a technique for surface modification which works on the principle of electrostatic attractions between oppositely charged particles. By customizing topography using LbL and titanium nanoparticles researchers
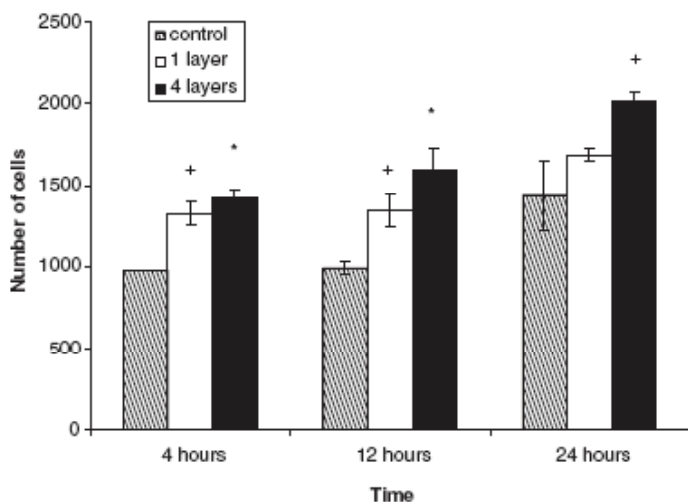
FIGURE 6.22 Number of cells attached on control, one-layer and four-layer substrates (*po0.01, +po0.05 compared with control). (Courtesy of Kommireddy *et al.*, 2006; reprinted with per mission.)

can design matrices with optimal surface attachment characteristics that are unique for different types of cells. David Mills' group in the Institute for Micromanufacturing at Louisiana Tech University in Ruston assembled titanium oxide (TiO_2) thin films and studied mouse mesenchymal stem cell attachment, proliferation and spreading as a function of layer numbers. In general, the higher layer numbers yielded increasing surface roughness that correlated with increased attachment and spreading (Kommireddy *et al.*, 2006 and Figure 6.22).

Along similar lines, Thomas Webster's group, whose research was discussed previously in this chapter and Chapter 3, investigated osteoblasts function on patterned titanium substrates containing alternating regions of micron rough and nano-rough surfaces prepared by novel electron beam evaporation techniques. The goal was to determine if surface orientation of features can mediate osteoblast adhesion and morphology. The results indicated greater osteoblasts adhesion on the nano-rough regions of the patterned substrates (Puckett *et al.*, 2008 and Figure 6.23). It should be noted that a number of groups have revealed toxicity and allergic reactions to titanium-based nanoparticulates.
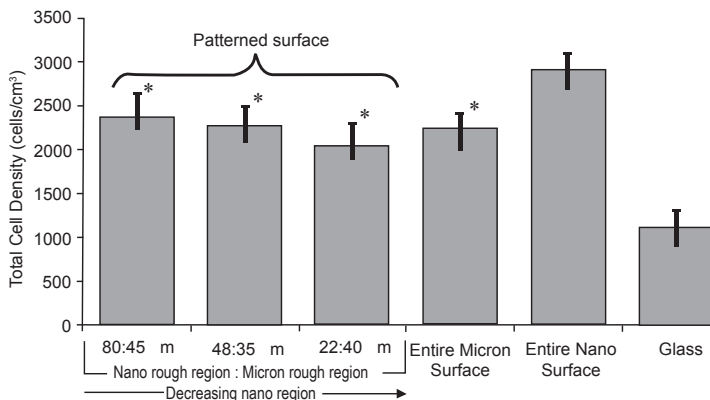
FIGURE 6.23 Increased osteoblasts adhesion on nano-rough Ti surfaces. Data are mean +/− SEM, $n = 3$, *$p < 0.01$ compared to the entire nano-Ti surface. Indicated on this graph is the total number of cells on each substrate irrespective of location of attachment. (Courtesy of Puckett *et al.*, 2008; reprinted with permission.)

Magnetic Nanoparticle-Based Systems

Heterotypic 3D co-culture of cells is essential for the mimicking of tissues and organs *in vitro*. Several groups have employed magnetism as a mechanism for creating these 3D environments using magnetic nanoparticles. Researchers at Kyushu University in Fukuoka, Japan have applied a magnetic force on magnetic cationic liposomes (MCLs) to construct a 3D co-culture system of HepG2 and NIH 3T3 cells that recapitulates in tissue culture the interactions of liver and mesenchymal cells *in vivo*. Cells were specifically labeled with MCLs and seeded onto attachment plates. Upon magnetic exposure cells formed a multilayered sheet resembling the *in vivo* 3D environment (Ito *et al.*, 2007 and Figure 6.24).

The same group expanded upon these studies to include an RGD targeting motif attached to the MCL phospholipid shell. The human keratinocyte cell line HaCaT demonstrated clustering, cell patterning and fibronectin expression when exposed to a magnetic field (Ito *et al.*, 2007 and Figure 6.25).

Finally, Nicholas Simpson and colleagues in the Department of Medicine at the University of Florida College of Medicine in Gainesville
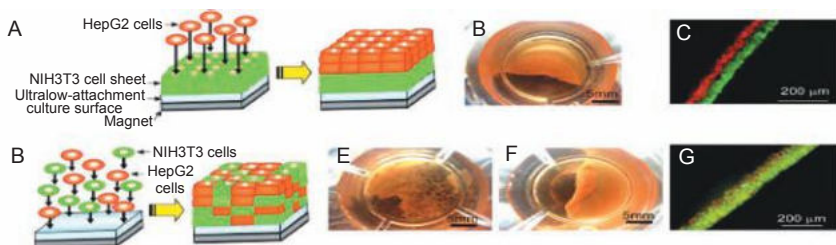
FIGURE 6.24 Heterotypic cell sheet of HepG2 and NIH3T3 cells constructed by magnetic exposure. (A) Schematic illustration of construction of layered cell sheet. (B) Harvested cells. (C) Fluorescence image of HepG2 cells (red) and NIH3T3 cells (green). (D) Schematic illustration of construction of mixed cell sheet. (E) Culture in the absence of a magnet. (F) Culture in the presence of a magnet. (G) Fluorescence image of HepG2 cells (red) and NIH3T3 cells (green). (Courtesy of Ito *et al.*, 2007; reprinted with permission.)

utilized monocrystalline iron oxide nanoparticles (MION) to distinguish between cells and monitor **glucose consumption rates (GCR)**, which is the rate of glucose consumption and metabolism by a particular cell, of pancreatic cells encapsulated in beads (Constantinidis *et al.*, 2010 and
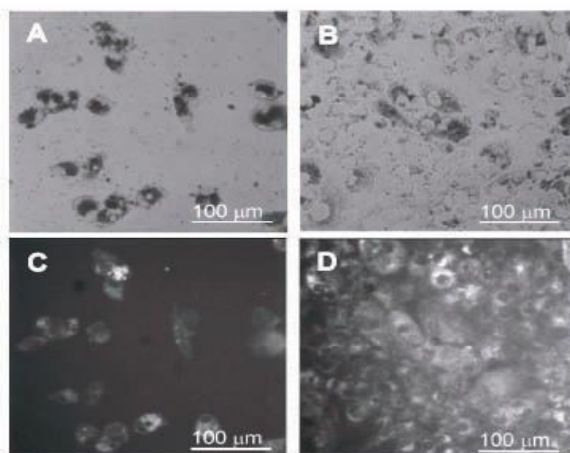
FIGURE 6.25 Fibronectin production of HaCaT cells cultured using RGD-MCLs. HaCaT cells were cultured for 3 (A, C) or 5 days (B, D) in the presence of RGD-MCLs and immunostained for fibronectin. Bright-field (A, B) and fluorescence (C, D) photography demonstrated cellular morphology and marker expression. (Courtesy of Ito *et al.*, 2007; reprinted with permission.)
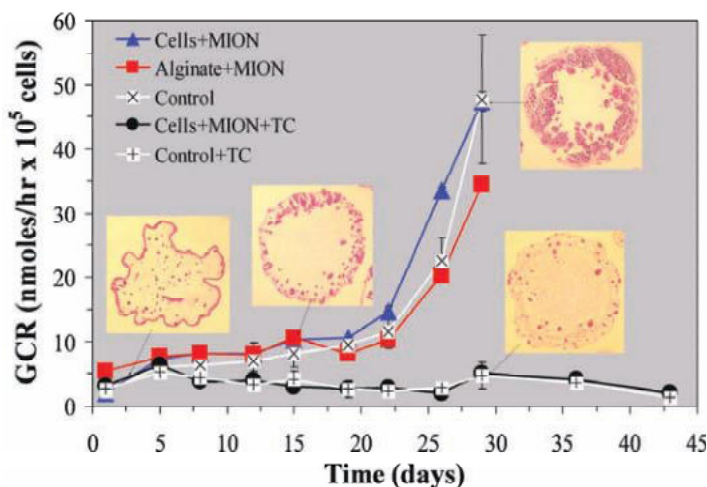
FIGURE 6.26 Temporal profiles of glucose consumption rates (GCR) by βTC-tet cells encapsulated in APA beads and cultured either in the absence or in the presence of tetracycline (TC). Five groups of APA cultures are depicted in the graph. GCR measurements from cultures that were not labeled with MION (i.e., Control) are depicted either by white squares with a black × for non-TC treated cultures, or by white squares with a black + for TC treated cultures. Measurements from MION-labeled cells encapsulated in APA beads and cultured in the absence of TC are depicted by triangles, while measurements from MION-labeled cells encapsulated in APA beads and cultured in the presence of TC are depicted by circles. Finally, measurements from APA beads made with MION-labeled alginate are depicted by squares. Representative histology cross-sections from beads cultured in the absence and presence of TC are shown to illustrate the effect of TC to regulate growth. (Courtesy of Constantinidis *et al.*, 2010; reprinted with permission.)

Figure 6.26). This is a prime example of a nanotechnology application for distinguishing between different cells of the same phenotype in culture and subsequent monitoring a particular cellular function.

CHAPTER SUMMARY

A Brief History of Tissue (Cell) Culture

1. Sydney Ringer was the first recorded researcher to culture a living explant.

2. Ross Granville Harrison invented the hanging drop tissue culture method.
3. Cell culture is now widely used to produce therapeutic monoclonal antibodies, vaccines and drugs.

Types of Cells Cultured

1. There are literally thousands of types of cells that may be propagated and manipulated in culture.
2. Immortalized cells such as cancer cells or certain stem cell lines can be propagated indefinitely in culture.

Manipulation of Cultured Cells

1. The ultimate goal of cell culture is to mimic the biological environment of a living organism.
2. Key issues that must be addressed in cell culture include nutrient depletion, accumulation of dead cells/debris, cell-cell contact and physical constraints.

2D or Not 2D?

1. The three-dimensional organization of cells in the body provides efficient access to growth factors, ligands, nutrients and signaling molecules.
2. Disadvantages of two-dimensional cell culture include lack of gradient signaling, altered cellular morphology and inadvertent clustering of cell surface receptors.

3D Cell Culture and the Issues of Scale and Purity

1. Many synthetic microfiber 3D matrices exist, yet large fiber diameter does not mimic a true 3D environment.
2. Pore and fiber size must be significantly smaller than cell size to accurately mimic the extracellular environment.
3. Naturally derived cell culture matrices such as Matrigel often contain impurities and unwanted growth factors thus negatively influencing cells.

Synthetic Nanofiber Scaffolds

1. The advantageous physical properties of electrospun nanofibers include high surface area to volume ratio, customizable pore size and high porosity.
2. The use of Ultra-Web showed enhanced proliferation and self-renewal of mouse embryonic stem cells.
3. A blend of PLLA and collagen I nanofibers resulted in the activation of collagen and decorin genes in tendon-derived fibroblasts.
4. Adjusting the chemical and degradation properties of nanospheres in a PLLA nanoscaffold/PLGA nanosphere blend allowed for the control of growth factor release.
5. Imprinted carbon nanofibers on a polycarbonate urethane substrate promoted osteoblast adhesion and differentiation.

Natural Nanofiber Scaffolds

1. Advantages for the use of natural nanofiber scaffolds in cell culture include biocompatibility, ease of synthesis and processability.
2. Collagen nanofibers are widely studied for 3D cell culture.
3. Advantages of silks in cell culture include biocompatibility, controlled degradation properties and versatile processability.
4. Electrospun silk nanofiber matrices promoted proliferation, alignment, elongation and differentiation of HAECs and HCASMCs.
5. Thermally responsive chitosan nanofibers have been developed that degrade upon temperature lowering and allow for the creation of polymer-free cellular sheets.
6. The primary advantage of biocomposite in cell culture is their heterogeneous nature.
7. Self-assembling peptides such as RADA16-I can spontaneously form three-dimensional matrices and assembly can be controlled by varying the pH of the system.
8. Self-assembling peptide nanofiber scaffolds have been shown to support cellular attachment, survival and differentiation of a variety of cell types.

Cellularizing Nanofiber Scaffolds

1. Complete cellularization of a nanoscaffold is crucial to appropriate cell culture.

2. Interweaving of different nanofiber sizes is a method for promoting the cellularization of 3D nanoscaffolds.

3. Salt leaching combined with electrospinning allows for delaminations in nanoscaffold pore size to promote cellularization.

4. Laser ablation may be implemented to introduce grooves into a nanoscaffold to promote cellularization.

5. The introduction of ice crystals or sacrificial nanofibers are ways to increase nanoscaffold pore size.

6. Cells may be seeded onto nanoscaffolds during scaffold synthesis.

Other Nanotechnology-Based Cell Culture Systems

1. The advantage of titanium nanoparticle platforms for 2D cell culture is the ability to customize matrix topography and roughness.

2. Nano-rough regions of titanium substrates have been shown to promote osteoblast adhesion.

3. Magnetic cationic liposomes have been used along with exposure to a magnetic field to create multilayered sheets of HepG2 and NIH 3T3 cells.

4. MIONs have been used to distinguish cell types and monitor glucose consumption rates.

KEY TERMS

- Tissue Culture
- Cell Culture
- *Ex Vivo*
- Hanging Drop Cell Culture
- Embryoid Body
- Hybridoma
- Transfection
- Micropore
- *In situ*
- Matrigel™
- Basement Membrane
- Pluripotency
- Ultra-Web®
- Nanosphere
- Lower Critical Solution Temperature (LCST)
- Elliptivity, ε
- Biocomposite
- Cellularization
- Layer-by-Layer (LbL) Assembly
- Glucose Consumption Rate (GCR)

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. What are some of the cell types used cell types in cell culture today?
2. List the critical issues that must be addressed for effective cell culture and their corresponding solutions.
3. What are the disadvantages of 2D cell culture?
4. Why is micromolecular 3D cell culture still not effective at recapitulating the *in vivo* environment?
5. What physical properties of electrospun nanofibers make them ideal for 3D cell culture?
6. List at least three types of synthetic nanofiber scaffolds used in 3D cell culture.
7. Describe Peter Ma's PLLA/nanosphere 3D cell culture system.
8. List at least three types of natural nanofiber scaffolds used in 3D cell culture.
9. Why are collagen nanofiber systems so popular in the area of 3D cell culture?
10. Describe Oscar Lee's characterization of mesenchymal stem cells grown on a 3D matrix of collagen nanofibers.
11. How did Kam Leong and colleagues activate the degradation of hydroxybutyl chitosan in their 3D cell culture system?
12. Write the equation for nuclear ellipticity, ε.
13. Why are biocomposite-based 3D nanofiber matrices advantageous for cell culture?
14. How did Xiaojun Zhao's group control the assembly process and 3D architecture of their self-assembled peptide-based nanoscaffolds?
15. Why is proper cellularization key to effective 3D cell culture?
16. Describe at least two methods for optimizing nanofiber scaffold pore size.
17. How did Robert Mauck and colleagues improve cell infiltration of their PCL-based nanoscaffold matrix?
18. What is the primary advantage of using titanium nanoparticles for cell culture applications?
19. How can magnetic nanoparticles be used to construct a 3D cell culture system?

7

Nanoparticle-Based Drug Delivery

Drug delivery is defined as the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. A great deal of development effort in the area of drug delivery has focused on technologies, methodologies and core delivery systems that modulate drug release rates, absorption efficiency, biodistribution and pharmacokinetics characteristics with the ultimate goals of improving efficacy and safety as well as reducing or eliminating unwanted side effects. While the field of drug delivery encompasses gross introduction of pharmaceuticals into the body including, for example, oral, topical, injectable and transmucosal routes, this chapter is focused on delivery at the tissue and cellular level post-introduction into the human body. It is really the extracellular and intracellular targeting capabilities of nanoparticles that will have the most impact on drug delivery and that are thus the major topics of the following sections. This chapter covers the major categories of

drug delivery and cites examples of each. Delivery across the blood-brain barrier (BBB) is not discussed as it was previously covered in Chapter 4.

TARGETED DRUG DELIVERY: BASIC PRINCIPLES

Traditional, non-specific drug delivery results in the distribution of medication throughout the body with only a small portion of the pharmaceutical reaching the intended physiological target tissue or cell type. As a result, drug efficacy may be low or unwanted side effects, such as those seen in many chemotherapeutic treatments, may be observed. **Targeted drug delivery** is a method of delivering medication to a patient in a manner that increases its concentration in some parts of the body or to certain cell types relative to others. It allows for achieving higher drug concentrations within intended target regions and lower amounts in healthy tissue, thus theoretically increasing efficacy and reducing unwanted side effects. Targeted drug delivery can be utilized to treat a variety of physiological disorders and anomalies including, for example, diabetes and

---

Focus Box 7.1 Elwood V. Jensen and the discovery of the estrogen receptor

Elwood V. Jensen is a pioneer in the field of hormone signaling. His finding that estrogens act by way of a receptor protein, which is absent in 70% of breast cancers, led to a targeted therapeutic for cancer. By measuring the receptor content of breast cancers one can predict which will not respond to endocrine therapy and should be placed directly on chemotherapy. He is considered "father" of the nuclear receptor field. Of his 44 prizes and honors, he received the prestigious Lasker Award in 2004. He is a Distinguished University Professor and the Wile Chair for Cancer Research at the University of Cincinnati College of Medicine. (Photo courtesy of the University of Chicago; reprinted with permission.)

cardiovascular diseases, yet perhaps its most promising application is in the area of cancer treatment. Discussed below are the two types of targeted drug delivery: active and passive targeting.

Active Targeted Drug Delivery

Active targeted drug delivery most often occurs as a result of tight binding between receptors present on the surface of a target cell and a targeting agent that is often the actual naturally occurring **ligand** (a molecule that binds to a receptor) specific for the target receptor or has been designed to mimic ligand binding effects. Monoclonal antibodies and peptides, for example, interact with cell surface receptors through amino acid recognition sequences, often in a molecular manner similar or identical to that for the receptor's naturally occurring protein ligand. Examples of entities which specifically interact with target cells include, but are not limited to, viruses, glycoproteins, bacteria, enzymes, hormones, antibodies and lectins (carbohydrate-binding proteins) (Figure 7.1). Targeted drug delivery methodologies are most often tailored to mimic interactions between these entities and their corresponding cell surface receptors.

An **antigen** is defined as any molecule that can be specifically bound by an antibody, although many antigens also bind other ligand types.
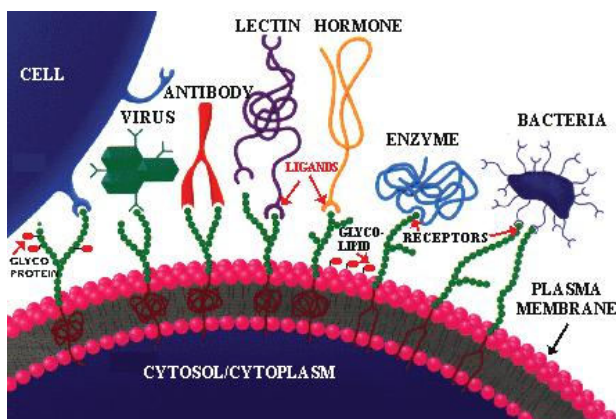
FIGURE 7.1 Diagrammatic illustration of ligand/receptor interactions on the cell surface. (Courtesy of R.E. Hurlbert, Washington State University; reprinted with permission.)

In the field of immunology antigens are characterized as binding major histocompatibility complexes (MHCs) and driving subsequent interaction with T cells to ultimately illicit antibody production, although this is beyond the scope of this text and will not be discussed here. In addition, as this chapter is focused on targeted therapies, the emphasis is on cell surface antigens, although many intracellular or secreted target examples exist. Researchers have now exploited cell surface antigenic properties to target drugs to a variety of specific cell types through antigen recognition. The vast majority of this effort has been directed at the targeting and eradication of cancer cells given that they tend to express surface markers, often in the form of antigens, which are unique to, or much more abundant in, cancer cells than normal cells. These are known as **tumor-specific antigens**, or TSAs. In the realm of cancer, there are now numerous confirmed examples of cancer cell types that express unique cell surface receptors and proteins which may allow for specific homing of anti-cancer drugs to these cells. There are also many more examples of **tumor-associated antigens (TAAs)**, which are antigens expressed by both normal and cancer cells but tend to be higher in level in cancer cells. Table 7.1 below is a compendium of both TSA and TAA examples.

A great deal of effort has thus been directed at utilizing TAAs and TSAs for drug targeting. For example, researchers at Purdue University have developed drug/ligand conjugates that specifically bind to the prostate-

Table 7.1 Examples of tumor-associated (TAA) and tumor-specific (TSA) antigens and their correspondence with certain cancers

Antigenic Determinant	Type of Antigen	Type of Cancer
Epidermal Growth Factor Receptor (EGFR or ERBB)	TAA	Lung, glioblastoma multiforme
Estrogen Receptor (ER)	TAA	Breast
Her2/Neu	TSA	Breast, ovarian, stomach
Plac1	TSA	Hepatocellular
Prostate Specific Antigen (PSA or PSMA)	TSA	Prostate
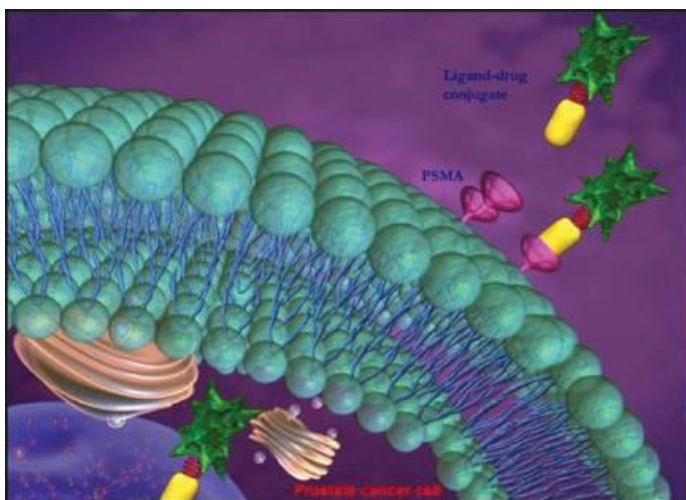
FIGURE 7.2 Diagrammatic illustration of drug targeting to prostate cancer cells. (Courtesy of *LabGrab*; reprinted with permission.)

specific membrane antigen (PSMA or PSA), which is a cell surface protein expressed exclusively by prostate cancer cells (Figure 7.2).

Active targeted drug delivery can thus be defined as the delivery of a drug to a particular tissue or cell type through specific and precise binding of the drug to the target tissues or cells. This binding is most often accomplished through interactions between outer moieties attached to the drug and specific antigens, surface markers or receptors that are unique to, and present on, the surface of target cells. Table 7.2 below lists examples of moieties that have been used successfully to drive drugs to intended target cancer cells, often by mimicking ligand/receptor interactions that have evolved over thousands, and perhaps millions, of years. It also outlines therapeutic mechanism of action and examples of cancer types treated.

Passive Targeted Drug Delivery

Passive targeted drug delivery is a mechanism and process by which certain sizes of molecules tend to preferentially accumulate in tumor tissues and is also known as the enhanced permeability retention (EPR) effect (also discussed in Chapter 2). It is based upon the considerable blood supply

Table 7.2 Examples of drug targeting agents as they apply to cancer therapeutics

Targeting Agent	Mode of Action	Cancer Application	Known Example(s)
Monoclonal Antibody	Cell Surface or Internalized	Breast, Colorectal, Head and Neck, Lymphoma	Herceptin, Erbitux, Avastin, Rituximab
Small Molecule	Cell Surface, Intracellular	Lung	Tarceva, Iressa
Aptamer	Cell Surface, Intracellular	Renal, Lung	AS1411 (in dev.)
Peptide	Cell Surface, Intracellular	Breast, Thyroid	RGD (in dev.)

requirements of tumors. As tumors grow and tumor cells divide new vasculature is generated locally to support this growth. Newly formed blood vessels are usually abnormal in form and architecture, with poor alignment of endothelial cells resulting in wide fenestrations lacking a smooth muscle layer. In addition, lymphatic drainage is usually hindered proximal to tumor tissues. These factors all contribute to fluid transport dynamics that promote the preferential uptake of macromolecules of a certain size by tumor tissues (Figure 7.3). It is referred to as "targeted" because of preferential accumulation of drug constructs within tumorigenic tissues as opposed to other areas of the body. Known as the EPR effect, it has now been exploited by a number of researchers and companies seeking to target the delivery of anti-cancer therapeutic agents to tumors and specifically to tumor cells. The EPR effect has been perhaps most successfully exploited for the delivery of nanoparticles that provide a mechanism for near infrared-based thermal ablation of cancer cells. This has been discussed in more detail in Chapter 2.

NANOPARTICLES FOR DRUG DELIVERY: BASIC REQUIREMENTS

The delivery of drugs and other therapeutic agents via the use of nanoparticles seeks to accomplish several primary goals which overlap with targeted drug delivery in general and include:

   i. More specific drug targeting and delivery
  ii. Reduction in toxicity while maintaining efficacy
 iii. Increased biocompatibility
  iv. Faster development of new therapeutic strategies
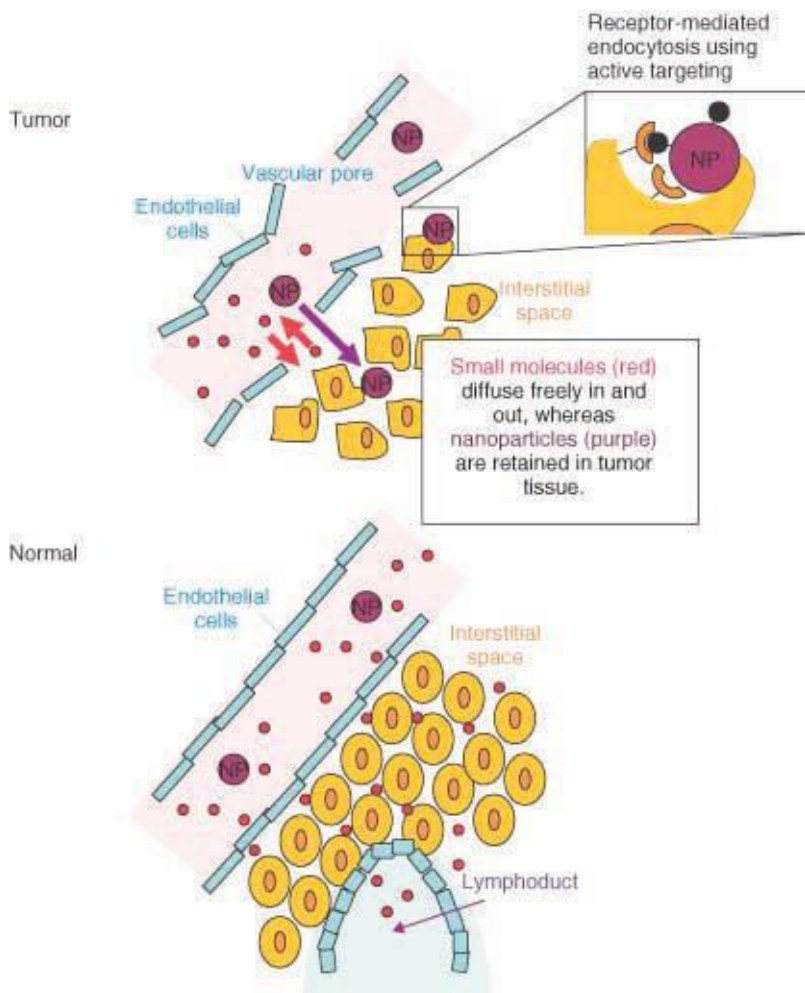
FIGURE 7.3 Diagrammatic illustration of the enhanced permeability retention effect. (Top) Small molecule flow from newly formed vasculature surrounding a tumor. (Bottom) Normal molecular flow. (Courtesy of John Wiley and Sons, Inc.; reprinted with permission.)

In order to properly and effectively design a novel nanoparticle-based targeted drug delivery platform there are basic prerequisites that must be taken into consideration including knowledge on:

   i. Drug incorporation and release
  ii. Nanoparticle/drug complex formulation stability and shelf-life
 iii. Biocompatibility
 iv. Biodistribution and targeting efficiency
  v. Functionality

In the majority of the cases the targeted nanoparticle-based drug delivery platform will not meet *all* of the primary goals nor will all of the knowledge parameters be fulfilled, yet some exciting examples listed below go a long way towards fulfilling the most critical milestones which are decreased toxicity, increased efficacy, biocompatibility and fewer side effects.

TYPES OF NANOPARTICLE-BASED SYSTEMS FOR DRUG DELIVERY

There are a variety of nanoparticle-based platforms currently under study for drug delivery. These include natural as well as synthetic and other man-made nanomaterials which possess the characteristics needed to either actively or passively target a therapeutic agent to a particular cell type or tissue. Table 7.3 lists some examples and corresponding delivered drugs. The major categories of nanomaterial- and nanoparticle-based drug delivery platforms are discussed below citing more notable unique examples for each. These are categorized as natural or man-made and as nanopolymers or nanoparticles for ease of classification. Several examples of some of the more exciting research in nano-based drug delivery have been cited, some of which emphasize targeting capabilities and others focus on non-targeted general drug delivery.

Synthetic Polymer-Based Nanoparticles
Polyethylene Glycol (PEG)
Polyethylene glycol (PEG) is perhaps one of the most widely studied and utilized polymers with respect to biomedical applications. It is an oligomer of ethylene oxide soluble in a variety of solvents, most notably water. It has exhibited low toxicity and can be synthesized to form nanoparticles for efficient *in vivo* drug delivery. Mark Davis' group in the Chemical Engineering Department at California Institute of Technology in Pasadena

Table 7.3 Examples of nanomaterials under study as drug delivery agents

Nanomaterial or Nanoparticle	Example of Delivered Drug	Indication
Albumin	Paclitaxel	Squamous cell carcinoma
Cetyl alcohol/polysorbate	Paclitaxel	Brain tumors
Chitosan	Insulin	Diabetes
Gelatin	Methotrexate	Various cancers
Gold	Radiation	Various cancers
Hydrogels	siRNA	Various cancers
Magnetic iron oxide	Daunorubicin	Leukemia
Poly(ethylene glycol)/poly (e-caprolactone) (PEG/PCL)	siRNA	Various cancers
Polyalkylcyanoacrylate (PACA) composites	Doxorubicin	Various cancers
Poly(D,L-lactic-co-glycolic) acid (PLGA)	Cisplatin	Prostate cancer
Solid lipid formulations	Clotrimazole	Fungal infections

has recently performed a phase I clinical trial for a systemic nanoparticle PEG-based RNAi delivery system to treat solid tumors. The nanoparticles consisted of (1) a linear, cyclodextrin-based polymer (CDP), (2) PEG as a hydrophilic nanopolymer for water solubility and stability in biological fluids, (3) a human transferrin protein targeting ligand to target the nanoparticles to cancer cells expressing the transferrin receptor and (4) siRNA molecules. The siRNA was designed to reduce the expression of the RRM2 gene which is an established anti-cancer target. The nanoparticles were administered intravenously into patients with malignant and metastatic melanoma tumors and ascertained for targeting via confocal microscopy. The authors showed a dose-dependent accumulation of targeted nanoparticles in tumors. In addition, it was demonstrated that the presence of RRM2 mRNA transcripts was drastically reduced in targeted tumors.

Poly(D,L-lactic-co-glycolic) Acid (PLGA)

As discussed in Chapter 3, poly(d, l-lactic-co-glycolic) acid (PLGA) is a synthetic polymer that has been studied extensively for use as a nano-scaffold for tissue regeneration and wound healing. Recently a number of groups have studied it in the context of individual nanoparticles for use as drug or therapeutic delivery vehicles. Researchers at the Koch Institute for Integrative Cancer Research at MIT in Cambridge studied a combination of PLGA and PEG nanoparticles as a platform for the targeted delivery of the anti-cancer drug cisplatin to prostate cancer cells. PLGA-PEG nanoparticles, average diameter size 150 nm, were synthesized by **nanoprecipitation**, which is defined as the formation of nanoparticles
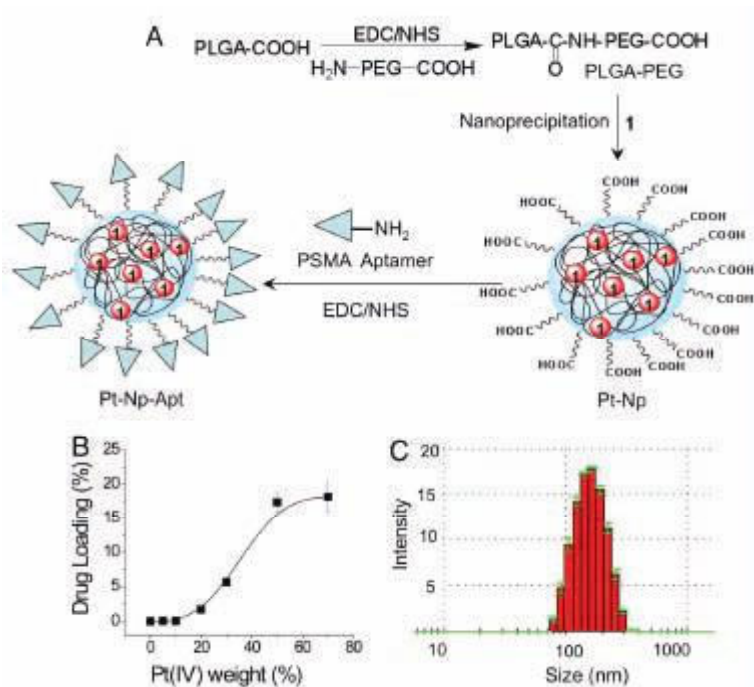
FIGURE 7.4 Construction and properties of aptamer-functionalized Pt(IV) nanoparticles. (A) Synthesis of Pt(IV)-encapsulated PLGA-*b*-PEG-COOH nanoparticles by nanoprecipitation and conjugation of PSMA aptamer to NP. (B) Loading of **1** in the PLGA-*b*-PEG-COOH nanoparticles. (C) Size of the Pt(IV)-encapsulated nanoparticles. (Courtesy of Dhar *et al.*, 2008; reprinted with permission.)

by precipitation of a water-insoluble polymer. This was accomplished by dissolving the polymer in a water-miscible organic solvent to which water was subsequently added followed by conjugation of prostate-specific membrane antigen (PSMA) aptamers (discussed in Chapter 2) to the final nanoparticles (Compound 1). Average drug loading capacity, LC, was ~6% (Dhar *et al.*, 2008 and Figure 7.4).

Cells were shown to be specifically targeted by the nanoparticles and to take up the drug cargo by **endocytosis**, which is defined as the transport of solid matter or liquid into a cell by means of a coated vacuole or vesicle. Uptake occurred specifically in LNCap human prostate epithelial cells which express high levels of PMSA but not the negative control cell line PC3 (Dhar *et al.*, 2008 and Figure 7.5). The efficacy of the system was confirmed to be approximately an order of magnitude greater than that of free cisplatin. As discussed in Case Study 7.1, other researchers have also developed PLGA-PEG-based nanoparticles systems for the delivery of therapeutics to treat prostate cancer.

Viral infections have also been targeted using nanoparticle-based drug delivery systems. Christopher Destache and colleagues at Creighton
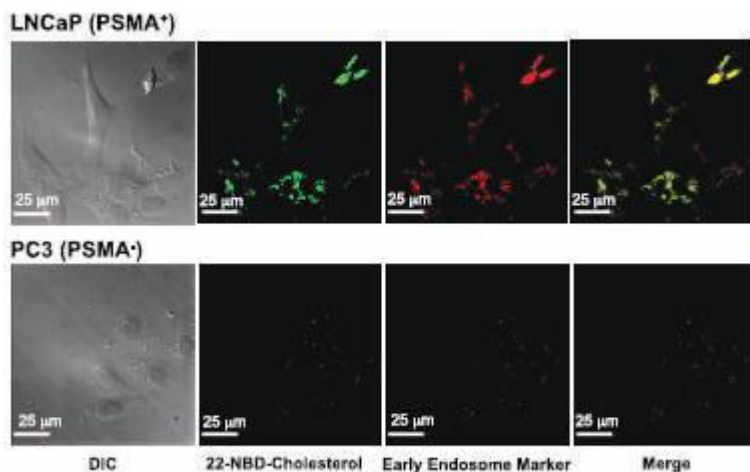
FIGURE 7.5 Detection of endosome formation and cellular uptake of Pt-NP-Apt in LNCaP cells by fluorescence microscopy. Green fluorescent 22-NBD-cholesterol and **1** were encapsulated in the PLGA-*b*-PEG nanoparticles and PSMA aptamers were conjugated to the surface of the particles. The early endosomes were visualized in red by using the early endosome marker EEA-1. (Courtesy of Dhar *et al.*, 2008; reprinted with permission.)

Case Study 7.1: Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo

Robert Langer's group at the Massachusetts Institute of Technology–Harvard Center for Cancer Nanotechnology Excellence in Boston has conducted studies utilizing a PLGA-PEG-based nanoparticulate system for the delivery of the anti-cancer drug docetaxel (Dtxl) to treat prostate cancer *in vivo*. An aptamer-based system targeting the PSMA protein expressed on the surface of prostate cancer cells was exploited to demonstrate remarkable efficacy and reduced peripheral cell toxicity. After a single intratumoral injection of the functionalized nanoparticles complete tumor reduction was observed in five of seven LNCaP xenograft nude mice. All of these animals survived the 109-day study in comparison to Dtxl alone which had a survivability rate of only 14% (Farokhzad *et al*., 2006 and Figure 7.6).
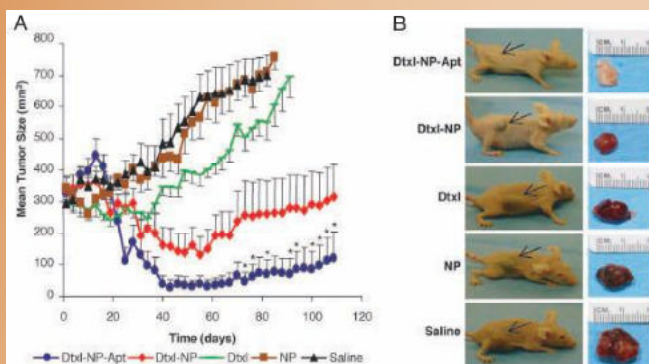
FIGURE 7.6 Comparative efficacy study in an LNCaP xenograft nude mouse model of prostate cancer. (*A*) Prostate cancer was induced in mice by implanting LNCaP prostate epithelial cells subcutaneously in the flanks of nude mice and allowing the tumors to develop to appreciable size over 21 days. The comparative efficacy study of single intratumoral injection (day 0) of (*i*) saline (black); (*ii*) pegylated PLGA NP without drug (NP, brown); (*iii*) emulsified Dtxl (Dtxl, green), (*iv*) Dtxl-encapsulated NPs (Dtxl-NP, red), or (*v*) Dtxl-encapsulated NP-Apt bioconjugates (Dtxl-NP-Apt, blue) was evaluated over 109 days and demonstrated that targeted NPs are significantly more efficacious in tumor reduction as compared with other groups. Data represent mean +/- SEM of seven mice per group. *Data points for the Dtxl-NP-Apt group that were statistically significant compared with all other groups. (*B*) Representative mice at end points for each group are shown (*Left*) alongside images of excised tumors (*Right*). For the Dtxl-NP-Apt group, which achieved complete tumor regression, the scar tissue and underlying skin at the site of injection are shown. Black arrows point to the position of the implanted tumor on each mouse. (Courtesy of Farokhzad *et al*., 2006; reprinted with permission.)
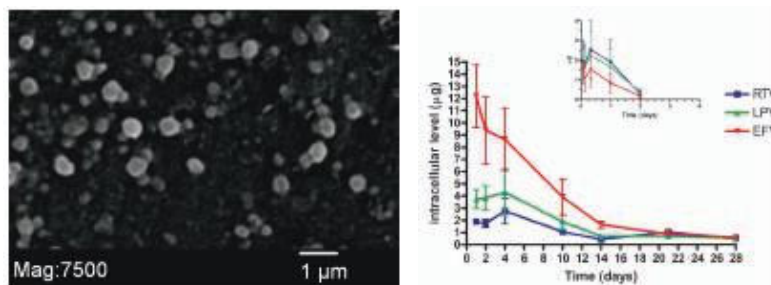
FIGURE 7.7 PLGA nanoparticles and AR drug release. (Left) Scanning electron microscopy (SEM) of fabricated antiretroviral nanoparticles (Mag × 7500). (Right) *In vitro* ART release from NPs incubated in PBMCs. Intracellular ritonavir, lopinavir, and efavirenz levels in PBMCs over time. The insert figure is the intracellular free drug levels in PBMCs over time. (Courtesy of Destache *et al.*, 2009; reprinted with permission.)

University in Omaha, Nebraska designed and synthesized a PLGA polymeric nanoparticulate system that contained three anti-retroviral (AR) drugs including ritonavir (RTV), lopinavir (LPV) and efavirenz (EFV) by an emulsion solvent evaporation procedure. Final nanoparticle-drug conjugate diameters averaged 262 nm with a loading capacity, LC, averaging 4%. The system was tested for AR drug release in the presence of human peripheral blood mononuclear cells (PBMCs) which demonstrated sustained release and high drug levels in the cells a full month after initial exposure with no cytotoxicity (Destache *et al.*, 2009 and Figure 7.7).

Polylactic Acid (PLA)

The slowly degrading **aliphatic** (carbon atoms linked in open chains) chains known as polylactides, or PLAs, are biocompatible and can be custom synthesized to meet both size and conformational requirements that allow for the encapsulation of a variety of drugs. PLA degradation allows for drug release and its rate can be controlled by the polymer's molecular weight, conformation and overall composition. These properties make PLA nanoparticles an advantageous choice for the intracellular delivery of therapeutic small molecules, proteins or nucleotide-based platforms. Francine Behar-Cohen's group in the Department of Ophthalmology, Hotel-Dieu of Paris, France studied the kinetics of polylactide nanoparticle

localization and encapsulated material release within the intraocular tissues. Nanoparticles containing the dyes Nile red (Nr) or Rh-6G (Rh) were injected intravitreously and studied for dye release. A rapid settling of the nanoparticles on the internal limiting membrane was observed with no toxic effects. Transretinal movement of the nanoparticles was observed followed by localization within RPE (retinal pigment epithelial) cells. This localization was sustained for at least 4 months post-injection and free dye diffusion from the PLA nanoparticles was also observed (Bourges *et al.*, 2003 and Figure 7.8).
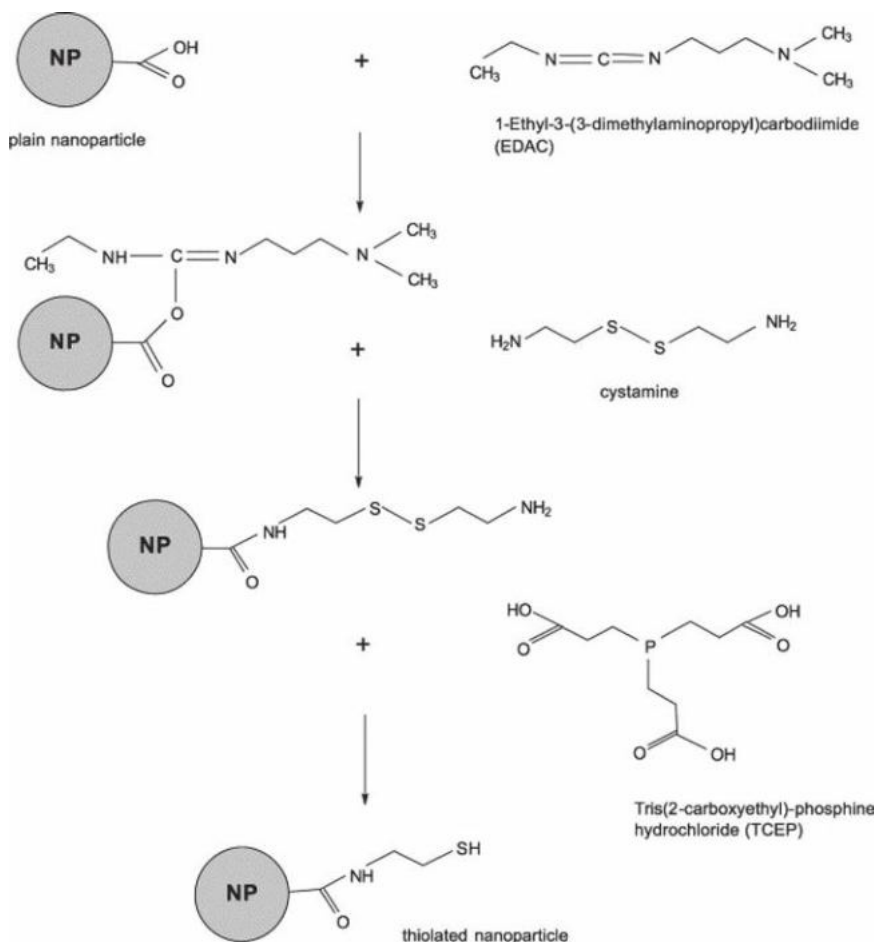
FIGURE 7.8 Diagrammatic flowchart of labeling PLA nanoparticles for active targeting. (Courtesy of *Science Direct*; reprinted with permission.)

Jinming Gao and colleagues in the Department of Pharmacology at the University of Texas Southwestern Medical Center in Dallas developed a PEG-PLA composite nanoparticle system for the delivery of the anti-cancer drug β-lapachone (β-lap), which is **bioactivated** (activation of an inert drug for use in a biological setting) by NADP(H):quinine oxidoreductase 1 (NQO1), an enzyme overexpressed in a variety of tumors. The PEG-PLA system was used to circumvent β-lap's poor aqueous solubility. A film sonication method yielded micelles with a loading capacity, LC, of ~4.7% and an optimal size of 29.6 nm in diameter. β-lap was sequestered internally, surrounded by the hydrophobic ends of hyodrophobic-hydrophilic polymer blocks (Blanco *et al.*, 2007 and Figure 7.9). The researchers performed *in vitro* studies on tumor cell lines expressing NQO1 which revealed significant β-lap activation and cytotoxicity.

Polycaprolactone (PCL)

Polycaprolactone (PCL) is yet another synthetic nanopolymer that has been studied extensively as a nanoparticle-based drug delivery vehicle. It can be custom synthesized into monodisperse nanoparticles of discrete size and makeup for the precise control of drug release. **Electrohydrodynamic atomization (EHDA)** is one method by which the polydispersity of PCL nanoparticles is kept to a minimum. It entails a process of filtration and spray drying synthesis as well as a stir/freeze dry procedure. Ventilation and residual discharge can be customized to increase yield, in this case to ~80%. It has now been successfully implemented to fabricate PCL nanoparticles containing the anti-cancer therapeutic agent Taxol® (Ding *et al.*, 2005 and Figure 7.10).

Most research on PCL with respect to its use as a drug delivery vehicle has been focused on its application to composite nanoparticles encompassing poly ethylene oxide (PEO). Researchers in the Department of Pharmaceutical Sciences at Northeastern University in Boston have developed a PCL-PEO nanoparticle delivery system that allows for the modulation of intracellular levels of the secondary lipid messenger ceramide in order to lower the apoptotic threshold of multidrug-resistant ovarian adenocarcinoma (SKOV3) cells. Nanoparticle dispersions were generated by solvent displacement and loaded with the drugs paclitaxel or tamoxifen which are known to drive an increase in intracellular ceramide
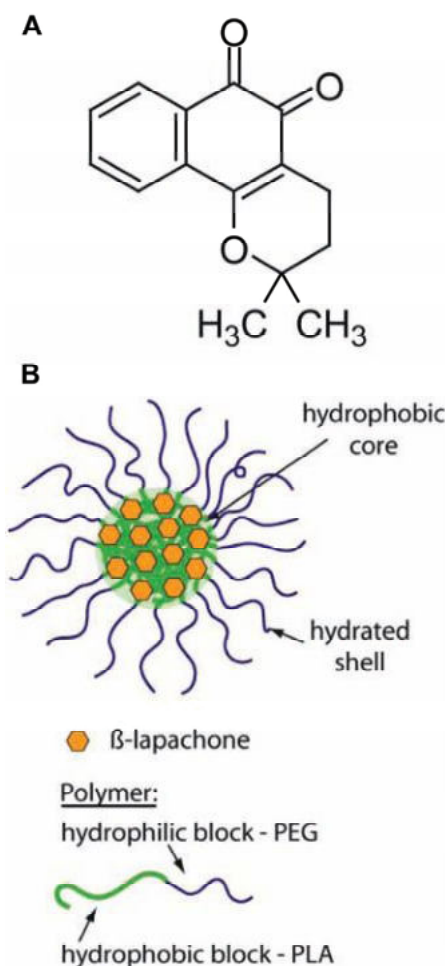
FIGURE 7.9 Schematics of β-lapachone and PEG-PLA nanoparticle delivery system. (A) Chemical structure of β-lap (MW = 242 Da). (B) Schematic of a β-lap-containing polymer micelle and constituent components. (Courtesy of Blanco *et al.*, 2007; reprinted with permission.)

levels. Oregon Green dye-labeled drugs were shown to be delivered efficiently into the cytoplasm of SKOV3 tumor cells *in vivo* (Devalapally *et al.*, 2008 and Figure 7.11). Increased tumor cell cytotoxicity was observed that was far superior using the PCL-PEO nanoparticle system as compared to naked drug delivery alone.
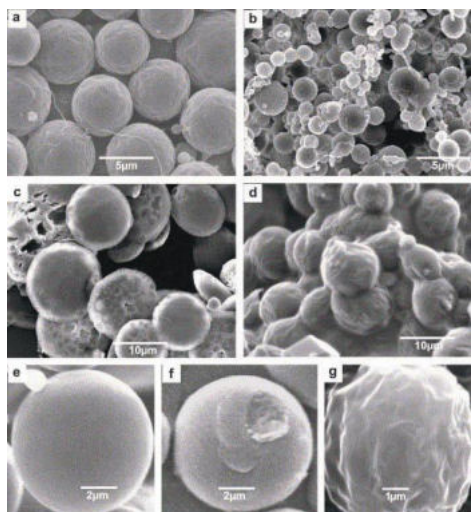
FIGURE 7.10 SEM images of Taxol loaded PCL nanoparticles. (a) PCL particles collected from the filter; (b) PCL particles fabricated from a spray drying technique; (c) PCL particles collected ultrasonic stirring and freeze drying treatment; (d) PCL particles after 45 days *in vitro* release; (e) Morphology of PCL particle collected from the filter; (f) Morphology of PCL particle collected from the side wall; (g) Morphology of PCL particle collected from the filter. (Courtesy of Ding *et al.*, 2005; reprinted with permission.)

Polyacrylate (PACA)

Polyacrylate (PACA) nanoparticles have become increasingly popular as potential drug delivery vehicles, primarily due to superior water-soluble characteristics which aid in the solubility of drug molecules in a biological setting. Many drugs can be **acrylated** (converted to a salt or ester of an acrylic acid) and thereby made water-soluble using polyacrylate nanoparticles. Edward Turos and colleagues at the Center for Molecular Diversity in Drug Design, Discovery, and Delivery, University of South Florida in Tampa did just this for penicillin. Specifically, the researchers converted the water-insoluble antibiotic to an acrylated form followed by dissolving it in a mixture of liquid monomers (butyl acrylate and styrene). The mixture was subsequently emulsified with a **surfactant** (a substance that when dissolved in water or an aqueous solution reduces its surface tension or the interfacial tension between it and another liquid) and polymerized into nanoparticles exhibiting diameters of between
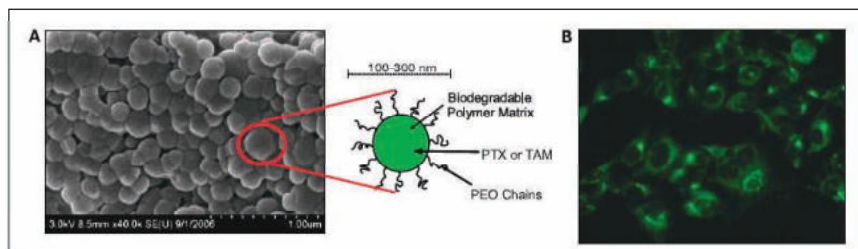
FIGURE 7.11 Polymer-based engineered nanoparticle formulations. (A) Scanning electron micrograph and schematic illustration of the PEO-PCL nanoparticle system with encapsulated paclitaxel (PTX) and tamoxifen (TAM) for single and combination therapy in SKOV3 ovarian adenocarcinoma model. (B) Fluorescence microscopy analysis of intracellular delivery of Oregon Green dye-labeled paclitaxel in PEO-PCL nanoparticles to SKOV3 cells. (Courtesy of Devalapally *et al*., 2008; reprinted with permission.)

25 and 40 nm. **Kirby-Bauer antibiotic testing** (KB testing), which is a classical test using antibiotic-impregnated wafers to discern bacterial killing efficiency, was performed revealing that the polyacrylate nanoparticle-based formulations retained activity even in the presence of the penicillin-degrading enzyme penicillinase (beta-lactamase), which is a characteristic enzyme produced by the methicillin-resistant form of *S. aureus*, MRSA (Turos *et al*., 2007 and Figure 7.12). As outlined by the authors, the features of this system include a simple one-step synthesis procedure, the ability to rigidly control nanoparticle size, absence of cytotoxic effects and an ability to incorporate drugs covalently into the nanopolymers framework or non-covalently via encapsulation.

Yet another example of the use of polyacrylate nanoparticle systems for increasing the water-solubility and therefore delivery efficiency of drugs has been developed by Anirban Maitra's group in the Department of Pathology at The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins School of Medicine in Baltimore. In these studies the researchers chose to focus on oral delivery of cancer chemotherapeutics using nano-delivery systems based on polyacrylates to increase water solubility in this case of the anti-cancer agent rapamycin. A copolymer of N-isopropylacrylamide and acrylic acid, with either methylmethacrylate or vinylpyrrolidone, was synthesized through free radical polymerization
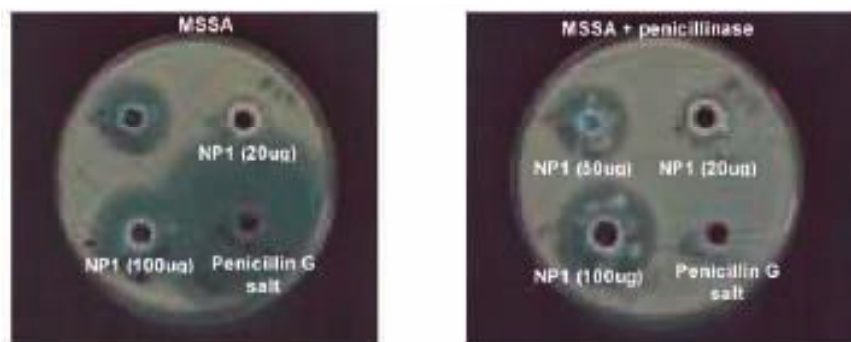
FIGURE 7.12 Kirby-Bauer studies of NP1 using *S. aureus*. Assays were performed first in the absence of added penicillinase protein (left image) and then in the presence of 100 μg of penicillinase added to the agar (right image). The control, penicillin G, lost all of its activity in the presence of the enzyme while the nanoparticulate system, NP1, retained its original activity at all 3 drug amounts, as noted. (Courtesy of Turos *et al.*, 2007; reprinted with permission.)

and loaded with rapamycin via a post-polymerization method in which the drug was dissolved after the copolymer formation has taken place, and rapamycin was directly loaded into the hydrophobic core of nanoparticles by physical entrapment. In *in vitro* studies, the efficiency of rapamycin release from the nanoparticles was characterized according to the following equation:

$$\text{Release } (\%) = \frac{(\text{Rapamycin})_{\text{rel}}}{(\text{Rapamycin})_{\text{tot}}} \times 100$$

where $[\text{Rapamycin}]_{\text{rel}}$ is the concentration of released rapamycin collected at time, t, and $[\text{Rapamycin}]_{\text{tot}}$ is the total amount of rapamycin initially entrapped in the nanoparticles. They observed a **"burst" effect** (a large drug volume is quickly released from a complex) of release at acidic pH and 100% release after 168 hours in culture. The researchers also demonstrated superior anti-cancer effects in xenograft murine animal models of pancreatic cancer in comparison to non-nanoparticle systems and controls (Bisht *et al.*, 2008 and Figure 7.13).
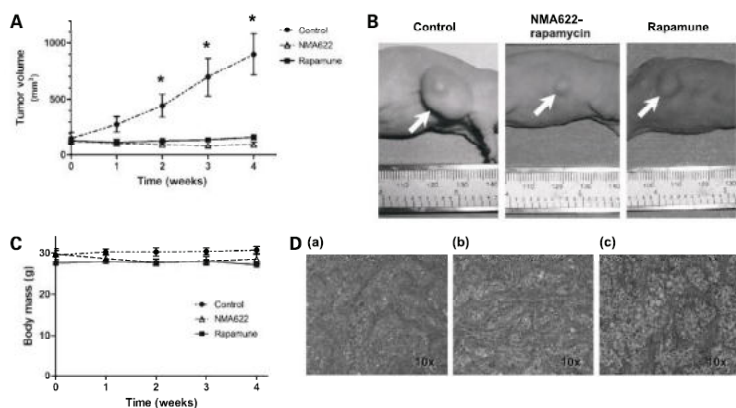
FIGURE 7.13 *In vivo* efficacy of oral nanorapamycin and oral Rapamune in the rapalogue-sensitive Panc198 pancreatic cancer xenograft. (A) Treatment of s.c. Panc198 xenografts with oral nanorapamycin and oral Rapamune results in significant growth inhibition as compared with mock-treated controls. X axis: Weeks of therapy; Y axis: Average tumor volumes for each time point of measurement (seven Panc198 xenografts per arm). (B) Representative photographs of Panc198 xenograft–bearing mice treated with oral nanorapamycin, oral Rapamune, and control tumor, taken at the end of the 4-wk treatment course. (C) No loss of body weight was observed in mice in the oral nanorapamycin treatment arm, comparable to the Rapamune and control arms, underscoring the absence of incidental toxicities. (D) Representative photomicrographs of control (a), NMA622-nanorapamycin–treated (b), or Rapamune-treated (c) Panc198 xenografts. There is an increase in the intervening stromal component, and smaller islands of neoplastic cells are seen, in the xenografts receiving oral nanorapamycin compared with control xenografts; this alteration was less appreciable in the Rapamune group. (Courtesy of Bisht *et al.*, 2008; reprinted with permission.)

Dendrimers

Dendrimers are perhaps the most widely studied synthetic nanopolymers as they apply to the development of platforms for drug delivery. As discussed in Chapters 1 and 4, dendrimers are synthetic polymers exhibiting branched-like configurations that achieve structural perfection. It is the branched nature of dendrimers that lends them well to drug delivery, both for the attachment of targeting moieties as well as side-chains to promote the aqueous solubility of therapeutic agents. For example, Gong Wu and

colleagues in the Department of Pathology at Ohio State University in Columbus targeted dendrimers containing the drug methotrexate to implanted glioma tumors in rats and observed an increased survival rate upon introduction of the dendrimer-drug complex (Wu *et al.*, 2006). **Methotrexate** is an anti-metabolite and anti-folate drug used in the treatment of cancers and autoimmune diseases. Its mechanism of action is to inhibit the enzyme dihydrofolate reductase (DHFR) to prevent folate synthesis in cancer cells. Their system targeted epidermal growth factor receptors (EGFR) present on the surface of tumorigenic cells via the use of the EGFR-specific monoclonal antibody cetuximab (trade name Erbitux). Specifically, cetuximab was covalently linked to fifth-generation (G5) polyamidoamine dendrimers containing the cytotoxic drug methotrexate according to the following schematic:

$$\text{Cetuxmab (C225)} \xrightarrow{\text{NaIO}_4} \text{C225-CHO}$$

$$\text{SPDP-G5} \xrightarrow[\text{EDC, NHS}]{\text{MTX}} \text{SPDP-G5-MTX} \xrightarrow[\text{KMUH}]{\text{DTT}} \text{KMUH-G5-MTX}$$

$$\left.\begin{array}{c} \\ \\ \end{array}\right\} \text{C225-G5-MTX}$$
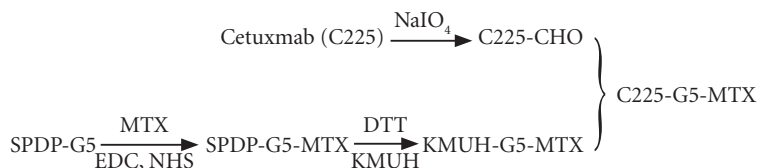
where MTX=methotrexate, EDC=1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride, NHS=N hydroxysuccinimide and KMUH= N-(n-maleimidoundecanoic acid) hydrazide. Roughly 12.6 molecules of methotrexate were present per unit of dendrimer. Dendrimer complexes were targeted to glioma tumors in rats and survival rate increases were assessed through the use of **Kaplan-Meier survival curves**, which are graphs of percent survival (y-axis) to time (x-axis). Within statistical error Wu's system was at least as effective as methotrexate alone with respect to survival rates but did reveal more extensive tumor cell necrosis (Wu *et al.*, 2006 and Figure 7.14).

Lajos Balogh and colleagues at the NanoBiotechnology Center, Roswell Park Cancer Institute in Buffalo, New York developed a dendrimer-based system for the delivery of radioactive gold (Au) nanoparticles for **brachytherapy**, which is defined as radiotherapy in which the source of radiation is placed in or close to the area being treated. The team fabricated poly[198]Au-radioactive gold/dendrimer composites, which they refer to as "nanodevices," for targeted radiopharmaceutical delivery to tumors. Fabrication was accomplished by irradiation of aqueous solutions of
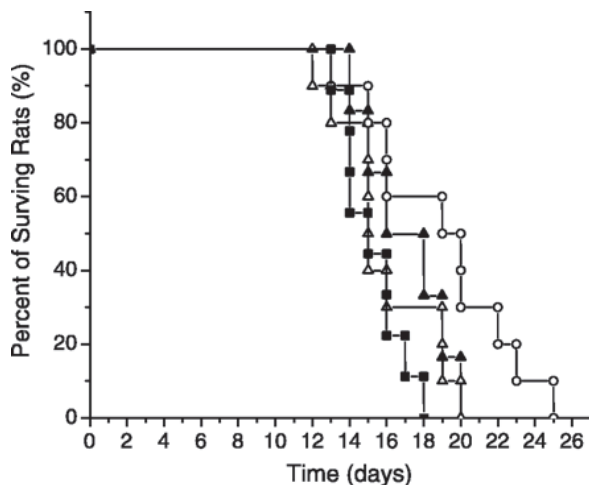
FIGURE 7.14 Kaplan-Meier survival curves for F98EGFR glioma-bearing rats treated with a targeted dendrimer/methotrexate complex. Rats were untreated (■) or treated with cetuximab (C225; ▲), C225-G5-MTX (△), and free methotrexate (o) 1 week after implantation of 10^5 F98$_{EGFR}$ cells. (Wu, *et al.*, 2006; reprinted with permission.)

^{197}Au-containing poly(amidoamine) dendrimer tetrachloroaurate salts or ^{197}Au^0 gold/dendrimer nanocomposites in a nuclear reactor which resulted in the formation of positively charged and soluble poly ^{198}Au^0 radioactive **composite nanodevices (CNDs)** (Khan *et al.*, 2008 and Figure 7.15). The nanodevices were of two sizes, 10 nm or 29 nm in diameter and were tested on a mouse model of melanoma. It was observed that a single intratumoral injection of the nanodevices resulted in a 45% reduction in tumor volume when compared with controls including non-radioactive dendrimers.

Hu Yang's group in the Department of Biomedical Engineering at Virginia Commonwealth University in Richmond took a different approach to create a dendrimer-based hybrid vehicle for hypoxia-targeted drug delivery. **Hypoxia** is defined as a deficiency in the amount of oxygen that reaches a tissue. It is well known that tumors often contain hypoxic regions that result from a shortage of oxygen due to poorly organized tumor vasculature. Cancer cells in hypoxic regions are often resistant to radiation and chemotherapy thus limiting treatment efficacy. Yang's
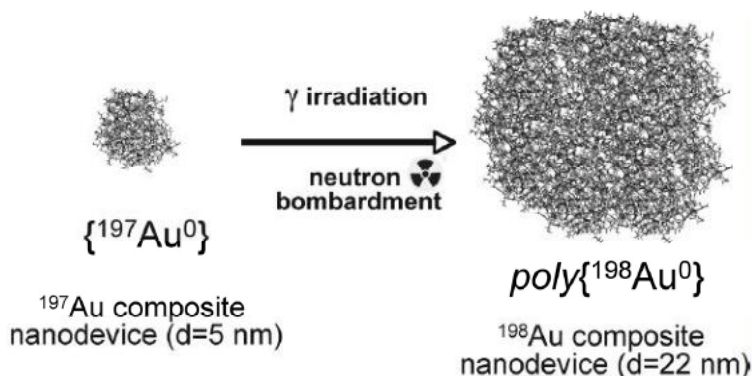
FIGURE 7.15 Schematic of polymerized composite nanoparticle formation. Composite nanoparticles were formed through radiation polymerization of a dendrimer network of templates with simultaneous neutron activation of ^{197}Au in gold composite nanoparticles into ^{198}Au. Dark dots represent gold atoms and the organic network is grey. (Courtesy of Khan *et al.*, 2008; reprinted with permission.)

group noted that **macrophages**, which are large white blood cells that ingest foreign particles and infectious microorganisms by phagocytosis (cellular eating) tend to target hypoxic tissues and thus may act as ideal anti-cancer drug delivery vehicles. They designed a novel cyborg-like targeting platform by hybridizing living macrophages with nanoparticles through cell surface modification. Nanoparticles, specifically PAMAM dendrimers, immobilized on the cell surface provided numerous sites for anti-cancer drug loading. Macrophage cell surfaces were modified with sialic acid residues to allow for the attachment of dendrimer nanoparticles via a **Schiff base linkage**, which is a linkage formed by the condensation of an aldehyde and a ketone. Dendrimers with surface-conjugated PEG were attached via an amino linkage and had multiple sites for drug loading (Holden *et al.*, 2010 and Figure 7.16). Quantum dot-labeled hybrids were analyzed and it was demonstrated that the majority of the nanoparticle components remained on the surface of the macrophages and was not internalized for an extended period of time. Ultimately, however, internalization of surface nanoparticles was inevitable due to the inherent phagocytic properties of macrophages. It will be necessary to address this before the hybrids may be effectively tested for *in vivo*
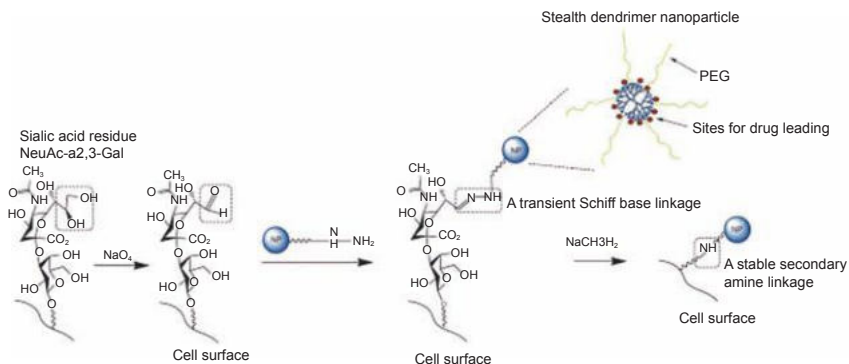
FIGURE 7.16 Hybridization of nanoparticles and macrophages through cell surface modification. Sialic acid residues on the cell surface are modified with sodium periodate to generate aldehydes. Aldehydes react with amine group of PEG conjugated to the nanoparticle surface to form Schiff bases. Schiff bases can be further reduced to stable secondary amine linkages using sodium cyanoborohydride. Abbreviation: PEG, polyethylene glycol.

therapeutics efficacy, yet the work is promising and demonstrates a truly novel approach to using a combination of both nanotechnology and live cells for cancer therapeutics applications.

Finally, James Baker's group at the University of Michigan's Center for Biologic Nanotechnology in Ann Arbor used folic acid receptor targeting to home PAMAM dendrimers carrying either methotrexate or tritium to tumors which overexpress the receptor. Generation 5 (G5) PAMAM dendrimers with an average of 110 surface primary amine groups were used on this study. Dendrimers were acetylated to reduce nonspecific binding and to allow for conjugation to methotrexate, folic acid or fluorescent/dye markers for monitoring of targeting efficiency. The **polydispersity index (PDI)**, which is defined as a measure of the distribution of molecular mass in a given polymer sample, of the G5 dendrimers was observed to be 1.032, indicating a very narrow distribution of nanoparticle size and high purity (an ideal sample has a PDI of 1.0). When introduced into 6–8 week old athymic nude mice harboring KB human tumors in which the cells overexpress the folate receptor specific targeting and significant decreases in tumor volume was observed over a two-month period (Kukowska-Latallo *et al.*, 2005 and Figure 7.17).
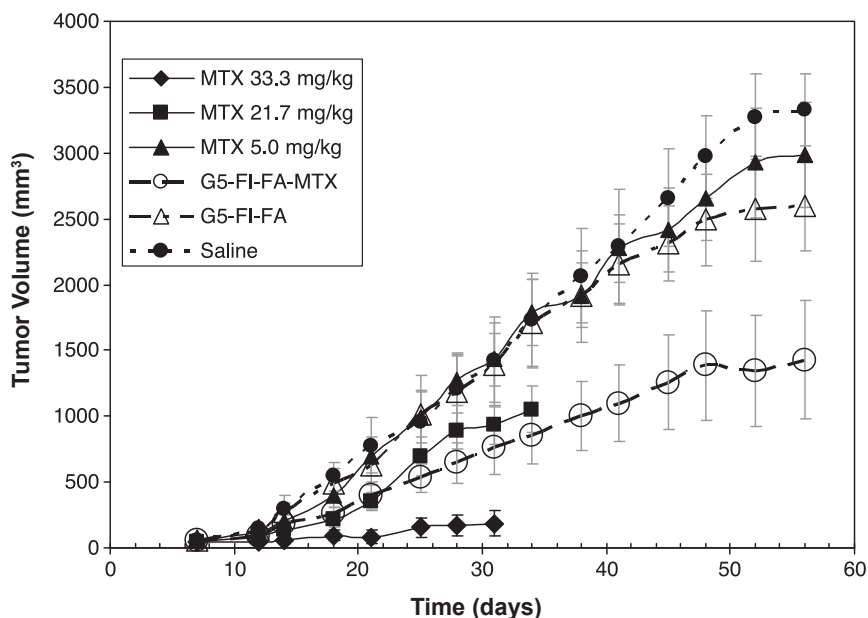
FIGURE 7.17 Tumor growth in SCID mice bearing KB xenografts during treatment with G5-FI-FA-MTX conjugate and free methotrexate (MTX). Tumor volume (mm^3) was calculated using the formula for a standard volume of an ellipsoid. The dose of the conjugate (55.0 mg/kg) equivalent to the lowest dose of free methotrexate (5.0 mg/kg) is as effective in tumor growth delay as the intermediate dose of free methotrexate (21.7 mg/kg). The lowest dose of free methotrexate (5.0 mg/kg) does not inhibit the tumor growth. During 15 biweekly injections (56 days) of the experiment, the highest dose of methotrexate (33.3 mg/kg) affected ~30% of animal body weight and was lethal (LD50) at 32 days. The intermediate dose of free methotrexate (21.7 mg/kg) was lethal (LD50) at 39 days, affecting ~30% of animal body weight. The remaining dose of free methotrexate (5.0 mg/kg) and all of the doses of the conjugates and control treatments were not toxic. (Courtesy of Kukowska-Latallo *et al.*, 2005; reprinted with permission.)

Synthetic Metal-Based Nanoparticles

Iron Oxide

In addition to applications in imaging and diagnostics as discussed in the next chapter, iron oxide nanoparticles have also been studied as potential carriers of therapeutic agents to sites of disease, most notably

cancer. In a unique study, Bao-an Chen and colleagues at Southeast University in Nanjiing, China coupled the anti-cancer drug daunorubicin (DNR) to iron oxide nanoparticles in a strategy aimed at addressing multi-drug resistance (MDR). The goal was to attack the effectiveness of p-glycoprotein and its removal of drugs from cells that express it (see Chapter 4). The researchers postulated that iron-oxide-conjugated DNR complexes are likely to be too large to be transported effectively by P-gp out of cells thus becoming trapped within the cells resulting in cytotoxicity. DNR-conjugated Fe_3O_4 nanoparticles were synthesized by electrochemical deposition under oxidizing conditions and capped with tetraheptylammonium, which acts as a stabilizer of colloidal nanocrystallites. Ultrasound was subsequently used to distribute the magnetic nanoparticles in saline to obtain a suspension solution. DNR was conjugated to the suspended nanoparticles by mechanical absorption polymerization. Final conjugates were introduced into Balb-c nude mice bearing K562-n/VCR multi-drug resistant tumors by vena caudalis injection and resulted in apoptosis of these cells and a corresponding reduction in tumor size that was greater than controls, including that of DNR alone (Chen *et al.*, 2009 and Figure 7.18).
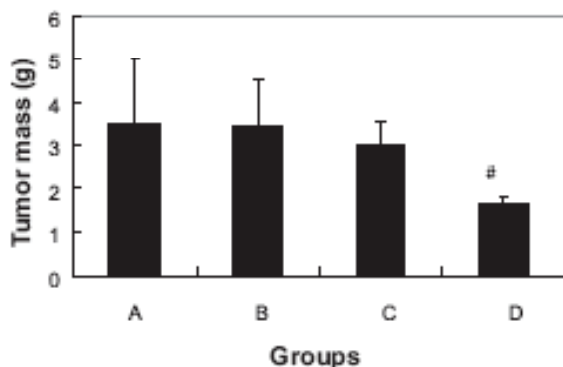
FIGURE 7.18 Tumor weight change in four groups. (A) negative control, (B) MNPs-Fe_3O_4, (C) DNR; (D) MNPs-Fe_3O_4 + DNR. Notes: A: K562-n/VCR tumor mass; #P || 0.05, the average K562n/VCR tumor weight was less in group D than that in groups A, B and C; B: k562-n tumor mass. #P || 0.05, the average K562-n tumor weight was less in groups C and D than that in groups A and B. Abbreviations: DNR, daunorubicin; MNPs-Fe_3O_4, magnetic nanoparticles of Fe_3O_4.

Fullerenes

Since their discovery in 1985, fullerenes have been studied extensively due to their unique physical and chemical properties. With respect to biomedicine, **functionalized fullerenes** are defined as those fullerenes that have been modified by various chemical and supramolecular approaches to enhance solubility or targeting agent/drug attachment for therapeutics applications. Some of the more high-profile examples of functionalized fullerenes as they apply to drug delivery are described below.

Buckyballs (C_{60})

Given their unique structure, buckyballs (C_{60}) have received a great deal of attention since their discovery by Richard Smalley and colleagues in 1985. Although there is considerable controversy surrounding C_{60} as it pertains to toxicity, specifically with respect to the promotion of reactive oxygen species formation, some groups have begun to study buckyballs as potential drug and gene delivery vehicles. David Engler and colleagues at the Richard E. Smalley Institute for Nanoscale Science and Technology at Rice University in Houston have developed a new class of water-soluble C_{60} transfecting agents by **Hirsh-Bingel chemistry** (use of malonate derivatives to introduce side chains onto fullerenes) and assessed their potential as gene delivery vehicles. Engel noted that most C_{60} derivatives are only slightly soluble in an aqueous environment and the use of solvents in their manufacture could contribute to observed cytotoxicity. The Hirsch-Bingel reaction is one of the simplest and highest yielding C_{60} functionalization methods known. It involves the attack of C_{60} by a nucleophilic anion followed by elimination of a negatively charged ion to yield the final derivatized products which may be positively charged, negatively charged or neutral, with charge considerably affecting water solubility (Sitharaman *et al.*, 2008 and Figure 7.19).

The C_{60} derivatives were tested for promoting cellular uptake of DNA in *in vitro* tests by **transfection**, which is defined as the insertion of DNA or RNA into a cell, and demonstrated to be successful in this respect. Genes coded by the corresponding DNA were also shown to be expressed in transfected cells as assayed by fluorescent markers. It should be noted that cellular toxicity increased with increasing concentrations of C_{60} used in the transfection studies. Researchers at the Petru Poni Institute of Macromolecular Chemistry in Lasi, Romania have taken studies of this

Compound I, n = 2
Compound II, n = 3
Compound III, n = 4
Compound VI, n = 6

Compound V
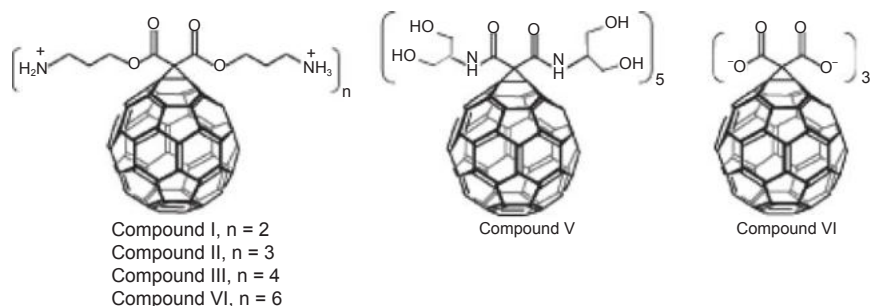
Compound VI

Figure 7.19 Depiction of the structures of the derivatized C_{60} vectors. The positively charged amino-C_{60} compounds (I-IV), the neutral serinolamide-C_{60} compound (V), and the negatively charged C_3-C_{60} compound (VI). Chemical structures are depicted at neutral pH in aqueous solution. Counterions in solution not shown. (Courtesy of Sitharaman *et al.*, 2008; reprinted with permission.)

nature a step further and generated the world's first fullerenol $C_{60}(OH)_{24}$-DNA complex. In this study the researchers generated fullerenol molecules containing 24 hydroxyl groups thus allowing for multiple hydrogen bonding-based double-stranded DNA binding sites. The three-dimensional architecture of double-stranded DNA consists of a major groove and a minor groove, each of which have different dimensions and steric hindrances which might come into play when interacting with nanoparticles. It is the phosphate backbone of DNA that is suggested to participate in hydrogen bonding with fullerenol. Given its diameter of 9.8 Angstroms, fullerenol is not predicted to fit within the minor groove but rather favors juxtaposition within the major groove. Depending upon the experimental conditions fullerenol may bind either within the major groove, as in the presence of sodium salt (Figure 7.20A) or on the outside of DNA as in the absence of sodium salt (Figure 7.20B). These studies are significant as they begin to elucidate nanoparticle-nucleic acid interactions at the molecular level under various physiological conditions which could impact DNA damage-based toxicity or even gene therapy delivery design (Pinteala *et al.*, 2009).

Buckysomes

Buckysomes are defined as self-assembled, spherical nanostructures composed of the **amphiphilic** (a molecule having a polar, water-
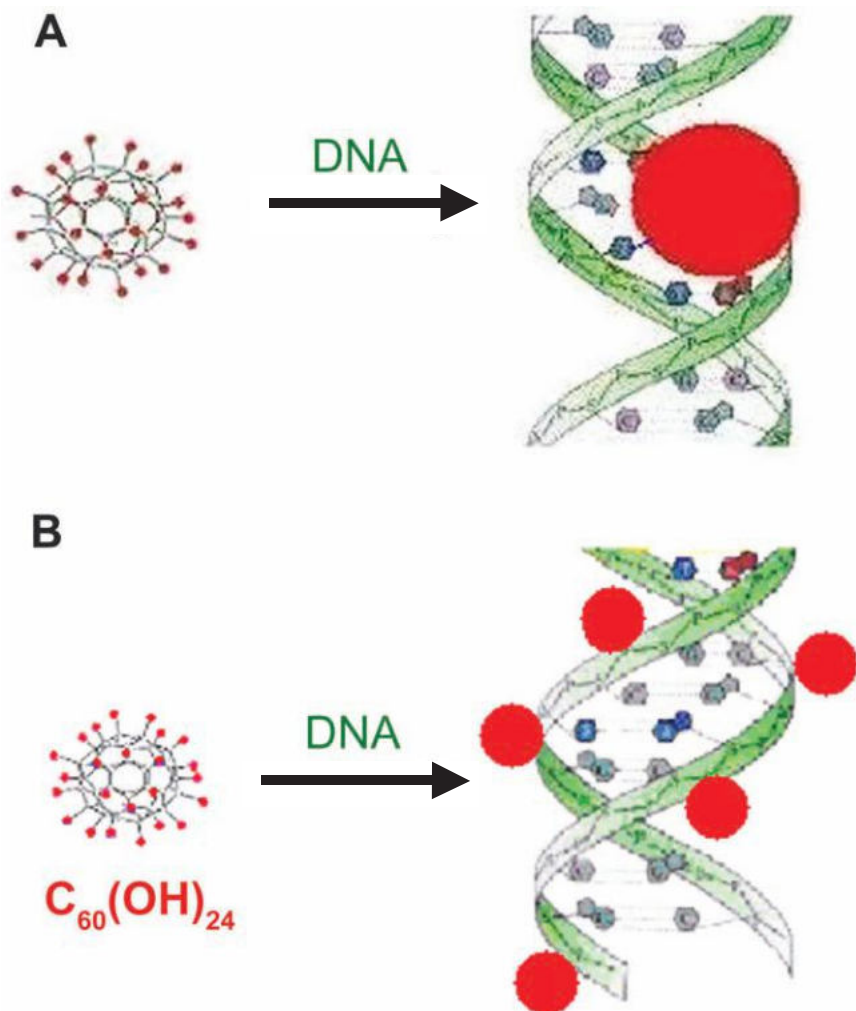
FIGURE 7.20 Binding fullerenol $C_{60}(OH)_{24}$ to dsDNA. (A) Binding fullerenol to the major groove of sodium salt of dsDNA. (B) Binding fullerenol to the outside of the dsDNA.

soluble group attached to a non-polar, water-insoluble hydrocarbon chain) fullerene AF-1. Paclitaxel-embedded buckysomes (PEBs) are spherical nanostructures around 100–200 nm in diameter composed of AF-1 with a hydrophilic surface containing embedded paclitaxel
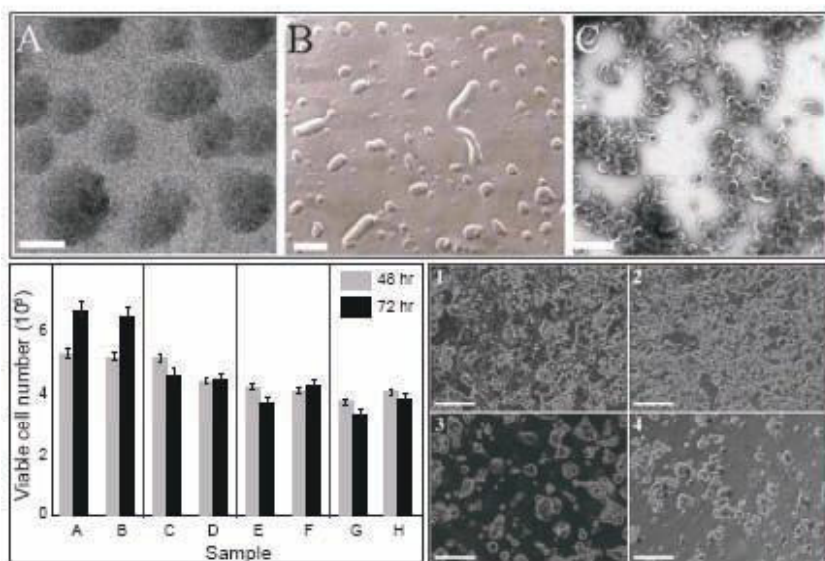
FIGURE 7.21 Functionalized fullerenes can function as drug-delivery agents for chemotherapy. Top. (A) Cryo-EM image showing the solid, dense spherical structures obtained upon self-assembly of AF-1 monomers at an elevated temperature of 70 °C; scale bar = 100 nm. (B) Freeze-fracture micrograph (scale bar = 200 nm) and (C) transmission electron micrograph (scale bar = 500 nm) confirm the spherical morphology of the buckysomes in panel A and also indicate a similar size profile. All three images were obtained from the same buckysome sample. Bottom. (Left) Trypan blue dye-based cell viability assay of MCF-7 cells. Samples were incubated for a period of 48 (gray bars) and 72 h (black bars) at 37 °C. Negative controls were 0.48 mM citrate buffer (A) and empty buckysomes (B). The concentration of paclitaxel in the PEBs was 28.6 (C), 143 (E), and 714 ng/ml (G). The comparative positive control was Abraxane, with identical paclitaxel concentrations in columns D, F, and H, respectively. Bottom (Right) Microscopic visualization of the morphology of live MCF-7 cells incubated with citrate buffer (1), empty buckysomes (2), PEBs (3), and Abraxane (4) for a 72-h time period. The concentration of paclitaxel in images 3 and 4 is 714 ng/ml. These images were collected from the same samples used in the assay shown in left. The scale bars in all four images are 250 μm. Copyright © 2008, American Chemical Society. Reprinted with permission from Partha R, Mitchell LR, Lyon JL, Joshi PP, Conyers JL. Buckysomes: fullerene-based nanocarriers for hydrophobicmolecule delivery. ACS Nano. 2008; 2(9): 1950–1958.

Focus Box 7.2 **Hongjie Dai and CNT-based drug delivery**

Hongjie Dai's group in the Department of Chemistry at Stanford University has taken a unique approach to the interfacing of chemistry, physics, nanomaterials and biophysics for the development of new therapeutic strategies. Their research emphasizes the importance of understanding the chemical and physical properties of carbon nanotubes and how those properties might be exploited for cancer treatment, drug and gene delivery and diagnostic imaging. See Case Study 7.2 for an example of the Dai lab's research. Dr. Dai is a rising star in the field of nanomedicine and in 2009 was elected to the American Academy of Arts and Sciences. (Photo courtesy of Stanford University.)

inside a hydrophobic core. The water-soluble nature of PEBs allow for the uptake of the drug without the need for potentially toxic non-aqueous solvents. Ranga Partha and Jodie Conyers in the Center for Translational Injury at the University of Texas Health Science Center, Houston developed PEBs and demonstrated them to be effective chemotherapeutic drug delivery agents, inducing MCF-7 human breast cancer cell death *in vitro* (for review see Partha *et al.*, 2009 and Figure 7.21).

Carbon Nanotubes (CNTs)

In addition to their applications in the area of thermal ablation-mediated cancer treatment via exposure to external fields such as radiofrequency waves and near infrared light, carbon nanotubes have shown promise as drug delivery vehicles. The ultra-high surface area-to-volume ratio of these poly-aromatic molecules allows for the efficient loading and conjugation of many types of drugs. As illustrated in Case Study 7.2, Hongjie Dai's group in Stanford University's Department of Chemistry has chosen to exploit this phenomenon to deliver the anti-cancer therapeutic paclitaxel (PTX) to cancer cells *in vivo*.

Case Study 7.2: Drug delivery with carbon nanotubes for in vivo cancer treatment

Chemically functionalized single-walled carbon nanotubes (SWNTs) have demonstrated potential as efficient drug delivery vehicles. In this study, drug-loaded carbon nanotubes were developed and tested as follows. SWNTs were solubilized and sonicated in the presence of non-covalent phospholipid-branched PEG followed by conjugation of succinic anhydride-modified PTX to form cleavable ester bonds. This was followed by linkage to the PEG termini via amide bond formation. Average CNT length following preparation was 100264 nm. Drug loading was confirmed by UV/VIS spectroscopy and effect on cell viability assessed by *in vitro* treatment of 4T1 murine breast cancer cells. Cellular toxicity was comparable to Taxol alone or PEGylated PTX and conjugation to the CNTs did not result in a loss of cancer cell destruction ability. In addition, significant endocytosis of the CNT drug complex was observed and no noticeable toxic effects were apparent from SWNTs alone (Liu, Z. *et al*., 2008 and Figure 7.22).
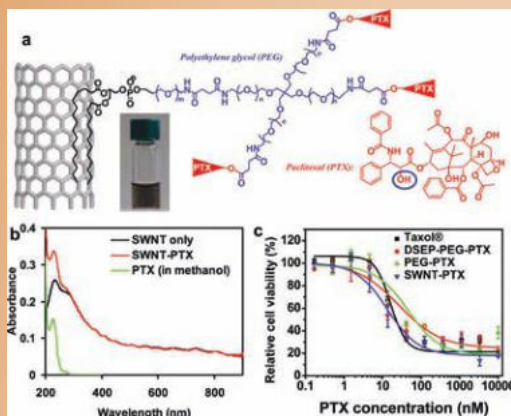
FIGURE 7.22 Carbon nanotubes for paclitaxel delivery. (a) Schematic illustration of paclitaxel conjugation to SWNT functionalized by phospholipids with branched-PEG chains. The PTX molecules are reacted with succinic anhydride (at the circled OH site) to form cleavable ester bonds and linked to the termini of branched PEG, via amide bonds. This allows for releasing of PTX from nanotubes by ester cleavage *in vivo*. The SWNT-PTX conjugate is stably suspended in normal physiological buffer and serum without aggregation. (b) UV-VISNIR spectra of SWNT before (black curve) and after PTX conjugation (red). The absorbance peak of PTX at 230 nm (green curve) was used to measure the PTX loading on nanotubes and the result was confirmed by radiolabel based assay. (c) Cell survival vs. concentration of PTX for 4T1 cells treated with Taxol®, PEG-PTX, DSEP-PEG-PTX or SWNT-PTX for 3 days. Plain SWNTs (no PTX conjugated) are non-toxic. (Courtesy of Liu, Z. *et al*., 2008; reprinted with permission.)

Case Study 7.2 illustrates the development and application of a carbon nanotube-based carrier system for cancer therapeutics delivery and the system was confirmed as effective *in vitro*. *In vivo* studies by Dai's group on the same delivery platform were much more telling. Local injections of the final preparations were performed intra-tumorally in Balb/c mice carrying 4T1 tumors. The CNT-PTX complex was observed to be significantly more effective in reducing tumor size than controls, including Taxol and PEG-PTX (Liu *et al.*, 2008 and Figure 7.23).
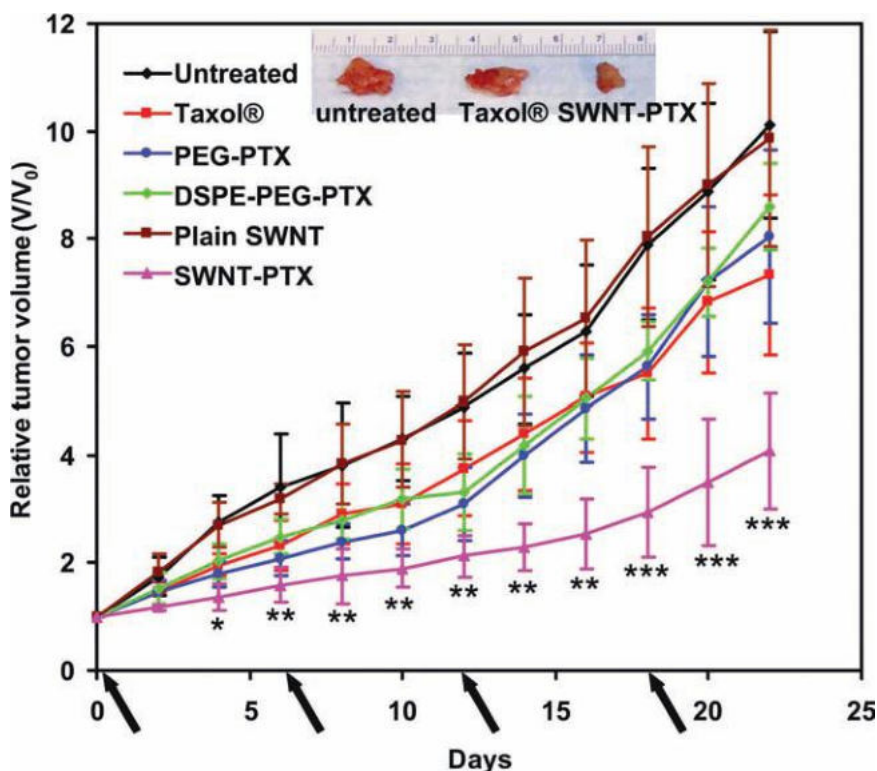
FIGURE 7.23 Nanotube paclitaxel delivery suppresses tumor growth in a 4T1 breast cancer mice model. Tumor growth curves of 4T1 tumor bearing mice received different treatments indicated. The same PTX dose was injected (on day 0, 6, 12 and 18, marked by arrows) for Taxol®, PEGPTX, DSEP-PEG-PTX and SWNT-PTX. Inset: a photo of representative tumors taken out of an untreated mouse (left), a Taxol® treated mouse (middle) and a SWNT-PTX treated mouse after sacrificing the mice at the end of the treatments. (Courtesy of Liu, Z. *et al.*, 2008; reprinted with permission.)

James Rusling's team in the Department of Chemistry at the University of Connecticut in Storrs have taken the use of SWNTs for anti-cancer drug delivery a step further through active targeting of the EGF receptor, which is overexpressed by certain head and neck squamous carcinoma cells. In this study the researchers attached cisplatin and epidermal growth factor (EGF), which binds specifically to the EGF receptor, to SWNTs. In the attachment process SWNTs were oxidized in acid to provide carboxylate groups on the SWNT ends and sidewalls to which drug and EGF were attached by **amidization** (covalent tethering by carbodiimide-mediated condensation) using EDC as a catalyst. Electron microscopy images show the targeting of EGF and cisplatin on the individual tubes (Bhirde *et al.*, 2009 and
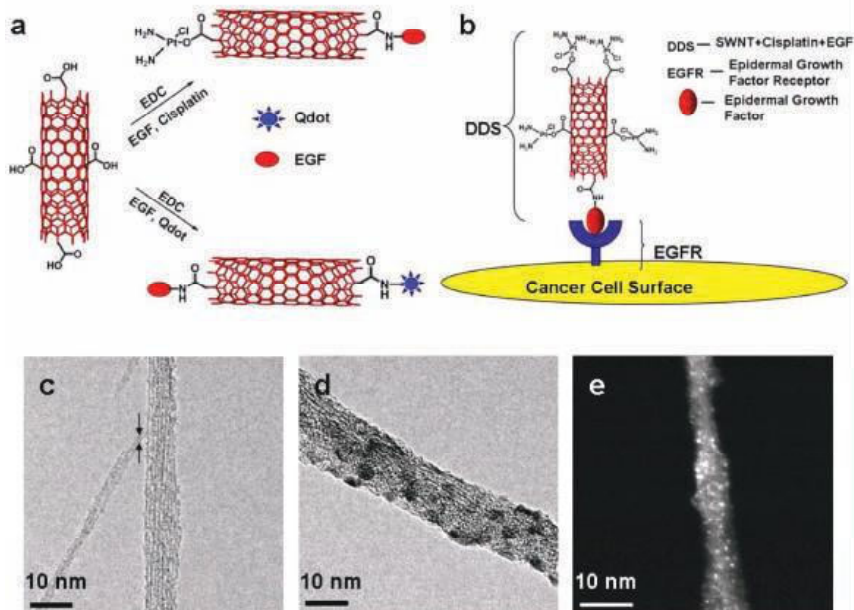
FIGURE 7.24 Nanotube-based delivery system. (A) Illustration of chemical reactions used to attach EGF, cisplatin and Qdots onto carboxylated SWNTs (in red) using EDC as the coupling agent. (B) Schematic showing SWNT bundles bioconjugated with EGF and cisplatin targeting the cell surface receptor EGFR on a single HNSCC cell. Transmission electron micrographs of (c) oxidized SWNT bundles with arrows showing a single SWNT (d) SWNT-Qdot-EGF bioconjugate bundle (e) STEM image of SWNT bundle showing cisplatin as the bright spots. (scale bar = 10 nm). (Courtesy of Bhirde *et al.*, 2009; reprinted with permission.)

Figure 7.24). Rusling's team applied this CNT-EGF-cisplatin complex to target HNSCC tumor cells *in vitro* and *in vivo* and demonstrated significant endocytic uptake and superior cell killing in comparison to controls.

Natural Material-Based Nanoparticles

Liposomes

Among the nanoparticulate drug carriers, liposomes are some of the most extensively studied and, along with polymer-based systems, possess the most suitable characteristics for the **encapsulation** (the encasing of a therapeutic in a delivery vehicle) of many drugs and genes. It is perhaps the ability to surface modify liposomes that provides a unique advantage in drug delivery. This functionality allows for easily customizable liposomal systems that can encapsulate a variety of therapeutic agents and target to numerous tissues and cell types within the body.

Liposomal Nanoparticles and Targeting Inflammation

The suppression of inflammation and inflammatory responses has been one of the major areas of focus in the medical and scientific communities for a number of decades. Now researchers are seeking to exploit nanotechnology as a way to more effectively deliver anti-inflammatory agents. Motomu Shimaoka's group at the Immune Disease Institute of Harvard Medical School in Boston developed a strategy for selectively silencing the cell cycle regulatory molecule cyclin D1 (CyD1), which is strongly upregulated at sites of inflammation. Liposome-based targetable, stabilized nanoparticles (tsNPs) were synthesized and loaded with CyD1-small interfering RNA (siRNA) to create I-tsNPs. Synthesis was accomplished by attaching hyaluronan to liposomal nanoparticles, ~80 nm in diameter, via covalent linkage. A monoclonal antibody (FIB504) that specifically binds integrins was covalently linked to the nanoparticles. siRNAs were condensed with **protamine**, a positively charged protein that has been previously used to enhanced nucleic acid delivery, and tsNPs were loaded with the siRNA cargo by rehydration of lyophilized nanoparticles in the presence of the condensed siRNAs. An 80% entrapment efficiency was observed with around 4000 siRNA molecules per nanoparticle (Peer *et al.*, 2008 and Figure 7.25).

I-tsNPs were targeted to β7 integrin which is expressed at high levels by leukocytes. Systemic application of I-tsNPs reversed experimentally
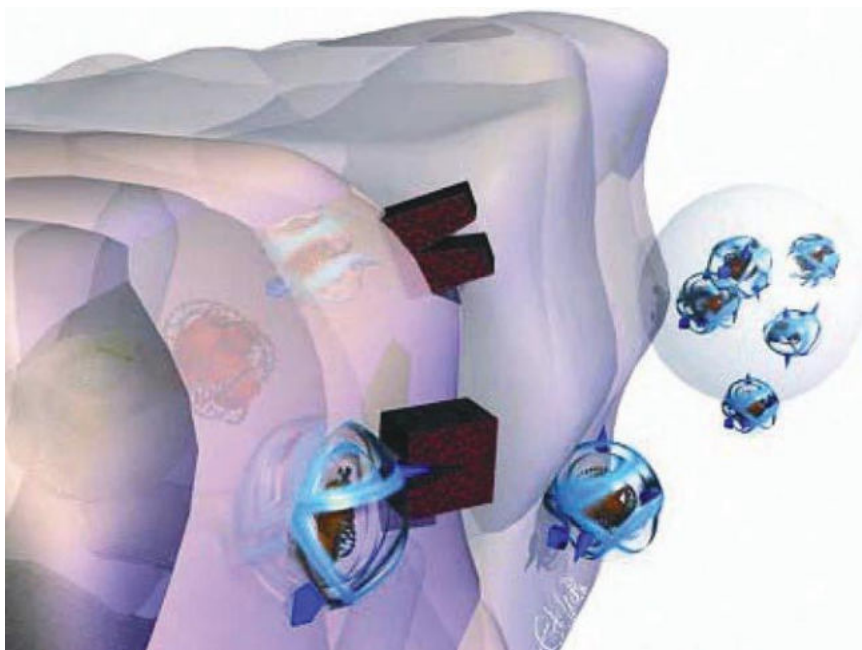
FIGURE 7.25 Diagrammatic illustration siRNA molecules encapsulated into nanoparticles and targeted to a cell surface. (Courtesy of Interdisciplinary Nanoscience Center; reprinted with permission.)

induced colitis (inflammation of the colon) in mice by suppressing leukocyte proliferation and T helper cell 1 cytokine expression. In a separate study, Renu Vermani, Medical Director at CVPath, Inc. in Gaithersburg, Maryland and colleagues employed the liposomal nanoparticle complex TRM-484 to inhibit restenosis. TRM-484 is a pegylated 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) liposomal complex with encapsulated prednisolone phosphate (a steroid). The complex specifically binds to chondroitin sulfate proteoglycans (CSPGs) expressed within the sub-endothelial matrix but not vascular endothelial cells. This allows for targeting of drugs to regions of the vasculature experiencing, for example, **restenosis** (a narrowing or constriction of a blood vessel, often due to inflammation) after stenting. As a cationic lipid-based system the nanoparticles are endocytosed with subsequent steroid release inside the cell. TRM-484 was administered

to New Zealand white rabbits in which atherosclerosis had been induced and subsequently treated via stenting. The researchers observed significant intimation of the nanoparticles of the blood vessel interstitial space as well as considerable anti-restenotic effects via steroid-dependent inhibition of inflammation. The targeted delivery allowed for considerably reduced dosing and no delay in healing was observed compared to controls.

Chitosan

Chitosan has been widely accepted for a number of years as a nasal delivery system for a variety of therapeutics, most notably for those that address seasonal allergic conditions. Due to their inherent physical properties which promote the binding and stabilization of nucleic acids, chitosan nanoparticles have received the most attention as gene delivery vehicles. Researchers in the Tissue and Therapeutic Engineering Lab, Johns Hopkins Division in Singapore studied the use of chitosan nanoparticles to deliver DNA constructs to the liver through retrograde **intra-biliary infusion** (introduction into the bile duct). They demonstrated high levels of liver expression of the DNA marker luciferase in the liver and negligible levels in other organs such as the spleen and lungs (Dai *et al.*, 2006 and Figure 7.26). Although not a truly targeted system, this demonstrates the possibility of using non-viral nanoparticles for the delivery of gene therapy constructs to the hepatic system.

Maria Alonzo and colleagues in the Department of Pharmacy and Pharmaceutical Technology at the University of Santiago de Compostela, Santiago de Compostela, Spain also pursued the development of gene therapy methodologies using chitosan-based nanoparticles, yet they instead chose to focus their efforts in on its application as a delivery vehicle for the ocular region. Their nanoparticle delivery system was composed of bioadhesive polysaccharides, hyaluronic acid (HA) and chitosan. They used a mild ionotropic gelation technique for nanoparticle synthesis followed by loading with EGFP (enhanced green fluorescent protein) DNA constructs to monitor gene activity in cells *in vitro* post-delivery. Nanoparticle diameters ranged from 100–225 nm. High levels of DNA construct uptake and expression were demonstrated in two separate cell lines previously immortalized from human cornea and conjunctiva. (de la Fuente *et al.*, 2008 and Figure 7.27). It will be interesting to determine if
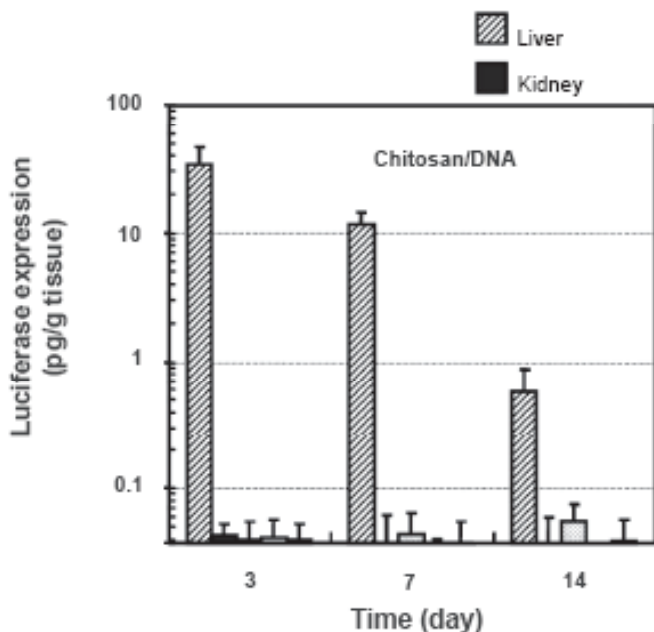
FIGURE 7.26 Luciferase expression in major organs of the rats after receiving intrabiliary injection of chitosan-DNA nanoparticles or PEI-DNA nanoparticles containing 200 μg of DNA. Each bar represents mean ± standard deviation (n = 5).

the system allows for efficient gene delivery at ocular sites *in vivo* such as the cornea and conjunctiva.

A discussion of chitosan-based nanoparticles would be incomplete without citing at least one example of cancer drug delivery *in vivo*. Li-li Wu's group in the Department of Gastroenterology at Fudan University in Shanghai prepared chitosan nanoparticles by **ion gelification** (formation of a gel catalyzed by the presence of ions) loaded with the anti-cancer drug fluorouracil and tested the system's anti-carcinoma effects as well as toxicity in **nude mice**, which are mice derived from a genetic mutation that results in a deteriorated or absent thymus, thus lowering T cell counts and inhibiting immunorejection of transplanted tumors as well as therapeutics. During the preparation of the delivery system drug encapsulation efficiency (EE) and loading capacity were calculated as follows:
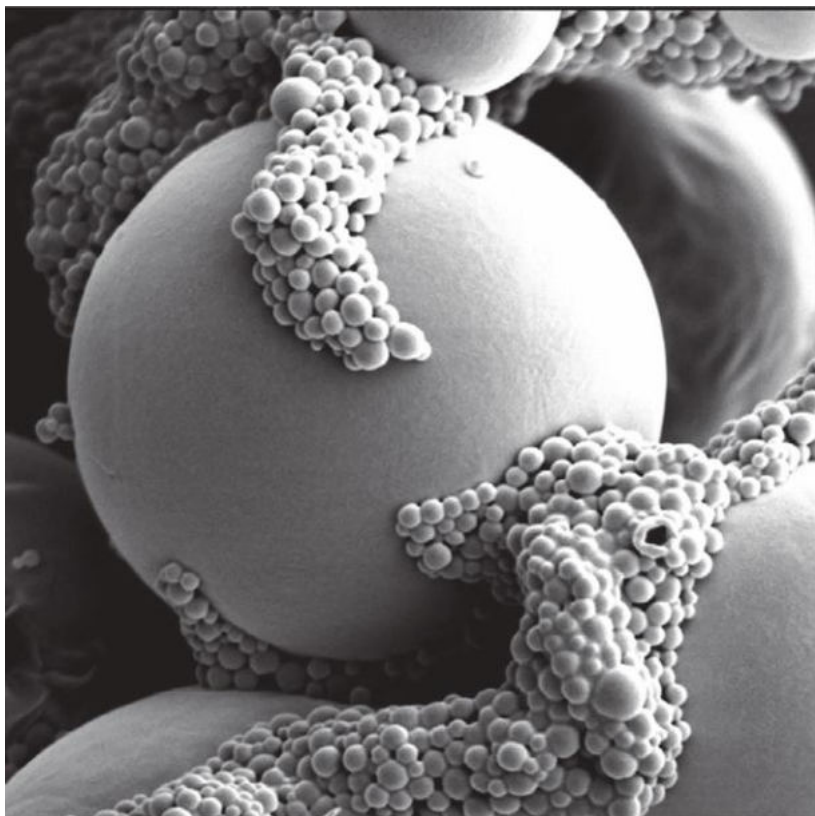
FIGURE 7.27 SEM image of chitosan-based microsphere for gene delivery. (Courtesy of Farahidah Mohamed Kulliyyah of Pharmacy International Islamic University Malaysia; reprinted with permission.)

$$EE = \frac{\text{The amount of 5-Fu in the nanoparticles}}{\text{Total amount of 5-FU}} \times 100\%$$

$$LC = \frac{\text{The amount of 5-Fu in the nanoparticles}}{\text{Total amount of nanoparticles weight}} \times 100\%$$

This allowed for a determination of the efficiency of the synthesis procedure. Upon achieving appropriate values for EE and LC, tumor inhibition rates were compared for the chitosan-based nanoparticle system and fluorouracil alone. The data revealed much higher rates of tumor

Table 7.4 Tumor volume and growth inhibition rate following chitosan-fluorouracil nanoparticle treatment. NS: negative control; 5-Fu: drug alone; CTS-Pasp: nanoparticle alone; CTS-Pasp-5Fu: nanoparticle plus drug) (Courtesy of Zhang *et al.*, 2008; reprinted with permission)

Groups	Before Treatment	After Treatment						
		3 d	7 d	IR	10 d	IR	14 d	IR
NS	0.11 ± 0.03	0.19 ± 0.05	0.33 ± 0.10		0.62 ± 0.28		0.80 ± 0.23	
5-Fu	0.12 ± 0.03	0.12 ± 0.04	0.24 ± 0.11	27.76%	0.31 ± 0.19	50.26%	0.33 ± 0.20	58.69%
CTS-Pasp	0.12 ± 0.03	0.19 ± 0.04	0.32 ± 0.10	2.80%	0.54 ± 0.10	13.49%	0.76 ± 0.26	5.58%
CTS-Pasp-5-Fu	0.10 ± 0.01	0.11 ± 0.04	0.13 ± 0.05	60.76%	0.19 ± 0.05	68.99%	0.23 ± 0.07	70.82%

growth inhibition when the nanoparticle carrier system was used (Zhang *et al.*, 2007, Table 7.4 and Figure 7.28).

Gelatin

Gelatin nanoparticles are yet another platform which has been extensively studied for drug delivery potential. Gelatin nanoparticles can be easily
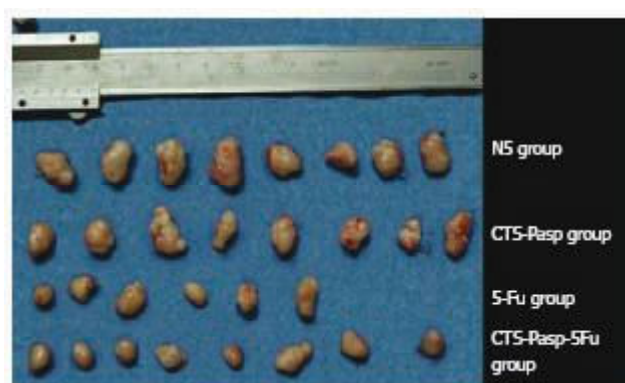
FIGURE 7.28 Tumor size following treatment with chitosan-fluorouracil nanoparticles. NS: negative control; 5-Fu: drug alone; CTS-Pasp: nanoparticle alone; CTS-Pasp-5Fu: nanoparticle plus drug). (Courtesy of Zhang *et al.*, 2008; reprinted with permission.)

customized to attach a variety of therapeutics and/or targeting moieties. Padmaja Magadal and Mansoor Amiji in the Department of Pharmaceutical Sciences at Northeastern University in Boston have developed a targeted gelatin-based engineered nanocarrier system (GENS) for the oral delivery of gene therapies using the **solvent displacement method** of synthesis, which is a procedure for the large-scale preparation of gelatin nanoparticle dispersions. This method allows for customization of the nanoparticles by controlling such parameters as temperature, pH and stirring conditions (Magadal *et al.*, 2008 and Figure 7.29).

Specifically, the researchers targeted the epidermal growth factor receptor (EGFR) which is preferentially overexpressed on the surface of certain pancreatic cancer cell lines. An EGFR targeting peptide was grafted onto the surface of the nanocarriers using a PEG spacer. DNA encoding enhanced green fluorescent protein (EGFP) was utilized as a marker for activity and delivered via targeting to PANC-1 human pancreatic adenocarcinoma pancreatic cancer cells. Carole Bourquin and colleagues in the Department of Internal Medicine, Ludwig at Maximilian University of Munich in
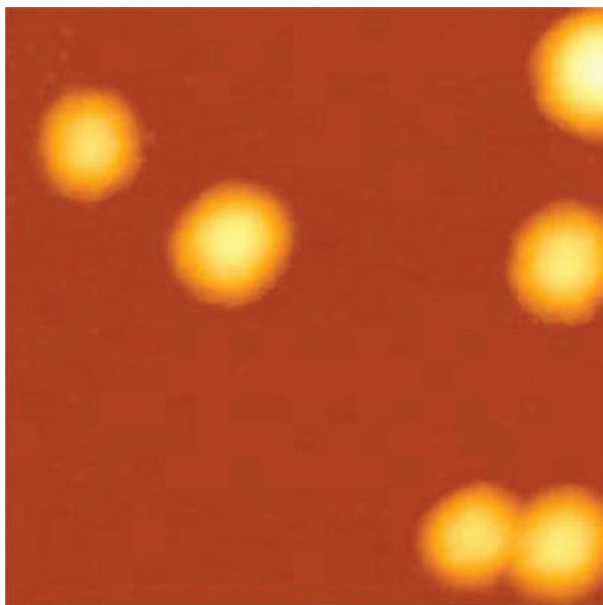
FIGURE 7.29 Microscopy of gelatin nanoparticles. (Courtesy of JPK Instruments; reprinted with permission.)
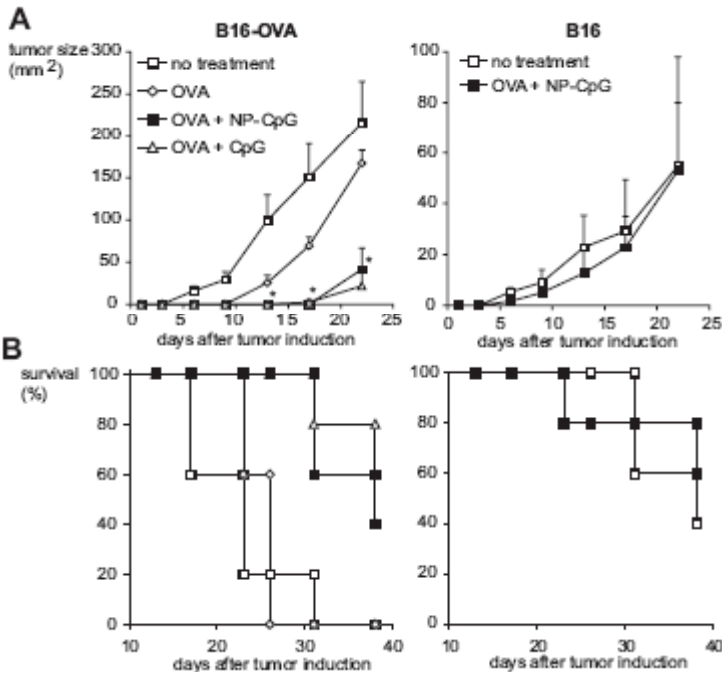
FIGURE 7.30 CpG-loaded nanoparticles elicit an OVA-specific antitumor response. B16-OVA or B16 tumors were implanted s.c. in C57BL/6 mice after immunizations with OVA and free CpG or NP-bound CpG ($n = 5$). (A) Immunization with OVA and NP-CpG significantly reduced growth of B16-OVA tumors compared to untreated mice or to mice treated with OVA alone. No effect of immunization was seen in the wild-type B16 tumors. (B) In mice with B16-OVA tumors, immunization with OVA and NP-CpG increased survival times compared with untreated mice or to mice treated with OVA alone. No effect of immunization on survival was seen in the wild-type B16 tumors. Similar results were obtained in two independent experiments. (Courtesy of Bourquin *et al.*, 2006; reprinted with permission.)

Germany focused their efforts on targeting of CpG oligonucleotides to the lymph nodes of mice for the promotion of anti-tumoral immunity. The antigenic response was due to CpG oligonucleotide binding to a receptor known as TLR9 resulting in specific cellular activation, maturation and migration to the lymph node followed by T cell immunogenic response and protective immunity. A two-step desolvation method was used to produce gelatin nanoparticles of a discrete diameter, average size 272 nm, that were cationized and loaded with CpG oligonucleotides. A clear

anti-tumor immune response was observed using this CpG delivery platform in mice (Bourquin *et al.*, 2006 and Figure 7.30).

Albumin

Some cancer therapeutics agents require the use of solvents in synthesis, such as Cremphor-EL, which contribute to some of the toxicities observed during treatment. Albumin is a naturally occurring protein that has been used to formulate some solvent-free cancer therapeutics in order to circumvent solvent toxicity issues. Albumin nanoparticles, on average smaller than a red blood cell, have been used previously in intra-arterial administrations and can be infused even into small arteries without causing discomfort. In a rare example of human albumin nanoparticle applications in a clinical setting, researchers in the Department of Radiology at the Institute Nazionale Tumort in Milan, Italy conducted a study to evaluate the effectiveness of intra-arterial infusion of paclitaxel (Taxol)-containing albumin nanoparticles (ABI-007) for the treatment of squamous cell carcinoma of the tongue. The formulation was introduced into patients either through trans-femoral catheterization via the external carotid artery, the lingual artery or the faciolingual trunk (Damascelli *et al.*, 2003 and Figure 7.31). After multiple infusions the patients also received
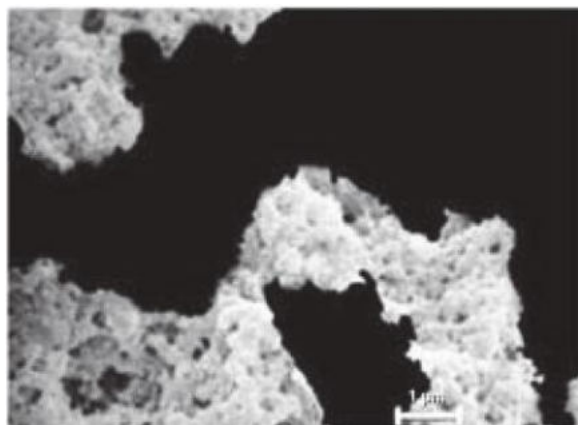
Serum Albumin Nanoparticles

FIGURE 7.31 SEM image of serum albumin nanoparticles. (Courtesy of PharmaInfo.net; reprinted with permission.)

either radiotherapy, chemotherapy or both. Patient response was positive, with tumor regression observed and minimal toxicity to tissues and cells surrounding tumorigenic regions. It should be noted that ABI-007 is now in clinical trials for the treatment of a variety of cancers.

CHAPTER SUMMARY

1. Much effort has been focused on developing new technologies for more efficient and specific drug delivery platforms.
2. The ultimate goals of drug delivery are to drive high therapeutics efficacy and eliminate unwanted side effects all while maintaining the safety of the delivery process.
3. Extracellular and intracellular targeting capabilities will have the most significant impact on the next generation of drug delivery vehicles.

Targeted Drug Delivery: Basic Principles

1. Traditional, non-specific drug delivery often results in unwanted side effects and low efficacy.
2. Targeted drug delivery increases therapeutic concentrations within certain tissues or cell types.
3. Active targeted drug delivery is often via attachment to cell receptors through the use of ligands or other receptor-specific entities as the targeting agent.
4. Cell surface antigens, which may or may not be receptors, have been exploited to target drugs to particular cell types.
5. Cancer cells express tumor-specific and tumor-associated antigens that act as ideal targets for delivering therapeutics.
6. Prostate-specific membrane antigen is a good example of a cancer cell-specific target for drug delivery.
7. Targeting moieties may include antibodies, small molecules, aptamers, and peptides.
8. The Enhanced Permeability and Retention (EPR) effect is a mechanism of passive targeted drug delivery to tumors due to abnormal angiogenesis as tumorigenic sites.
9. The EPR effect has been exploited to deliver nanoparticles for the thermal ablation of cancer cells.

Nanoparticles for Drug Delivery: Basic Requirements

1. An ideal nanoparticle drug delivery system must be: more specific, reduce toxicity, increase biocompatibility and allow for the faster development of new therapeutic strategies in comparison to more conventional delivery methods.

2. The effective design of a nanoparticle-based targeted drug delivery platform requires knowledge on: drug incorporation and release, stability and shelf life, biocompatibility, biodistribution and targeting efficiency and functionality.

Types of Nanoparticle-Based Systems for Drug Delivery

1. Both natural and man-made nanoparticle-based systems for drug delivery exist.

2. Polyethylene glycol (PEG) has been used to deliver RNAi molecular therapeutics to solid tumors.

3. Cisplatin as well as docetaxel has been delivered to cancer cells using a combination of PEG and PLGA nanoparticles as the carrier and the entire complex was demonstrated to enter cells via endocytosis.

4. Polylactic Acid (PLA) nanoparticles are slowly degrading, biocompatible, can be customized for control of drug release and are advantageous for intracellular drug delivery.

5. Polycaprolactone (PCL) nanoparticles can be custom synthesized by electrohydrodynamic atomization (EHDA) in discrete sizes allowing for precise control of drug release.

6. PCL/PEO heterogeneous composites have been studied as a nanoparticle system for modulating intracellular levels of the secondary lipid messenger ceramide through the delivery of paclitaxel and tamoxifen.

7. Polyacrylate (PACA) nanoparticles have superior water-solubilization characteristics due to their acrylated nature.

8. PACA nanoparticles were demonstrated to exhibit a burst effect of rapamycin release at acidic pH.

9. The branched nature of dendrimers lends them well to drug delivery due to multiple sites of therapeutic and targeting moiety attachment.

10. Dendrimers, especially PAMAM dendrimers, have been widely studied for the *in vivo* delivery of both drugs and imaging agents.

11. Dendrimer/macrophage hybrids have been developed and it was shown that the cells presented dendrimers on their surface for extended periods of time.

12. In addition to applications as imaging agents, iron oxide nanoparticles have been studied as drug carriers.

13. Ultrasound was used to disperse iron oxide-drug conjugates in a saline solution for delivery to tumors.

14. Despite their controversial potential toxicity, buckyballs (C_{60}) have been studied as potential drug and gene delivery vehicles.

15. The Hirsch-Bingel reaction is a simple yet high yielding method for functionalizing buckyballs.

16. Buckyballs have been demonstrated to promote cellular uptake of nucleic acids.

17. Paclitaxel-embedded buckysomes (PEBs) are water-soluble, solvent-free and have been shown to efficiently deliver chemotherapeutics *in vitro*.

18. The ultra-high surface area-to-volume ratio of carbon nanotubes allows for efficient loading and conjugation of many types of drugs.

19. CNTs have been used to delivery drugs such as Taxol and paclitaxel to cancer cells *in vitro*.

20. CNTs carrying cisplatin have been targeted to carcinoma cells via the EGF receptor both *in vitro* and *in vivo* resulting in extensive cell killing.

21. Liposomes are used to encapsulate drugs and genes for efficient delivery to cells and tissues.

22. Inflammatory pathways have been successfully controlled via the use of liposome-based targetable, stabilized nanoparticles (tsNPs) delivering siRNA to specific cell types through targeting of the b7 integrin receptor.

23. Chitosan nanoparticles favorably bind nucleic acids and have been studied extensively as gene delivery vehicles.

24. Chitosan nanoparticles prepared by ion gelification were used to deliver fluorouracil to cancer cells *in vivo* and tumor size was shown to be significantly reduced.

25. Gelatin nanoparticles can be easily customized to attach therapeutics, imaging or targeting agents.

26. The solvent displacement method allows for the large-scale synthesis of gelatin nanoparticle dispersions which can be customizable by varying temperature, pH and stirring conditions.

27. Gelatin nanoparticles loaded with oligonucleotides and designed to target the EGF receptor exhibited considerable anti-tumor activity in mice carrying human pancreatic cancer.

28. Albumin nanoparticles eliminate the need for solvent dispersion of some drugs and can be infused into small arteries without causing discomfort.

KEY TERMS

- Drug Delivery
- Targeted Drug Delivery
- Ligand
- Antigen
- Tumor-Specific Antigen
- Tumor-Associated Antigen
- Electrohydrodynamic Atomization (EHDA)
- Acrylation
- Surfactant
- Kirby-Bauer Antibiotic
- Testing
- Burst Effect
- Methotrexate
- Kaplan-Meier Survival Curve
- Brachytherapy
- Composite Nanodevice (CND)
- Hypoxia
- Macrophage
- Schiff Base Linkage
- Active Targeted Drug Delivery
- Passive Targeted Drug Delivery
- Nanoprecipitation
- Endocytosis
- Aliphatic
- Bioactivation
- Polydispersity Index (PDI)
- Functionalized Fullerene
- Hirsh-Bingel Chemistry
- Transfection
- Buckysome
- Amphiphilic
- Amidization
- Encapsulation
- Protamine
- Restenosis
- Intrabiliary Infusion
- Ion Gelification
- Nude Mice
- Solvent Displacement Method

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. Compare and contrast active verses passive targeted drug delivery (EPR).

2. What is the difference between a tumor-associated antigen and a tumor-specific antigen? List some examples of each.

3. What are the primary goals of targeted drug delivery?

4. What are the prerequisites of targeted drug delivery?
5. List five examples of nanomaterials currently being studied as drug delivery vehicles and cite examples of payloads and the disease they treat.
6. How did Robert Langer's group treat prostate cancer in mice?
7. What properties make polylactide nanoparticles useful for drug delivery?
8. What sole property of polyacrylate nanoparticles make them useful for drug delivery?
9. Write the formula Anirban Maitra's group used to calculate the efficiency of rapamycin release for polyacrylate nanoparticles.
10. Why does the branched nature of dendrimers make them advantageous for drug delivery?
11. Write the equation for Gong Wu's synthesis of a dendrimer-methotrexate complex.
12. How might dendrimers and macrophages be used together to treat cancer?
13. How can buckyballs be made water soluble?
14. Why might carbon nanotubes be good drug delivery vehicles?
15. What are the advantages of using liposomes for drug delivery?
16. Write the formulas for encapsulation efficiency (EE) and loading capacity (LC).
17. Write the chemical reaction scheme for Magadal's and Amiji's synthesis of EGFR peptide-modified gelatin nanoparticles.

8

Nanodiagnostics

This chapter explores the use of nanoparticles, nanomaterials and other nanotechnologies for the diagnosis of illness or disease. Particular focus is paid to nanoparticle technologies that provide unique contrast agent characteristics for the imaging of tumors. **Nanodiagnostics** can be defined as the application of nanotechnology for the diagnosis of a physiological anomaly or disease in humans or animals. Numerous types of nanotechnologies are currently being explored for use in nanodiagnostics applications including, but not limited to:

- Nanoscale visualization
- Nanoparticle bio-labels
- Nanotechnology-based biochips/microarrays
- Nanoparticle-based nucleic acid diagnostics
- Bio-barcode assays
- Nanopore technology
- DNA nanomachines for molecular diagnostics
- Nanoparticle-based immunoassays
- Nanobiosensors

Applications of nanotechnology as it applies to clinical diagnostics can be categorized as either *in vitro* or *in vivo* in nature and the design of the diagnostics system depends upon the nanotechnological platform exploited as well as the desired diagnostics application. This chapter outlines in detail numerous examples of nanotechnology and its application primarily to clinical diagnostics. As in other parts of the book there is a heavy focus on nanotechnology as it application towards tackling the problem of cancer as it is in this realm that most of the scientific advances have taken place.

IN VITRO NANODIAGNOSTICS

Nanobiochips and Nanobiosensors

Nanotechnology on a chip is rapidly emerging as a new paradigm in the area of large-scale or total chemical analysis. The use of nanoscale components enables higher precision in diagnostics while considerably reducing the cost of the platform. **Nanobiochips** are essentially miniaturized laboratories that can in some cases perform hundreds or thousands of biochemical reactions for the identification of a particular molecular signature unique to the diagnosis in question. They are formally defined as a collection of miniaturized test sites arranged on a solid substrate that permits many tests to be performed at the same time in order to achieve higher output and speed. The terms "nanobiochip" and "biochip" are often used interchangeably but biochips, which do not incorporate nanotechnology, will not be discussed here. The collection of miniaturized test sites is referred to as a **microarray** and has now been employed by numerous diagnostics companies for the *in vitro* identification of disease hallmarks such as antigens or even genetic mutations. It is covered in the next section.

Perhaps one of the most intriguing and promising nanobiochip technologies involves nanofluidics arrays. **Nanofluidics** can be defined as is the study of the behavior, manipulation, and control of fluids that are confined to structures of nanometer (typically 1–100 nm) dimensions. If a nanofluidics system is properly designed it can allow for the efficient and precise isolation and analysis of individual biomolecules. In 1965 the researchers C.L. Rice and R. Whitehead published a seminal paper on the theory behind fluidics physics in a nanoscale capillary which resulted in the derivation of the following equation for the radial distribution of the

velocity of a liquid, $v_z(r)$ as follows:

$$v_z(r) = \frac{\varepsilon\phi_0}{4\pi\eta} E_z \left[1 - \frac{(kr)}{(ka)} \right]$$

where ε is the dielectric constant, k is the Boltzmann constant, ϕ is the potential, n is the ion number density and E_z *is* the electric field. It is from this final equation that fluid flow in nanosized capillaries is governed by both the pore size (radius) and the **Debye Length** (the distance over which significant charge separation can occur). These two parameters thus allow for a custom tailoring of nanofluidics devices to meet fluid flow requirements for various diagnostics applications.

Most nanofluidics structures are fabricated as cylindrical channels or nanoslits through methods such as photolithographic etching on silicon, glass or polymers as discussed in Chapter 1. The precision of photolithography allows for fabrication at Angstrom-level precision. For example, Peidong Yang's group in the Department of Chemistry at the University of California, Berkeley constructed silicon nanowires on a substrate using photolithographic etching followed by oxidization to convert the nanowires into hollow nanotubes. This allowed for the creation of nanotubes with diameters as small as 10 nm. They demonstrated successful DNA translocation through the nanotubes as a function of ionic current (applied by electrodes) and could change the current and rate of translocation by altering buffer concentrations and components. This suggested an interplay between both electrostatic charge and geometric blockage of the translocation phenomenon (Fan *et al.*, 2005 and Figure 8.1).

Kristian Helmerson's group in the Physics Laboratory at the National Institute of Standards and Technology (NIST) in Gaithersburg, Maryland
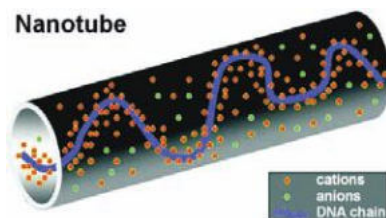
FIGURE 8.1 Diagrammatic illustration of an inorganic nanotube microfluidics device for single DNA molecular sensing. (Courtesy of Fan *et al.*, 2005: reprinted with permission.)

has taken a different approach to the synthesis of polymer-based nanotubes. In their studies they literally pull the membranes off **polymersomes**, which are polymer vesicles composed of amphiphilic diblock copolymers, using either optical tweezers or a micropipette to create polymer nanotubes that have an aqueous core connected to the aqueous interior of the polymersome. The pulled nanotubes were stabilized by chemical cross-linking and were demonstrated to be extremely robust. In addition, networks of polymer nanotubes could be generated by subsequent optical manipulation (Reiner *et al.*, 2005 and Figure 8.2).

The study of nanofluidics and the development of nanoscale tubes has now opened the door to the development of even further refined nanosystems for molecular detection and/or sieving. A **nanopore** is defined as a small electrically insulated hole that can be used as a single-molecule detector upon passage of that molecule through the pore. Detection may occur as a result of changes in the ionic current of an electrolyte solution containing the molecules in question which results in a change in electrical current. This is known as a **translocation event signal**. Nanopores have been studied extensively as the possible basis behind the next generation of DNA sequencers. As DNA is composed of four unique bases, adenine, guanine, cytosine and thymidine, each has a unique molecular structure
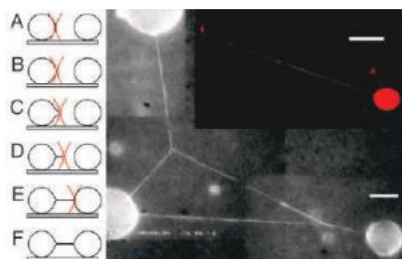
FIGURE 8.2. Images illustrating the creation of polymer nanotube–vesicle networks. (*Left*) Sequence of images illustrating the pulling of a nanotube from the membrane of a polymersome using optical tweezers (shown in red) and the attachment of the nanotube to another polymersome. (*Right*) Composite image from video fluorescence microscopy of a network of polymer nanotubes and polymersomes, containing the membrane dye DiO-C16, assembled using optical tweezers. (Scale bar: 10 μm.) (*Inset*) Scanning confocal microscopy image of a nanotube pulled from a polymersome encapsulating sulforhodamine B dye in buffer. Variations in the intensity are due to movement of the nanotube during the scan. (Scale bar: 10 μm). (Courtesy of Reiner *et al.*, 2005; reprinted with permission.)

and thus will cause a unique translocation event signal upon passage through a nanopore. One of the leaders in this field of research, Amit Meller in the Department of Biomedical Engineering at Boston University, has developed a DNA sequencing system that involves the optical detection of specific DNA sequences (bases) translocating through a nanopore. This system relies on the mapping of each base of the target DNA onto a 2-unit code by biochemical conversion into what are known as Designed DNA Polymers (DDPs). The 2-unit codes are hybridized to fluorescently labeled complementary molecular beacons that results in self-quenching. The theory behind this system is that as the molecular beacons are "unzipped" from the complementary hybrid during translocation through a 2 nm-wide nanopore their fluorescent tags are unquenched and detected. The method involves 3 steps: (*a*), biochemical conversion of the target DNA to DDP format, (*b*) hybridization of the design polymers with molecular beacons, and (*c*), optical readout of the design polymers using a nanopore-assisted method. Detection is by a dual-color total internal reflection fluorescence (TIRF) microscope, with the 2-color optical signal thus correlated to the target DNA sequence (Soni *et al.*, 2007 and Figure 8.3).

Apart from the sequencing of nucleic acids, nanopores may be used in other applications such as the identification of unrelated molecules and the separation of double-stranded DNA or determination of DNA/RNA length. It is clear that incorporation of nanotechnologies such as nanofluidics and nanopores into biochips will enable high-throughput detection and quantification of many different types of molecules and open the door to the next generation of chip-based *in vitro* diagnostics.

A **nanobiosensor** is defined as a device that combines a biological indicator with an electrical, mechanical or chemical sensing system on the nanoscale. There are numerous types of nanobiosensors currently under development including optical, electrical, chemical and/or electrochemical in nature. Applications of nanobiosensors for the detection of various agents are virtually limitless and some of the more high-profile examples are outlined in Table 8.1.

Nanowires have long been contemplated as unique and advantageous components for the development of a nanobiosensing device. Nanowire field effect nanobiosensors, for example, incorporate the use of nanowires constructed from carbon nanotubes, metal oxides or silicon to create a nanoscale channel through which current is passed. Surface detectors such
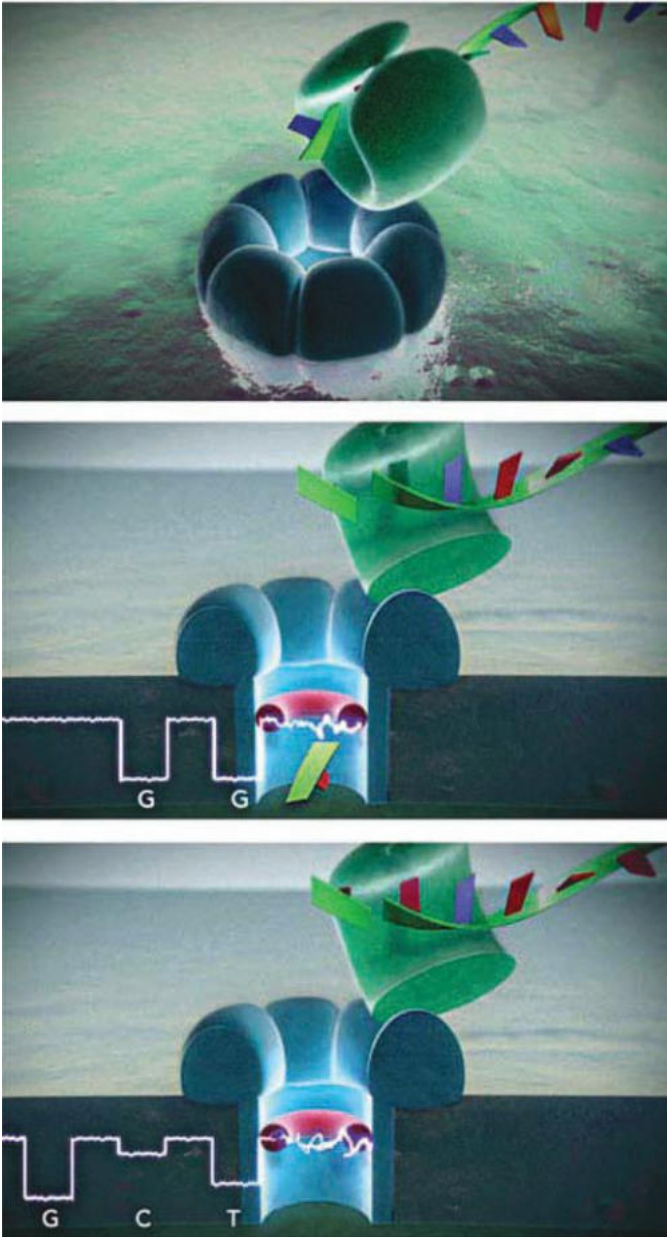
FIGURE 8.3 Diagrammatic illustration of DNA sequencing using a nanopore. (Courtesy of Chemical & Engineering News; reprinted with permission.)

Table 8.1 Examples of nanobiosensors and their applications

Type of Sensor	Use
DNA-Based	Genetic monitoring, disease
Immunosensors	HIV, hepatitis and other viral diseases, drug testing
Cell-Based	Drug testing
Point-of-care Sensors	Blood and urine testing
Bacterial Sensors	Bacterial infection identification
Enzyme-Based	Diabetes, drug testing

as antibodies, when bound to biological targets, change conformation and result in a change in the current passage through the nanowire allowing for discrete and extremely sensitive detection due to the nanoscale of the system (Figure 8.4).
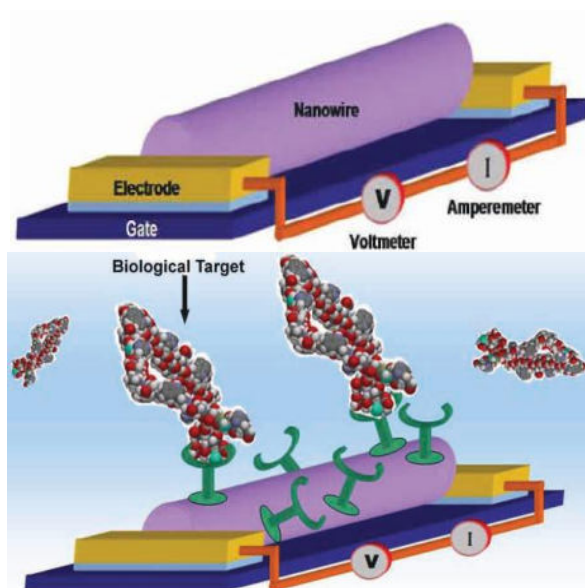
FIGURE 8.4 Diagrammatic illustrations of nanowire field effect nanobiosensors. (Top) Before detection. (Bottom) During detection. (Courtesy of Cagri Ozge Topal, Oklahoma State University; reprinted with permission.)

Xiaohui Tang and colleagues in the Microelectronics Laboratory at the Universite catholique de Louve in Louvain-la-Neuve, Belgium developed a protein bionanosensor by replacing the gate of a metal oxide semiconductor field effect transistor (MOSFET) with a nano-interdigitated array (nIDA). The sensor was able to detect the binding reactions of typical antibodies/antigens at concentrations below 1 ng/ml. The researchers demonstrated that the electrical drain current of the bionanosensor is significantly increased with successive binding and that sensitivity can be tuned and optimized by changing the geometrical design of the nIDA-gate MOSFET (Tang *et al.*, 2009 and Figure 8.5).

Kimberly Dick and colleagues in the Solid State Physics Department at Lund University in Sweden created a novel **nanotree** (branched nanorod structures used in the creation of a detection reactor) acetylcholine esterase enzyme reactor for the detection of trace amounts of acetylcholine. The reactors were designed for use in regional ion sensitive field effect transistors (RISFET) and were constructed of gold-tipped nanorods structures grown on silicon nitrate-covered wafers. Enzymatic activity was measured by calculating the amount of choline formed via application of a chemiluminescent luminal reaction. The researchers demonstrated significantly higher sensitivity in the nanotree enzyme-based reactors as compared to nanorod reactors and it was speculated that this was due
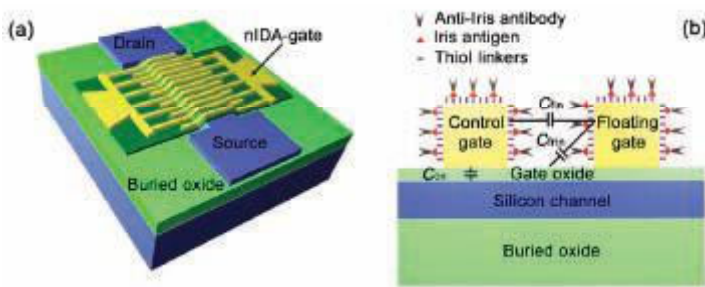
FIGURE 8.5 A schematic nIDA-gate MOSFET sensor. (a) Three-dimensional structure, showing source and drain regions connected by a silicon channel (blue) capacitively coupled with a nano-interdigitated array gate (yellow). (b) Cross-section of two fingers, showing the successive binding of the thiol linker, Iris and anti-Iris biomolecular layers, as well as, the gate oxide capacitance (Cox), inter-finger capacitance (Cfin) and fringing capacitance (Cfrin). (Courtesy of Tang *et al.*, 2009; reprinted with permission.)
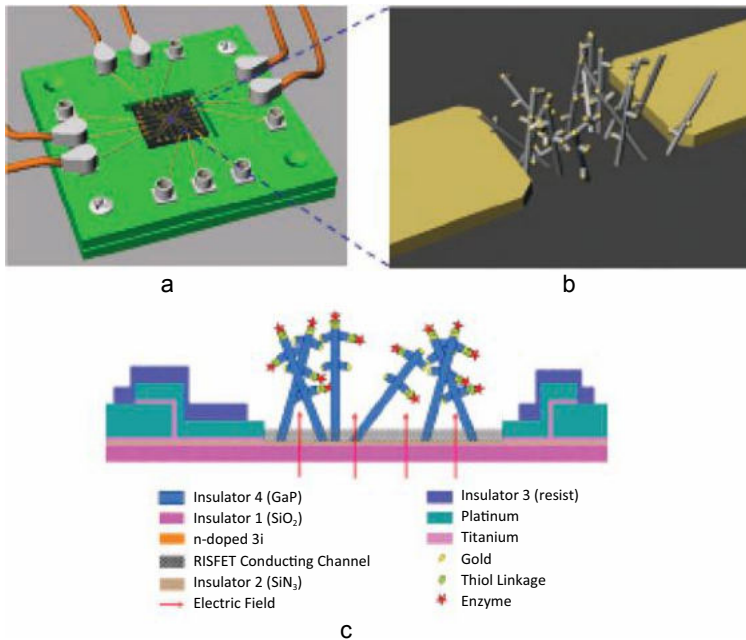
FIGURE 8.6 Schematic illustrations of a RISFET system based on nanotree enzyme reactors. (a) a RISFET sensor chip inserted in a probe station with mini coax connections, (b) a future RISFET-based biosensor chip with a nanotree-based nanoreactor between the RISFET sensing electrodes, and (c) illustration of the conducting channel in a nanotree-based RISFET biosensor chip. The principle behind the RISFET sensor involves the on-chip concentration of an analyte (or a product generated from the analyte enzymatically). The analyte (or product) is focused by a charged field to become concentrated in a narrow region—a conducting channel—located between two sensing electrodes. The focusing process leads to significant changes in conductivity, increasing the level of the current between the sensing electrodes and the ability to sense extremely low concentrations of specific analytes by means of an amplifying process. (Courtesy of Risveden *et al.*, 2010; reprinted with permission.)

to increased gold surface area thus allowing for higher enzyme binding capacity (Risveden *et al.*, 2010 and Figure 8.6).

BioNEMS is defined as nanoelectromechanical systems, made up of components between 1 and 100 nm in size, that integrate electrical and mechanical functionality at the nanoscale for use in biological applications. Giuseppe Maruccio's team at the National Nanotechnology Laboratory

in Lecce, Italy developed a bioNEMS nanoarray-based biosensor for DNA sequencing that electrically detects nucleic acid hybridization events. In this system, oligonucleotides are conjugated to gold nanoparticles and target-probe binding events generate a conductive bridge between two electrodes resulting in a quantized change in electrical conductivity. The device was constructed using **molecular beam epitaxy**, which is a process for the deposition of single crystals under high vacuum. It consists of two main parts: a nanojunction array as the transducer and the molecular probes immobilized on the electrodes which have been specifically designed for the recognition event. Immobilization of probes was accomplished using alkanethiol (C_6-SH) groups attached to 5' termini. Each target nucleotide sequence is marked with conductive gold nanoparticles. The researchers observed a change in I-V curves after hybridization of targets to probes. The system is sensitive enough to detect single hybridization events and could potentially be applied to other binding events such as ligand-receptor interactions. In addition, the application of polymerase chain reaction (PCR), a staple of current DNA sequencing methods, is no longer necessary using this method.

Silicon nanowires have also been explored as devices for bionanosensing. The functional integration of nanomaterials with membrane proteins is speculated to be an important step towards building bionanoelectronic interfaces. Lipid membranes represent crucial structural and protective elements of cells but also harbor a large number of proteins that can be exploited for ionic-based signal transduction. Aleksandr Noy's team at Lawrence Livermore National Laboratory in Livermore, California have done just that to create a bioelectronic silicon nanowire devices incorporating functional cellular membrane proteins which serve as transmembrane peptide pores to drive ionic flux which is converted to an electrical signal. The **Donnan Potential**, which is defined as the distribution of an ion species between two ionic solutions separated by a semi-permeable membrane is given by the equation below.

$$\Delta\phi = \frac{RT}{F}\ln\frac{[Cl]_i}{[Cl]_o}$$

where F is the Faraday constant and [Cl]i(0) denotes the concentration of mobile ions on the inner (outer) surface of the membrane, respectively, and [Cl]I is given by:

$$[Cl]_i = \frac{[M]}{2} \cdot \left(\sqrt{1 + \frac{4C_0^2}{[M]^2}} - 1 \right)$$

where [M] is the effective concentration of the immobile anions on the inner side of the membrane and C_0 is the concentration of the background electrolyte on the outside of the membrane. In this system lipid bilayer membranes were incorporated into SiNW transistors by covering the nanowires with a continuous lipid bilayers shell that forms a barrier between the nanowire surface and the solution. Transport of ions across the nanopores was shown to result in a change in the electronic signal (Misra *et al.*, 2009 and Figure 8.7). This biomimetic device could thus enable new applications in areas such as nanobiosensing and bioelectronics.

John McDevitt's group in the Departments of Chemistry and Bioengineering at Rice University in Houston, Texas (see Focus Box 8.1 and Case Study 8.1 below for additional information) has taken a unique approach to the diagnosis of oral cancer through the creation of a state-of-the-art bio-nano-chip sensor which measures biomarkers in exfoliative cytology specimens. In this system oral lesions from patients were sampled using a non-invasive brush biopsy technique, immunolabeled and characterized for both the expression of the epidermal growth factor receptor (EGFR) biomarker and **cytomorphometry**, which is defined as

Focus Box 8.1 John McDevitt and bio-nano-chip sensors

Rice University researcher John McDevitt is a pioneer in the development of bio-nano-chip sensors. His lab has authored over 170 scientific publications and secured over 150 patents in the area. His cumulative research was recently selected as Science Coalition's Best Scientific Advances for Year as well as the Popular Science's Best of What's New in the medical device category. See Case Study 8.1 for an example of his research.

(Photo courtesy of Rice University; reprinted with permission.)
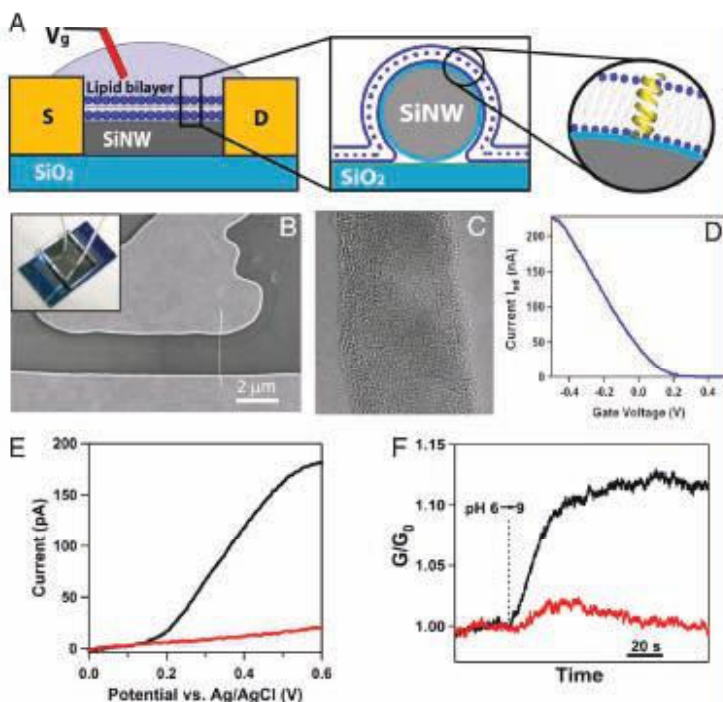
FIGURE 8.7. Bionanoelectronic devices incorporating lipid-coated SiNWs. (A) Device schematics showing a NW connected to micro- fabricated source (S) and drain (D) electrodes. (*Insets*) The configuration of the lipid bilayer and a pore channel placed in the bilayer membrane. (*B*) An SEM micrograph of the NW transistor showing a NW bridging the source and drain electrodes. (*Inset*) A photograph of the device chip covered with a PDMS flow channel. (*C*) TEM micrograph of the as-synthesized SiNW. (*D*)Atypical IV characteristic of the SiNW transistor in fluid. (*E*) Cyclic voltammetry curves measured for an uncoated SiNW device (black line) and a device coated with the lipid bilayer (red line). Fe(CN)6 solution (10 mM)was used as a redox agent. (*F*) Time traces of the normalized conductance of a non-coated SiNW transistor (black line) and SiNW transistor coated with the lipid bilayer (red) as the pH of the solution in the fluid cell was changed from 6 to 9. (Courtesy of Misra *et al.*, 2009; reprinted with permission.)

the measurement of morphological changes at the cellular level. The bio-nano-chip sensor is a multilayered structure built on a $22 \times 30 \times 8.6$ mm poly-methyl methacrylate base containing a mm diameter fluid inlet and outlet port. A polycarbonate screen filter membrane was embedded within the base. A 1 mm $\times$ 125 µm $\times$ 8.2 mm long microfluidics

channel creates a channel volume of 1 uL and samples are introduced by peristaltic pump. Cells retained on the surface of the membrane filter were analyzed for protein and/or nucleic acids using fluorescent labeling techniques (Weigum *et al.*, 2010 and Figure 8.8).

This system was able to detect significant changes of six parameters in biopsy samples obtained from oral squamous cell carcinoma (OSCC) patients. These include:
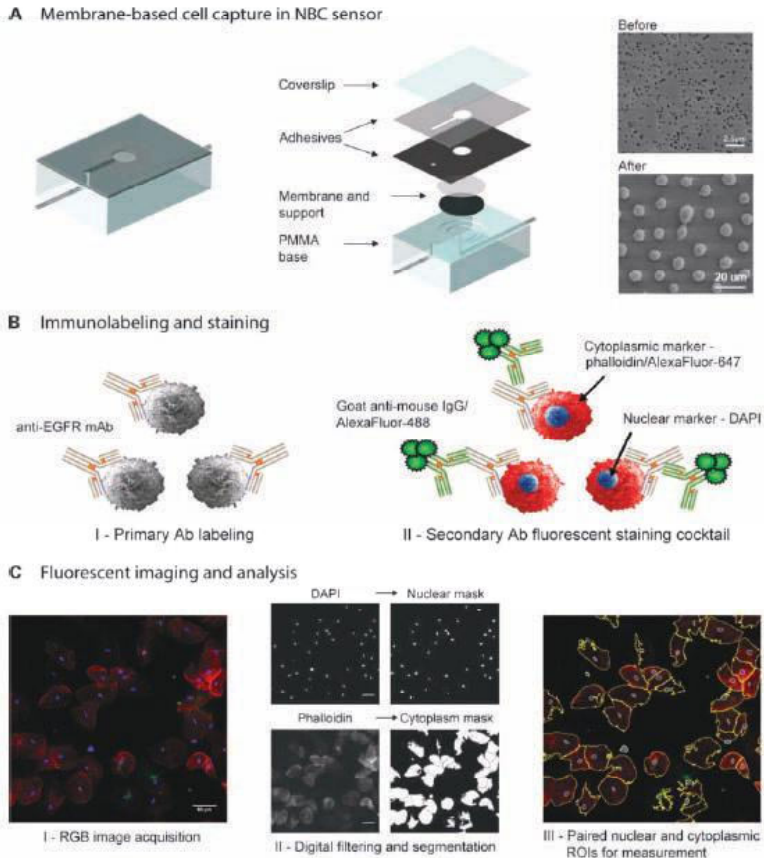
FIGURE 8.8 The NBC oral cytology assay. It consists of three primary steps: (A) Cell capture on the membrane filter; (B) EGFR/AlexaFluor-488 immunolabeling and staining with phalloidin/AlexaFluor-647 and DAPI; and (C) Fluorescent imaging and analysis, where paired cytoplasmic/nuclear ROIs are defined for each cell. (Courtesy of Weigum *et al.*, 2010; reprinted with permission.)

Case Study 8.1: Integration of semiconductor quantum dots into nano-bio-chip systems for enumeration of CD4+ T cell counts at the point-of-need

John McDevitt and colleagues at Rice University have created a quantum dot-based bio-nano-chip system for the characterization of CD4+ lymphocyte counts in HIV-infected patients. The cell capture device is constructed from a custom-machined PMMA base fitted with stainless steel inlet and outlet ports. The base structure is fitted with a polycarbonate track etched membrane for lymphocyte capture and stabilization. This silicon chip based bionanosensor is capable of collecting and analyzing specific lymphocyte cell populations in whole blood. The device demonstrates reduced optical requirements compared to other platforms such as molecular fluorophores and is suggested to be suitable for point-of-need diagnostics and in resource-scarce settings (Jokerst *et al.*, 2008 and Figure 8.9).
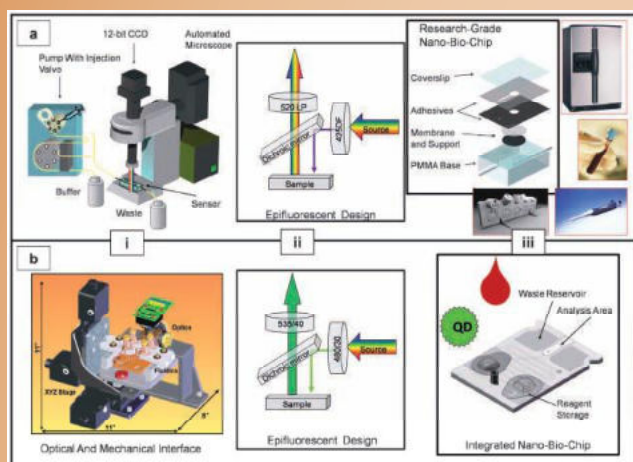
FIGURE 8.9 Schematics illustrations of the NBC lymphocyte detection system. (a) The bench top model employing an automated microscope (i), in tandem with support components including Hg lamp, CCD camera, and peristaltic pump. The adapted microscope used herein contains an epi-fluorescent arrangement customized for QD image capture (ii) and long-pass filter (iii). Insets: Refrigeration for reagents, fluid metering secondary hardware, and a reliable power supply. (b) Battery power supply, optics, and image analysis software combined into a portable device (i). Singly selective fluorescent filter cubes specific to each color channel (ii). Sample metering, on-board reagent supply, and waste disposal are all contained within this highly integrated NBC system (iii). (Jokerst *et al.*, 2008; reprinted with permission.)

- Nuclear area
- Nuclear diameter
- Cellular area
- Cellular diameter
- Nuclear-to-cytoplasmic ratio
- EGFR biomarker expression

The McDevitt bionanosensor platform synergistically utilizes components and achievements from nanotechnology, molecular diagnostic biomarker discovery and microfluidics to create an extremely sensitive and convenient small diagnostics device. As evidenced in Case Study 8.1 below, McDevitt's group has also developed a silicon chip quantum dot-based bio-nano-chip sensor detection system and applied it for the enumeration of CD4+ lymphocyte levels in HIV-infected patients.

Cantilever Biosensors

Cantilever biosensors are based on the mechanical detection of an object's three-dimensional properties and can transform these characteristics from a simple interaction into a mechanical motion on the nanometer scale that is converted to hard data characteristic of and unique to the object's surface. Measurement occurs, within the precision of about 10 nm, by deflecting a light beam from the cantilever surface. In the area of DNA sequence identification and genotyping, cantilever nanobiosensing provides an alternative to polymerase chain reaction (PCR) and at the minimum complements if not has the capability to replace some microarray detection methodologies. The advantages of cantilevers are that they provide fast, label-free detection of biomolecules. In the area of genotyping, this allows for the identification of single-nucleotide polymorphisms and oncogenes. Nanocantilevers could represent the next generation of ultrasmall sensors for the detection of viruses, bacteria and other pathogens. Researchers at the Birck Nanotechnology Center in West Lafayette, Indiana used silicon cantilever beams with length 3–5 mm, width 1.4–1.5 μm and thickness ~30 nm for the resonant detection of vaccinia virus particles. Three different receptor attachment schemes were employed on three different chips. The biotin-streptavidin system was used to link antibodies to the cantilevers. The **resonant frequency**, which is defined as the tendency of a system to oscillate at larger amplitudes for certain frequencies, of antibody-coated cantilevers was measured in

air by using a **laser Doppler vibrometer (LDV)**. An LDV is a scientific instrument that is designed to make non-contact vibration measurements of a surface. After LDV measurement, the cantilevers were immersed in solution to activate the antibody molecules and treated with a mixture of antigens. This was followed by cantilever drying and re-measurement of resonant frequencies in air. Use of this system allowed for the clear detection of single vaccinia virus nanoparticles which was confirmed by scanning electron microscopy (Gupta *et al.*, 2006 and Figure 8.10). In addition, the development of a real-time nanocantilever-based detection system could provide continuous point-of-reference monitoring of clinically relevant parameters in personalized medicine.

Nanolaser Scanning Confocal Spectroscopy

Nanolaser scanning confocal spectroscopy is an extremely precise system for the laser-based spectroscopic measurement of subcellular organelles.
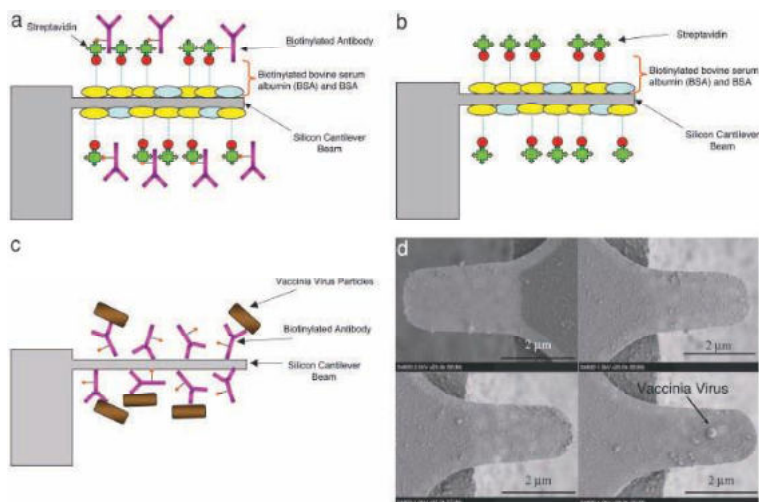
FIGURE 8.10 Protein attachment schemes and the corresponding images of the functionalized cantilevers. (*a–c*) Schematic diagrams depicting protein attachment for schemes 1 (a), 2 (b), and 3 (c). (d) Field emission scanning electron micrograph representing the interaction of a mixture of virus particles with protein-coated cantilever beams using schemes 1. Scheme 1 shows the capture of vaccinia virus. (Courtesy of Gupta *et al.*, 2006; reprinted with permission.)

The high-throughput detection of cancerous phenotypes in individual cells has long been a goal of pathologists which currently rely on labor-intensive microscopic examination of individual tumor cells using various staining techniques. A system which provides accurate, real-time high-throughput screening of tumor cells without invasive and damaging chemical agents has the potential to transform this area of cancer-based pathological characterization at the cellular level. Robert Naviaux's team in the Departments of Medicine and Pediatrics at the University of California - San Diego have created just such a system and demonstrated proof-of-concept in characterizing the respiratory health of single cells, changes of which are hallmarks for the cancerous phenotype. **Mitochondria** are membrane-based enclosed organelles that generate most of the cell's supply of ATP. Changes in mitochondrial function have long been suspected to at least indirectly contribute to the development and progression of cancer. The differences in mitochondrial energy metabolism between normal and cancer cells can allow for the selective identification of cancer cells due to their altered respiratory efficiencies. Naviaux's team based their diagnostics platform on the theory of light scattering by mitochondria in cells, with abnormal mitochondria exhibiting on average 15% larger size and loss of network structure resulting in altered light scattering. The theory is based heavily on the **structure factor**, which, in condensed matter physics and crystallography, is a mathematical description of how a material scatters incident radiation. It is calculated according to the following equation:

$$S(q) = 1 + (\rho) \int dr \exp(iq \cdot r)[g(r) - 1]$$

where q is the change in momentum of the incident and scattered photon, (ρ) is the average particle density and g(r) is the correlation function describing the fluctuation of the particle density from the mean value. The structure factor is related to light scattering, imaging and spectroscopic measurements. Thus a modification of the effective differential cross section scattering of light via a structure factor allows for sensitive detection of differences in mitochondrial light scattering. Confocal nanolaser spectroscopy employing mitochondrial membrane sensing dyes was used to apply this theory and resulted in the successful detection of light scattering differences between normal and cancerous liver cells.

Naviaux's associate Paul L. Gourley took this technology a step further and created a **"Biocavity Laser" chip** that encompasses a gallium-arsenide laser emitting in the near-infrared range integrated with a microfluidic chip about the size of a dime and designed to flow cells one at a time through the laser. When cells flowed through the cavity they acted as individual internal waveguides with peak spacing, intensity and spectral shift dependent upon all components of the cells, most notably also the mitochondrial makeup (Gourley *et al.*, 2005b and Figure 8.11).
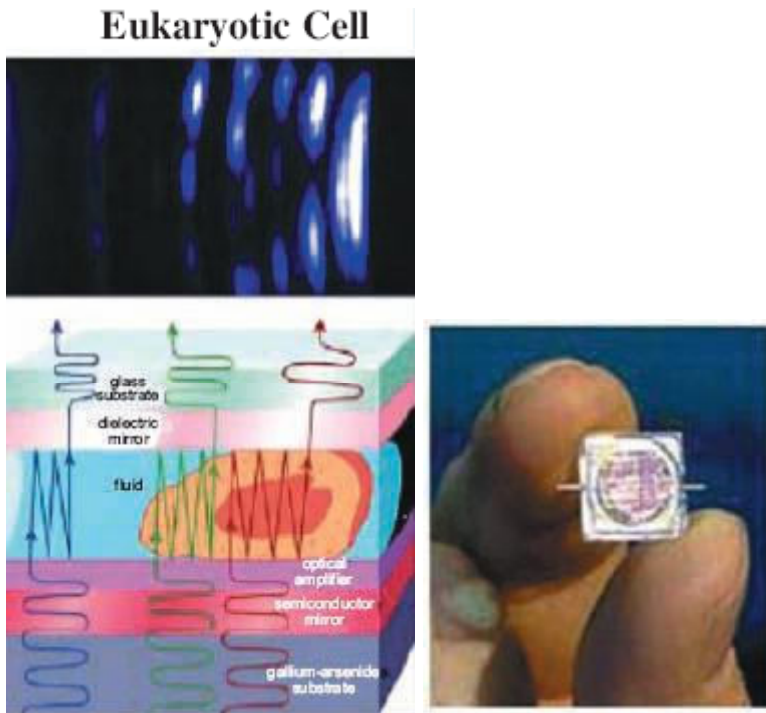
FIGURE 8.11 The Biocavity Laser. (Left) Illustration of the interrogation of eukaryotic cell and a typical spectrum. The layers of the chip are from bottom to top: gallium-arsenide substrate (gray), semiconductor mirror (red), optical amplifier (purple), fluid cavity (blue), dielectric mirror (pink), and glass substrate (green). (Right) The size of the biocavity laser and integrated, microfluidics flow chip are illustrated. (Upper Left) Actual image output. (Courtesy of Gourley *et al.*, 2005b; reprinted with permission.)

Mass Spectroscopy and Nanoproteomics

Nanoproteomics is defined as the application of nanobiotechnology to proteomics and can enable the detection of a single molecule of protein in a sample. Shu-Hui Chen's team in the Department of Chemistry and Institute of Bioinformatics at National Cheng Kung University in Tainan, Taiwan developed an integrated proteomics approach using chemically functionalized gold nanoparticles (AuNP) as probes for affinity purification of large protein complexes. These complexes were dissected using standard mass spectroscopy (MS) techniques to comprehensively identify individual proteins present in a sample. In their approach, nucleotide sequences that specifically bind protein complexes in the nucleus of cells were linked to the AuNPs and were functionalized with PEG. Functionalized AuNPs were incubated with whole cell lysate extracts (extracts of protein/DNA complexes from the entire cell, nucleus and cytoplasm) of the cell line MCF-7 and characterized for the presence of protein binding complexes by MS. This system was designated as **quantitative nanoproteomics (QNanoPX)**, which is defined as the quantitative application of nanobiotechnology to proteomics. It was applied to globally map the transcriptional activation complex of the **estrogen response element (ERE)** which is a nucleotide sequence bound by the estrogen receptor (ER) during the activation of ER target gene transcription.

Quantum dots have also been applied in the field of nanoproteomics for the detection of **micrometastases**, which are defined as the spread of cancer cells from a primary site and the formation of microscopic tumors at secondary sites. The coupling of antibodies specific for antigens that mark micrometastatic events to quantum dots allows for efficient fluorescence-based detection of these markers at the nanoscale. In an excellent summary of research in this field Igor Nabiev and colleagues at the Basque Foundation for Science, Bilbao, Spain outlined the basic principles and concepts for the simultaneous detection of multiple parameters signaling micrometastases. This is accomplished by increasing the number of functionally active antibodies linked to each quantum dot, which may be routinely accomplished using the biotin-streptavidin linkage system (Mahmoud *et al.*, 2010 and Figure 8.12).
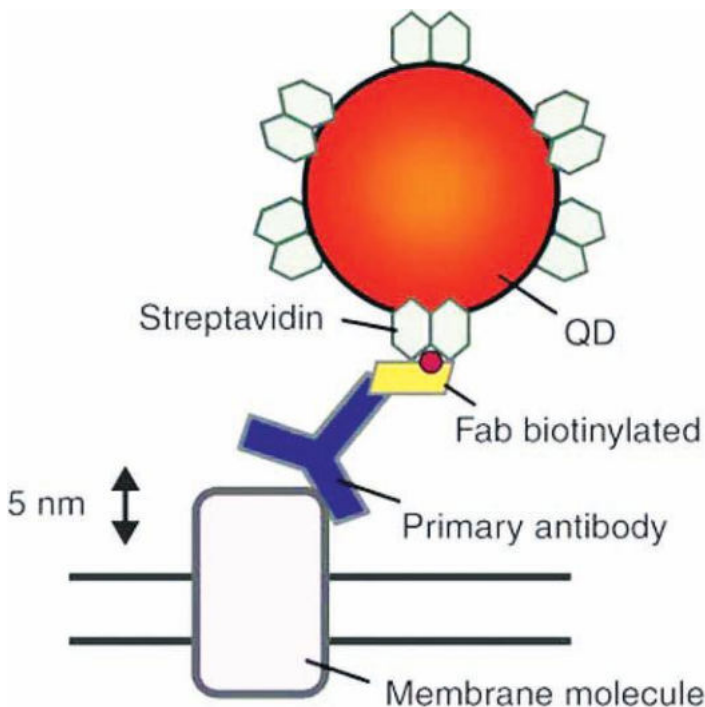
FIGURE 8.12 Diagrammatic illustration of a quantum dot targeted to a cell via membrane molecule attachment through an antibody interaction. (Courtesy of Nature Protocols; reprinted with permission.)

Surface-Enhanced Raman Scattering Nanobiosensors

Surface plasmons are coherent electron oscillations that exist at the interface between any two materials where the dielectric function changes sign across the interface. **Surface plasmon resonance (SPR)**, discussed in Chapter 2 and defined as the excitation of surface plasmons by light on a planar surface, is one of the best-known and widely used examples of nanotechnology applied to optical nanobiosensing. **Surface-enhanced Raman scattering (SERS)** is a technique that results in the enhancement of molecular photon scattering effects on rough metal surfaces. Tags that are optically detectable can be formed by SERS at the interface of glass and metal with each tag representing the Raman spectrum of a different small molecule, referred to as a **Raman shift** (Figure 8.13).
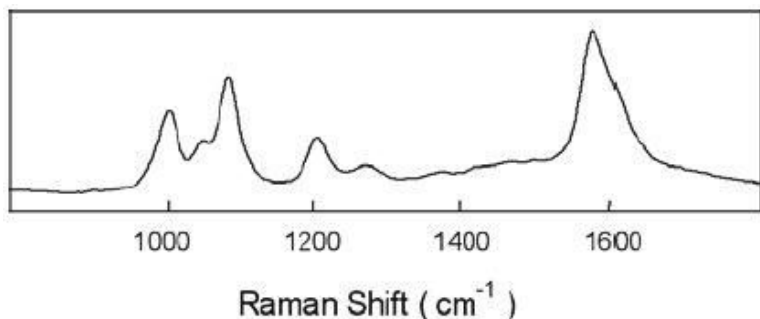
FIGURE 8.13 Raman shift. The difference in energy between the incident photon and the Raman scattered photon is equal to the energy of a vibration of the scattering molecule. A plot of the intensity of scattered light versus energy difference is a Raman spectrum. (Courtesy of Oxonica; reprinted with permission.)

As SERS banding patterns are roughly 1/50 the width of fluorescent bands and enable a greater degree of multiplexing than current fluorescence-based techniques, the obvious advantage of SERS with respect to nanobiosensing is the degree of sensitivity. In addition, the proportion of SERS-based tag spectral intensity to the number of particles is linear allowing not only for analyte identification but also quantification. SERS nanobiosensing has been applied extensively over the last several years for the identification and quantification of biomarkers in various samples. The UK-based nanotechnology company Oxonica specializes in providing SERS-active metal nanoparticles for biomarker analysis. Referred to as Nanoplex™ technology, the biotags consist of a gold core with a known characteristic Raman spectrum encapsulated by silica and conjugated to a biological recognition molecule for detection (Figure 8.14).

Researchers at Oxonica claim the following advantages to using their system including:

- High-level multiplexing and simultaneous internal calibration
- Minimal interference from highly colored and/or scattering samples such as blood
- Environment-insensitive signal generation and simple biofunction-alization

By applying the basic principles of SERS technology for nanobiosensing a variety of sensors have been developed to detect the presence of chemical
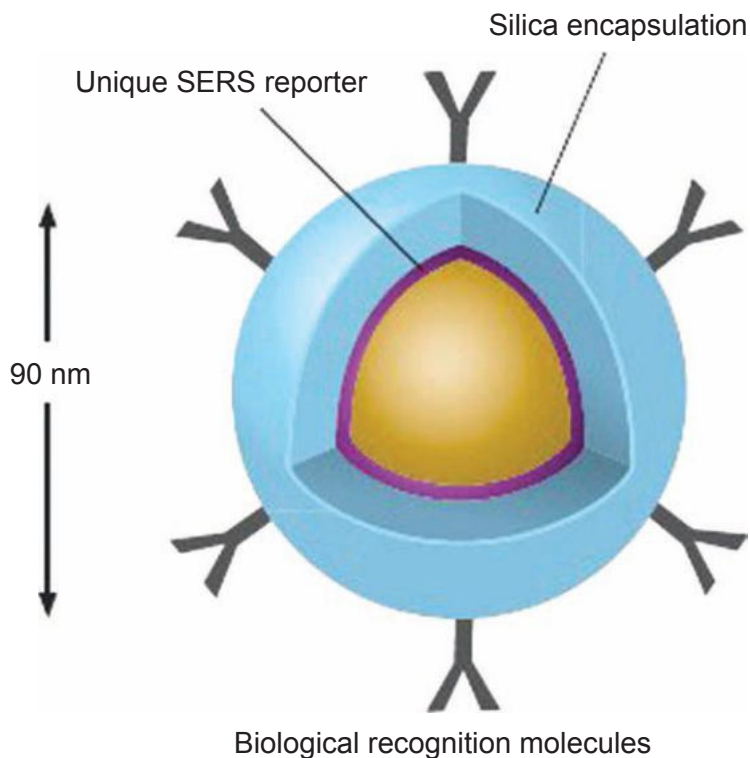
FIGURE 8.14. Illustration of the composition of a Nanoplex biotag. (Courtesy of Oxonica; reprinted with permission.)

agents and/or biological species. In addition, a DNA-based version has been used to detect gene targets through complementary strand hybridization. Tuan Vo-Dinh's group at the Center for Advanced Biomedical Photonics, Oak Ridge National Laboratories in Tennessee (now at Duke University) has described the use of plasmonics-based nanoprobes that act as specific molecular recognition sentinels to identify unique DNA sequences in a given sample. These **molecular sentinels (MS)**, are comprised of a metal nanoparticle conjugated to a stem-loop DNA molecule which is Raman label-tagged. In the unbound state, the stem loop maintains the Raman label in close proximity to the metal nanoparticle, thus inducing an intense SERS effect and strong Raman signal. The SERS signal is quenched upon target binding due to disruption of the stem loop configuration and removal of the Raman label from the metal nanoparticle. This system was used to

detect the *gag* gene sequence of the human immunodeficiency virus type 1 (HIV-1) in **PCR amplicons** (polymerase chain reaction amplification products) (Wabuyele *et al.*, 2005 and Figure 8.15). The use of Raman over more conventional fluorescence-based systems provides the advantages of much narrower emission lines and molecular-specific vibrational bands. It's enhancement as a SERS DNA hairpin molecular sentinel system now provides the sensitivity and signal intensity needed for the identification of rare gene sequences in a variety of samples.
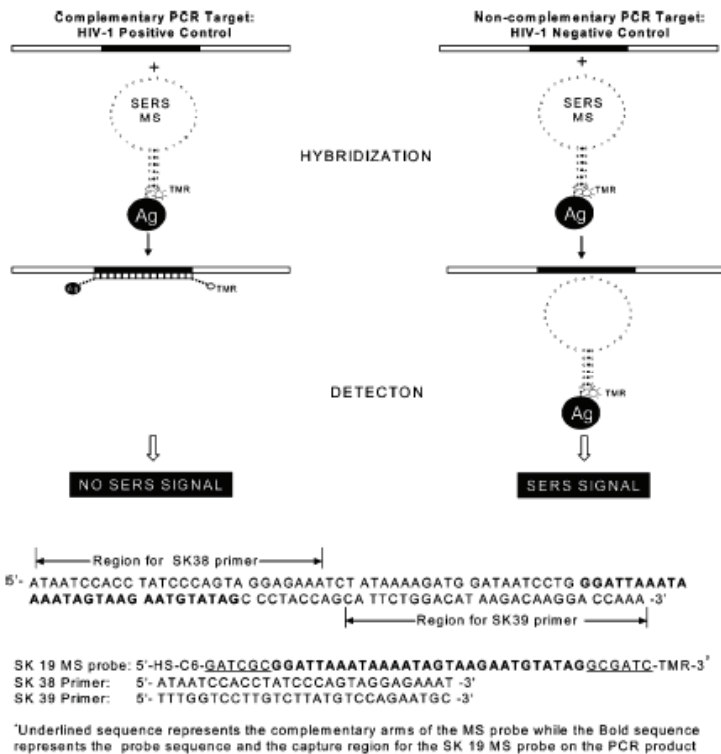
FIGURE 8.15 Schematic representation of the SERS molecular sentinel hybridization and optical detection scheme. Binding of SK19 MS nanoprobes to a capture region of the 115-bp PCR product quenches the optical signal whereas unbound nanoprobes generate a detectable SERS signal. The sequence of the PCR product of the 115-bp gag region of the HIV-1 genome, the primers, and SK19 MS nanoprobe are shown. (Courtesy of Wabuyele *et al.*, 2005; reprinted with permission.)

IN VIVO NANODIAGNOSTICS

This section focuses on nanodiagnostics as it applies to the detection and characterization of biomarkers in the body. Particular emphasis is placed on nanotechnologies in which nanoparticles targeted to a particular site are acted upon by an external field such as magnetic resonance imaging (MRI), SERS or computed tomography (CT) to provide superior contrasting as compared to more conventional platforms currently utilized in diagnostics applications.

Gold Nanoparticles

Computed tomography (CT) is a medical imaging method employing *in vivo* sectional imaging by computer processing of X-ray images. It is one of the most useful diagnostic tools in terms of cost and availability. It is used worldwide for internal imaging of patients but to date is not considered a specific molecular imaging platform since appropriate molecularly-targeted contrast agents for X-ray analysis have not been developed to the point of wide acceptance by the medical community. It is well known that gold induces strong X-ray attenuation. In addition, gold nanoparticles have unique physical properties that may make them ideal as *in vivo* contrast agents, most importantly including their flexibility with respect to functional group modification for the efficient attachment of targeting species. The fact that gold nanoparticles are generally accepted as non-toxic is also an important consideration when designing nanoparticle-based contrast agents. Raul Kopelman's group in the Department of Chemistry at the University of Michigan in Ann Arbor has now combined targeted gold nanoparticles with CT to enable molecular *in vivo* imaging of head and neck cancer. In their studies they synthesized gold nanorods (AuNRs) using a seed-mediated growth method to produce nanorods with a mean length of 45 nm and mean diameter of 15 nm and subsequently coated these with polyacrylic acid (PAA). UM-A9 antibodies, which specifically bind to head and neck cancer cells, were conjugated to the AuNRs through an amidation reaction with PAA. Addition of these conjugates to UM-SCC-1 and UM-SCC-5 human head and neck cancer cells followed by applied CT allowed for effective and highly sensitive imaging of these cells *in vitro* (Popovtzer *et al.*, 2008 and Figure 8.16).

The combination of gold nanoshells (GNS) and narrow band near-infrared (NIR) light is yet another platform for the application of gold
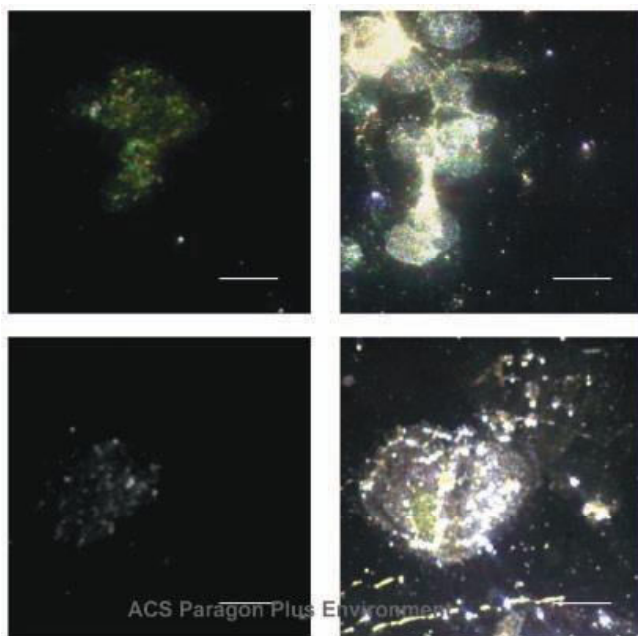
FIGURE 8.16 Imaging of head and neck cancer cells using targeted gold nanoparticles and CT. Dark field microscope images of SCC head and neck cancer cells (oral cancer upper images, larynx cancer lower images) after incubation with non-matching antibody-coated gold nanorods (left) vs. matching UM-A9 antibody-coated gold nanorods (right). Scale bar: 10 μm. (Courtesy of Popovtzer *et al.*, 2008; reprinted with permission.)

nanoparticles as *in vivo* contrast agents. This can be accomplished by optically tuning the ratio of the dielectric silica core to the outer gold shell for sensitivity to light of the NIR spectrum. This customizable tuning trait allows for the efficient fabrication of GNS to strongly absorb in the NIR wavelength range, where there is minimal absorption of light by biological chromophores and optimal penetration of light through most tissues in the body. Researchers in the Department of Biomedical Engineering at the University of Texas in Austin lead by James Tunnell have reported the use of GNS as exogenous contrast agents in combination with narrow-band imaging (NBI) for the enhanced *in vivo* visualization of tumors. GNS were prepared by growing gold colloids over a silica internal core and reacted with $HAuCl_4$ causing the gold surface to coalesce thus forming a complete
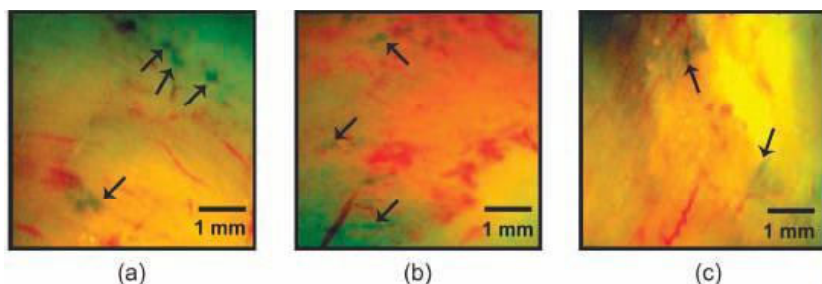
FIGURE 8.17 Composite imaging of human colon tumors using gold nanoshells and near IR light. The black arrows indicate gold nanoshells present in the tumor. (Courtesy of Puvanakrishnan *et al.*, 2009; reprinted with permission.)

shell. The GNS had a core size of 120 nm and a shell thickness of 15 nm. PEG was further coated on the outside of the GNS for passive targeting via the enhanced permeability retention effect (see EPR in Chapter 2). GNS were introduced into colorectal tumor xenograft animal models via intravenous tail vein injections. Tumors were excised 24 hours later for NBI and it was demonstrated that GNS acted as effective contrast agents to distinguish tumor cells from tumor vasculature (Puvanakrishnan *et al.*, 2009 and Figure 8.17). It should be noted that this was *ex vivo* detection and the system will need to be further optimized for deep tissue *in vivo* imaging before successful application in diagnostic medicine.

"Golden" Carbon Nanotubes

An interesting fusion of nanotechnologies for *in vivo* diagnostics involves the use of **golden carbon nanotubes (GNTs)** as multimodal photoacoustic and photothermal high-contrast molecular agents. Vladimir Zharov and colleagues at the Institute for Nanoscale Materials Science and Engineering, University of Arkansas in Fayetteville have addressed the relatively low absorption and toxicity concerns of carbon nanotubes using gold-plated CNTs as an *in vivo* contrast agent for the photothermal (PT) and photoacoustic (PA) targeted visualization of lymphatic vessels in mice by enhanced near-infrared light. Antibodies which bind the lymphatic endothelial receptor were conjugated to golden carbon nanotubes and these formulations exhibited minimal toxicity *in vitro*, most likely due to the shielding of the CNT from the biological environment by the plated gold

FIGURE 8.18 Illustration of serpentine "golden" carbon nanotubes. (Courtesy of Weizmann Institute of Science; reprinted with permission.)

coating. The GNTs were single-walled in composition having a diameter of 1.5–2 nm, a length of 11 nm and coated with a thin gold layer 4–8 nm in thickness. They exhibited size uniformity and were considerably water-soluble due to minimization of van der Waals attractive forces between individual SWNTs by the gold coating. Following coating of the GNTs with antibodies specific for the LYVE-1 receptor, expressed preferentially in the lymphatic system, the conjugates were delivered to the lymphatic system of nude mice and exposed to laser pulses using an integrated intravital microscope (Kim *et al.*, 2009 and Figure 8.18). The degree of PA or PT contrast sensitivity was compared to other nanoparticle-based contrast agents such as gold nanospheres and nanoshells as well as uncoated carbon nanotubes and shown to be considerably superior to these other agents.

Magnetic Nanoparticles

Superparamagnetic iron oxide (SPIO) nanoparticles are nanosized magnetic chemical compounds composed of iron and oxygen. SPIO nanoparticles have long been studied as *in vivo* contrast agents for the detection of cancer and pathogens in the body. Combined with the application of magnetic resonance imaging (MRI), SPIO nanoparticles

have shown tremendous promise as the next generation of contrast agents for magnetic resonance imaging applications as well as for magnetic hyperthermia therapeutic applications. They are considered to be superior contrast agents to that of more conventional dadolinium chelates as they provide for longer delineation of tumor margins which is a result of both increased cellular internalization and slower clearance from the site of tumorigenesis. Miqin Zhang and colleagues in the Department of Materials Science & Engineering at the University of Washington in Seattle developed a PEG-coated iron oxide nanoparticle-conjugated to the targeting peptide chlorotoxin (CTX), that was demonstrated to be capable of specifically targeting and thereby marking glioma tumors for MRI-based imaging. PEG-coated target-specific nanoparticles of 10–15 nm in size were prepared with PEG serving not only to drive nanoparticle solubility and stability *in vivo* but also as a linking agent for CTX attachment with ~30 attachment sites per nanoparticle. Conjugation of CTX to each nanoparticle was accomplished in a three-step process to create stable thioester linkages between the nanoparticle and the peptide. Introduction of the conjugates into nude mice bearing 9L xenograft tumors followed by MRI revealed clear and preferential accumulation of the nanoparticles in sites of tumorigenesis (Sun *et al.*, 2008 and Figure 8.19).

Zharov's group at the University of Arkansas (discussed above for golden carbon nanotube diagnostics) have taken *in vivo* magnetic imaging using magnetic nanoparticles (MNPs) a step further and devised a way to capture rare tumor cells circulating in the bloodstream, a hallmark of metastasis, and subsequently detect their presence photo-acoustically. Zharov's system is based on combining *in vivo* multiplexed targeting with multicolor and magnetic enrichment to allow for the concentration of circulating tumor cells (CTCs) from a large volume of blood which may then be detected photo-acoustically. To achieve appropriate sensitivity levels, two-color nanoparticles were used that could be illuminated by laser pulses. These were applied along with multiplexed targeting and a multicolor detection strategy. The magnetic nanoparticles were conjugated to the amino-terminal fragment of the urokinase plasminogen activator, a ligand for receptors highly expressed in circulating cancer cells but not present on the surfaces of normal blood and endothelial cells. The intrinsic absorption properties of the Fe_2O_3 nanoparticle cores allowed for either magnetic or photoacoustic detection. The second molecular detection
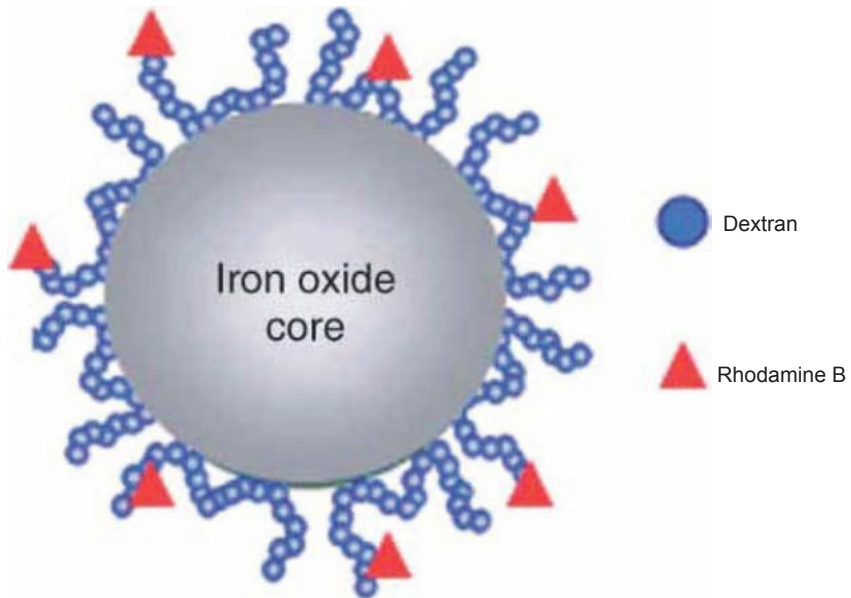
FIGURE 8.19 Illustration of SPIO nanoparticles with rhodamine molecules attached via dextran. (Courtesy of Wiley Online Science Library; reprinted with permission.)

agent used was golden carbon nanotubes (discussed earlier in this chapter). Intravenous injections of these multiplexed contrast nanoparticles into nude mice bearing circulating human breast cancer cells was carried out followed by photoacoustic exposure. Detection of circulating breast cancer cells was observed very rapidly, roughly 8–10 minutes after introduction of the contrast agents.

As evidenced by the above examples, while numerous groups have been successful at applying magnetic nanoprobes in the context of imaging, the results are often far from optimal. Signal enhancement via the use of SPIO nanoparticles, for example, is still unsatisfactory compared with that obtained using florescence and PET. Jin-Suck Suh's group at the Nanomedical National Core Research Center, Yonsei University in Seoul, South Korea have addressed the issue of magnetic nanoparticle signal enhancement limitations by using an innovative approach to develop **magnetism-engineered iron oxide (MEIO) nanoparticles** which are novel metal-doped nanoparticles engineered to possess exceptionally high and

tunable nanomagnetism. The nanoparticles were synthesized under high temperature in an organic medium for control of purity, size uniformity, crystallinity, stoichiometry and magnetism. The researchers then performed an analysis and full characterization of metal (manganese, iron, cobalt or nickel) doped MEIO nanoparticles to elucidate the magnetic characteristics that drive magnetic resonance signal enhancement. Thus, based on magnetic spin as well as the size and type of nanoparticles, a superior nanocrystalline system was developed. This was confirmed via targeting and MRI *in vivo* imaging of tumors in nude mice using manganese MEIO Herceptin conjugates.

Cancer is not the only physiological area for the use of magnetic nanoparticles in cellular tracking. The *in vivo* identification and tracking of therapeutic cells, such as stem cells, is a valuable tool for determining the nature of stem cell migration and differentiation into terminal lineages. Researchers in the Radiology and Imaging Sciences Clinical Center at the National Institute of Biomedical Imaging and Bioengineering in Bethesda, MD have developed an *in vivo* stem cell tracking platform based on electrostatically assembled fluorescent SPIO nanoparticle-peptide complexes. Iron oxide nanoparticles were synthesized under high temperature and coated with a carboxylic acid functionalized biocompatible amphiphilic triblock copolymer. After purification of the nanoparticles, having a diameter of ~10 nm, the fluorescent label Texas Red® was attached via an amine reaction to generate the final tracking complexes. The nanocomplexes were introduced intracellularly in tissue culture. Seven days after introduction of both labeled and unlabeled stem cells into the flanks of athymic nude mice both MRI and fluorescence imaging were performed with clear visualization of the tracked cells (Lee *et al.*, 2009 and Figure 8.20). The advantages of this system are that it allows for MRI-based cellular imaging at the single cell level due to the combination of negatively charged fluorescent mono-disperse SPIO nanoparticles with positively charged peptides in a complex to improve the magnetic resonance properties of labeled stem cells.

Perfluorocarbons

As discussed in Chapter 1, perfluorocarbons (PFCs) are compounds derived from hydrocarbons via the replacement of hydrogen atoms with fluorine
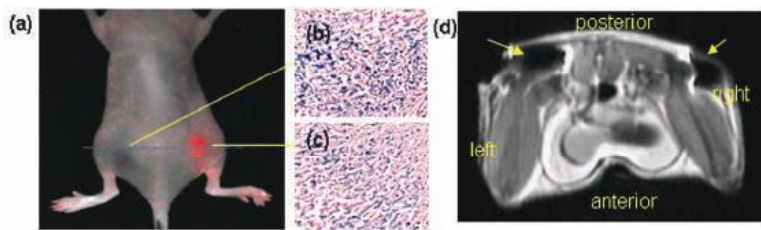
FIGURE 8.20 Stem cell tracking via SPIO nanoparticle-peptide complexes. (a) *In vivo* fluorescence imaging of subcutaneous flank tumor bearing mice 7days later after co-injection of Texas HTIO15-Pro labeled hMSC and unlabeled C6 gliomas (right side), and co-injection of FE-Pro labeled hMSC and unlabeled C6 gliomas (left side). The line represents the approximate MRI scanning line through the mouse. (b) Histology of left side of flank tumor and (c) right side of flank tumor. (d) Corresponding magnetic resonance image through a cross section slice of the mouse indicates hypointense (dark) voxels on right and left top sides (arrows). (Courtesy of Lee *et al*., 2009; reprinted with permission.)

atoms. They are made up of carbon and fluorine atoms only which can be arranged in a linear, cyclic or polycyclic shape. In *in vivo* diagnostic applications, PFCs may be detected by a variety of methods but the most commonly studied are ultrasound and magnetic resonance imaging. Ultrasound detects microbubbles of gaseous perfluorocarbons which oscillate and vibrate when a sonic energy is applied thus characteristically reflecting the ultrasound waves and distinguishing the PFC microbubbles from surround tissues. The advantages of PFC microbubbles over that of air include:

- Stability
- Inertness
- Low diffusion rates
- Solubility

In magnetic resonance imaging (MRI) applications the imaging system is tuned to detect 19-fluorine nuclei. Given the fact that there is no fluorine naturally present in the body this allows of ease of targeted PFC detection *in vivo*. In addition, PFCs may be used in radiographic imaging if the derivative perfluorooctyl bromide (PFOB) is employed given its higher degree of opaqueness to X-rays.

In order to effectively utilize perfluorocarbons as diagnostics agents, a correct calculation of particle concentration is essential. Without knowing the concentration of PFCs prior to *in vivo* administration it is difficult if not impossible to drive the appropriate number of nanoparticles to targeted sites for effective contrast imaging. The concentration of PFC nanoparticles can be calculated according to the following formula:

$$[NP] = \frac{V_{NP}}{V_{np} \cdot V_{E} \cdot N_{av}}$$

where V_{NP} is the total volume of the components used to create the nanoparticles (such as oils, PFCs and any surfactants used for emulsification), v_{np} is the volume of each nanoparticle, assumed to be a sphere, using the measured particle size, V_{E} is the total volume of the final emulsion and N_{av} is Avagadro's number (Morawski *et al.*, 2004).

Quantum Dots

As mentioned with respect to Nabiev's *in vitro* quantum dot (QD) diagnostics technology review above, quantum dots are excellent fluorescent nanoprobes due to their unique size-dependent optical and electronic properties. Discussed in Chapter 1, these nanoparticles possess

Focus Box 8.2 Sam Wickline, Greg Lanza and perfluorocarbons for cancer diagnostics

Samuel Wickline (right) and Gregory Lanza at Washington University in St. Louis, Missouri have dedicated their careers to the early detection of cancer and cardiovascular disease using targeted perfluorocarbons combined with magnetic resonance imaging. They have invented perfluorocarbon emulsions that can incorporate various classes of target-specific ligands that can be imaged in the body with MRI, CT or ultrasound methods for picomolar-sensitive detection of epitopes. They are now pursuing similar technologies for the targeted treatment of cancer and CV disease. See Case Study 8.2. (Photo courtesy of *St. Louis Commerce Magazine*; reprinted with permission.)

Case Study 8.2: Detection and quantification of angiogenesis in experimental valve disease with integrin-targeted nanoparticles and 19-fluorine MRI/MRS

Abnormal angiogenesis is one of the hallmarks of early atherosclerotic plaque development and may contribute significantly to aortic valve stenosis. Samuel Wickline, Gregory Lanza and colleagues at Washington University in St. Louis, Missouri (also see Focus Box 8.2) have developed a perfluorocarbon-based procedure for the *in vivo* monitoring of neovasculature formation in rabbit models of aortic valve disease. Utilizing ^{19}F integrin-targeted perfluorocarbons, which home in on and detect early angiogenesis, the researchers demonstrated neovasculature detection in atherosclerotic heart valves of rabbits fed high cholesterol diets. Targeting was accomplished via the attachment of a peptidomimetic vitronectin antagonist to the emulsified PFC nanoparticles and detection was based on magnetic resonance imaging (MRI) of PFCs targeted to sites of angiogenesis proximal to the aortic valve (Figure 8.21). The researchers demonstrated a 220% increase in PFC presence in aortic valves of rabbits with targeted vs. non-targeted PFCs. Immunohistochemical staining for the endothelial markers PECAM and ab3 integrin confirmed the presence of neovasculature in the valve leaflets (Waters *et al.*, 2008).
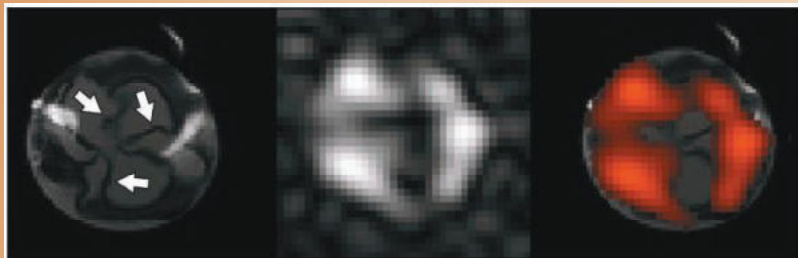
FIGURE 8.21 ^{19}F-nanoparticle imaging of aortic valve angiogenesis. (Left) Co-registered proton image. (Center) Fluorine image. (Far Right) The fluorine image is false colored and overlaid on the proton image, showing strong signal at the base of the valve leaflets, as well as signal from the body of the leaflets. (Courtesy of Waters *et al.*, 2008; reprinted with permission.)

unique optical properties in comparison with traditional organic dyes including size- and composition-dependent tunable emission, narrow emission spectra and long-term photo-stability. Bioconjugated versions of quantum dots have long been contemplated as diagnostic platforms for

the detection of specific agents. Ralph Tripp's team in the Department of Infectious Diseases at the University of Georgia in Athens has shown that functionalized quantum dots conjugated to monoclonal antibodies can be used to rapidly and specifically detect respiratory syncytial virus (RSV) *in vitro* and *in vivo*. In these studies cadmium telluride (CdTe) quantum dots of emission peaks at 585 nm and 540 nm were conjugated to monoclonal antibodies which specifically bind the RSV-specific F protein present on the surface of viral particles. The bioconjugates were introduced intravenously into RSV-infected Balb/c mice via tail vein injection followed by lung biopsy immunohistochemical analysis. The system easily detected the presence of RSV viral particles in the lungs of infected mice as compared to controls (Tripp *et al.*, 2007 and Figure 8.22). Although quantum dot toxicity issues still need to be addressed, their targeted use
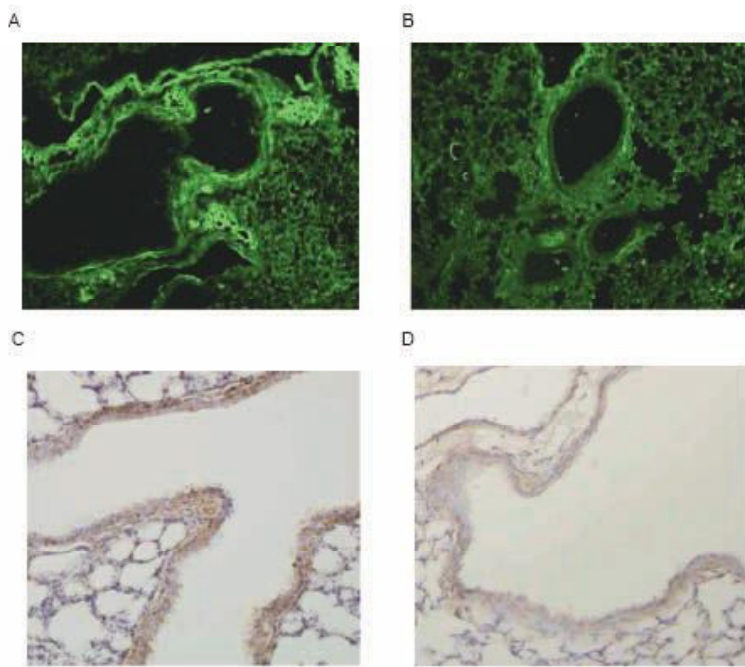
FIGURE 8.22 RSV-NPs detection of RSV-infected lung tissue. Lung tissue sections from RSV-infected (A, C) or naïve, mock-treated BALB/c mice (B, D) were stained by IH Cusing RSV-NPs (A, B), or by conventional IHC (C, D), and analyzed using an immunofluorescence microscopy. Abbreviations: IHC, immunohistochemistry; RSV-NP, respiratory syncytial virus-nanoparticles. (Images courtesy of Tripp *et al.*, 2007; Reprinted with permission.)

as nanobiosensors for *in vivo* detection of pathogens may someday bridge the gap between current cumbersome and time consuming viral detection assays and the need for more rapid and sensitive real-time detection of viral agents.

Liposomes and Micelles as Diagnostic Metal Nanoparticle Carriers

Liposomes and micelles, described previously in Chapter 1, have received much attention over the past ten years as *in vivo* carriers for nanoparticles due to their good pharmacological characteristics, low toxicity and ease of synthesis. Given their unique physiological makeup, liposomes may incorporate contrast agents in either the internal aqueous core or the outer hydrophobic membrane itself. For gamma irradiation or magnetic resonance, liposomes containing contrast nanoparticles, often in the form of metals, are prepared by either of two methods. The metal may be chelated into a soluble chelate such as diethylenetriamine pentaacetate (DTPA) and subsequently included in the interior of the liposome. Alternatively, DTPA or another compound which exhibits similar chelating effects may be chemically derived through the incorporation of a hydrophobic group which would act as an anchor for the chelating moiety on the liposome surface, either during or after liposomal synthesis. Different chelators and different hydrophobic core compositions must be used for the various types of metal nanoparticles to be incorporated in the liposomes including ^{111}In, ^{99m}Tc, Mn and Gd nanoparticles. With respect to magnetic resonance imaging, to increase signal intensity it is recommended that all reporter atoms are freely exposed for interaction with water. Thus membranotropic chelating agents that consist of a paramagnetic atom incorporated into the polar head of the liposomal molecule are most ideal for this application.

In micelles a hydrophilic chelate can be localized on the hydrophilic shell of the micellular structure while the lipid portion of the molecule can be anchored within the hydrophobic core. PEG-lipid hybrid micelles have several advantages for diagnostic imaging applications, the most important of which is their small size allowing for high levels to be accumulated at diagnostic sites, especially within sites of tumorigenesis.

Polychelating amphiphilic polymers (**PAPs**) are macromolecules containing both hydrophobic and hydrophilic domains that sequester metal ions at multiple sites on each molecule. In order to increase the contrast agent nanoparticle payload of either liposomes or micelles PAPs have
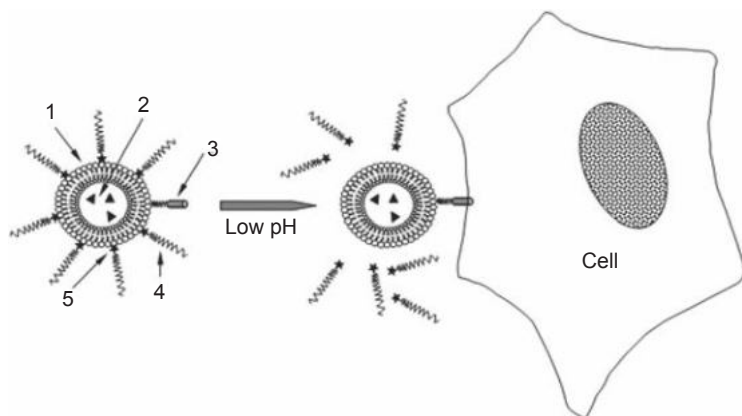
FIGURE 8.23 Diagrammatic illustration of a micelle polychelating polymer complex targeting the surface of a cell. (Courtesy of The AAPS Journal; reprinted with permission.)

been synthesized which consist of a main chain with multiple chelating groups attached. These groups allow for the binding of many contrast agent nanoparticles and the inclusion of a hydrophobic terminal group that enables polymer adsorption onto hydrophobic nanoparticles. These groups also allow for incorporation of the hydrophobic nanoparticles into the hydrophobic cores of liposomes and micelles (Torchilin *et al.*, 2007 and Figure 8.23).

There are numerous examples of liposome- and micelle-based encapsulation of metal nanoparticle contrast agents for diagnostic applications based on the above synthesis methodologies. As early as 1984, researchers were attempting to target radioactively labeled, negatively charged liposomes for imaging of the lymphatic system. Richard Vaughan-Jones' group in the Department of Biochemistry at Charing Cross Hospital in London subcutaneously injected 99 mTc and ^{125}I-labeled polyvinylpyrrolidone liposomes into rats as contrast agents for **lymphoscintigraphy**, which is defined as a diagnostic technique in which a two-dimensional picture of the lymphatic system is produced through the detection of radiation emitted by a radioactive substance administered into the body (Patel *et al.*, 1984). Vladimir Torchilin is one of the pioneers in this field, having created amphiphilic biocompatible PEO-based micelles of 10–50 nm in diameter that incorporate indium-111 (^{111}In) and

gadolinium chelates as a nanoparticulate contrast agent for percutaneous lymphography (Trubetskoy *et al.*, 1996). Other studies dating back as early as 1989 were focused on the use of liposomes loaded with chelated paramagnetic nanoparticles including Gd, Dy, Mn, Fe to visualize macrophage-rich tissues in rats via magnetic resonance contrast. Evan C. Unger and colleagues in the Department of Radiology at Fox Chase Cancer Center in Philadelphia, Pennsylvania used liposomes encapsulating gadolinium diethylene-triaminepentaacetic acid (DTPA) as a contrast agent for magnetic resonance imaging of hepatic metastases in rats. In these studies, egg phosphatidylcholine cholesterol liposomal vesicles, ~70 nm in diameter, were prepared by standard freeze-thaw extrusion methods at a molar ratio of 6:4 with Gd-DTPA. Transformed C5 rat liver epithelial cells were introduced into the superficial lobe of Fisher rat livers and allowed to grow to a certain size followed by tail vein injections of liposome contrast agent. Magnetic resonance imaging following injection revealed a significant increase in signal intensity and sustained vascular enhancement using liposomal formulations as opposed to free Gd-DTPA (Unger *et al.*, 1989 and Figure 8.24). The researchers speculated that this superior contrast agent performance was due to preferential targeting of liposomes to the liver and spleen due to their natural accumulation in Kupffer cells and hepatocytes, which are the normal cells of these systems. Thus the liposome-based system provides normal tissue contrast to tumorigenic tissues.

More recently, in order to generate Gd-liposome complexes with increased signal intensity, researchers at the University of Texas Health Science Center in Houston have created dual Gd-labeled immunoliposomes in which gadolinium was attached to the surface of the liposome as well as integrated internally within the core (Figure 8.25).

The goal of these studies was to increase the **spin-lattice relaxation time, T1**, which is a time constant that characterizes the rate at which the longitudinal, Mz, component of magnetization recovers. It is related to signal intensity when magnetic resonance imaging is applied and affects both detection and anatomic demarcation during the imaging process. Higher T1 relaxivity correlates with improved imaging of small features by increasing signal intensity, especially within the vascular space. In these studies, dual Gd liposomal contrast agents were synthesized under vacuum, followed by hydration, extrusion and filtration to remove unencapsulated Gd chelate molecules. A plot of relaxation rates to liposome concentration
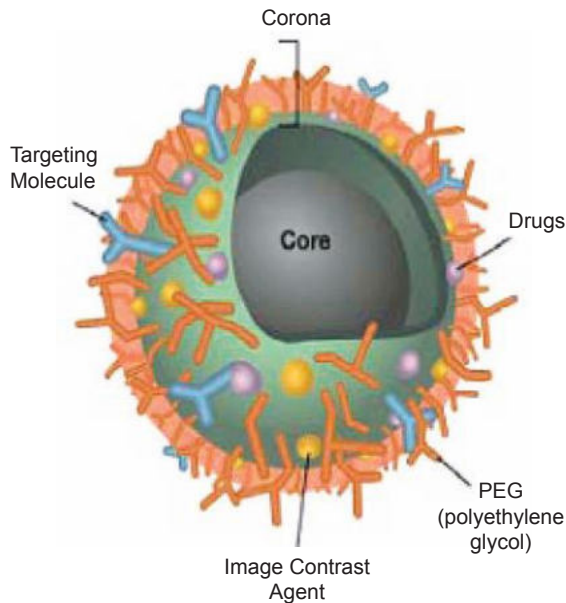
FIGURE 8.24 Illustration of a magnetic liposome. (Courtesy of the National Research Council of Canada; reprinted with permission.)
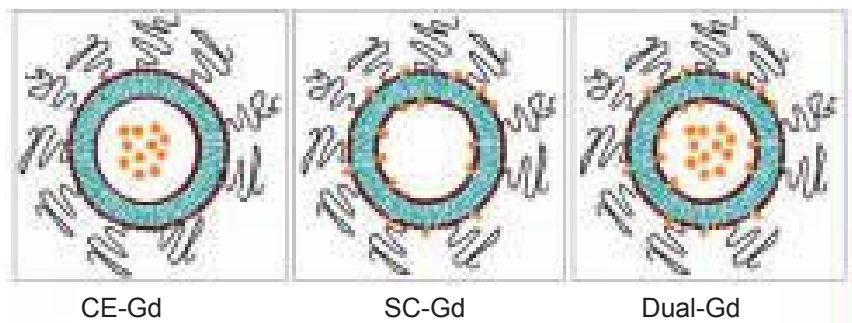
FIGURE 8.25 Schematic of various types of liposomal-Gd agents for imaging. Core-encapsulated gadolinium (CE-Gd) liposomes contain conventional low molecular-weight Gd-chelates in the core interior of the liposomes, surface-conjugate gadolinium (SC-Gd) liposomes contain Gd-chelates conjugated on the internal and external surface of the liposome bilayer. Dual-Gd liposomes contain both core-encapsulated and surface-conjugated Gd-chelates. The stars represent Gd-chelates. (Courtesy of Ghaghada *et al.*, 2009; reprinted with permission.)
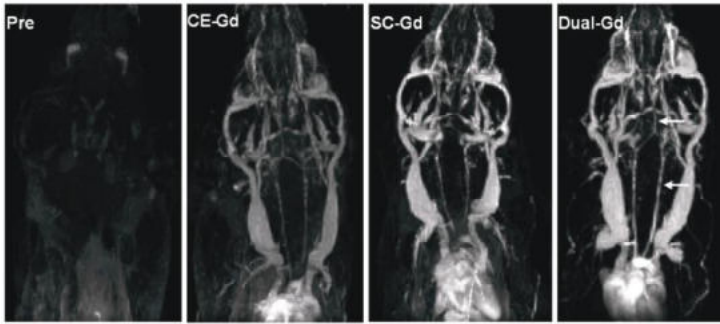
FIGURE 8.26 *In vivo* comparison of liposomal-Gd contrast agents. Coronal maximum intensity projection (MIP) images of the head and thorax in mice acquired pre-contrast, post CE-Gd liposomes, post SC-Gd liposomes and post Dual-Gd liposomes were analyzed. The contrast agents were administered intravenously. Note the increased signal in the vessels compared to background and the high vessel conspicuity for smaller vessels (arrows in the Dual-Gd image). All images were acquired in different animals using the 3D-FSPGR sequence. The MIP images are presented at identical gray-scale levels. (Courtesy of Ghaghada *et al.*, 2009; reprinted with permission.)

yielded a straight line with the slope defined as the nanoparticle-based T1 relaxivity, with dual complexes yielding the highest T1 values. When these Gd-liposomal complexes were used as *in vivo* MRI-based contrast agents in mice the dual system also gave the highest signal intensity and strongest anatomical visualization (Ghaghada *et al.*, 2009 and Figure 8.26).

Liposome-based contrast agents have also been delivered to the heart for imaging of the vascular endothelium as well as monitoring of drug and gene delivery efficacy. Katherine Ferrara's group in the Department of Biomedical Engineering at the University of California in Davis combined radioactive/fluorescently labeled liposomes with positron emission tomography (PET) to specifically image vascular target regions surrounding the hearts of FVB mice. In this study, the short linear peptide CRPPR, which has been shown to specifically bind to target cells present within the heart endothelium, was coupled to PEG via **pegylation** to create lipo-PEG-peptides (LPPs). Pegylation is defined as the process of covalent attachment of polyethylene glycol polymer to another molecule. This was followed by mixture with either ^{18}F FDP or the fluorescent dye Alexa 555® and extrusion to result in the final target-specific liposomal nanoparticles.

These formulations were introduced into mice by catheterization procedures to ensure the administration of proper amounts and PET imaging performed. Targeted accumulation of the radioactive version of the LPPs was demonstrated in the endothelium of the heart. A startling 44% of the injected LPPs accumulated in the cardiac endothelium and was calculated to be over 30-fold greater in concentration compared to that in the skeletal muscle.

As discussed in Chapter 4, drug delivery to the brain is especially challenging due to the existence of the nearly impermeable blood-brain barrier (BBB). Thus the ability not only to deliver drugs to specific sites within the brain but also to monitor delivery efficiency is a major focus of clinical neuroscience. Florence Gazeau and colleagues at the Laboratoire Matiere et Systemes Complexes, Universite Paris have developed a unique **magnetic fluid-loaded liposomal (MFL)** delivery and diagnostics system based on the use of a magnetic field to target nanoparticles to specific regions of the brain. In this system, monodisperse maghemite anionic nanocrystals with an average diameter of 8 nm were combined with liposomes labeled with the fluorescent dye rhodamine to generate MFLs with a high load of internal magnetic fluid. The solution was further concentrated by magnetic separation to yield final MFLs with a high iron load, estimated at 1%–4%, corresponding to 60–300 magnetic nanoparticles per liposome. MFLs were injected intravenously into C57BL/6 mice and tracked via laser-scanning confocal fluorescence microscopy (LSCFM) through a closed cranial window implanted above the left parieto-occipital cortex. MFLs were targeted to the cortical region of the brain by placing a magnet on the cranial window and it was demonstrated that significant MFL accumulation occurred in the brain microvasculature exposed to the magnet. While it is clear that a cranial window is not viable for tracking drug targeting and diagnostics in humans, Gazeau has demonstrated the basic proof-of-concept behind a unique magnetically based targeting system that may be refined for both drug delivery and diagnostics.

CHAPTER SUMMARY

Nanodiagnostics Technologies and Applications

1. Many types of nanotechnologies are now being explored for use in the diagnostics medical field.
2. There are two categories of diagnostics: *in vitro* and *in vivo*.

3. Nanotechnology applied to chip diagnostics enables higher precision while reducing costs due to less testing specimen and reagents/instrumentation needed.

4. Microarrays are employed to identify markers of disease such as antigens or even genetic mutations.

5. Nanofluidics can allow for the efficient and precise isolation and analysis of individual molecules.

6. Fluid flow in nano-capillaries is controlled by both pore size and Debye Length.

7. Most nanofluidics systems use cylindrical channels or nanoslits and are fabricated by photolithographic etching.

8. Nanotubes can be fabricated by literally pulling them from polymersomes with the use of optical tweezers.

9. A translocation event signal results from the passage of a molecule through an electrically insulated nanopore.

10. Nanopores may represent the core technology behind next-generation DNA sequencers.

11. Nanobiosensors may be optical, electrical, chemical and/or electrochemical in nature.

12. Nanowires coated with sensing molecules may be implemented as components in a nanobiosensing device.

13. Nanotree enzyme reactors used in RISFETs have been demonstrated to allow for the sensitive measurement of enzymatic activity.

14. BioNEMS combines both biological and electromechanical components for use in biological applications such as those pertaining to diagnostics and may one day replace PCR.

15. Cellular lipid membranes harbor many types of proteins that may be exploited for ion-based signal transduction based on the Donnan Potential.

16. Bionanosensor chips have been developed for the non-invasive *in vitro* diagnosis of oral cancer.

17. Cytomorphometry may be used to diagnose a patient's risk for, or the presence of, certain diseases.

18. Cantilever biosensor-based systems convert an object's three-dimensional properties to hard data uniquely characteristic of that object.

19. The primary advantages of using cantilever biosensors are that they provide fast, label-free detection of biomolecules.

20. Nanocantilevers are being studied for the detection of ultrasmall pathogens such as viruses.

21. Nanolaser scanning confocal spectroscopy provides accurate, real-time high-throughput screening of samples without invasive chemical agents.

22. Differences in mitochondrial energy metabolism between those of cancerous and normal cells can now be measured with precision by nanolaser scanning confocal spectroscopy.

23. Nanoproteomics allows for the detection of a single molecule of protein in a sample.

24. A combination of mass spectroscopy with chemically functionalized gold nanoparticles has allowed for the identification of individual proteins present in whole cell lysate extracts.

25. The phenomenon of surface plasmon resonance (SPR) is routinely exploited in optical nanobiosensing.

26. Surface-enhanced Raman scattering is a process which results in the enhancement of photon scattering effects and unique optically detectable tags can be formed by SERS.

27. SERS tagging provides improved qualitative sensitivity over fluorescence-based tagging techniques and quantification based on SERS tags may also be undertaken.

28. Molecular sentinels have been created using SERS technology and can allow for the detection of rare gene sequences such as those viral in nature.

In vivo Nanodiagnostics

1. Targeted gold nanoparticles can be coupled with computed tomography (CT) to enable molecular *in vivo* imaging of specific cell types.

2. Gold nanoshells (GNS) can be used as *in vivo* contrast agents for near infrared light applications.

3. Tuning of GNS cores to the outer shell allows for an enhancement of sensitivity to detection by light in the near IR spectrum.

4. Golden carbon nanotubes (GNTs) address the low absorption and toxicity concerns of naked CNTs and may act as *in vivo* contrast agents

for either photothermal (PT) or photoacoustic (PA) visualization of lymphatic vessels.

5. Superparamagnetic iron oxide (SPIO) nanoparticles combined with magnetic resonance imaging (MRI) in cancer are considered to be superior to other markers due to improved delineation of tumor margins.

6. Magnetic nanoparticles can be modified to enhance signal intensity through metal doping.

7. Stem cells may be tracked in the body using superparamagnetic (SPIO) nanoparticle-peptide complexes that target specific receptors on the stem cell surface.

8. Using SPIO nanoparticle-peptide complexes for tracking stem cells allows for MRI imaging as the single cell level *in vivo*.

9. Perfluorocarbons may be detected by a variety of methods *in vivo* but the most popular are ultrasound and magnetic resonance imaging.

10. Knowing the correct concentration of PFCs prior to administration is crucial to their proper usage in diagnostics applications.

11. Quantum dots have been used to detect RSV *in vitro* and *in vivo*, yet their potential for toxicity remains an issue.

12. Contrast agents for diagnostics may be incorporated into the internal core or outer hydrophobic membrane of liposomes.

13. Chelators must be used to efficiently incorporate certain metals into liposomes.

14. Hydrophilic metal chelates can be incorporated on the hydrophilic shell of micelles.

15. Polychelating amphiphilic polymers (PAPs) have been used to increase contrast agent nanoparticle payload as they are designed to allow for the binding of many contrast agent molecules.

16. Both radioactive and magnetic contrast agents have been successfully delivered *in vivo* using liposomes and micelles.

17. Increasing the spin-lattice relaxation time, T1, of gadolinium increases magnetization signal intensity.

18. Liposome-based contrast agents have successfully been delivered to the heart and brain and shown to be effective contrast agents in these organs.

KEY TERMS

- Nanodiagnostics
- Nanobiochip
- Microarray
- Nanofluidics
- Debye Length
- Polymersome
- Nanopore
- Translocation Event Signal
- Nanobiosensor
- Nanotree
- BioNEMS
- Molecular Beam Epitaxy
- Donnan Potential
- Cytomorphometry
- Resonant Frequency
- Laser Doppler Vibrometer (LDV)
- Nanolaser Scanning Confocal Spectroscopy
- Mitochondria
- Structure Factor
- Biocavity Laser Chip
- Nanoproteomics
- Quantitative Nanoproteomics (QNanoPX)
- Estrogen Response Element (ERE)
- Micrometastases
- Surface Plasmons
- Surface Plasmon Resonance (SPR)
- Surface-Enhanced Raman Scattering (SERS)
- Raman Shift
- Molecular Sentinel (MS)
- PCR Amplicon
- Computed Tomography (CT)
- Golden Carbon Nanotube (GNT)
- Superparamagnetic Iron Oxide (SPIO) Nanoparticle
- Magnetism-Engineered Iron Oxide (MEIO) Nanoparticle
- Polychelating Amphiphilic Polymer (PAP)
- Lymphoscintigraphy
- Spin-Lattice Relaxation Time, T1
- Pegylation
- Magnetic Fluid-loaded Liposome (MFL)

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. List at least five types of nanotechnologies currently explored for use in nanodiagnostics applications.
2. Write the formula for the distribution of the velocity of a liquid, $v_s(r)$, as it applies to nanofluidics.
3. How are detections made using nanopores in nanofluidics?

4. List at least five types of nanobiosensors and describe their use in diagnostics.
5. Write the equation for the Donnan Potential, $\Delta\sigma$.
6. What are the six cytomorphometric parameters for which significant changes were observed in oral squamous cell carcinoma by John McDevitt's bionanosensor system?
7. What are the advantages of using a cantilever-based detection system over fluorescence?
8. How does Robert Naviaux's diagnostics system detect cancerous cells?
9. Write the equation for the structure factor, $S(q)$.
10. Describe the basics behind Shu-Hui Chen's QNanoPX diagnostics system and draw a schematic supporting this description.
11. What are the advantages of SERS vs. fluorescence signals in diagnostics?
12. Diagram Tuan Vo-Dinh's molecular sentinel system for SERS-based detection of gene sequences.
13. Why are gold nanoparticles considered good contrast agents for computed tomography?
14. List two reasons why coating carbon nanotubes with gold is advantageous for using CNTs in diagnostics applications.
15. Why are SPIO nanoparticles considered to be superior contrast agents to more conventional chelates such as dadolinium?
16. How did Jin-Suck Suh's group address the issue of magnetic nanoparticle signal enhancement limitations?
17. What are the most common detection methods for use of perfluorocarbons in *in vivo* diagnostics?
18. What are the four advantages of using PFC microbubbles over air for ultrasound detection?
19. Write the formula for calculating the concentration of PFC nanoparticles prior to *in vivo* administration.
20. What are the three unique optical properties of quantum dots that may make them good diagnostics agents?
21. How may contrast agents be incorporated into liposomes?
22. Diagram the incorporation of an amphiphilic polychelating polymer into both a liposome and micelle.

This page intentionally left blank

9

Government Influence on Nanotechnology

Worldwide investment in nanotechnology-based research increased more than ten-fold, from $432 million to an estimated $6.4 billion during the six-year period from 1997 to the beginning of 2004, with a larger percentage of this originating from government sources. The vast majority of this funding has been allocated for the implementation of basic research to study and characterize the fundamental physics and science behind nanotechnology in order to gain a firm understanding of its potential in benefitting many aspects of society, most notably medicine. Expectations for translational applications of basic research in nanomedicine are high. The Asia-Pacific Economic Cooperation (APEC) Center for Technology Foresight, for example, predicts the development of usable nanosensors and drug delivery systems within the next several years and the application of advanced medical diagnostics as well as the nanotechnology-based targeting of human cells for organ

repair by 2013. The U.S. National Nanotechnology Initiative (NNI and see below), funded at an expected $1.8 billion USD for fiscal year 2011, expects nanomedicine to produce advanced drug delivery systems, including implantable devices that automatically administer drugs and monitor drug levels to be available for FDA approval in the very near future. Government influence, however, has not only impacted the financial aspect of nanotechnological research but also its application in society through various efforts at oversight and regulation. This chapter outlines some of the more high-profile worldwide government efforts at both funding and regulating the emerging field of nanotechnology and, where possible, a clear distinction is made between nanotechnology in general and nanomedicine in particular.

GOVERNMENT PROMOTION OF ADVANCEMENTS IN NANOMEDICINE

U.S. GOVERNMENT FUNDING AND INITIATIVES

The following section dissects funding and active initiatives implemented by the United States government with respect to advancing the field of nanotechnology in general and nanomedicine in particular.

U.S. Federal Funding for Nanomedicine

As of the publication of this text federal funding for nanomedical research and development is at the highest levels ever, despite economic conditions that favor a reduction in federally funded programs. This section outlines some of the major initiatives undertaken by the U.S. government, both in terms of dollars dedicated as well as action items implemented to promote advances in nanomedicine.

U.S. Presidential Influence

President Bill Clinton was the first U.S. president to formally advocate the need for a strong national effort in nanotechnological research. In an historic speech on the campus of the California Institute of Technology in January of 2000 he announced that his 2001 fiscal budget "will include a

$2.8 billion USD increase in the "Twenty-First Century Research Fund"—investments that will support advances in biomedical research, information technology, nanotechnology, university-based research, and cleaner energy." Three years later President George W. Bush signed the **21st Century Nanotechnology Research and Development Act** (Public Law 108–153). This Act specifically authorized a staggering $3.63 billion USD over four years for nanoscience, nanoengineering and nanotechnology research. It is important to note, however, that these funds were appropriated across multiple agencies in a manner different to that proposed by the original authorization Act.

The Federal Government's National Nanotechnology Initiative (NNI)

In fiscal year 2001 the Federal government started the **National Nanotechnology Initiative (NNI)** to help shape a major research effort at developing new nanotechnology-based tools to improve human health. Cumulative government investment in NNI now totals almost $14 billion USD. It is one of nine initiatives that encompass the National Institutes of Health's Roadmap and is discussed in detail below. The four main goals of the NNI are to:

1. Advance a world-class nanotechnology research and development (R&D) program
2. Foster the transfer of new technologies into products for commercial and public benefit
3. Develop and sustain educational resources, a skilled workforce, and the supporting infrastructure and tools to advance nanotechnology
4. Support responsible development of nanotechnology.

Currently 15 agencies participate in the NNI from the perspective of active research and development, and as mentioned above the total budget for fiscal year 2011 is $1.8 billion USD for this initiative. Much of these funds are allocated to various "Centers and Networks of Excellence" associated with the following Federal departments and institutes:

- Department of Defense (DoD)
- Department of Energy (DoE)
- National Aeronautics and Space Administration (NASA)

- National Institutes of Health (NIH)
- National Institute for Occupational Safety and Health
- National Institute of Standards and Technology (NIST)
- National Science Foundation (NSF)

Some of these are discussed in more detail below, particularly with respect to the NIH given its relevance to nanomedicine.

The U.S. National Institutes of Health (NIH)

Logo courtesy of the National Institutes of Health

In 1999 the trans-NIH Bioengineering Consortium (BECON) officially announced a **Bioengineering Nanotechnology Initiative** to specifically invite grant applications for nanotechnologies useful to biomedicine. That same year the NIH launched a second initiative aimed at enhancing nanoscience and nanotechnology research approaches that have the potential to make valuable contributions to biology and medicine. The Bioengineering Nanotechnology Initiative has continued over the past eight years and provided millions of dollars in research funding support specifically for the study of nanotechnology as it applies to biology and medicine.

In 2005 the NIH officially launched its **Roadmap on Nanomedicine Initiative** by establishing a national network of eight nanomedicine development centers. These centers serve as both the intellectual and technological centerpiece of the nanomedicine roadmap initiative.

They include:
- **The Nanomedicine Center for Mechanobiology Directing the Immune Response** at New York University

- **The Center for Engineering Cellular Control Systems** at the University of California, San Francisco and the University of California, Santa Barbara

Logos courtesy of the National Institutes of Health

- **The National Center for Design of Biomimetic Nanoconductors** at the University of Illinois at Urbana-Champaign

- **The Center for Protein Folding Machinery** at Baylor College of Medicine in Houston, Texas

- **The Nanomedicine Development Center for Optical Control of Biological Function** at the University of California, Berkeley and Lawrence Livermore National Laboratories

- **The Center for Cell Control** at the University of California, Los Angeles

- **The Phi29 DNA-Packaging Motor Center for Nanomedicine** at the Purdue University and the University of Cincinnati

- **The Nanomedicine Center for Nucleoprotein Machines** at Georgia Institute of Technology in Atlanta

These centers have each received significant government funding in the form of grants to support their infrastructure as well as the hands-on research in nanomedicine conducted at each center. Each of these centers is staffed with researchers from a variety of scientific disciplines including biology, computer science, mathematics and medicine. Nanomedicine is

just one of nine major initiatives that encompass the roadmap of the NIH that seeks to both improve and accelerate biomedical research. Currently, the primary objective of each of the nanomedicine centers is to gather extensive information about the chemical and physical properties of biological structures. Some examples of areas of study promoted by the NIH Nanomedicine Roadmap include:

- A study of the molecular events inside cells in real-time, i.e., on the order of milliseconds, microseconds or nanoseconds
- The design of artificial systems for engineering within living cells
- The ensurement of nanodevice biocompatibility
- The development of nanodevices that may reduce the cost of health care

In addition to the basic research goals of the initial roadmap, a second phase has recently been approved that will focus more on translational research initiatives. The acquired fundamental knowledge and developed tools from the original plan will be applied to understanding and treating disease. In this phase the centers will continue to expand knowledge of the basic science of nanostructures in living cells and will gain the capability to engineer biological nanostructures. Finally, these centers will be expected to apply gleaned knowledge, capabilities and developed devices to focus on specific target diseases.

Logo courtesy the of United States Federal Government

The **American Recovery and Reinvestment Act (ARRA)**, otherwise known as the Stimulus Package, was formulated in 2009 as a direct response to an economic crises and a way to kick-start the economy during a time of severe economic recession by:

- Creating new jobs and saving existing ones
- Spurring economic activity and investing in long-term growth
- Fostering unprecedented levels of accountability and transparency in government spending

$10.4 billion USD of the total $787 billion USD package was directed as a two-year infusion into the NIH specifically to be used for "empowering the nation's best scientists to discover new cures, advance technology, and solve some of our greatest health challenges." More than 150 projects have now been funded by the ARRA from that pool of capital that focus on nanomedicine-related initiatives. This subset of funding is intended to advance the development of nanomedicine-based medical treatments as well as address issues such as ethics and risk. Examples of projects funded are listed in Table 9.1.

Table 9.1 American Recovery and Reinvestment Act funded nanomedicine initiatives (Source: National Institutes of Health)

Research Topic	Investigator(s) and Institution
Nanoparticle Standardization	Junghae Suh, Rice University
Nanopore Development	Mark Akeson and David Deamer, the University of California, Santa Clara
Building Research and Ethics Oversight	Susan M. Wolf, the University of Minnesota
Research on the Health and Safety of Nanomaterials	James Christopher Bonner, North Carolina State University, Raleigh
Targeting Tumors with Nanoparticles	Shuming Nie, Emory University
Nanoparticle Tracking of Stem Cells	Emerson Perin, Texas Heart Institute

While the ARRA is a temporary act designed to drive both job creation and spur economic recovery, the infusion of cash into nanomedicine-focused research as a result of this program will play a major role in driving advances in this area.

The U.S. National Institute of Standards and Technology (NIST)

Logo courtesy of the National Institute of Standards and Technology

The U.S. National Institute of Standards and Technology (NIST) has long been a proponent of nanotechnology research. In recent years it has issued a number of financial awards for research in nanomedicine to scientists and institutions in both academia and industry. Some examples of funded projects include the following:

- Precision microassembly for the development of nanomaterial composites for biomedical implants and 3D Microsystems for miniature instrumentation
- Development of a DNA sequencing platform for obtaining the first entire human genome sequence for $100 USD
- Development of a MEMS-based portable drug infusion system
- Development of targeted fluorescent nanoparticles for early cancer detection

In addition, NIST has its own active research programs in nanomedicine and NIST scientists have made significant discoveries directly impacting the advancement of nanomedical technologies. A prime example of this is the research of Limin Sun and Laurence Chow of NIST's Paffenbarger Research Center in Gaithersburg, Maryland. Drs. Sun and Chow developed a calcium fluoride (CaF_2) nanoparticle composite as an effective anticaries agent that promotes tooth remineralization (Sun *et al.*, 2008 and Figure 9.1).
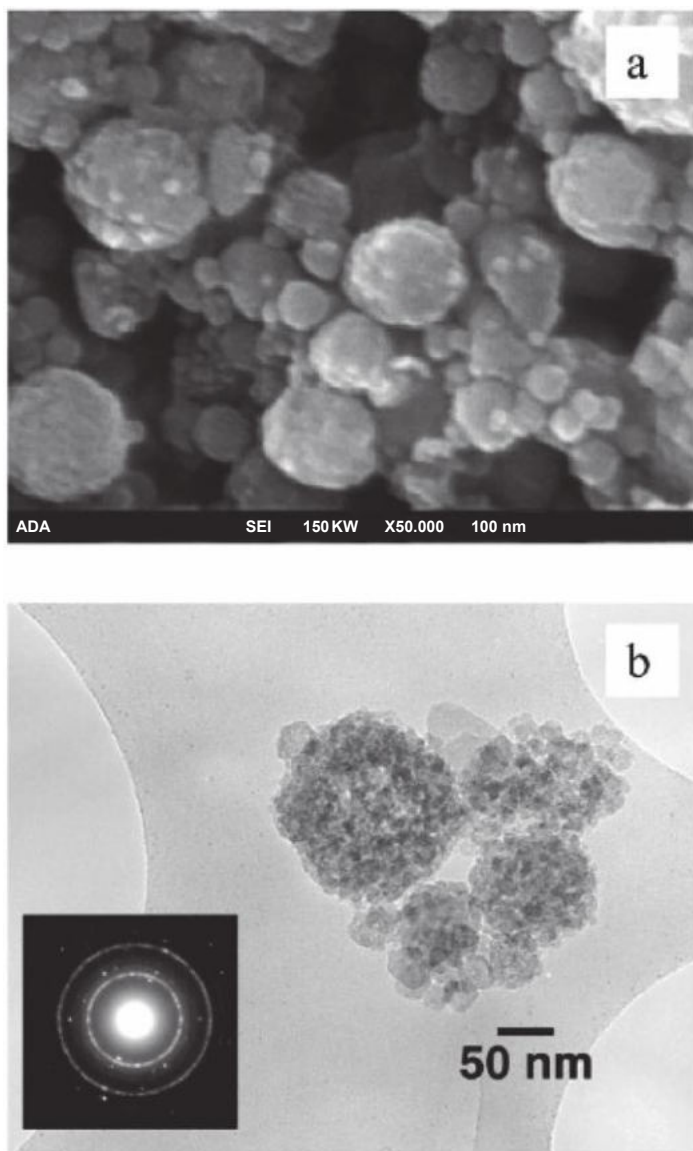
FIGURE 9.1 Calcium fluoride nanoparticle conglomerates for the promotion of tooth remineralization. SEM (a) and TEM (b) of nano CaF_2 showing conglomerates consisting of particles of about 10–15 nm in size. (Courtesy of Sun *et al.*, 2008; reprinted with permission.)

The U.S. National Science Foundation (NSF)

Logo courtesy of the National Science Foundation

The National Science Foundation (NSF) has dramatically increased the number of awards issued for research in nanotechnology with a considerable percentage of these awards directed in the sub-field of nanomedicine. Correspondingly, the number of publications in this area has increased along with the availability of these funds (Figure 9.2).
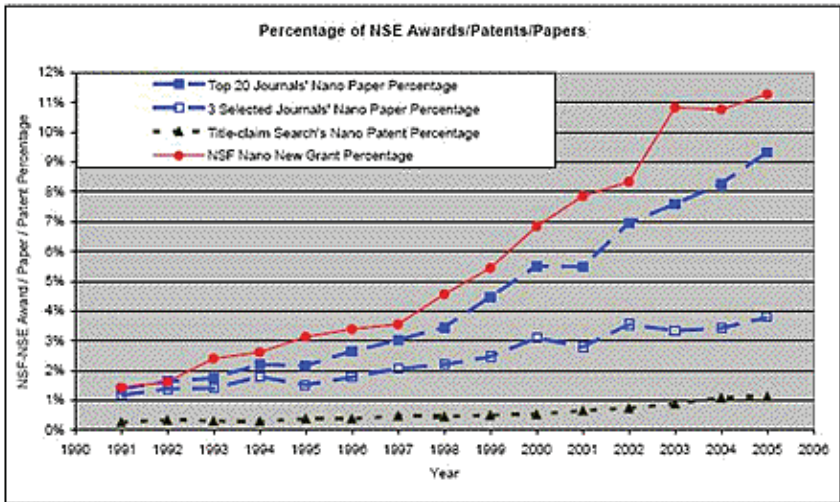
FIGURE 9.2 Trends in the growth of nanotechnology research at the National Science Foundation (NSF). (Courtesy of ISPE Boston; reprinted with permission.)

The U.S. National Cancer Institute (NCI) Alliance for Nanotechnology in Cancer

Logo courtesy of the National Cancer Institute

Championed by Dr. Mauro Ferrari (see Focus Box 9.1), a 5-year initiative focused on the application of nanotechnology to research and treat cancer was introduced in 2004 by the U.S. National Cancer Institute (NCI), a division of the NIH. The $144 million USD program was designed to coalesce scientists and clinicians for the translation of nanotechnology-based research in cancer to aid the patient. It was also designed to bring together both public and private organizations in the form of a broad-based alliance of multiple institutions across the United States. The mission of the **NCI Alliance for Nanotechnology in Cancer** is "to accelerate the application of the best capabilities of nanotechnology to cancer." The Alliance's goals are to develop:

- Research tools to identify new biological targets
- Agents to monitor predictive molecular changes and prevent precancerous cells from becoming malignant
- Imaging agents and diagnostics to detect cancer in the earliest, most easily treatable, pre-symptomatic stage
- Multifunctional targeted devices to deliver multiple therapeutic agents directly to cancer cells
- Systems to provide real-time assessments of therapeutic and surgical efficacy
- Novel methods to manage symptoms that reduce quality of life

To date nine Centers of Cancer Nanotechnology Excellence (CCNEs) have been formed and are located at the following universities.

- **The University of California, San Diego**
- **Stanford University**, Stanford, California
- **California Institute of Technology,** Pasadena, California
- **The University of North Carolina,** Chapel Hill, North Carolina

- **Massachusetts Institute of Technology,** Cambridge, Massachusetts
- **Northwestern University,** Chicago, Illinois
- **Georgia Institute of Technology and Emory University,** Atlanta, Georgia
- **Harvard University, Massachusetts General Hospital,** Cambridge, Massachusetts
- **Washington University,** St. Louis, Missouri

In addition, the NCI Alliance for Nanotechnology in Cancer has driven the formation of twelve Cancer Nanotechnology Platform Partnerships (CNPPs) that engage in directed, product-focused research that "aim to translate cutting-edge science and technology into the next generation of diagnostic and therapeutic tools." These CNPPs focus on six primary key nanomedical platform technologies which include:

- Molecular imaging and early detection
- *In vivo* nanotechnology imaging systems
- Reporters of efficacy
- Multi-functional therapeutics
- Prevention and control
- Research enablers

U.S. Department of Defense (DOD) Defense Advanced Research Projects Agency (DARPA)

Logo courtesy of the Defense Advanced Research Projects Agency

The U.S. Defense Advanced Research Projects Agency (DARPA) is a branch of the U.S. military created in 1958 to fill a need for the formulation and execution of research and development projects that would expand the frontiers of technology beyond the immediate and specific requirements of the military services and their laboratories. Within recent years this mission has come to include the development and application of new

Table 9.2 Examples of DARPA-funded nanomedical research projects

Research Focus	Awardee and/or Institution	Amount (USD)	Year
Development of implantable, wireless peripheral nerve interface with nanocomposite surface for neural control of advanced arm prosthetics	Gareth Hughes of Zyvex Corporation	$3.5 million	2005
Development of nanoscale, low-power synapse like devices	IBM	$4.9 million	2008
Development of nanoparticle-based targeted breast cancer therapies	Mauro Ferrari of the University of Texas Health Science Center at Houston	$7 million Innovators Award	2009

nanotechnologies that may aid the military on the battlefield. This mission has since expanded to cover research and development in nanomedicine to address future cutting-edge methods of treating soldiers and other military personnel in need of medical care with, of course, wider implications for the treatment of the broader U.S. population. Some examples of DARPA-funded awards are listed in Table 9.2.

U.S. State Funding and Initiatives

While the vast majority of funding and support for nanomedicine-related research and development comes at the federal level, there are some examples of state funding and initiatives worthy of mention. These are primarily tailored to promote collaborative and collegial interactions among leaders in the field within the states as well as to foster education at the local level of the next generation of nanomedical scientists and doctors.

Missouri

In 2009 the Missouri Life Sciences Research Fund, created as part of the controversial 1998 tobacco settlement, has provided $1.5 million USD in funding for the establishment of the **St. Louis Institute of Nanomedicine**

Logo courtesy of Missouri Life Sciences Research Board

Working Group. This is a collaborative regional effort to apply advances in nanotechnology for the treatment of human diseases. This Institute's mission is to bring together the skills and expertise from other local institutions and promote joint research projects to create novel nanomedicine-based solutions for complex healthcare problems. The Institute's three primary areas of focus are:

- The development and evaluation of new nanotechnologies for health care
- The facilitation of commercialization and testing in patients
- The education of a new workforce and the public at large

Regional research efforts in nanotechnology supported by the Institute include the development of synthetic ion channels that function as antibiotics, nanocapsules for targeted drug delivery, nanoparticles for imaging diseased sites within the body and nanosensors imaging technology to study live cancer cells.

New York

The **Alliance for Nanomedical Technologies (ANMT)** was established in 2001 as a partnership between Cornell University, the University of

Logos courtesy of the Alliance for Nanomedical Technologies and Cornell University
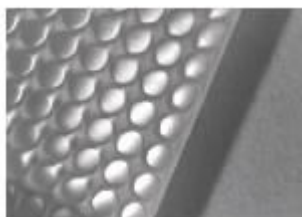
FIGURE 9.3 Replicated microlens array created by soft lithography. (Courtesy of Kunnavakkam *et al.*, 2002; reprinted with permission.)

Rochester, the Wadsworth Center and Tompkins Courtland Community College to help nucleate and invest in nanomedical research programs in the state of New York. In 2004 the New York State Office of Science, Technology and Academic Research (NYSTAR) awarded $2.8 million USD to the ANMT for the development of biomedical nanodevices. The mission of the Alliance for Nanomedical Technologies is to bring together academia and private industry to develop the next generation of medical devices. It is the Alliance's goal to exploit the interface between engineering and biology while harnessing microfabrication techniques to build integrated devices for biomedical research and diagnosing disease. Through this initiative the Alliance will create **nanoBioFab**, a state-of-the-art fabrication facility that will be built specifically for handling biomaterials. An example of research conducted at the ANMT towards the fabrication of medical nanoprecise medical devices is the creation of low-cost, low-loss microlens arrays by soft lithography spearheaded by Madanagopal V. Kunnavakkam (Kunnavakkam *et al.*, 2002 and Figure 9.3).

Texas

The **Alliance for Nanohealth (ANH)** is the first Texas-based collaborative research endeavor that uses nanotechnology to bridge gaps between

Logos courtesy of the Texas Alliance for Nanohealth and the Texas Emerging Technology Fund

medicine, biology, materials science, computer technology and public policy. The ANH's mission is to "develop nanotechnology solutions to address unresolved problems in medicine." Founded in 2005, the ANH has received a total of over $16 million USD in funding from both the state of Texas and the federal government to support its mission and goals. $2.5 million of this funding was from the Texas Emerging Technology Fund (TETF) Subchapter specifically for the recruitment of Dr. Mauro Ferrari (see Focus Box 9.1) to the Texas Medical Center located in Houston, Texas. Other funds have been specifically utilized for:

- Infrastructure development & equipment
- Seminars, workshops, & conferences
- Undergraduate student summer internships with ANH institutions
- Graduate student scholarships to attend nanomedicine workshops abroad
- Fellowships for both new and more experienced post-doctoral researchers
- Seed grant funding for high-risk collaborative research projects
- Planning grants intended to prepare multi-institutional teams for future Center of Excellence funding opportunities
- Recruitment of world-class nano-bio researchers

Focus Box 9.1 Mauro Ferrari and next-generation nanomedicine

Mauro Ferrari is a pioneer in nanomedicine. His research spans multi-stage drug delivery systems, implantable therapeutic devices, nanochips for early detection of cancer, and nanoscaffolds to treat bone fractures. Nanofluidics started with his nanochannels for cell transplants. He has received numerous awards, including the NSF's National Young Investigator Award, the Walter H. Coulter Award, and the DoD's Innovator Award in Breast Cancer. He was the founding Chairman of the Department of Nanomedicine and Biomedical Engineering at the University of Texas Health Science Center, Houston, which was the first department of nanomedicine in a medical school. He is now President and CEO of The Methodist Hospital Research Institute. (Photo courtesy of Nature; reprinted with permission.)

FIGURE 9.4 Researchers at the University of Houston Nanofabrication Facility. (Courtesy of the University of Houston; reprinted with permission.)

The ANH has defined **nanohealth** as understanding and addressing the molecular origins of diseases that originate within a human cell and applying nanotechnology's power to control individual molecules for the detection, diagnosis, and treatment of these debilitating and incurable illnesses. It is the position of the ANH that the barriers between nanotechnology and medical research can only be overcome through the formation of such an alliance. One unique example of the infrastructure created by the ANH is the **University of Houston Nanofabrication Facility**, which is a state-of-the-art cleanroom equipped with an extensive toolset for nano/micro-device prototype development and characterization (Figure 9.4).

In addition to the United States other governments have initiated major funding and research initiatives in the field of nanomedicine. The following sections of this chapter outline these international efforts.

AUSTRALIAN GOVERNMENT FUNDING AND INITIATIVES

In early 2010 the **National Enabling Technologies Strategy** implemented by the Australian government's Department of Innovation, Industry, Science and Research had A\$38.2 million (US\$32.3 million) committed to it as part of the *Powering Ideas—An Innovation Agenda for the 21st Century* program.

Australian Government

**Department of Innovation
Industry, Science and Research**

Logo courtesy of the Australian Government Department of Innovation, Industry, Science and Research

The Strategy is meant to provide funding and a framework for developing enabling technologies, most notably of which is nanotechnology. In addition, the strategy has been formulated to "provide balanced and factual information to support evidence-based policy and regulatory practice, and increase community awareness and understanding of nanotechnology and biotechnology." Also with respect to nanotechnology, the National Measurements Institute (NMI) has received A$18.2 million (US$15.4 million) to develop measurement infrastructure, expertise and standards.

Victorian Government

The government of Australia has long been a strong proponent of nanotechnology research. Across the country estimates are that up to A$100 million (US$82 million) per year have been infused into

Logo courtesy of Nanotechnology Victoria, Ltd

nanotechnology development by federal, state and territorial governments along with private investment. A considerable portion of this is dedicated to research and development in the nanomedical area, particularly with respect to diagnostic and therapeutic strategies using nanoparticles. **Nanotechnology Victoria, Ltd.** is a primary organization created in 2003 that promotes nanotechnological advancements in the Victorian region of Australia. Its goal is to ensure that Victoria benefits from advances in nano- and microtechnology-related sciences by combining the resources and knowledge of both industry and government. Founding consortium member institutes include the following:

- Monash University
- Swinburne University
- Royal Melbourne Institute of Technology University
- Australian Commonwealth Scientific and Research Organization

The consortium has been established specifically to promote three primary areas which include research and development, investment and

commercialization activities. Nanotechnology Victoria has been awarded A$28 million (US$23.1 million) from consortium members and the Victorian government to develop a strategically focused nanotechnology directive and associated infrastructure. On a scientific level, one of the major areas of focus for Nanotechnology Victoria, Ltd. is the development of nanomedical technologies for the early diagnosis of cardiovascular disease, which affects roughly 4 million Australians. Australian research in this area is focused on the development of biocompatible nanospheres loaded with contrast agents that may allow for the detection of diseased blood vessels.

In 2005 the Australian Department of Industry, Tourism and Resources established the **National Nanotechnology Strategy Task Force (NNST)** to compile a formal strategic review and recommendation for the country with respect to nanotechnology. The task force delivered their report appropriately titled "Options for a National Nanotechnology Strategy" in 2006. The main recommendations from the report are listed below.

- The establishment of a dedicated office within a federal department responsible for developing and coordinating the implementation of a National Nanotechnology Strategy
- The coordination of regulatory systems and assessment of whether Australia's current regulatory framework is appropriate in light of nanotechnology are priorities
- A coordinated public awareness and engagement campaign that addresses social and ethical issues
- The establishment at the National Measurement Institute of physical standards and instruments, and the establishment of a laboratory for nanoparticle standards
- A federal/state ministerial forum to coordinate efforts amongst the federal and state's/territories' jurisdictions
- Supporting the access to specialized prototyping, production and fabrication facilities enabling Australian firms to research and develop nanotechnology enhanced products

CANADIAN GOVERNMENT FUNDING AND INITIATIVES

The Canadian Institutes of Health Research (CIHR) launched the **Regenerative Medicine and Nanomedicine Initiative (RMNI)** in 2003 as a way to support the development of new and emerging areas of integrative

biomedical research including the study of stem cells, tissue engineering, rehabilitation sciences and nanomedicine. The CIHR's efforts through the RMNI are focused on local and international partnership formation, an example of which is a partnership with the U.S.-based non-profit Juvenile Diabetes Research Foundation (JDRF). This jointly funded effort combined Canadian and American research capacities to develop strategies for regenerating or repairing insulin-producing cells in the body. Other external partners include:

- ALS Society of Canada
- Canadian Space Agency
- Canadian Stroke Network
- Foundation Fighting Blindness
- Heart and Stroke Foundation of Canada
- Jacob's Ladder
- Neuroscience Canada
- Ontario Neurotrauma Foundation
- Stem Cell Network

Artwork courtesy of Canadian Institutes of Health Research;
reprinted with permission

The RMNI, coupled with other Canadian funding programs such as the National Sciences Engineering Research Council's (NSERC) Nano Innovation Platform Awards, has supported a number of high risk, high reward research endeavors in nanomedicine and allowed for low-cost tuition at major Canadian universities making it possible to recruit a
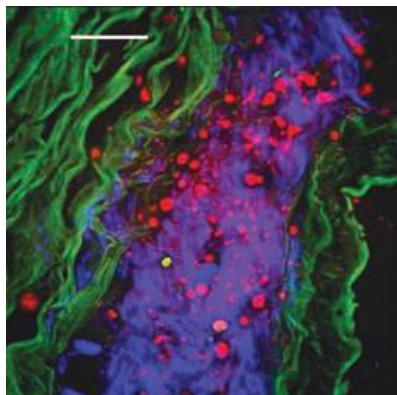
FIGURE 9.5 Image of a live aorta by CARS microscopy. (Courtesy of NRC-Olympus Microscopy Facility; reprinted with permission.)

highly talented pool of young scientists. An example of research funded under the RMNI is that led by Albert Stolow, Principal Researcher with the National Research Council of Canada. He and his colleagues at the NRC have developed a new multimodal microscope module that greatly simplifies laser technology used to capture label-free images of living cells and tissues. It is based on a phenomenon known as Coherent Anti-Stokes Raman Scattering (CARS) (Figure 9.5).

The Alberta Energy Research Institute (AERI) and the Alberta Agricultural Research Institute (AARI) also both support nanomedicine-related projects on a smaller scale. It is evident, however, that while funding is beginning to flow into research laboratories across Canada for the study of nanomedicine, there is still no coordinated Canadian effort to track and champion its cause.

EUROPEAN FUNDING AND INITIATIVES
Nano2Life

In 2004 twenty three public and non-profit research organizations from twelve European Member states and associated states joined forces to

Logo courtesy of Nano2Life

support **Nano2Life** as the first European Network of Excellence to be supported by the 6th Framework Programme. Funding for the four-year term of the program was allocated at EU$13.04 million (USD$16.83 million) with the European Union contributing EU$8.8 million of that amount. The primary goal of Nano2Life is to keep Europe competitive with the United States and Asia in the field of nanobiotechnology with a long-term objective of becoming a leader in nanobiotechnology within 4 years after inception. The five primary objectives Nano2Life strives for in order to accomplish this task are as follows:

- Reducing fragmentation in European nanobiotech
- Interfacing the world of life sciences and nanotechnology
- Making Europe an international leader in nanobiotech
- Translating nanobiotech into economic benefits
- Educating society about nanobiotech

Nano2Life involved more than 200 scientists across Europe with the focus of promoting research on the future trends emerging in nanobiotechnology, education to allow young scientists proper training environments and innovation to promote public/private collaborations across the European Union. Figure 9.6 illustrates an example of research supported by Nano2Life. Under this program Severine Le
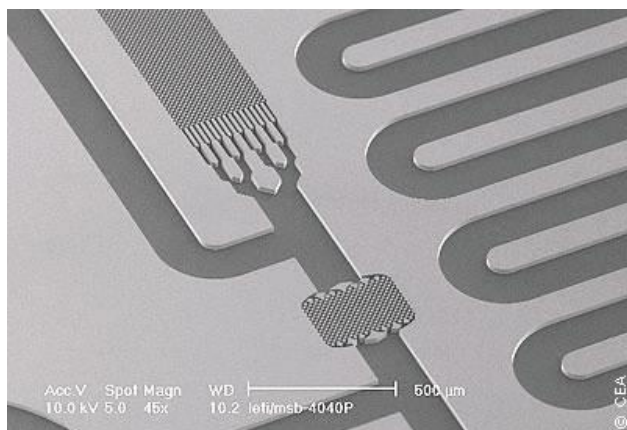
FIGURE 9.6 Concentration module of the BioChipLab© on a chip designed for the presentation of peptidic samples before mass spectrometry analysis. (Courtesy of Nano2Life; reprinted with permission.)

Gac and Rolando Christian at the Laboratory of Organic Chemistry and Biochemistry, University of Science and Technology of Lille, in Villeneuve d'Ascq, France developed a new analytical device dedicated to proteomic studies. Referred to as BioChipLab® on a chip the device was design as a mechanism for the presentation of peptide samples before mass spectrometry analysis.

European Technology Platform (ETP) in Nanomedicine

Difficulties coordinating efforts in nanomedical research at the national level has hindered progress on this front in Europe until several years ago with the European Commission's formation of the **European Technology**

Logos of courtesy the European Union

Platform (ETP) in Nanomedicine in 2005. The 7th Framework Programme announced in 2007, which is the main instrument of research funding at the European level, had nanomedicine as a strategic theme and a collaboration between industry and the European Commission including 53 original stakeholders as participants drove the development of the ETP in Nanomedicine. Membership has now been expanded to more than 150 participants and a strategic research agenda has been formulated to address nanomedical advances through the year 2020. The policy objectives of this program are as follows:

- Establish a clear strategic vision in the area resulting in a Strategic Research Agenda
- Decrease fragmentation in nano-medical research
- Mobilize additional public and private investment
- Identify priority areas
- Boost innovation in nanobiotechnologies for medical use

Achieving these objectives will be the basis behind three key priorities outlined by the ETP in nanomedicine stakeholders. These include nanotechnology-based diagnostics, targeted drug delivery and release and regenerative medicine.

Logo courtesy of the European Union

Another significant program that fosters the promotion of nanomedical research in Europe is the **Euronanomed** ERA (European Research Area)-Net Initiative announced in late 2009. Euronanomed is also supported by the European Commission's 7th Framework Programme (FP7). It is comprised of 24 partners from 18 countries or regions which include the Basque region (Spain), France, Germany, Hungary, Iceland, Israel, Latvia, Lithuania, Poland, Portugal, Romania, Spain, Sweden, Switzerland, Turkey, Walloon Region (Belgium). The goal of this program is to support trans-national collaborations of academia, clinical/public health communities and small to medium-sized companies for nanomedicine-related research and technology development. The priorities and areas of nanomedical technology focus for Euronanomed are similar to that for the ETP in Nanomedicine: regenerative medicine, diagnostics and targeted delivery systems. The current budget of Euronanomed fluctuates between 40 million to 60 million Euros (USD$51 million to 78 million). Much of these funds are distributed across Europe in the form of grants designed to promote strong partnership formation as a minimum of 3 partners from 3 separate countries are required for each proposal to be awarded funding. One example of research funded under the Euronanomed Initiative is that spearheaded by Ling Peng at the Center National de la Recherche Scientifique in Marseille, France. His work focuses on the use of PAMAM dendrimers (see Chapter 4) to delivery siRNA molecules to specific genes for transcriptional inactivation.

SINGAPOREAN GOVERNMENT FUNDING AND INITIATIVES

Singapore has taken its efforts at establishing world-class research endeavors in nanomedicine a step further than most countries by funding a bricks-and-mortar research institute specifically focused on merging the disciplines of biomedicine and nanotechnology. The Singaporean Agency for Science, Technology and Research (A*STAR) was formed in 2002 to foster scientific research and top talent recruitment for the promotion of scientific advancement at the local level. The agency oversees 14 biomedical sciences, physical sciences and engineering research institutes, one of

*Logo courtesy of the A*STAR Institute of Bioengineering and Nanotechnology*

which is the **Institute for Bioengineering and Nanotechnology (IBN)**. The primary goals of the IBN are to:

- Provide international leadership in bioengineering and nanotechnology
- Conduct innovative research and create intellectual properties
- Play an active role in technology transfer
- Foster an exciting multidisciplinary research environment for the training of students and young researchers

Some examples of cutting-edge advances in nanomedicine developed at the IBN include those in drug and gene delivery, cell and tissue engineering and biosensors. An example of this research is that of scientists Yu Han and Jackie Ying at the IBN which resulted in the development of a nanoparticle synthesis technique that allows for the creation of fluorocarbon nanoparticles with tunable pore sizes in the range of 5–30 nm in size (Figure 9.7).
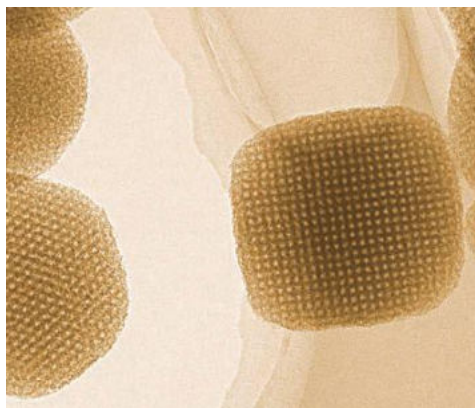
FIGURE 9.7 Fluorocarbon nanoparticles with tunable pore sizes. (Courtesy of Yu Han and Jackie Ying, the Institute for Bioengineering and Nanotechnology; reprinted with permission.)

MEXICAN GOVERNMENT FUNDING AND INITIATIVES

Even developing countries and countries not considered to be at the forefront of medical or nanomedical research have begun to make inroads into establishing the necessary infrastructure to promote advancements in nanomedicine. Mexico, for example, announced in late 2009 it will fund the construction of a world-class biomedical and nanomedical research complex. Referred to as **Campus Biometropolis** (Figure 9.8), it will be a center for medical research and development and will be integrated with the National Autonomous University of Mexico, which is the top university in the world with Spanish as its primary language. This campus will act as a research and development cluster to attract pharmaceutical and biomedical companies and organizations from around the world and is expected to provide a fertile ground for nanomedicine-focused R&D as well as to drive major commercialization initiatives. Campus Biometropolis is a significant component of a five-year general development plan, activated in 2007, aimed at promoting equality through better health, education and technology.

GLOBAL INSTITUTIONAL COLLABORATIONS

Currently there is one primary global initiative aimed at promoting nanomedicine. Referred to as GEM[4] and discussed below, it is the first

FIGURE 9.8 Artist's rendering of Mexico's Campus Biometropolis. (Courtesy of Foster + Partners Designs; reprinted with permission.)

of what is expected to be numerous globally coordinated efforts at advancing the cause of nanomedical R&D to be formulated over the next ten years.

Global Enterprise for Micro-Mechanics and Molecular Medicine (GEM[4])

In 2005 a broad-based global effort was undertaken to launch an international collaboration to promote the development and use of nanotechnology for global health and medical research. The primary

Logo courtesy of GEM[4]

members include the National University of Singapore and the Institut Pasteur of France. The collaboration, referred to as the **Global Enterprise for Micromechanics and Molecular Medicine** (**GEM**[4]), has brought together both researchers and engineering as well as medical professionals across the globe to "address significant problems at the intersections of select topics of engineering, life sciences, technology, medicine and public health." Other participating Core Institutions include:

- **Massachusetts Institute of Technology,** Cambridge, Massachusetts
- **National University of Singapore**
- **The University of Illinois** at Urbana-Champagne
- **California Institute of Technology,** Pasadena, California
- **The University of California**, San Diego
- **Georgia Institute of Technology,** Atlanta, Georgia
- **Harvard University,** Cambridge, Massachusetts
- **The University of Cambridge,** Cambridge, Massachusetts
- **Imperial College,** London, United Kingdom
- **Tohoku University,** Miyagi, Japan
- **Erasmus Medical College,** Rotterdam, Netherlands
- **Centro Investigacion Biomedica en Red—Bioingenieria, Biomateriales y Nanomedica (CIBER-BBN),** Zaragoza, Spain

Although designated as a micro-mechanics initiative, it is clear that nanotechnology in general and nanomedicine in particular is both a part of and has a significant impact on GEM[4]. It is anticipated that GEM[4] researchers will implement the use of tools such as atomic force microscopes and laser tweezers for the study of changes in human cells undergoing a transition to the cancerous phenotype as well as the study of malaria, sickle cell anemia and cardiovascular disease. In addition, GEM[4] has implemented a major initiative at the promotion of education in the area of nanomedicine by providing a series of short courses focused on the interdisciplinary research of cell/molecular biology and engineering (Figure 9.9). It is anticipated that these short courses allow for the training and influencing of a new generation of researchers with a strong fusion of knowledge in engineering and biology that will drive translational
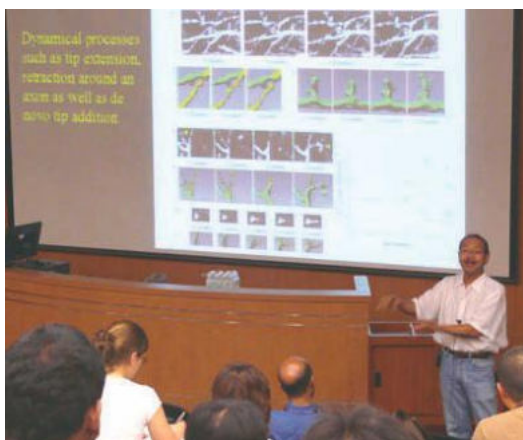
FIGURE 9.9 Lecture by Professor Peter So of Massachusetts Institute of Technology at a GEM[4] summer school course. (Courtesy of the National University of Singapore; reprinted with permission.)

developments in micro- and nanomechanical engineering approaches to medicine.

GOVERNMENT EVALUATION, POLICY AND REGULATION OF NANOTECHNOLOGY

Since the inception of utilizing nanoparticles and nanomaterials as *in vivo* therapeutics platforms a great deal of controversy has surrounded the issue of associated short-term and chronic toxicity. Concerns surrounding detrimental effects of introducing nanoparticles within the body but also into the environment have resulted in considerable efforts to classify the toxic nature of various types of nanoparticles including gold nanoshells, carbon nanotubes, fullerenes, quantum dots and dendrimers just to name a few. This section focuses on the governmental regulatory aspects of nanomedicine while also giving some insight into and examples of government-based evaluation, policy and efforts at regulating other areas of nanotechnology. While some of the more high profile efforts at governmental oversight of nanotechnology and nanomedicine are described, the list is by no means comprehensive and it is highly recommended that the reader consult the comprehensive work of Beth Dunning at Carleton University for further information in this area (Dunning, 2010).

THE UNITED STATES FEDERAL OVERSIGHT

The U.S. Food and Drug Administration Nanotechnology Task Force

In 2006 the United States Food and Drug Administration announced the formation of an internal **Nanotechnology Task Force** to focus on "determining regulatory approaches that encourage the continued development of innovative, safe, and effective FDA-regulated products that use nanotechnology materials." In addition, the Task Force was given the responsibility to identify and recommend ways to address any knowledge or policy gaps that exist so as to better enable the agency to evaluate possible adverse health effects from FDA-regulated products that use nanotechnology-based materials. Interestingly, in its first official report, released in July, 2007, the Task Force reported that the potential use of nanoscale materials includes most product types already regulated by the FDA and that those materials present regulatory challenges similar to other emerging technologies. In addition, the report recommended:

- Consideration of guidance that would clarify what information manufacturers should give FDA about products, and also when the use of nanoscale materials may change the regulatory status of particular products.
- That manufacturers using nanotechnology or nanomaterials contact the FDA early in the product development process. In addition, the report recommends that the agency should assess data needs for regulated nanotechnology products, including biological effects and interactions of nano-particles.

Logos courtesy of the United States Food and Drug Administration

- That the FDA develops in-house expertise and ensures the consideration of new information on nanotechnology as it becomes available. The FDA also should evaluate current testing approaches to assess the safety, effectiveness, and quality of nanoscale materials.

To date the FDA has treated most products made with nanotechnology similarly to the way it handles others. That is, the FDA currently regulates nanotechnology by applying existing regulation. This is similar to the stance the FDA took a number of years ago when formulating regulatory policy around the emerging field of biotechnology. With respect to drugs derived from or containing nanoparticles, this means that companies and researchers involved in the development of those drugs much provide the FDA with safety and efficacy data in a manner identical to that for more classical drugs such as small molecules (which, interestingly, could theoretically be defined as nanotechnology due to their size!). It is important to note that cosmetics and dietary supplements are not subject to FDA oversight and regulation raising the concerns of some regarding the release of untested products in the marketplace which contain or are derived from nanotechnology.

Two issues with the strategy to apply existing regulatory structure to the emerging field of nanotechnology are 1) correctly placing nanotechnology-based products within the regulatory scheme and 2) amassing appropriate regulatory scientific expertise in this area. It will be interesting to note U.S. policy changes and possible additional regulatory oversight of nanotechnology in general and nanomedicine in particular as this field rapidly matures over the next decade.

Logo courtesy of the Nanotechnology Characterization Laboratory

The FDA also plays a major role in promoting the active testing of nanoscale materials. In 2005 the FDA partnered with the National Institute of Standards and Technology (NIST) and the National Cancer Institute (NCI) to form the **Nanotechnology Characterization Laboratory (NCL)** based in Frederick, Maryland. The mission of the NCL is to standardize and perform the pre-clinical characterization of nanomaterials intended
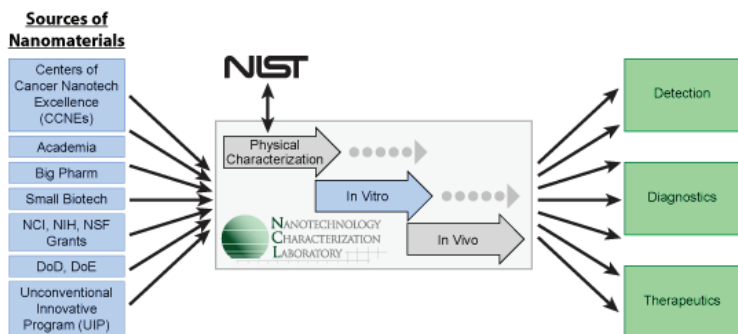
FIGURE 9.10 Flow chart representing the Nanotechnology Characterization Laboratory's role in testing nanomaterials. (Courtesy of the Nanotechnology Characterization Laboratory; reprinted with permission.)

for cancer therapeutics and diagnostics developed by researchers from academia, government, and industry (Figure 9.10).

To accomplish this mission, the NCL has developed the following six objectives:

1. Establish and standardize an analytical cascade for nanomaterial characterization
2. Facilitate the clinical development and regulatory review of nanomaterials for cancer clinical trials
3. Identify and characterize critical parameters related to nanomaterials' absorption, distribution, metabolism, excretion, and toxicity profiles of nanomaterials using animal models
4. Examine the biological and functional characteristics of multi-component/combinatorial aspects of nanoscaled therapeutic, molecular and clinical diagnostics, and detection platforms
5. Engage and facilitate academic and industrial-based knowledge sharing of nanomaterial performance data and behavior resulting from pre-clinical testing (i.e., physical characterization, *in vitro* testing, and *in vivo* pharmaco- and toxicokinetics)
6. Interface with other nanotechnology efforts

All characterization data gleaned at the NCL is presented in the form of technical reports which include the physical characteristics of the nanomaterials as well as *in vitro* and *in vivo* testing results. Examples of

nanomaterials tested at the NCL include ceramide-containing liposomes, functionalized fullerenes and various dendritic nanotechnologies.

Finally, the FDA is actively networked with other government agencies as a member of the Nanoscale Science and Engineering Technology (NSET) Subcommittee of the National Science and Technology Council (NSTC). This allows the FDA to have a direct impact on the coordination of knowledge and U.S. national policy as it pertains to nanotechnology. In early 2010 for the first time the FDA requested funding directed specifically for nanotechnology and nanomedicine-related efforts under the NNI.

The U.S. Environmental Protection Agency

In 2004 the U.S. Environmental Protection Agency (EPA) created a **Nanotechnology Working Group** to examine the potential environmental implications of nanotechnology. This Group issued a white paper two years later which outlined the potential benefits of nanotechnology while emphasizing current challenges inherent in nanotechnology risk assessment. This was followed up the following year through a collaboration with the National Science Foundation (NSF) to award funding for the **Center for Environmental Implications of Nanotechnology (CEINT)**, located on the Duke University campus, to monitor interactions between nanomaterials, the environment, plant and animal life. In late 2008 the EPA issued a notice that carbon nanotubes are considered to be a chemical substance distinct from other carbon-based allotropes such as graphite. This announcement effectively places CNTs in the category of new substances and thus the nanomaterial now falls under different regulatory requirements. Also in 2008 Significant New Use Rules (SNURs) were implemented for certain nanoforms under the Toxic Substances Control Act (TSCA) requiring

Logo courtesy of the U.S. Environmental Protection Agency

persons intending to manufacture, import or process nanomaterials for a defined New Use to notify the EPA at least 90 days in advance. That same year the EPA announced new regulatory policies focused specifically on nanotechnology. The EPA defined nanotechnology as "research and technology development at the atomic, molecular or macromolecular levels, in the length scale of approximately 1–100 nanometer range; the creation and use of structures, devices and systems that have novel properties and functions because of their small size; and the ability to control or manipulate matter on an atomic scale." Yet despite the definition of nanotechnology the EPA appears to be regulating actual nanosubstances in this realm in a nature similar to that for other substances currently covered under the TSCA. According to this legislation the definition of a new chemical substance under the TSCA did not specifically take into account that substance's size. In April of 2010 this policy was changed as EPA announced a new definition of a nanomaterial as "an ingredient that contains particles that have been intentionally produced to have at least one dimension that measures between approximately 1 and 100 nanometers." This new definition will most certainly affect the way nanomaterials are classified and thus how they are regulated by the EPA. As an addendum to this new regulatory effort, the EPA proposed new strict reporting rules for chemical companies who manufacture, process or import carbon nanotubes with the finalization of these rules expected at the end of 2010, thus giving the EPA broad authority to oversee and regulate this specific type of nanomaterial.

The U.S. National Science and Technology Council (NSTC)

The National Science and Technology Council (NSTC) was established by Executive Order in 1993 to organize and coordinate the U.S. government's science and technology policy across the plethora of research and enterprise initiatives of the federal government. It is headed by the President and Director of the Office of Science and Technology Policy. In 2006 the NSTC's **Nanoscale Science, Engineering and Technology Subcommittee**, an interagency body responsible for coordination of the National Nanotechnology Initiative, released at document titled "Environmental, Health and Safety Research Need for Engineered Nanoscale Materials." The purpose of this document was "to identify for the Federal Government environmental, health, and safety (EHS) research and information needs related to understanding

Logo courtesy of the United Stated National Science and Technology Council

and management of potential risks of engineered nanoscale materials that may be used, for example, in commercial or consumer products, medical treatments, environmental applications, and research." The document is now actively used by Federal agencies participating in the National Nanotechnology Initiative (NNI and see above) to both formulate and guide various research programs under this initiative.

The U.S. National Research Council

The United States National Research Council (NRC) was organized almost 100 years ago as a response to increased demand for scientific and technical services caused by the first World War. Its role has expanded vastly over the years and now includes comprehensive reviews of emerging technologies and the federal government's role in their regulation. In late 2008 the NRC released a report titled "Review of the Federal Strategy for Nanotechnology—Related Environmental Health and Safety Research." This report specifically addresses the National Nanotechnology Initiative and paints a critical picture of the Federal government's lack of effort and oversight pertaining to the possible effects of nanotechnology on environmental, health and safety issues. As a direct response to this report and mounting criticism of the federal government, the U.S. House of Representatives issued the National Nanotechnology Initiative Amendments Act of 2009, which contains measures for additional funding and research into the EHS risks of nanotechnology. In early 2010 the U.S. Senate followed the House's lead by issuing the "Nanotechnology Safety Act of 2010" which proposes a program under the guidance of the FDA to assess nanotechnology safety in consumer products and definitively outlines a best-practices action plan for companies using nanotechnology.
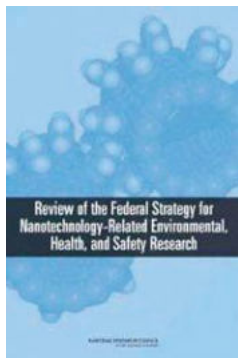
Image courtesy of the National Research Council

The U.S. Patent and Trademark Office

In 2004 the United States Patent and Trademark Office (USPTO) created a new classification for nanotechnology patents. Termed **Class 977**, its purpose is to provide a cross-reference for examiners and others to search prior art. Before the establishment of Class 977, examiners relied solely on keyword searches to find relevant information in patent applications and issued patents. In order for a patent to be classified under the 977 category, it has to meet the following two requirements:

- Relate to research and technology development in the length scale of approximately 1–100 nm in at least one dimension
- Provide a fundamental understanding of phenomena and materials at the nanoscale and create and use structures, devices, and systems that have size-dependent novel properties and functions

Class 977 now allows patent examiners the use of an efficient and streamlined cross-referencing system for scouring nanotechnology-based

Logo courtesy of the United States Patent and Trademark Office

prior art and should speed the process of patent examination and ultimately patent issuance in the field of nanotechnology.

The Project on Emerging Nanotechnologies

In April of 2005 the Woodrow Wilson International Center, which is focused on "fostering research, study, discussion, and collaboration among a full spectrum of individuals concerned with policy and scholarship in national and world affairs," collaborated with the Pew Charitable Trusts to form the **Project on Emerging Nanotechnologies (PEN)**. The Project's mission was to ensure that as nanotechnologies advance, possible risks are minimized, public and consumer engagement remains strong, and the potential benefits of these new technologies are realized. The PEN actively collaborates with researchers, government and industry to identify gaps in knowledge and regulatory processes and to develop strategies (i.e., regulations) for closing those gaps.

The Project on
Emerging Nanotechnologies
at the Woodrow Wilson International Center for Scholars

Logo courtesy of the Woodrow Wilson International Center

STATE AND LOCAL OVERSIGHT

Over the last several years numerous state and local governments have begun to take notice of the emerging discipline of nanotechnology and its potential impact on the environment as well as the health and well-being of humans. Discussed below are some of the more high-profile examples of governmental efforts at these levels to exert regulatory oversight and ultimately control over various aspects of nanotechnology.

California State Regulation of Carbon Nanotubes

While not directly related to nanomedicine, it is important to note efforts by the state of California to regulate nanotechnology in general.

Image courtesy of the State of California

In October of 2008 the California Environmental Protection Agency's Department of Toxic Substances Control (DTSC) announced that it will request information regarding the analytical testing methods, fate and transport and any other information it deems relevant from manufacturers of carbon nanotubes. The responsibility to provide this information is placed solely on the manufacturer or importer of CNTs. The request was officially enforced in January, 2009 with a formal letter sent to all CNT manufacturers located in the state of California as well as to those who export CNTs into the state.

Berkeley, California and the Regulation of Nanoparticles

Within the state of California, the City of Berkeley is the first city in the state, and in the nation, to officially regulate nanotechnology. In December of 2006 the city council passed nanotechnology regulation that requires all manufacturers or users of nanoparticles submit a written disclosure of the current toxicology of the material that is known at the time and how the user will safely handle, monitor, contain, dispose, track inventory, prevent releases and mitigate such materials.
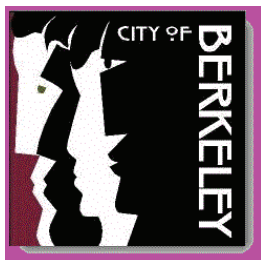
Logo courtesy of the City of Berkeley, California

THE EUROPEAN UNION

While the regulation of nanotechnology in Europe more or less mirrors that of the United States, some recent developments in this area should be noted. In late 2009 the European Parliament approved an update on legislation regulating cosmetics containing nanoparticles. In effect, all cosmetics of this nature must be labeled as such and must undergo a safety assessment, which "could lead to a ban on a substance if there is a risk to human health." The details of the safety assessment procedure are not yet available. Also that year the European Parliament recommended that food produced by nanotechnological processes undergo specific risk assessment before being approved for use and labeled. Interestingly, the risk assessment must not involve the use of vertebrate animals.

Logo courtesy of the European Union

In a separate effort, in 2004 the European Commission formed a study group referred to as **The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR)**. This group was formed to provide the Commission with unambiguous scientific advice on the safety of technology which requires a comprehensive assessment of risks. Nanotechnology in general and nanomedicine in particular fall within the scope of this group's responsibility for evaluation. In 2006 this group issued a formal opinion regarding nanotechnology on the appropriateness of existing methodologies to assess its potential risks. Unexpectedly the committee determined that to date there was insufficient data available to allow for the formation of systematic rules that govern the toxicological characteristics of all products of nanotechnology. The opinion concluded that there is an urgent need for comprehensive exposure data on humans (consumers and workers) and environmental species, including microorganisms. In 2005 the Commission adopted the communication titled "Nanosciences and Nanotechnologies: An Action Plan for Europe, 2005–2009" which emphasizes a number of commitments with respect

to European collaborations on nanosciences and nanotechnology and notes that risk assessment of human health and the environment must be integrated at all stages of the nanotechnology development lifecycle.

Image courtesy of the European Union

Several years later, in late 2006, a 21-month project termed Nanologue aimed at establishing a common understanding concerning social, ethical and legal aspects of nanotechnological applications and facilitating a European-wide dialogue among science, business and civil society about its benefits and impacts was commissioned and funded by the E.U. One of the outputs of this project was a pamphlet distributed across Europe titled "The Future of Nanotechnology: We Need to Talk" which promotes open dialogue regarding the potential risks and rewards of nanotechnology. In February of 2008 the European Commission produced the document titled "Recommendation on a Code of Conduct for Responsible Nanoscience and Nanotechnologies Research." This document's primary purpose is to drive the implementation of integrated, safe and responsible nanoscience and nanotechnology research in Europe for the benefit of society as a whole. The intended goal of this document is to guide Member states in the formulation of nanoscience and nanotechnology-based research strategies.

THE UNITED KINGDOM

Over the last several years the government of the United Kingdom has implemented numerous efforts at assessing the potential risks associated

with nanotechnology. Yet like the U.S. federal government, the U.K. has stopped short of announcing new strategies and passing formal laws of the regulatory control of nanotechnology and its associated products/ materials. Discussed below are some of the more significant efforts within the U.K.'s governmental structure to analyze and assess nanotechnological risks.

The Royal Society's Analysis on the Safety of Nanoparticles

In 2004 the U.K.'s Royal Society issued a massive report titled "Nanoscience and Nanotechnologies: Opportunities and Uncertainties" which highlights an immediate need for research to address uncertainties about the health and environmental effects of nanoparticles. In addition, it also recommends regulation to control exposure to nanoparticles. Some other specific recommendations outlined in this report are listed below.

Image courtesy of the United Kingdom's Royal Society

- Nanoparticles and nanotubes should be treated as hazardous materials
- Nanoparticles and nanotubes should be treated as new substances under existing Notification of New Substances (NONS) regulations
- The use of nanoparticles in environmental applications be prohibited until further research can be conducted on their safety
- All nanoparticle ingredients in food undergo a full safety assessment
- All nanoparticle ingredients in food should be listed on the label

Image courtesy of the United Kingdom's Royal Society

In late 2006 the Royal Society came together with the Nanotechnology Industries Association (NIA), the Nanotechnology Knowledge Transfer Network and Insight Investment to formulate the **Responsible Nano Code**, which outlines seven principles ranging from oversight of worker health and safety to transparency and disclosure. These principles are primarily directed at business entities and outline how to minimize risks to human health and the environment as well as to reduce negative social or ethical implications of nanotechnology development and usage.

The Council for Science and Technology (CST)

The Council for Science and Technology (CST) is the U.K. Prime Minister's top-level independent advisory body on science and technology policy issues and its purpose is to advise the Prime Minister and the First Ministers of the devolved administrations on strategic issues that cut across the responsibilities of individual government departments. In early 2007 the CST published an independent review titled "Nanosciences and Nanotechnologies: A Review of the Government's Progress on its Policy Commitments." This was a scathing report that criticized the U.K. government's efforts at promoting research on the toxicology and the health and environmental impacts of nanomaterials. The governmental of the United Kingdom has since taken multiple actions at addressing this criticism including actively funding comprehensive toxicological research on nanomaterials.

Logo courtesy of the Council for Science and Technology

Nanotechnology Engagement Group (NEG)

The **Nanotechnology Engagement Group (NEG)** was founded in 2005 to "document the learning from a series of groundbreaking attempts to

Image courtesy of the United Kingdom Nanotechnology Engagement Group

involve members of the public in discussions about the development and governance of nanotechnologies." It was funded by the U.K.'s Office of Science and Innovation (OSI)'s *Sciencewise Programme*. In 2007 the Group released its findings in a report titled "Democratic Technologies: The final report of the Nanotechnology Engagement Group." The report analyzed six projects undertaken in the U.K. which were meant to actively engage the general public in a dialogue on nanotechnology. It was concluded that early public engagement in science and technology can produce impressive results with respect to alleviating concerns and aspirations while informing the public so that citizens can more proactively participate in policy-making decisions.

The Department for Environment, Food and Rural Affairs

In 2008 the U.K.'s Department of Environment, Food and Rural Affairs commissioned a report lead by the Institute of Occupational Medicine to review the worldwide progress made towards the environment, health and safety research objectives which were set by the Nanotechnologies Research Co-ordination Group (NRCG) in 2005. This report considered the completed and ongoing research into nanomaterials and nanotechnology

Logo courtesy of the United Kingdom Department for Environment, Food and Rural Affairs

between 2004 and 2008, making specific recommendations for new research to further increase our understanding of engineered nanomaterials and their effects. The five areas covered in this report including the following:

- Metrology, characterisation, standardisation and reference materials
- Exposure – sources, pathways and technologies
- Human health hazard and risk assessment
- Environmental hazard and risk assessment
- Social and economic dimensions of nanotechnologies

The main conclusions from this report were mostly focused on the amassing of data needed to establish a baseline and standardization for the testing of nanoparticles. They are listed below.

- Progress has been made in identifying candidate materials which may be used to develop characterised reference nanoparticles for toxicology
- It is now clear that filters, such as those used in respiratory protective equipment and in air cleaning systems, are highly effective in removing nanoparticles from the air
- The lack of mass balance toxicokinetics for any nanoparticle and the patchy nature of the published toxicokinetic data is a severe impediment to identifying extra-pulmonary hazards
- Studies have improved the understanding of kinetics of nanoparticle uptake in invertebrate and vertebrate models and have related this to ecotoxicity

As evidenced by the four points above, the report gave a mixed review of current nanoparticle standardization initiatives.

British House of Lords

The British House of Lords published a critical report in early 2010 titled "Nanotechnologies and Food" criticizing the U.K. food industry for failing to be more open and transparent regarding its research into the uses of nanotechnology and nanomaterials in food. The report suggests that the British government should fund research into the potential safety and health risks of nanotechnology and nanomaterial applications in the food industry. The report also recommends that the Food Standards

Logo courtesy of the British House of Lords

Agency maintain a food register for public access that lists foods and food packaging which contains nanomaterials.

The Government of the United Kingdom

The government of the U.K. responded to yet another critical report by the Royal Commission on Environmental Pollution by publishing "Nanotechnologies Strategy: Small Technologies, Great Opportunities" in March of 2010. The report outlines how the U.K. government will ensure that British society can benefit from the societal and economic opportunities that nanotechnology and nanomedicine offer. The report also addresses challenges of nanotechnology and how those might be met in the future. Key actions outlined include:

- Government Chief Scientific Advisers to review coordination of nanotechnologies research across government including research on safety issues
- A new website to keep the public informed about government work on nanotechnologies

Image courtesy of the government of the United Kingdom

- A new Nanotechnologies Collaboration Group to facilitate ongoing communication and collaboration between government, academia, industry and other interested parties
- A new Ministerially led Nanotechnologies Leadership Group to address barriers to commercial growth in this area
- Government to explore a new industry reporting scheme with a broader scope covering nanomaterials as well as products containing them

AUSTRALIA

Australia is relatively new to the field of nanotechnology in general and nanomedicine in particular, thus there are limited examples of, or perhaps even a need for, governmental oversight of research, development and commercialization in this area. Some examples of Australian government and university influence on the regulation of nanotechnology are cited below.

Australian Government Department of Health and Ageing

In early 2006 the Australian Government Department of Health and Ageing issued a voluntary Call for Information to industry requesting information on the application and quantification of nanomaterials being imported or manufactured for industrial or cosmetics use. The Call was implemented under the National Industrial Chemicals Notification and Assessment Scheme (NICNAS). This was the first international governmental call for a voluntary reporting scheme. Only roughly 20 Australian companies responded to this call due to its voluntary nature.

Logo courtesy of the Australian Government Department of Health and Aging

The same department released the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) **Proposal for Regulatory Reform of Nanomaterials** in late 2009 as a public consultation on a nanomaterials regulatory strategy. It is a public discussion paper that

addresses the current regulatory efforts for "nanoforms" and proposes an approach for the regulation of industrial nanomaterials under the NICNAS. It strongly promotes a "permit" framework for mandatory notification of the government with respect to the manufacture, import and use of nanoforms.

Monash University and Its Influence on Australia's Regulatory Framework

An independent review conducted by the Centre for Regulatory Studies at Victoria, Australia's Monash University was concluded and released titled "A Review of Possible Impacts of Nanotechnology on Australia's Regulatory Framework" in mid-2008. This review outlines six regulatory issues which need addressing including:

- The definition of "new" or "existing" substances or products
- Proper quantification of nanomaterials at the nanoscale
- Knowledge of the presence of nanomaterials in products and their risks
- Outlining of standard risk assessment protocols and techniques
- Research and development exemptions
- Analysis of international documentation adequacy

It is not clear what influence this review will have on Australian governmental policy regarding the regulation of nanotechnology.

Logo courtesy of Monash University

CANADA

Two primary areas addressed by the Canadian government with respect to regulatory oversight of nanotechnology are its potential effects on the environment and human health. Much of the governmental regulatory efforts in Canada have been centered on manipulating the definition of new substances or nanomaterials to further clarify their possible classification as being "nano" in nature. Examples of these unclear and even overly broad definitions outlined by both Environment Canada and Health Canada are cited are discussed below.

Environment Canada

Environment Canada is a government office tasked with "protecting the environment, conserving the country's natural heritage and providing weather and environmental predictions to keep Canadians informed and safe." In 2007 the New Substances Division of this office issued an advisory note which outlines and clarifies the requirements for nanomaterials under New Substances Notification Regulations (NSNR). Titled "Requirements for Nanomaterials under the New Substances Notification Regulations (Chemicals and Substances)" this advisory note states that substances already on the Domestic Substances List (DSL) as well as those not on the DSL are considered to be new substances if they have "unique structures or molecular arrangements." Many have speculated that this considerably vague definition is meant as a catch-all mechanism for Environment Canada to exert broad control over a multitude of substances that may or may not be considered "nano" in composition.
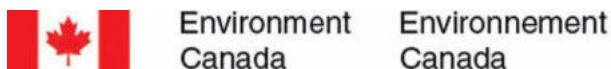
Image courtesy of Environment Canada

Environment Canada took its efforts at regulating nanomaterials a step further later that same year by jointly issuing the document titled "Proposed Regulatory Framework for Nanomaterials under the Canadian Environmental Protection Act, 1999" with Health Canada. The proposal's primary purpose is to promote the development of an effective regulatory framework for nanomaterials in Canada that manages risks while allowing for the proper introduction of nanomaterials into the Canadian market.

Health Canada

Health Canada is a federal department whose mission it is to be "responsible for helping the people of Canada maintain and improve their health." In early 2010 Health Canada announced the adoption of a policy document titled "Interim Policy Statement on Health: Canada's Working Definition for Nanomaterials." The purpose of this document is to formally announce an intentionally broad definition of nanomaterials that will enable other departments strengthened and wide-ranging regulatory oversight.

Image of courtesy Health Canada

According to this policy, the formal definition of a nanomaterial is "any manufactured product, material, substance, ingredient, device, system or structure if:

1. It is at or within the nanoscale in at least one spatial dimension, or;
2. It is smaller or larger than the nanoscale in all spatial dimensions and exhibits one or more nanoscale phenomena.

It is clear that theoretically many materials could be classified as nanomaterials under this definition and therefore possibly be subject to regulation by a variety of Canadian governmental departments.

INTERNATIONAL EFFORTS AT NANOTECHNOLOGY REGULATION

Like the efforts of most individual governments, the majority of internationally organized efforts at nanotechnology regulation have been in the form of assessment and analysis panels and have not resulted in the passage of any international law governing nanotechnology or, more specifically, nanomedicine. Below some of the more high profile initiatives are described.

The International Risk Governance Council (IRGC)

Founded in 2003, the International Risk Governance Council (IRGC) is an independent organization whose purpose is "to help the understanding

Logo courtesy of the International Risk Governance Council

and management of emerging global risks that have impacts on human health and safety, the environment, the economy and society at large." In July of 2006 the council released a report titled "The Risk Governance of Nanotechnology: Recommendations for Managing a Global Issue." This report was focused primarily on the assessment of the current government oversight of nanotechnology. It provided an overview of governance deficits and proposed overarching recommendations for the management of those deficits. Some of the key findings and suggestions of this report include the following:

- Further research monies are needed for risk related research and better understanding of the fundamental behaviors of nanoparticles
- Standardized measurement of and nomenclature for the nanoscale are urgently required
- Special governance approaches for safety should be considered for future applications if unexpected developments occur
- Need to adapt elements of existing regulatory regimes which are applicable to nanotechnology
- Use existing international channels through organizations such as OECD and ISO to accelerate the regulatory process

The International Council on Nanotechnology (ICON)

Logo courtesy of the International Council on Nanotechnology

The **International Council on Nanotechnology (ICON)** is a global partnership between the nanotechnology industry, government, academia and other select organizations. Its role is to promote effective nanotechnology stewardship, including the production and dissemination of information as well as the identification of knowledge gaps and how to fill those gaps. Formed in 2004, its primary focus has been on the environmental impact and risks associated with developing nanotechnology. While ICON has no authority to formulate or pass country-specific or international law regulating nanotechnology, it is generally accepted that the information produced by this organization is carefully noted by the international

community including government entities and it may indeed impact future law in this area. The reports generated by ICON are informational in nature, wide-ranging and cover virtually all aspects of nanotechnology.

The Organization for Economic Cooperation and Development (OECD)

Founded in 1961, the Organization for Economic Cooperation and Development (OECD) brings together governments from countries around the world. It currently has representatives from 30 industrialized countries in North America, Europe, Asia and the Pacific Region. It is committed to democracy and the market economy primarily to drive economic growth. In April, 2006, however, the OECD deviated from its original mission of promoting economic growth and released the "Report of the OECD Workshop on the Safety of Manufactured Nanomaterials: Building Co-operation, Co-ordination and Communication." The main recommendation of this report was to establish a Working Group to consider how best to organize future international activities to manage and characterize nanomaterials for impacts on the environment, health and safety. This Working Group was formed later that year as the "Working Party on Manufactured Nanomaterials (WPMN)" and as a separate "**Working Party on Nanotechnology**" in 2007. In July of 2008 the WPMN formally recommended a priority list of fourteen manufactured nanomaterials that should be the focus of further investigation and listed 60 endpoints for the full characterization of these materials. In late 2009 the OECD released a report titled "Preliminary Guidance Notes on Sample

ORGANISATION
FOR ECONOMIC
CO-OPERATION
AND DEVELOPMENT

OECD

Logo courtesy of the Organization for Economic Cooperation and Development

Preparation and Dose Symmetry for the Safety Testing of Manufactured Nanomaterials" which offers guidance for the preparation of samples for safety testing.

The International Center for Technology Assessment (ICTA)

The International Center for Technology Assessment (ICTA) is a non-profit international organization "committed to providing the public with full assessments and analyses of technological impacts on society." It was formed to assist the general public as well as governmental policy makers to better understand how technology and technological advancements affect society. In 2007 the ICTA released a report titled "Declaration Principles for the Oversight of Nanotechnologies and Nanomaterials." The report primarily addresses the health and safety of those working with nanotechnology and/or nanomaterials as well as that of the general public and recommends broad-based active governmental regulation of nanotechnology and, more specifically, nanomaterials. The primary conclusions of the report are listed below.

- Current legislation provides inadequate oversight of nanomaterials
- Nanomaterials must be classified as new substances for regulatory purposes
- Voluntary initiatives are wholly inadequate to oversee nanotechnology
- Adequate and effective nanomaterial oversight requires an immediate emphasis on preventing known and potential exposures to nanomaterials that have not been proven safe
- Full lifecycle environmental, health and safety effects must be assessed prior to commercialization
- Government funding of environmental, health and safety research must be increased dramatically and a strategic risk research plan delineated

Image courtesy of the International Center for Technology Assessment

- The public's right to know requires the labeling of all products containing nanomaterial ingredients
- Social impact, ethical assessment, equity, justice and individual community preferences should guide the allocation of public funding for research
- All who market nano-products must be held accountable for liabilities incurred from their products

This declaration was the result of a broad international collaboration between civil society, public interest, environmental and labor organizations and was signed by over 70 representatives from six continents. Should even a modest percentage of the recommendations contained within this report be realized, the research, development and commercialization of nanotechnology-related products will be significantly affected.

The International Union of Food, Farm and Hotel Workers (IUF)

In March of 2007 during their annual meeting in Geneva, Switzerland, the International Union of Food, Farm and Hotel Workers (IUF), which represents 12 million workers from more than 300 unions from over 120 countries, called for a moratorium on the use of nanotechnology in food and agriculture. It also called on the World Trade Organization (WTO) to "suspend the grant of patents related to nanotechnology

Logo courtesy of the International Union of Food, Farm and Hotel Workers

in the food industry and agriculture, until the countries affected and social movements can carry out an evaluation of their impact." This moratorium and request to the WTO were largely symbolic and to date has not resulted in a formal banning of foods or agricultural efforts involving nanotechnology.

US/EU Collaborative Efforts

In the Fall of 2009 a joint U.S. and E.U. analysis of the effective and convergent regulation of nanomaterials was published by Chatham House, the London School of Economics, the Environmental Law Institute and the Project on Emerging Nanotechnologies. Titled "Securing the Promise of Nanotechnologies," the massive 122-page document outlined current national and international regulatory frameworks and defined existing regulation imposed on the chemicals, food and cosmetics industries. Rather than yet another effort at suggesting broad international governmental control over nanotechnology, the conclusions were focused on promoting transatlantic cooperation and convergence in nanomaterials regulation. The report suggests that:

1. Increased funding is recommended for research in the environmental health and safety risks of nanomaterials;
2. Mandatory reporting requirement on the commercial use of nanomaterials should be strengthened; and
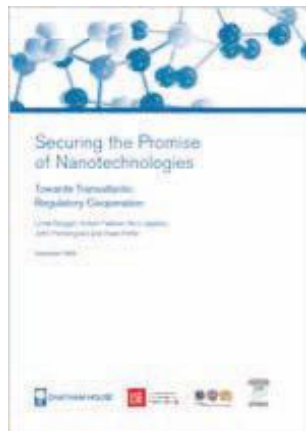
Image courtesy of Chatham House

3. There is no overwhelming case that the U.S. and E.U. should prioritize international efforts to create new, mandatory, labeling requirements.

CHAPTER SUMMARY

Government Promotion of Advancements in Nanomedicine

1. U.S. President Bill Clinton was a strong supporter of nanotechnological research.
2. U.S. President George W. Bush signed the 21st Century Nanotechnology Research and Development Act.
3. The U.S. National Nanotechnology Initiative (NNI) was formed to help shape a major U.S. research effort in nanotechnology-based efforts to improve human health.
4. The U.S. Bioengineering Nanotechnology Initiative was formed as a funding source for grant applications in nanomedicine.
5. The NIH's Roadmap on Nanomedicine Initiative encompasses eight intellectual and technological centers focusing on advancements in nanomedicine.
6. $10.4B of the $787B American Recovery and Reinvestment Act (ARRA) was allocated to the NIH for medical research.
7. The U.S. National Institute of Standards and Technology (NIST) has recently allocated numerous financial awards for research in nanomedicine and has its own research programs in this area.
8. The U.S. National Science Foundation (NSF) recently dramatically increased the number of grant awards issued for nanotechnology research.
9. The Alliance for Nanotechnology in Cancer is a U.S. National Cancer Institute (NCI) initiative designed to bring scientists and clinicians together to drive translational advancements in nanomedicine.
10. Twelve Cancer Nanotechnology Platform Partnerships (CNPPs) have been formed in the U.S. to focus on the development of the next generation of nanotechnology-based diagnostic and therapeutic tools.
11. The platform technologies addressed by the CNPPs include imaging and detection, reporters of efficacy, multi-functional therapeutics, prevention and control and research enablers.
12. The U.S. Defense Advanced Research Projects Agency (DARPA) has recently expanded its mission to cover R&D in nanomedicine.

13. Missouri's St. Louis Institute of Nanomedicine Working Group is a collaborative regional effort to promote nanotechnology research for the treatment of human diseases.

14. New York's Alliance for Nanomedical Technologies (ANMT) is a multi-institutional partnership created to help nucleate and invest in nanomedical research programs in the state of New York.

15. Texas' Alliance for NanoHealth (ANH) uses nanotechnology to bridge gaps between medicine, biology, materials science, computer technology and public policy.

16. The Australian government's National Enabling Technologies Strategy provides funding and a framework for developing enabling technologies with a major focus in nanotechnology.

17. Nanotechnology Victoria, Ltd is a consortium of institutes and promotes nanotechnological advancements in the Victorian region of Australia.

18. The National Nanotechnology Strategy Task Force (NNST) was formed by the Australian government to compile a formal strategic review and recommendation for the country with respect to nanotechnology.

19. The Regenerative Medicine and Nanomedicine Initiative (RMNI) launched by the Canadian Institutes of Health and Research (CIHR) is directed at supporting the development of new and emerging areas of integrative biomedical research including the study of stem cells, tissue engineering, rehabilitation sciences and nanomedicine.

20. *Nano2Life* was formed as the first European Network of Excellence supported under the 6th Framework Programme and has the mission of keeping Europe competitive with other countries in the field of nanobiotechnology.

21. The European Technology Platform (ETP) in Nanomedicine was formed under the 7th Framework Programme to promote European efforts in nanotechnology-based diagnostics, targeted drug delivery and release and regenerative medicine.

22. The 7th Framework Programme funded *Euronanomed* was formed to support trans-national collaborations of academia, clinical/public health communities and small to medium-sized companies for nanomedicine-related research and technology development.

23. Singapore's Institute for Bioengineering and Nanotechnology (IBN) was created to provide international leadership, conduct innovative

research, drive technology transfer and foster a conducive research environment in nanomedicine.

24. Campus Biometropolis was formed by the Mexican Government to be a center for medical research and development with a particular emphasis on nanomedicine and will be integrated with the National Autonomous University of Mexico.

25. The Global Enterprise for Micromechanics and Molecular Medicine (GEM[4]) was formed to promote the development and use of nanotechnology for global health and medical research.

Government Evaluation, Policy and Regulation of Nanotechnology

1. The U.S. Nanotechnology Task Force formed by the FDA focuses on "determining regulatory approaches that encourage the continued development of innovative, safe, and effective FDA-regulated products that use nanotechnology materials."

2. The FDA currently treats most products made with nanotechnology similarly to the way it handles others.

3. The Nanotechnology Characterization Laboratory (NCL) was formed by the FDA and NIST to standardize and perform the pre-clinical characterization of nanomaterials intended for cancer therapeutics and diagnostics developed by researchers from academia, government, and industry.

4. A Nanotechnology Working Group was created by the U.S. Environmental Protection Agency (EPA) in 2004 to examine the potential environmental implications of nanotechnology.

5. The Center for Environmental Implications of Nanotechnology (CEINT) was established through a collaboration between the EPA and NSF to monitor interactions between nanomaterials, the environment, plant and animal life.

6. 2008 saw considerable regulatory change with respect to carbon nanotubes and other certain nanoforms under the Significant New Use Rules (SNURs).

7. In April of 2010 the EPA changed the definition of a nanomaterial to a more broader version and proposed new strict reporting rules for chemical companies who work with carbon nanotubes.

8. The U.S. NSTC's Nanoscale Science, Engineering and Technology Subcommittee released a document on the environmental and safety impact of nanomaterials now actively used by Federal agencies to both formulate and guide various research programs.

9. The U.S. National Research Council (NRC) released a report in 2008 criticizing the Federal government's lack of effort and oversight pertaining to the possible effects of nanotechnology on environmental, health and safety issues.

10. Class 977 is a new classification for nanotechnology patents created by the U.S. Patent and Trademark Office that provides a cross-reference for examiners and others to search prior art.

11. The Project on Emerging Nanotechnologies (PEN) was formed by the Woodrow Wilson International Center to ensure that as nanotechnologies advance, possible risks are minimized, public and consumer engagement remains strong, and the potential benefits of these new technologies are realized.

12. In 2008 California's Environmental Protection Agency announced that it will request information regarding the analytical testing methods, fate and transport and any other information it deems relevant from manufacturers of carbon nanotubes.

13. In 2006 the city of Berkeley, California was the first city in the nation to officially regulate nanotechnology by requiring that all manufacturers or users of nanoparticles submit a written disclosure of the current toxicology of the material that is known at the time and how the user will safely handle, monitor, contain, dispose, track inventory, prevent releases and mitigate such materials.

14. The European Parliament has suggested new labeling requirements for cosmetics containing nanoparticles and recommended that food produced by nanotechnological processes undergo specific risk assessment.

15. The European Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) determined in 2006 that to date there was insufficient data available to allow for the formation of systematic rules that govern the toxicological characteristics of all products of nanotechnology.

16. *Nanologue* is a European project aimed at establishing a common understanding concerning social, ethical and legal aspects of nanotechnological applications.

17. So far the U.K. has stopped short of announcing new strategies and passing formal laws of the regulatory control of nanotechnology and its associated products/materials.

18. The U.K.'s Royal Society and Nanotechnologies Industry Association formulated the Responsible NanoCode which outlines seven principles ranging from oversight of worker health and safety to transparency and disclosure.

19. The U.K.'s Council for Science and Technology issued a scathing report that criticized the U.K. government's efforts at promoting research on the toxicology and the health and environmental impacts of nanomaterials.

20. The Nanotechnology Engagement Group (NEG) was founded in 2005 in the U.K. to "document the learning from a series of groundbreaking attempts to involve members of the public in discussions about the development and governance of nanotechnologies."

21. In 2008 the U.K.'s Department of Environment, Food and Rural Affairs commissioned a report focused on the amassing of data needed to establish a baseline and standardization for the testing of nanoparticles.

22. A Proposal for Regulatory Reform of Nanomaterials was released by Australian Government Department of Health and Ageing addressing the current regulatory efforts for "nanoforms" and proposes an approach for the regulation of industrial nanomaterials.

23. Environment Canada issued a broad definition of new substances as having "unique structures or molecular arrangements."

24. Health Canada issued a broad definition of a nanomaterial that theoretically could include a wide range of materials that would possibly be subject to regulation by the Canadian government.

25. The International Risk Governance Council (IRGC) issued a report on nanotechnologies in 2006 recommending more risk-related research, standardization and safety regulation.

26. The International Council on Nanotechnology (ICON) issues informational reports covering virtually all aspects of nanotechnology.

27. The international Organization for Economic Cooperation and Development (OECD) drove the formation of the Working Party on Nanotechnology which recommended a priority list of fourteen

manufactured nanomaterials that should be the focus of investigation and listed 60 endpoints for the full characterization of these materials.

28. The non-profit organization known as the International Center for Technology Assessment (ICTA) released a report in 2007 recommending a broad-based active governmental regulation of nanotechnology and, more specifically, nanomaterials.

29. A moratorium on the use of nanotechnology in food and agriculture was called for by the International Union of Food, Farm and Hotel Workers (IUF) in 2007.

30. Transatlantic cooperation and convergence in nanomaterials regulation was promoted by a report released jointly by the U.S. and E.U. in 2009.

KEY TERMS

- 21st Century Nanotechnology Research and Development Act
- National Nanotechnology Initiative (NNI)
- Trans-NIH Bioengineering Nanotechnology Initiative
- Roadmap on Nanomedicine Initiative
- American Recovery and Reinvestment Act (ARRA)
- NCI Alliance for Nanotechnology in Cancer
- St. Louis Institute of Nanomedicine Working Group
- Alliance for Nanomedical Technologies
- nanoBioFab
- Alliance for Nanohealth (ANH)
- Nanohealth
- University of Houston Nanofabrication Facility
- National Enabling Technologies Strategy
- Nanotechnology Victoria, Ltd.
- National Nanotechnology Strategy Task Force (NNST)
- Regenerative Medicine and Nanomedicine Initiative (RMNI)
- Nano2Life
- European Technology Platform (ETP) in Nanomedicine
- Euronanomed
- The Institute for Bioengineering and Nanotechnology (IBN)
- Campus Biometropolis
- GEM[4]
- U.S. Food and Drug Administration Nanotechnology Task Force
- Nanotechnology Characterization Laboratory (NCL)
- Nanotechnology Working Group of the EPA

- Center for Environmental Implications of Nanotechnology (CEINT)
- Nanoscale Science, Engineering and Technology Subcommittee of the NSTC
- Class 977
- Project on Emerging Nanotechnologies (PEN)
- The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR)
- Nanologue
- Responsible Nano Code
- Nanotechnology Engagement Group (NEG)
- Proposal for Regulatory Reform of Nanomaterials
- International Council on Nanotechnology (ICON)
- The OECD's Working Party on Nanotechnology

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. What are the four main goals of the U.S. National Nanotechnology Initiative?
2. Name the eight centers involved in the NIH's Roadmap on Nanomedicine Initiative as cite two examples of research at these centers.
3. How much of the $787 billion funding for the American Recovery and Reinvestment Act (ARRA) was dedicated to the NIH and for what purpose?
4. Cite at least two examples of nanomedical research funded by NIST.
5. What are the goals of the NCI Alliance for Nanotechnology in Cancer?
6. Cite two examples of DARPA-funded nanomedical research.
7. What are the three primary areas of focus for the St. Louis Institute of Nanomedicine Working Group?
8. What is the mission of the Texas Alliance for Nanohealth?
9. Name a major area of focus for Nanotechnology Victoria, Ltd.
10. What initiatives has the Canadian government undertaken to promote nanomedical research?
11. What are the five primary objectives of *Nano2Life?*
12. What are the five policy objectives of the European Technology Platform in Nanomedicine?

13. What is the goal of *Euronanomed*?
14. What types of research will scientists conduct under the GEM[4]?
15. What did the U.S. FDA Nanotechnology Task Force recommend in 2007?
16. What are the six objectives of the Nanotechnology Characterization Laboratory?
17. What efforts has the U.S. Environmental Protection Agency made with respect to nanomaterial characterization?
18. What were the findings of the U.S. National Research Council's 2008 report and what was the government's response?
19. Why did the U.S. Patent and Trademark Office create Class 977?
20. What is the first city to regulate nanotechnology and what are the details of the new law?
21. What document was produced by the European Commission in 2008 and what was its purpose with respect to nanoscience and nanotechnology?
22. Cite at least three specific recommendations of the Royal Society's document titled "Nanoscience and Nanotechnologies: Opportunities and Uncertainties."
23. What are the five areas covered by the report issued in 2008 by the U.S. Institute of Occupational Medicine and what are the main conclusions?
24. Cite at least three key actions outlined by the U.K. Royal Commission on Environmental Pollutions report titled "Nanotechnologies Strategy: Small Technologies, Great Opportunities."
25. How did Monash University influence nanotechnology policy of the Australian government?
26. What two actions did Environment Canada take to influence Canadian governmental policy on nanotechnology?
27. What is the primary focus of ICON?
28. What did the OECD's Working Party on Nanotechnology recommend in 2008?
29. Cite at least three primary conclusions of the ICTA's report titled "Declaration Principles for the Oversight of Nanotechnologies and Nanomaterials."
30. What two recommendations did the IUF make in 2007?

10

Future Concepts in Nanomedicine

In this chapter futuristic applications of nanotechnology as they apply to the field of medicine are discussed. The examples cited are mostly conceptual in nature but backed by sound reasoning and a strong foundation of recent nanotechnological advances which, conceivably, could drive towards their future routine applications in both therapy and diagnostics. While some of these may be considered science fiction at present, it is tempting to speculate that the related real nanotechnological advances described may indeed drive the development of new avenues for the diagnosis and treatment of disease.

NANOROBOTICS AND MEDICINE

When the general public thinks of nanotechnology and, more specifically nanomedicine, often the first thought that comes to mind are nanorobots (defined later in this chapter) swimming through the blood stream seeking out viruses and bacteria for destruction. While this is certainly a futuristic

concept, it is not altogether unrealistic and thus warrants discussion in this book. In order to consider the development of a nanoscale robotics-based device capable of *in vivo* travel and the performance of various therapeutic tasks it is necessary to understand the basic requirements for its size and functionality. The three primary components of an effective therapeutic nanorobot are:

1. Potential energy source
2. Mechanism for converting potential energy to kinetic energy
3. Ability to use kinetic energy for mobility and a therapeutic benefit

(Image courtesy Physorg.com; reprinted with permission)

This section describes some of the essential individual components that meet these criteria and are necessary for the development of therapeutic nanorobots. Cited are both real advances in the development of these components as well as theoretical examples of nanorobots performing therapeutic functions with the human body.

Nanomolecular Motors and Gears

In order to have a general understanding of the possibilities for *in vivo* nanorobotics applications in medicine it is important to grasp the concept of **nanomotors,** which are defined as devices, nanoscale in size and often made up of individual molecules, capable of converting energy into movement (potential energy into kinetic energy). The successful design and development of nanomotors or nanomolecular motors as they are sometimes referred to are essential if the medical application of nanobots is to become a reality. Not only will nanomotors need to be developed that are efficient at converting fuel into mechanical energy, but strategies for their successful incorporation and usage in the context of nanomachinery such as nanorobots will have to be undertaken. Nanomotors exist in nature, with perhaps the most notable discovery being that of dimeric motor proteins capable of undergoing transportation along microtubules
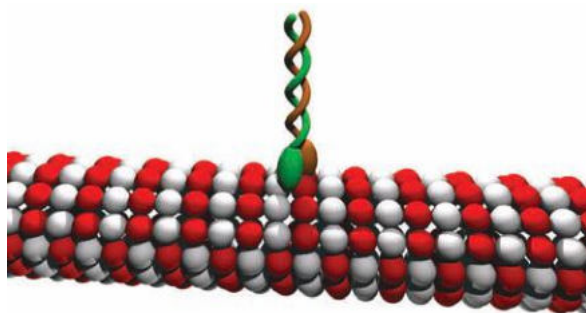
FIGURE 10.1 Diagrammatic illustration of a kinesin protein dimer moving along a microtubule. (Courtesy of Wikivisual.com; reprinted with permission.)

within living cells. **Kinesin**, for example, is a naturally occurring class of motor protein dimers found in biological cells capable of transporting cellular cargo most often along microtubular structures within cells (Figure 10.1).

There are also now a number of examples of prototype man-made nanomotors made from a variety of different types of naturally occurring and synthetic molecules. The basic requirements behind the construction of a biologically functioning nanomotor are generally accepted by the research community as:

- Less than ~300 nm in scale
- Low energy requirements
- Operates autonomously
- Operates under physiological conditions
- Exhibits long-term stability

Below are described some of the more high-profile research efforts at generating nanomotors from several different platforms that meet these criteria and may be the basis behind those to be incorporated in the larger context of nanomachinery for *in vivo* medical applications.

Rotaxane-Based Nanomotors

J. Fraser Stoddart's group at the California Nanosystems Institute of the University of California, Los Angeles has developed an artificial nanomotor powered by **optomechanical energy conversion** (the conversion of optical light to mechanical energy) that meets the basic requirements listed above.

Their system is based on **rotaxane**, which is a mechanically interlocked molecular compound consisting of a dumbbell-shaped molecule threaded through a ring. It is a linear molecular motor powered exclusively by visible light and exhibits autonomy by relying on intramolecular processes. The motor consists of an electron donor macrocycle ring enclosed around a dumbbell-shaped dual electron acceptor. The overall length of the system is ~5 nm and the distance between the two electron acceptor sites is ~1.3 nm. A photosensitizer drives electron transfer upon exposure of the system to visible light and powers rotaxane shuttling (Balzani *et al.*, 2006 and Figure 10.2). The authors highlighted and confirmed the following features of their system:

  i)   Powered by visible light
 ii)   Operates autonomously
iii)   No waste product generation
 iv)   Intramolecular operation
  v)   Operates at a frequency of 1 kHz
 vi)   Operates under mild conditions
vii)   Stable for at least 10^3 cycles
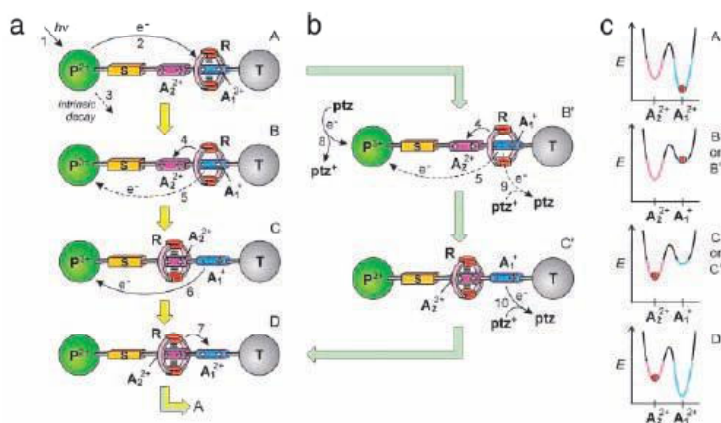
FIGURE 10.2 Mechanisms of the photochemically driven ring shuttling in 16+. (*a*) Intramolecular mechanism. (*b*) Shuttling assisted by an electron relay. (*c*) Graphical representation of how the energy profile of shuttling changes upon reduction of **A1**2+ and the location of the ring at **A1**2+ (blue) or **A2**2+ (magenta) for each molecular structure illustrated. (Courtesy of Balzani *et al.*, 2006; reprinted with permission.)

Nucleic Acid-Based Nanomotors

Nucleic acid-based nanomotors are nanoscale motors made of nucleic acids such as DNA or RNA. Given their unique intra- and intermolecular hybridization properties, a number of groups have studied nucleic acids in the context of nanomotor development. Weihong Tan and colleagues at the Center for Research at the Bio/Nano Interface, University of Florida in Gainesville have tapped in to the unique properties of DNA to develop yet another nanomotor driven by exposure to light. The researchers designed a self-hybridizing, hairpin-structured DNA molecule with azobenzene molecules incorporated at strategic locations along the nucleic acid strand. Azobenzene is capable of reversible isomerization between planar *trans* and non-planar *cis* forms upon exposure to UV and visible light. Thus a photoswitchable single-molecule DNA motor (PSMM) was constructed in which the hairpin exhibited an open or closed conformation depending upon the *trans* or *cis* forms of the azobenzene molecule (Kang *et al.*, 2009 and Figure 10.3). This is the first example of a fully reversible single-molecule DNA nanomotor driven by photons.
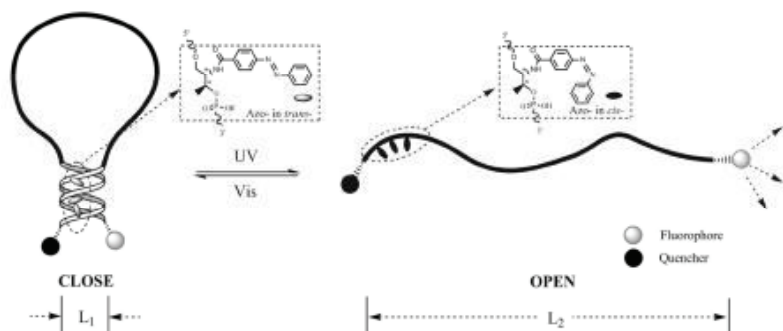
FIGURE 10.3 Schematic illustration of a photo-switchable single molecule DNA nanomotor. **T**hree azobenzene moieties are inserted to a DNA stem duplex on an arm labeled by quenchers. The structural change of DNA displays a contraction (CLOSE) state when tethered azobenzene moiety (Azo-) takes a *trans* conformation under visible light irradiation and an extension (OPEN) state when Azo- takes a *cis* conformation under UV light irradiation. L1 is the average size of the hairpin structure based on the distance between F/Q pair, and L2 is the average size of the extended molecule based on persistence length of a single DNA strand. (Courtesy of Kang *et al.*, 2009; reprinted with permission.)

Nanotube-Based Nanomotors

Nanotube-based nanomotors are nanoscale motors made of single-walled or multi-walled carbon nanotubes. The first carbon nanotube-based nanomotor was developed in 2003 by Alex Zettl's group at the University of California, Berkeley's Center of Integrated Nanomechanical Systems. It consists of a multi-walled carbon nanotube acting as an axis around which a plate of gold rotates as an actuator. Each end of the MWNT rests on a silicon-oxide layer and form electrodes at each contact point. There are three fixed stator electrodes which surround the assembly. Upon application of four independent voltage signals (one to each stator and one to the rotor itself) the rotor's position, velocity and direction may be controlled (Fennimore *et al*., 2003 and Figure 10.4).

Joseph Wang's group at the Biodesign Institute, Arizona State University in Tempe (now at the University of California, San Diego) has taken a different approach to the utilization of carbon nanotubes in nanomotors and applied them instead to induce acceleration of **nanowire-based nanomotors** (motors made of a wire with a diameter no larger than 100 nm) to produce a highly efficient and controllable nanomotor system. Their platform is based on the development of self-powered gold-platinum (Au/Pt) bimetal nanowire nanomotors which run on the decomposition of hydrogen peroxide to oxygen and water. The researchers incorporated carbon nanotubes within the platinum segment of catalytic nanowires based on the Au/Pt system as CNTs are well recognized for their unique electronic and mechanical properties which in some instances promote accelerated electron-transfer reactions and lower over-voltages. It was thus
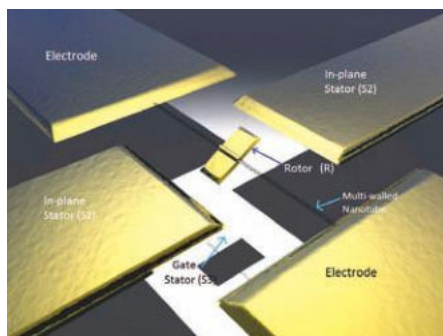
FIGURE 10.4 Schematic showing the basic layout of the Zettl MWNT nanomotor. (Courtesy of Wikipedia.org; reprinted with permission.)

speculated that coupling CNTs with the Pt surfaces in a nanomotor system might lead to improvements in electron transfer and increased acceleration and this was indeed demonstrated (Laocharoensuk *et al.*, 2008 and Figure 10.5). It should be noted that these nanomotors, while autonomous in nature and capable of speeds ~10 um/s, do not compare to the speed and efficiency of natural nanomotors.
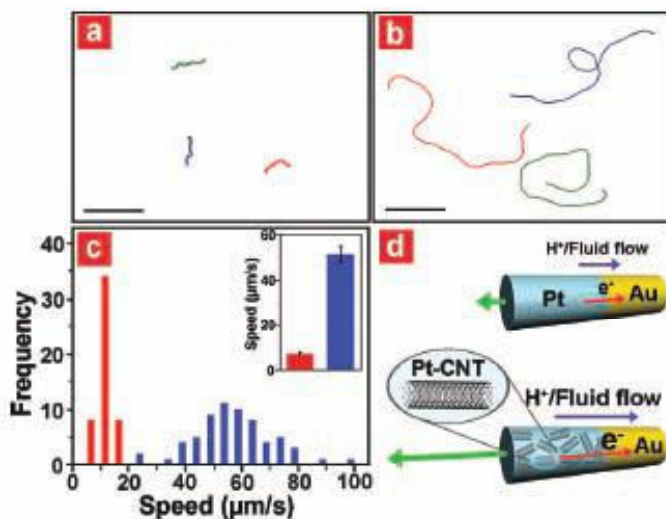
FIGURE 10.5 Carbon nanotube-induced high speed catalytic nanomotors. (a, b) Tracking lines illustrating a typical motion and moving distances of Au/Pt (a) and Au/Pt-CNT (b) nanomotors. Scale bar is 45 μm. (c) Histograms of average speeds of Au/Pt (red) and Au/Pt-CNT (blue) nanomotors measured from the movement of the nanomotors. Bar graphs with error bars (inset) represent the mean of average speeds (μm/s) of the corresponding nanomotors, respectively. (d) A schematic representation of the self-electrophoresis mechanism of Au/Pt (top) and Au/Pt-CNT (bottom) bipolar nanomotors. Hydrogen peroxide fuel is preferentially consumed/oxidized on the Pt (top, blue) or Pt-CNT (bottom, patterned blue) ends while oxygen is catalytically reduced on the Au (yellow) segment. The flux of electrons inside the nanomotors proceeds from one end to the other generating a local electric field, as well as the migration of protons and surrounding fluid outside the nanomotors resulting in the movement of the nanomotor in the opposite direction. The higher electrocatalytic activity of Pt-CNT compared with Pt provides a faster reaction rate, and hence a higher proton and fluid flow corresponding to an increased flux of electrons inside the nanomotors as indicated by the vectors. (Courtesy of Laocharoensuk *et al.*, 2008; reprinted with permission.)

Nanogears

Nanogears are defined as mechanical instruments for transmitting motion that are nanoscale in size. All of the nanomotors mentioned above would be useless without a mechanism to convert the kinetic energy generated into work performed. Thus a nanoscale gearing system will need to be employed for coupling of the aforementioned motors to, for example, mechanical flagella for movement. Eric Drexler (see also Chapter 1 and Focus Box 10.1) has contemplated this issue and devised a "**molecular planetary gear**" that might accomplish such a task. The gear would convert shaft power from one angular frequency to another. The casing is composed of a silicon shell terminated by sulfur. Each of nine separate planet gears is attached to the planet carrier by a carbon-carbon single bond (Drexler, 1992 and Figure 10.6).

Nanocomputers

Nanocomputers are defined as computation-capable devices which use fundamental parts not larger than a few nanometers or for which the total size is measured on the nanoscale. Nanocomputers may be built utilizing electronic, mechanical, biochemical or quantum technologies as the bases behind their computation capabilities. Nanocomputers will

Focus Box 10.1 K. Eric Drexler and mechanical nanocomputers

In addition to his pioneering theories on nanoscale assembly technology, Dr. Eric Drexler has focused much of his efforts in the area of mechanical nanocomputing. His ideas on "rod logic" applications (see text) for the creation of nanoscale mechanical computers have received much attention. Dr. Drexler received his Ph.D. in 1991 from MIT. His doctoral thesis was published as the book "Nanosystems Molecular Machinery Manufacturing and Computation" which received the American Association of Publishers award for Best Computer Science Book in 1992. He is currently Chief Technical Advisor to Nanorex, Inc. of Bloomfield, Michigan. (Photo courtesy of David Orban; reprinted with permission.)
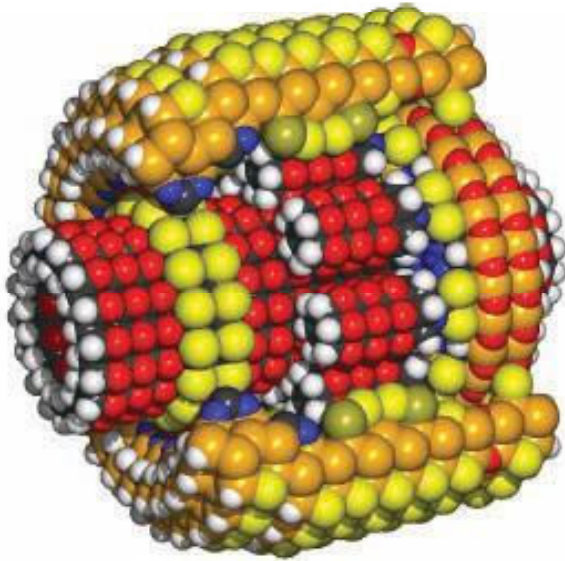
FIGURE 10.6 Diagram of a molecular planetary gear that is a mechanical component which might be found inside a medical nanorobot. The planetary gear shown here has not been built experimentally but has been modeled computationally. (Copyright 1995, Institute for Molecular Manufacturing (IMM) and courtesy of Drexler, 1992; reprinted with permission.)

eventually become essential on-board components of nanomachinery and nanorobotic platforms for *in vivo* medical applications. The successful creation of a fully functioning and useful nanocomputer will require the synthesis of logic circuits with molecular-scale components. As discussed below, the four main types are nanocomputers under study today are those of electronic, mechanical, chemical and quantum origin.

Electronic Nanocomputers

Electronic nanocomputers are computation-capable devices at the nanoscale that use electrical signals to store and process information. It is envisaged that electronic nanocomputers would operate in much the same way as more conventional microcomputers of present-day. The wealth of knowledge and information relating to electronic computing will most likely drive much of the research on nanocomputer development towards

utilizing an electronic design. Due to obvious size limitations, electronic nanocomputers will not operate through the use of transistors but will still perform computing applications via the storage of information based on electron positioning. There are six primary technologies currently under study for the replacement of conventional transistors for nanocomputing including:

- Resonant Tunneling Transistor
- Single Electron Transistor
- Quantum Dot Cell
- Molecular Shuttle Switch
- Atom Relay
- Refined Molecular Relay

Table 10.1 compares the current status, advantages and disadvantages of all six electronic nanocomputing technologies.

While electronic computing receives the most attention, it is the application of molecular shuttle switch, atom relay and refined molecular relay technology which show the most promise in this area as these technologies may be incorporated into nanodevices such as nanorobots (see below) at the nanoscale. **Molecular shuttles switches** are based on a ring-shaped molecule which encircles and slides along a secondary chain molecule. The chain contains biphenol and benzidine stations between which the shuttle moves (Figure 10.7).

A **refined molecular relay** relies on atom movement with the rotational aspects of a molecular group affecting the electrical current of the system (Figure 10.8).

An **atom relay switch** is composed of precisely patterned lines of atoms on a substrate in a cross formation with a mobile atom providing the switching capabilities (Figure 10.9).

Mechanical Nanocomputers

Mechanical nanocomputers are defined as computation-capable devices at the nanoscale that use nanosized gears to store and process information. The concept behind mechanical nanocomputers is very similar, almost identical, to that implemented during the time period from the 1940s to the 1970s for mechanical calculators. A slide rule is an example of a mechanical computer. **Rod logic**, defined as displacement of solid rods

Table 10.1 Comparison of nanoelectronic computing technologies (Recreated from presentation by Patrick Kennedy, John Maley and Sandeep Sekhon of the Catholic University of America)

Device	Status	Advantages	Disadvantages
RTT	Capable of large-scale fabrication	Logic compression semiconductor-based	Limits on scaling—similar to MEMS
SET	Operate only at very low temperatures	High gain operation	Low temperature; difficult to control
Quantum Dot Cell	Can be fabricated but still theoretical	Wireless; low energy dissipation	Difficult design rules; susceptible to noise
Molecular Shuttle Switches	Experimental; can only switch chemically	Small and robust	Slow switching speed; how to interconnect?
Atom Relay	Theoretical	Very high speed; Subnanometer size	Very unreliable
Refined Molecular Relay	Theoretical	Subnanometer size; More reliable than atom relay	How to fabricate and how to interconnect?
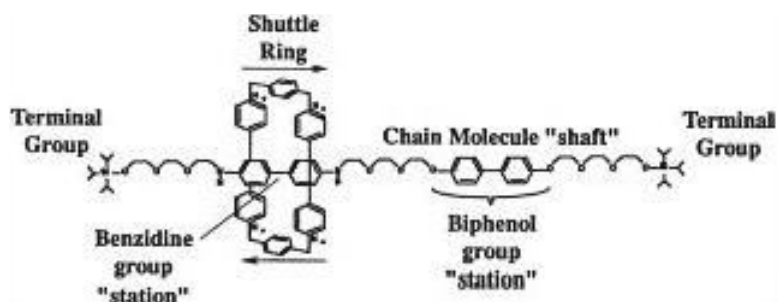
FIGURE 10.7 Diagrammatic illustration of a molecular shuttle switch. (Courtesy of Freitas, R.A., 1999; reprinted with permission.)
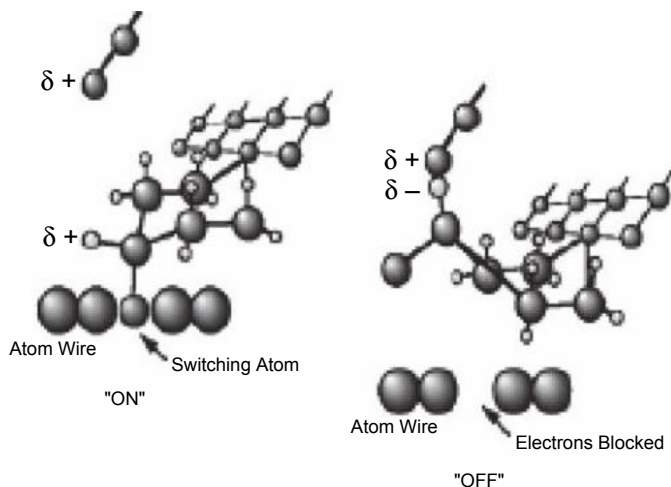
FIGURE 10.8 Diagrammatic illustration of a refined molecular relay. (Courtesy of Patrick Kennedy, John Maley and Sandeep Sekhon of the Catholic University of America; reprinted with permission.)

to represent the digital signal in theoretical mechanical nanocomputers, is the driving force behind their development. It is predicted that rod logic implemented in the form of precisely controlled molecular gates would enable the application of random access memory (RAM), mass storage capabilities and programmable logic arrays (Drexler *et al.*, 1989 and Figure 10.10). Eric Drexler (see Chapter 1 and Focus Box 10.1), and Ralph Merckle, pioneer theoreticists in molecular nanotechnology, are the leading pioneers behind the development of mechanical nanocomputers based on rod logic. Drexler has hypothesized that a mechanical nanocomputer could contain ~10^6 transistor-like interlocks within a 400 nm-sized cube. The speed of the system could theoretically reach 1 GHz and consume only ~60 nW of power.

There are issues with the theory behind mechanical nanocomputers, however. One of the primary concerns relates to the extremely slow process that would be required to assemble the computers. Parts would have to be assembled under atomically precise conditions, most likely through the use of a scanning tunneling microscope (bottom up approach; see Chapter 1). Thus large-scale production of mechanical nanocomputers is currently not realistic and many researchers believe the reliability of the parts at this scale would suffer.
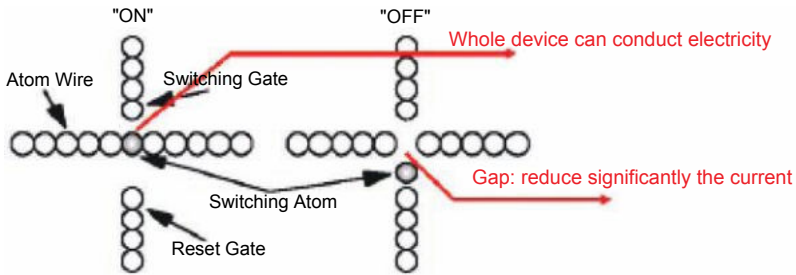
FIGURE 10.9 Diagrammatic illustration of an atom relay switch. (Courtesy of Patrick Kennedy, John Maley and Sandeep Sekhon of the Catholic University of America; reprinted with permission.)

Chemical and Biological Nanocomputers

Chemical nanocomputers are defined as computation-capable devices at the nanoscale that store and process information in terms of chemical structures and/or chemical interactions. Perhaps the most significant scientific advancement in the field of chemical nanocomputation was accomplished in 1994 by Leonard Adelman in the Department of Computer Science at the University of Southern California in Los Angeles who used fragments of DNA to compute the solution to a complex graph theory known as a Hamiltonian path problem. A **Hamiltonian path** is defined as a path in an undirected graph which visits each vertex exactly once. In the study a small graph was encoded in the molecules of DNA and the
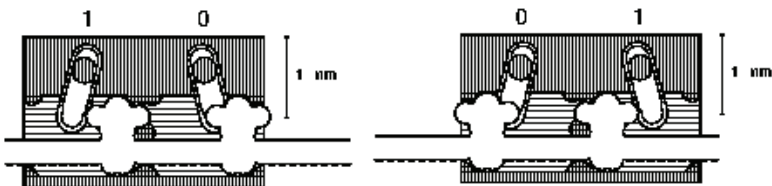
FIGURE 10.10 Diagrammatic illustration of a rod logic gate. (Upper) Mechanism for two nanocomputer gates, initial position. One control rod with two gate knobs is seen laterally; two more rods with knobs are seen end on. Each rod with associated knobs is a single molecule. (Lower) The lateral rod has been pulled to the left during computation. Notice that one of the end-on rods has now been blocked and the other one unblocked in mechanical mimicry of the transistor action. (Courtesy of Drexler, 1989; reprinted with permission.)

operations of the computation were performed with standard protocols and enzymes (Adleman *et al.*, 1994). Adleman's method utilized the unique identities of the molecular subunits to represent the vertices of a network. Combinations of these sequences formed randomly during large-scale parallel reactions in a test tube and thus represented random paths through the network or graph. Adleman was able to subsequently extract the correct answer to the graph theory from the plethora of random paths represented by the final product DNA strands (Adleman *et al.*, 1994 and Figure 10.11). His work led to the coining of the term **DNA computing**, which is defined as computation based on the use of DNA, biochemistry and molecular biology instead of traditional silicon-based technologies. While Adleman's system represents a very impressive demonstration of computation based on nucleic acid templates, the system is largely uncontrollable and currently lacks efficient input and output techniques to make it a plausible platform for computing at the nanoscale.

Quantum Nanocomputers

Quantum nanocomputers are the fourth and perhaps most intriguing prospect of information storage at the nanoscale. They are computers at the nanoscale that apply quantum mechanical phenomena, such as
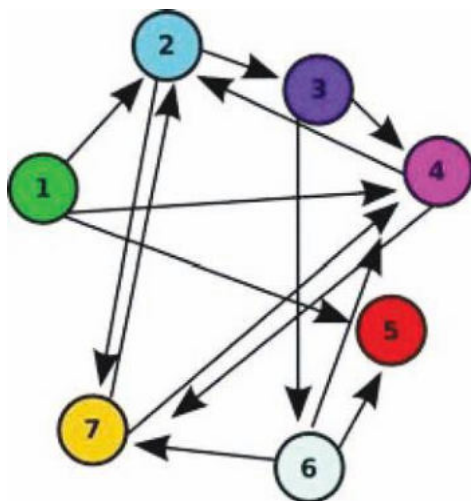
FIGURE 10.11 Example of a Hamiltonian path. (Courtesy of Suramya.com; reprinted with permission.)

superposition and entanglement, to store information. The brain child of Paul Benioff and Richard Feynman (see Focus Box 10.2 and Chapter 1) during the 1980s, quantum nanocomputers hold each bit of data as a quantum state of the computer, with information stored as the spin of the orientation or state of an atom. **Wave interference**, which is defined as the interaction of particle waves, would be utilized to calculate correct outputs by constructive interference thus eliminating incorrect outputs through wave-canceling destructive interference. The system is based on **qubits**, which are units of quantum information analogous to the classical bit. While bits can represent a value of 0 or 1, qubits can represent 0, 1 or a superposition of both. Qubits are defined by a state vector in a quantum mechanical system (Figure 10.12).

Perhaps the most significant disadvantage of quantum nanocomputers is instability, with instantaneous electron energy levels difficult if not impossible to predict and even more difficult to control. For example, an electron can easily fall to a lower energy level thus emitting a photon. These photons can strike atoms and thus drive one or more of its electrons to a higher energy level.

Each theoretical nanocomputing system described above represents significant advances towards the ultimate goal of creating a true
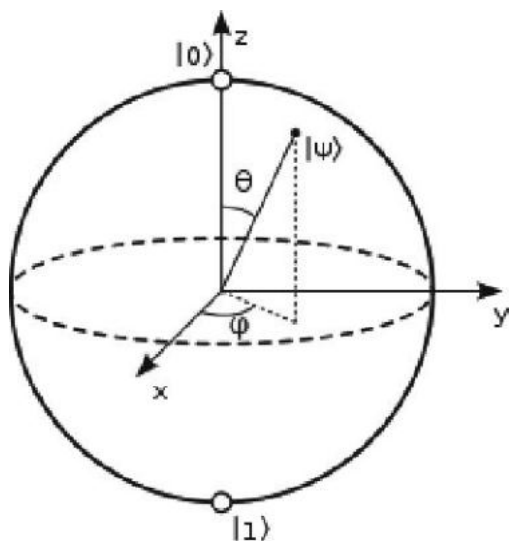
FIGURE 10.12 Bloch sphere representation of a qubit. (Courtesy of Wikipedia. org; reprinted with permission.)

nanocomputer, yet there are problems common to each technology used that must be addressed along with those issues that are specific to each. Perhaps the most significant is the problem of interconnectivity. How do external components interface with the nanocomputer? This will most likely be a central focus of scientists moving towards the development of a true nanocomputer that can be integrated on-board some or all of the futuristic nanorobots described below.

Therapeutic Nanorobots

The nanocomputing systems described above are crucial components of next-generation nanotechnology-based therapeutics machinery. This section describes some conceptual examples of **nanorobots** (also referred to as "**nanobots**"), which are defined as robotic structures, nanoscale in size or utilizing nanoscale parts, which perform complicated, often repetitive tasks at the nanoscopic level. Much of this theory and the detailed conceptual design of nanorobots for use in medicine can be credited to Robert A. Freitas Jr. (see Focus Box 10.3). Some of his more high-profile concepts on and examples of nanorobots as therapeutic platforms are described below.

Focus Box 10.3 Robert A. Freitas Jr., respirocytes and the future of nanomedicine

Robert A. Freitas Jr. is perhaps the world's foremost expert on futuristic visions regarding nanotechnological applications in medicine. He has conceived of numerous nanorobot-based therapeutic strategies, including in 1996 the popular "respirocyte" (see chapter description) which was the first detailed technical design study of a medical nanorobot ever published in a peer-reviewed mainstream biomedical journal. He serves on the Editorial Boards of 9 medical or nanotech journals, co-authored the book *Kinematic Self-Replicating Machines* (Landes Bioscience, 2004), co-founded in 2006 the Nanofactory Collaboration to build the first working nanofactory, won the prestigious 2009 Feynman Prize in nanotechnology for theory, and in 2010 was awarded the first U.S. patent on diamond mechanosynthesis. He is currently a Senior Research Fellow at the Institute for Molecular Manufacturing in Palo Alto, California. (Photo reprinted courtesy of R.A. Freitas Jr.)

Respirocytes

Respirocytes are hypothetical artificial red blood cells, roughly 1 micron in diameter, that can supplement or perhaps even replace the function of much of the human body's normal cellular respiratory system. A respirocyte has the capacity to mimic the function of hemoglobin-containing red blood cells (RBCs) to carry oxygen to, and carbon dioxide away, from cells in the body. The current design theory suggests that a typical respirocyte would be composed of 18 billion diamondoid atoms arranged as a microscopic pressure tank that could be filled with oxygen and/or carbon dioxide up to 1000 atmospheres (ATMs) (Freitas, 1998 and Figure 10.13). It would contain an on-board computer as well as numerous chemical and pressure sensors which would enable a physician to program it remotely via externally applied acoustic signals. Based on Freitas' calculated physics, in theory each respirocyte could store and thus transport up to 236 times more oxygen than a normal red blood cell. Thus if all RBCs in a human's circulatory system were replaced by fully functioning respirocytes that individual would have
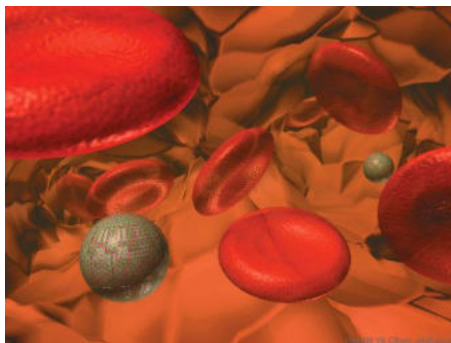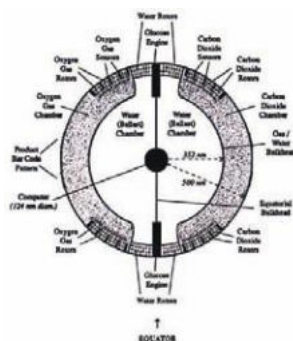
FIGURE 10.13 **Hypothetical respirocyte.** (Left) Equatorial cutaway view of respirocyte. The oxygen gas chamber is at left (south pole), the carbon dioxide gas chamber is at right (north pole), and the water (ballast) chamber occupies the center, surrounding the onboard computer system. The equatorial bulkhead separates the north and south hemispheres of the device. (Right) Artist's rendering of respirocytes (grey) alongside red blood cells in the circulatory system. (Courtesy of the Foresight Institute; reprinted with permission.)

the ability to hold his or her breath for four hours or sprint at full speed for fifteen minutes without taking a breath!

Clottocytes

Hemostasis is a complex process which results in the inhibition of bleeding and involves both blood clotting and coagulation, primarily through the activation of platelets. The clotting and coagulation of blood to stop bleeding internally or at the site of open wounds is critical to human survival. The development of new technologies to aid or speed this process is of intense focus, not only for applications to healthy individuals upon injury but could considerably benefit those with clotting and coagulation disorders such as hemophiliacs. **Clottocytes** are theoretical artificial mechanical blood platelets that may have the ability to promote hemostasis in as little as one second, which is on the order of 100 to 1000 times faster than the normal platelet response. They consist of biodegradable fiber mesh that is folded into a compact formation until needed at a site of bleeding. An on-board computer activates unfolding of the mesh at the site of an injuring blood vessel, with soluble thin films degrading in the presence of plasma water to expose a sticky mesh complementary to blood group antigens
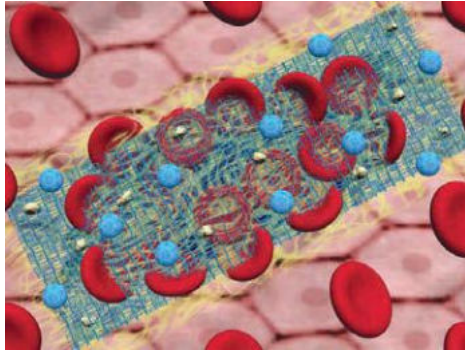
FIGURE 10.14 Artist's rendering of clottocytes in action. Eleven clot-inducing medical nanorobots with fully deployed netting are shown embedded in a patch-like growing clot with red cells and fibrin strands involved. (Courtesy of Robert A. Freitas Jr. and the Foresight Institute; reprinted with permission.)

present on the surfaces of RBCs (Figure 10.14). This allows for trapping of the red blood cells to create a netting composed of both cells and mesh to halt bleeding. Clottocytes may wirelessly communicate with one another to drive activation of mesh unfolding upon detection of factors denoting an injured blood vessel, which would enhance the repair response. It is predicted based on the size (~2 microns in diameter) and proposed efficiency of the system that clottocytes could theoretically perform a clotting function similar in efficiency to that of natural biological platelets but at only 0.01% concentration in the blood stream, or about twenty nanorobots per cubic millimeter of serum. This represents an increase in efficiency of ~10,000-fold over an equal volume of natural platelets (Bushko, 2002).

Microbivores

The development of new technologies for the elimination of blood-borne pathogens is a major focus of the research and medical communities. While vaccines allow for an immune response to attack, sequester and remove foreign invaders from the body, vaccination is often ineffective against certain pathogens, such as, for example, the human immunodeficiency virus. **Phagocytes** are white blood cells that protect the body by ingesting harmful foreign particles such as bacteria and dead or dying cells (**phagocytosis**). They are crucial in the body's constant fight against

infections and in the promotion of subsequent immunity. **Microbivores** are theoretical nanorobotic devices that could provide eradication of blood-borne pathogens in a manner similar to that of phagocytes. Robert A. Freitas Jr. has envisioned a microbivore as an oblong spherical nanorobot, 3.4 microns and 2 microns in diameter along its major and minor axes respectively, consisting of 610 billion precisely arranged structural atoms plus ~150 billion gas or water molecules. This size would allow for passage through capillaries of all sizes. Continuous power consumption is predicted to be on the order of 100–200 pW allowing for the digestion of trapped microbes at a rate of up to 2 microns^3 per 30 seconds. This is sufficient for the eradication of almost any species of bacteria (Figure 10.15).
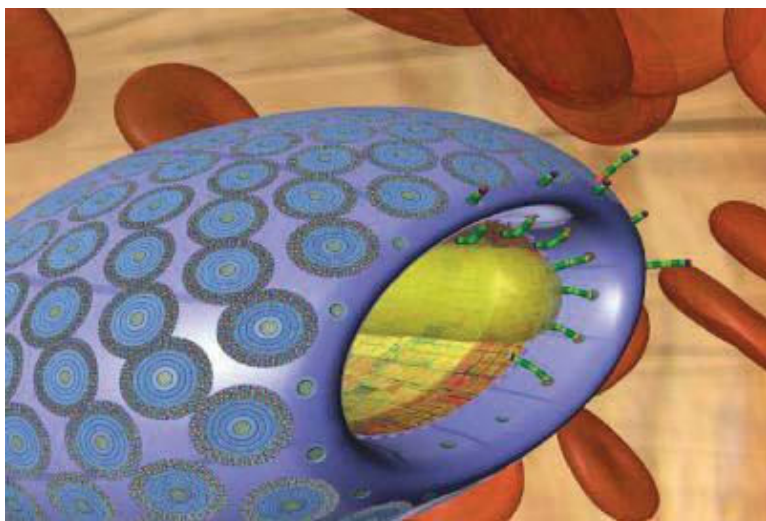
FIGURE 10.15 Artist's rendering of a microbivore. The microbivore, an artificial white cell, floats along in the bloodstream until it encounters a pathogen. In this still-theoretical medical nanorobot scenario, a rod-shaped bacterium has been trapped by binding site disks on the microbivore hull and then transported by tiny manipulator arms to the ingestion port, whereupon the microbe is digested inside the device. The binding site arrays appear as multicolored circular dapples on the blue sapphire-colored surface. In this image, the mouth of the microbivore is open and a few of the telescoping grapple arms are extending from the surface of the device to feed rod-shaped bacterium into the microbivore's mouth. (Courtesy of the Foresight Institute; reprinted with permission.)

The ingestion and digestion process would involve specific but reversible binding of bacterium to the microbivore surface via receptors with transport of the bacterium to an open port at one end of the microbivore by telescoping robotic grapples. Digestion occurs in two phases, mechanical mincing followed by exposure to degradation enzymes to yield harmless bi-products such as simple sugars, amino acids and mononucleotides.

Chromallocytes

DNA damage, whether the result of inherited genetic mutations or exposure to environmental mutagens, has resulted in a wide array of human anomalies including, for example, cancer and **Down's Syndrome**, which is a congenital disorder caused by having an extra chromosome 21. The ability to repair mutations resulting in these and other conditions would revolutionize many aspects of medicine as we know it. Yet, current efforts at gene therapy are rudimentary at best and have only shown mild promise with numerous cases of unwanted side effects observed. **Chromallocytes**, also the conceptualization of Robert A. Freitas Jr., are futuristic hypothetical cell repair nanorobots capable of performing chromosomal replacement therapy. Shaped like a lozenge, the typical chromallocyte would measure 4.18 microns and 3.28 microns along cross-sectional diameters and measure 5.05 microns in length. Continuous power consumption is estimated to be 50–200 pW with up to 1000 pW consumed in brief bursts when performing **outmessaging**, which is defined as communication from an *in vivo* nanorobot to the attending physician. Chromallocytes would be capable of vascular travel into target tissues and/or organs followed by cell membrane penetration to access the nucleus where genetic material is in need of repair and chromosomal replacement therapy (CRT) would occur. CRT is performed via the use of a funnel assembly and a **proboscis**, a large axially positioned manipulator that collects old chromatin and transfers new chromatin in its place. Actual CRT occurs when the funnel assembly and proboscis extend into the nucleoplasm with the proboscis binding the chromosome of interest via a chromophilic (chromosome-binding) adhesion surface based on sequence-specific DNA binding sites similar to those found in restriction endonucleases. Spooling of the proboscis results in a bolus of unwanted chromosomal material. Retraction of the proboscis allows for the removal

(A) Front View   (B) Side View

Retracted   *Extended*

(C) Funnel/Proboscis Extension   (D) Device w/Funnel Partly Extended
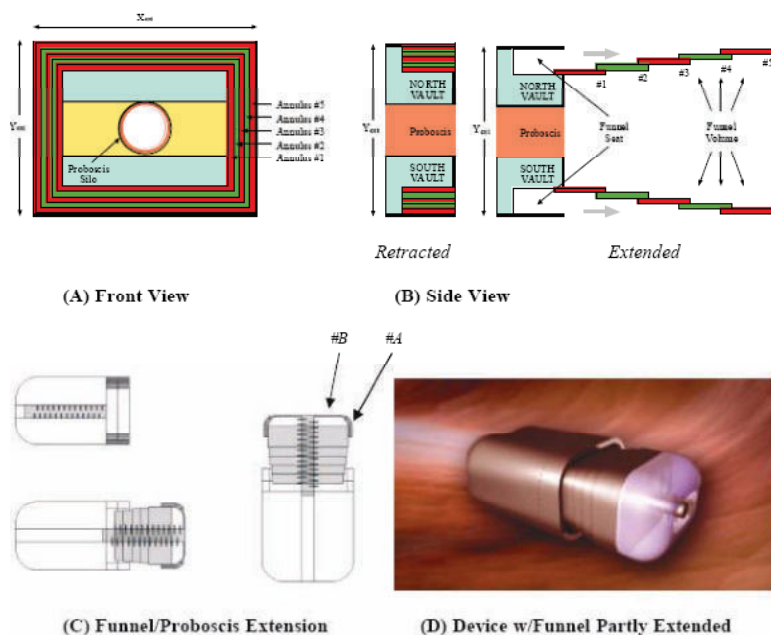
FIGURE 10.16 Schematics of telescoping funnel assembly and proboscis operation. Images (C) and (D) are © 2006 Stimulacra LLC and Robert A. Freitas Jr. (Courtesy of Freitas, 2007; reprinted with permission.)

of unwanted chromosomal material followed by replacement with new material via a repeat penetration of the nucleus (Freitas, 2007 and Figure 10.16).

PERSONALIZED NANOMEDICINE

Nanoparticle-Based Theranostics

Theranostics can be defined as the process involving a diagnostic therapy for an individual patient. These are often custom tailored for the patient as treatment efficacy is closely monitored during the treatment phase. Yet with the advent of nanotechnologies that may allow for simultaneous disease detection and treatment, a more appropriate definition for this term in the context of this book may be the simultaneous diagnosis and treatment of a disease or disorder. Recent advances in nanoparticle-mediated tagging and thermal ablation of cancer cells is

perhaps the most realistic and near-term application of theranostics in nanomedicine. As described in Chapter 2, examples of targeting tumor cells with nanoparticles followed by ablation of these cells through heat or drug release are numerous. One recent example of the development of a combination theranostic using nanotechnology is that of **plasmonic nanobubbles**, which are gold nanoparticles that generate transient photothermal vapor. A team of researchers lead by Dmitri Lapotko of the Joint US-Belarusian lab for fundamental and biomedical nanophotonics at Rice University has developed a novel cancer theranostics method based on gold nanoparticle-generated transient photothermal vapor nanobubbles (PNBs). The PNBs combine optical scattering for diagnosis with intracellular mechanical damage of target cells for therapy. The system works by clustering gold nanoparticles around molecular targets within cancer cells followed by exposure to a laser pulse, which results in light scattering by small PNBs and mechanical damage to cells via expansion and contraction of larger PNBs. Thus simultaneous detection and eradication of cancer cells is accomplished (Lukianova-Hleb *et al.*, 2010 and Figure 10.17). It is tempting to speculate that in the not-too-distant future first diagnoses of cancer can be coupled with immediate therapeutic intervention thus saving valuable time, reducing chances of metastasis and increasing patient survivability rates.

Whole-Genome Diagnostics

Whole-genome diagnostics is the comprehensive sequencing and analysis of an individual's entire genome for purposes of determining that individual's genetic predisposition to disease. Advances in technologies for nucleic acid sequencing are maturing at such a pace that many scientists and researchers envision a day when personalized medicine will mean a full analysis of an individual's genome. By assessing DNA sequence, doctors may implement short- or long-term medicinal preventative therapeutics to ward off inherited and often inevitable anomalies encoded by the human genome such as, for example, cancer. Indeed, as technological advances in this area become realized, such as nanopore-based DNA sequencing (see Chapter 8), the costs of whole genome analysis are decreasing. In 2007 two entire human genomes were successfully sequenced (that of Craig Venter and James Watson) at a cost of roughly $1,000,000 each. Just one year later, in 2008, the first human genome of a woman was sequenced at a cost of
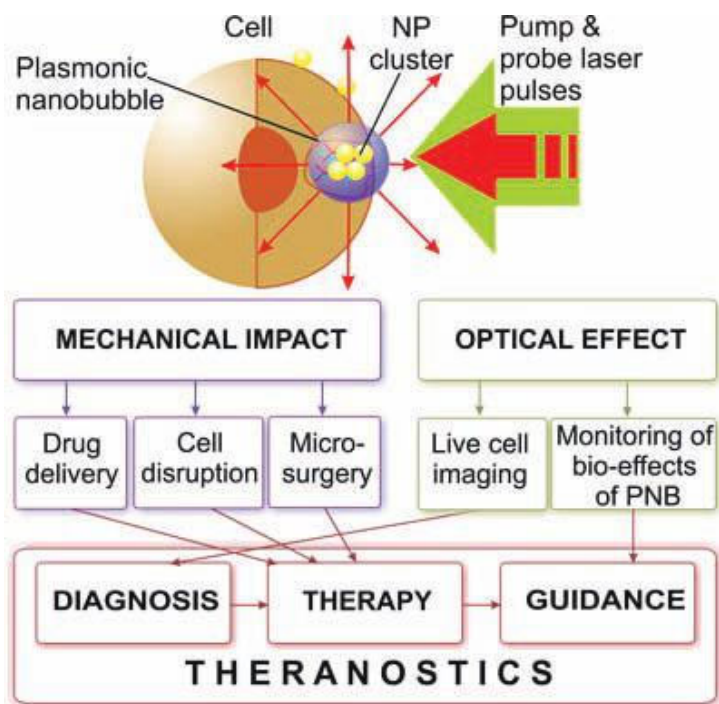
FIGURE 10.17 Diagrammatic illustration of the use of plasmonic nanobubbles in theranostics. (Courtesy of Dmitri Lapotko, Rice University; reprinted with permission.)

$60,000. A logarithmic plot of sequencing costs vs. time suggests that the cost of sequencing a single base-pair of DNA is cut in half roughly every 1.9 years (Kurzweil, 2005 and Figure 10.18). This suggests that at present time the total cost to sequence an individual's entire genome could be well under $1,000, making whole genome analysis for individuals well within financial reach.

While the costs for comprehensive DNA sequencing are rapidly decreasing, the speed of data output must correspondingly increase if whole genome diagnostics is to become a reality. Nanopore-based DNA sequencing platforms may address throughput by eliminating the need for nucleotides to be labeled as in the case of the **Sanger method** of sequencing DNA, which, developed by Frederick Sanger in 1975, is
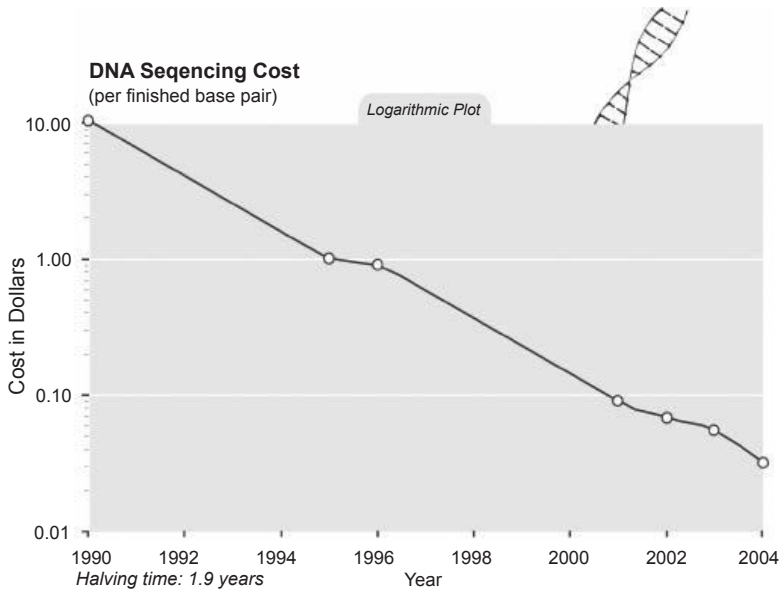
FIGURE 10.18 Graph of DNA sequencing costs vs. unit time. (Courtesy of Kurzweil, 2005 and reprinted with permission.)

a DNA sequencing procedure involving nucleic acid chain termination using dideoxynucleotide triphosphates. Nanopore diameters are small enough to allow passage of single nucleotides and, as each nucleotide has a unique molecular architecture, these may be easily identified. In a manner somewhat similar to that of Amit Meller of Boston University described in Chapter 8, researchers at Purdue's Birck Nanotechnology Center in West Lafayette, Indiana have demonstrated that nanopores can be used to rapidly and precisely detect specific DNA sequences and may be the basis behind a tool for personalized whole genome diagnostics. The research was led by Rashid Bashir in Purdue's School of Electrical and Computer Engineering and the Weldon School of Biomedical Engineering. Bashir's team fabricated nanopores in a thin silicone membrane which were subsequently immersed in DNA-containing solutions. Voltage application across the membrane allowed for negatively charged DNA to flow through the channel and it was discovered that single strands complementary (matching) to those in the channels flowed faster than
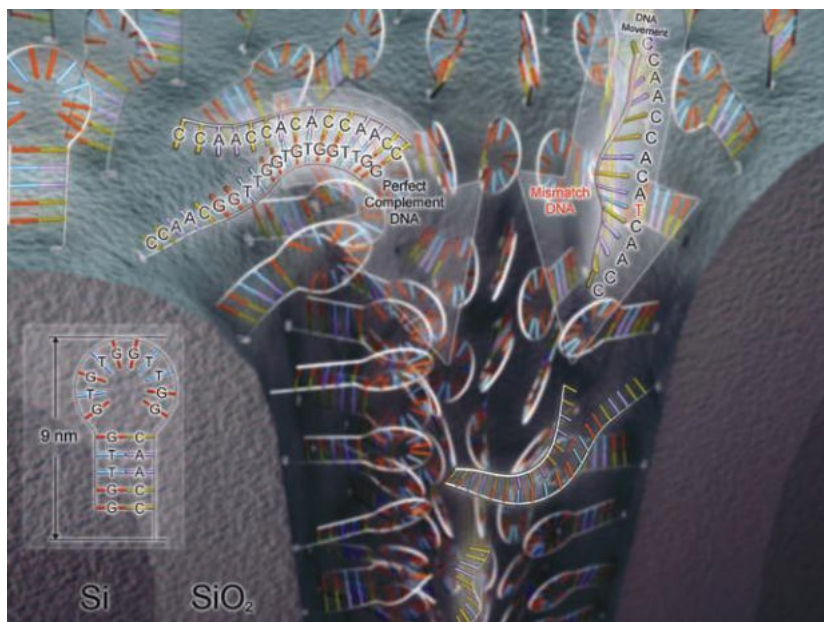
FIGURE 10.19 Artist's rendition depicts how "nanopore channels" can be used to rapidly and precisely detect specific sequences of DNA. The tiny channels, which are 10 to 20 nanometers in diameter and a few hundred nanometers long, were created by researchers from Purdue's Birck Nanotechnology Center. The Purdue researchers "functionalized" the channels so that single strands of DNA were attached inside each one. (Courtesy of Seyet LLC; reprinted with permission.)

non-complementary strands with a current difference clearly detectable. This allowed for the detection of different types of nucleotide sequences (Iqbal *et al.*, 2007 and Figure 10.19).

NANONEPHROLOGY

It is estimated that 11.5 percent of adults aged 20 and older have some form of kidney disease and 900,000 patients wo rldwide require dialysis annually. Thus the creation and development of next-generation diagnostic and therapeutic applications for kidney-related disease is a major focus of doctors and researchers. **Nanonephrology** is a broad, futuristic category of nanomedicine that can be defined as the study of kidney structures at the atomic level and the development of

nanotechnologies for the diagnosis or treatment of renal disorders. Some speculate that nanotechnology may be applied for the development of artificial kidneys. California-based Biophiltre, Inc. is a company that is developing a nanotechnology-based filtering system that mimics the function of the human kidney and specifically the basic structural and functional unit of the kidney known as the **nephron**. Biophiltre's platform, referred to as a human nephron filter (HNF) and based on the research of Dr. Allen Nissenson of the Department of Medicine's Division of Nephrology at the University of California, Los Angeles Medical Center, is a two-membrane system that mimics nephron filtration functionality. The first membrane (the G membrane) acts as the functional equivalent of the **glomerulus**, which is a capillary tuft that performs the first step in filtering blood to form urine. It generates a plasma ultrafiltrate containing all solutes up to the molecular weight of albumin. The second membrane (the T membrane) mimics the **renal tubule** which is the portion of the nephron containing the tubular fluid filtered through the glomerulus. Its function is to selectively reclaim certain designated solutes for the maintenance of body homeostasis by convection. The T membrane is composed of 1.6 quadrillion nanopores, several nanometers in size and 1 to 5 nanometers apart. Each membrane has been synthesized by atomically precise manufacturing procedures and consists of a polycarbonate wall surrounding a polymer matrix. The system is unique in that no dialysate is used in contrast to more conventional dialysis technology. Computer models have suggested this filtration design has the capacity to provide double the filtration of conventional dialysis with a continuous filtration rate of 30 ml/min. The plan is for the system to be packaged in a 3–4 pound wearable cartridge-based system running on a 12 volt battery and is expected to be far superior in performance to conventional dialysis systems while operating as a remote unit (Nissenson *et al.*, 2005 and Figure 10.20). This device may represent the first step in a transition towards an implantable nanotechnology-based filtration system that could transform the treatment of renal disorders.

NANONEURAL INTERFACES

A **nanoneural interface** is a biocompatible substance that interacts and intermingles with neurons for either the enhancement or transmission of neuronal signaling. The development and application of wireless MEMS-
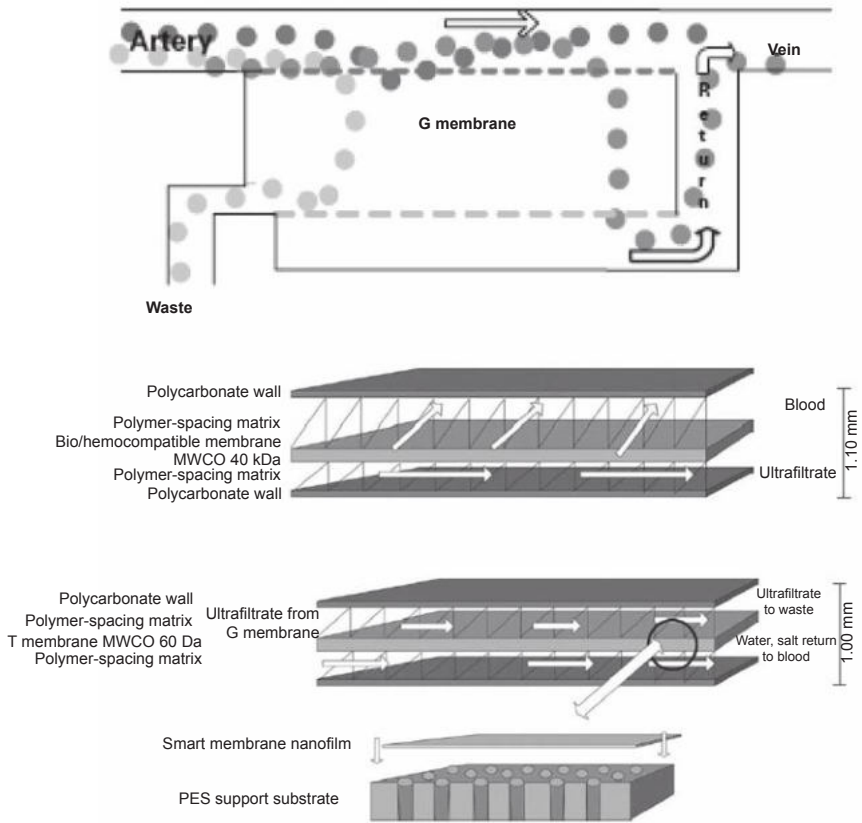
FIGURE 10.20 Diagrammatic outline of the Biophiltre artificial human nephron filter and its components. (Top) Outline of the system. (Middle) Diagram of the G membrane. (Lower) Diagram of the T membrane. (Courtesy of Nissenson *et al*., 2005; reprinted with permission.)

based devices for the detection and stimulation of neural activity has been a major focus in the field of bioengineering for the past twenty five years. The ability to enhance or possibly create electrical signals between neurons in damaged tissue using, for example, wireless neural transponder systems, would have major repercussions on the development of next-generation prosthetics and assisted robotics (Figure 10.21). An example of considerable progress made in this area is discussed in Case Study 10.1 below.
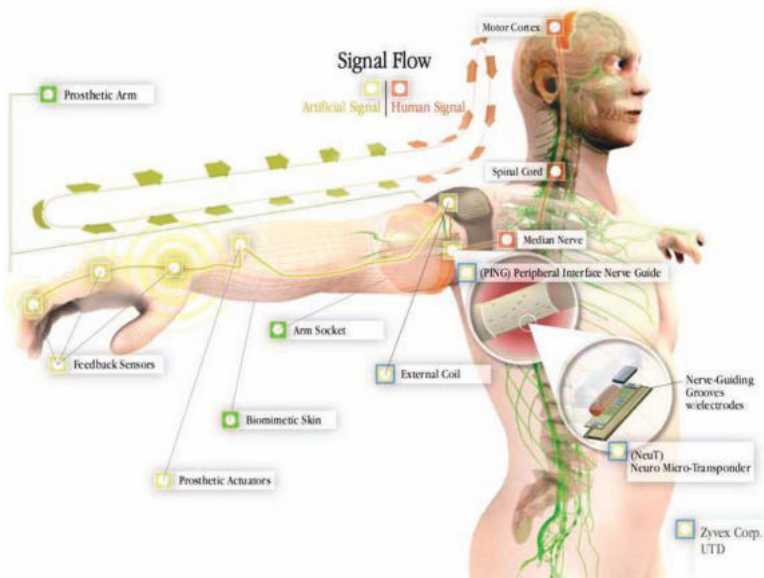
FIGURE 10.21 Diagrammatic illustration of the concept behind a wireless neural transponder. See text for details. (Courtesy of Zyvex Labs, LLC; reprinted with permission.)

OPTICAL IMAGING AT THE NANOSCALE

Endoscopy is defined as internal explorations of the body for medical reasons using an endoscope. It is implemented for diagnostic purposes to seek out lesions, tumors and other anomalies that require therapeutic intervention. Yet the current resolution capacity of the typical endoscope is considerably limited and thus abnormalities that occur on the nanoscopic or even microscopic scale go undetected. Researchers at the University of California, Berkeley have taken a major step towards addressing this issue by developing a "**hyperlens**" which takes them a step closer to nanoscale optical imaging. It is based on the capture of evanescent waves, which contain far greater detail and resolution than the propagating wave forms captured by optical imaging technologies. It consists of multiple layers of silver and aluminum oxide coated along the cavity of a quartz half-cylinder. Upon illumination of an object evanescent waves travel

Case Study 10.1: Biocompatible SU-8-based microprobes for recording neural spike signals from regenerated peripheral nerve fibers.

Gareth Hughes, formerly of Zyvex Corporation in Richardson, Texas and J.B. Lee's group in the Department of Electrical Engineering at the University of Texas, Dallas, also in Richardson have developed a biocompatible neuro-microprobe constructed using SU-8 microfabrication techniques. **SU-8** is an epoxy-novolac resin and a well-established negative photoresistor for microfabrication and microengineering. The microbe structure consists of bipolar longitudinal gold electrodes recessed below grooves designed to provide pathways for the growth of regenerative axons. The grooves also limit the number of neuronal fibers that come into contact with the longitudinal electrodes. The **Sciatic nerve** is the largest nerve in the vertebrate body and is a sensory and motor nerve originating in the sacral plexus and running through the pelvis and upper leg. After surgical implantation of the microprobes into transected Sciatic nerves of rats the researchers observed spike signals for periods from 4 to 51 weeks. (Cho *et al*., 2008 and Figure 10.22).
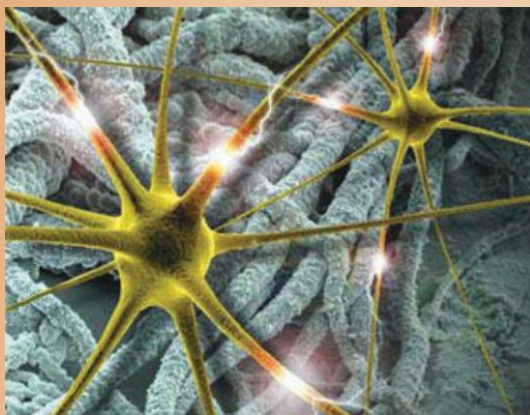
FIGURE 10.22 Illustration of a nanoneural interface. (Courtesy of TryNano. org; reprinted with permission.)

through the lens and are progressively compressed. This compression allows the image to be magnified by the time it reaches the outer layers of the lens and can subsequently be captured by a conventional optical lens (Figure 10.23). This mechanism is in effect a conversion of evanescent
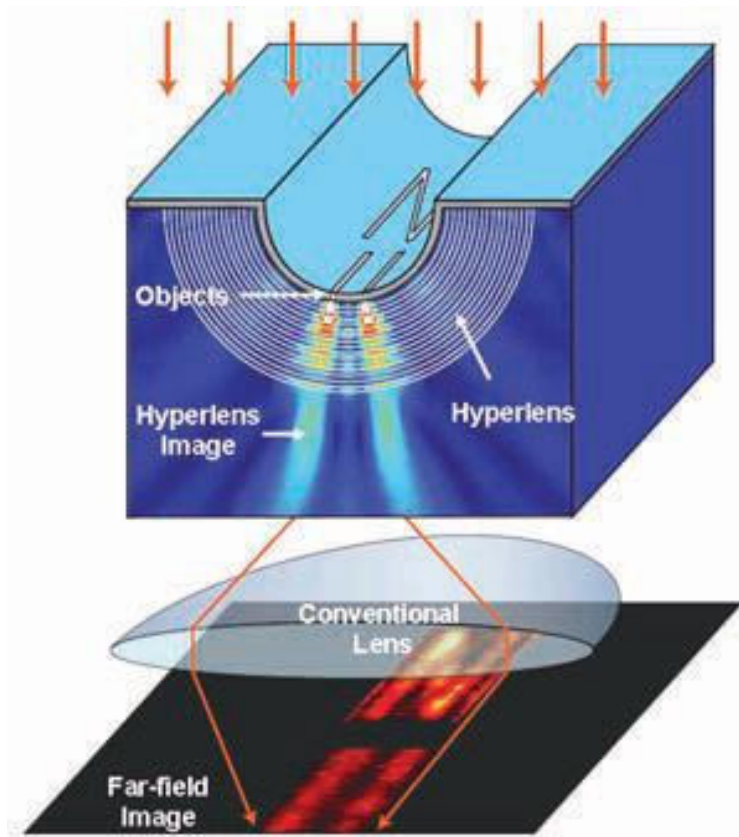
FIGURE 10.23 Diagrammatic illustration of a hyperlens capable of imaging at the nanoscale. (Courtesy of Xiang Zhang *et al.*, the University of California, Berkeley and Medgadget.com; reprinted with permission.)

waves to propagation waves. The hyperlens has the ability to beat the **diffraction limit** of a sample, which is the minimum angular separation of two sources that can be distinguished by a scopic instrument, and can image objects as small as 150 nm in size. This provides the possibility to endoscopically study individual cells in the body, the internal organelles of those cells and even cellular movement such as migration in real-time. It has the potential to revolutionize exploratory endoscopy and the real-time diagnosis of even the most minute of disorders only detectable at the nanoscale.
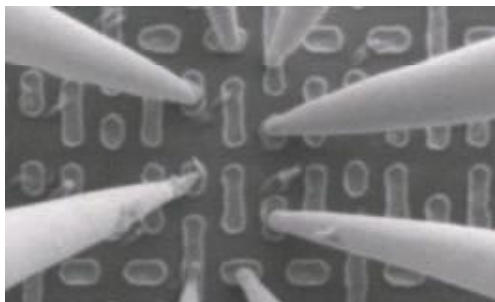
FIGURE 10.24 Eight probes characterize the stability and functionality of nodes on a 6T RAM bitcell. (Courtesy of Zyvex Instruments: reprinted with permission.) (www.zyvex.com)

ARTIFICIAL INTELLIGENCE AND "THE SINGULARITY"

Artificial intelligence is defined as the capacity for abstract thought, reasoning, planning, problem solving, communication and learning by machines and/or computers. Advancements in nanotechnology have driven artificial intelligence development to the point where it may play a role in the aiding of human cognition. As was described in Chapter 1, Moore's Law states that the number of transistors (nodes) that can be inexpensively placed on an integrated circuit chip doubles approximately every two years. This phenomenon has continued primarily as a result of advances in the ability to manufacture and analyze nodes on the sub-100 nm scale. With the development of nanoprecise machinery and failure analysis tools and equipment current node size continues to shrink and is now below 35 nm (Figure 10.24).

It is this precision at the nanoscale which will continue to drive node size and eventually integrated circuit chip size smaller, increasing computation efficiency and ultimately allowing for the use and application of integrated circuitry to drive the advancement of both human and artificial intelligence through a marrying of man and computer. It is forecast that this technological progress will become extremely fast and thus make the future of man and machine unpredictable and qualitatively different from today. This concept is known as the **Singularity**, and is the prediction and vision of the innovative scientist and entrepreneur Ray Kurzweil (see Focus Box 10.4). He envisions six epochs of evolution, four of which have

---

Focus Box 10.4 Ray Kurzweil and "the Singularity"

Ray Kurzweil is one of the world's leading inventors, futurists and thinkers with a twenty-year track record of accurate predictions regarding technological advances. He has pioneered such areas as music synthesis, speech and character recognition, virtual reality and cybernetic art. He was the principal developer of the first omni-font optical character recognition system, the first print-to-speech reading machine for the blind, the first CCD flat-bed scanner and the first text-to-speech synthesizer. His latest theories suggest a physical marrying of humans and machines to create a new level of intelligence driven primarily by advances in nanotechnology (see text for details). He is the recipient of the MIT-Lemelson Prize, the National Medal of Technology and has been inducted into the National Inventors Hall of Fame. (Photo courtesy of *the auters;* reprinted with permission.)
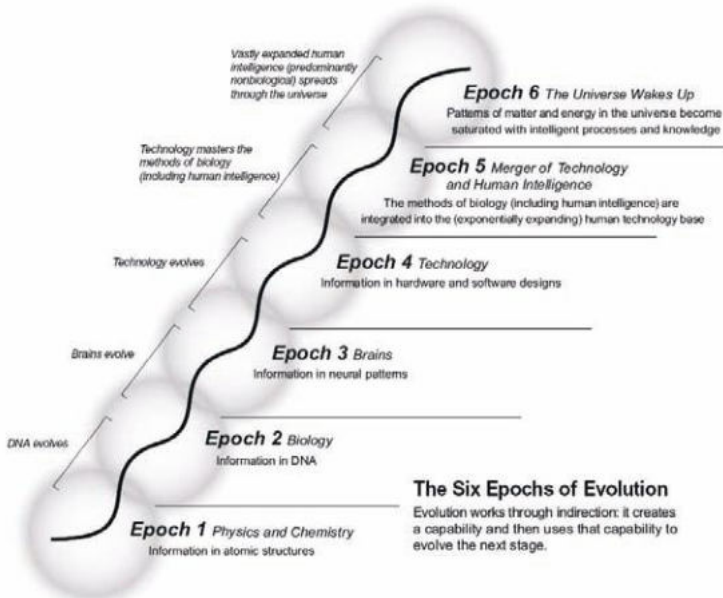
FIGURE 10.25 Illustration of the six epochs of evolution. (Kurzweil, 2005; reprinted with permission.)

FIGURE 10.26 Artist's rendering of the Transcendent Man. (Courtesy of *The Daily Galaxy*; reprinted with permission.)

already been reached. These epochs represent inflection points in the evolution of intelligence, both at the biological and artificial level. **Epoch 5** encompasses the joining of biological and artificial technology to bring forth the Singularity. It is an era in which human intelligence will become increasingly non-biological and trillions of times more powerful than it is today (Kurzweil, 2005 and Figure 10.25).

Kurzweil predicts that in this new world there will be no clear distinction between man and machine with a true merging of the two to create a cyborg-like entity he refers to as the **Transcendent Man** (Figure 10.26). As a result of achieving the Singularity, for all practical purposes human aging and illness will be reversed and world hunger as well as poverty will become extinct, with even death becoming a solvable problem. All of this, Kurzweil speculates, will be possible due to advancements in nanotechnology.

CHAPTER SUMMARY

Nanorobotics and Medicine

1. The three primary components of an effective therapeutic nanorobot are energy source, energy conversion mechanism and ability to convert used energy into valuable work.

2. Nanomotors exist in nature, the most notable example being kinesin.
3. The basic requirements for a biologically functioning nanomotor are appropriate scale, low energy requirements, autonomous operation, biological compatibility and long-term stability.
4. Rotaxane-based nanomotors are intramolecular in nature and powered by optomechanical energy conversion.
5. Nucleic acid nanomotors take advantage of inter- or intramolecular hybridization principles.
6. Nanotube-based nanomotors have been created in which the tube acts as an axis with metal rotating as an actuator.
7. Self-powered nanowire-based nanomotors have been constructed which run on the decomposition of hydrogen peroxide.
8. Nanogears are essential for converting nanomotor-generated kinetic energy into work.
9. A theoretical molecular planetary Nanogear has been conceptualized that converts shaft power from one angular frequency to another.
10. Nanocomputers may be built using electronic, mechanical, biochemical or quantum technologies.
11. Electronic nanocomputers will function via the storage of information based on electron positioning.
12. Molecular shuttle switch, atom relay and refined molecular relay technologies show the most promise for use in electronic nanocomputers.
13. Mechanical nanocomputers are based on rod logic.
14. A primary concern of mechanical nanocomputer development is the slow process that would be required for assembly.
15. Chemical nanocomputers based on DNA computing have been demonstrated to solve complex problems such as a Hamilton path.
16. Superposition and entanglement are used by quantum nanocomputers to store information.
17. qubits are the basis behind quantum nanocomputing and are analogous to classical computation bits.
18. The main disadvantage of quantum nanocomputing is electron energy level instability.
19. Nanorobots are currently theoretical in nature but could become a reality as the field of nanotechnology matures.
20. Respirocytes are conceptual nanorobots that could potentially replace red blood cells.

21. Theoretical clottocytes may aid or speed the process of blood clotting at a site of injury.
22. Clottocytes work by providing a biodegradable fiber mesh at the site of bleeding.
23. Theoretical microbivores act as nanorobotic phagocytes to eradicate blood-borne pathogens.
24. Chromallocytes are theoretical chromosome-repairing nanorobots that perform chromosomal replacement therapy via a proboscis.

Personalized Nanomedicine

1. Nanoparticle-mediated thermal ablation of cancer cells is a prime example of next-generation theranostics.
2. Plasmonic nanobubbles (PNBs) combine optical scattering for diagnosis with intracellular mechanical damage of target cells for therapy.
3. The costs of full-scale human genome sequencing are rapidly decreasing thus making whole-genome diagnostics more affordable.
4. Nanopore-based DNA sequencing may soon replace the Sanger dideoxy method.
5. A synthetic human nephron filter (HNF) has now been developed based on a two-membrane system that mimics nephron filtration functionality.
6. Nanoneural interfaces are of intense interest for improving inter-neuronal signaling efficiency.
7. An Su-8-based wireless neural transponder has been developed for recording neural spike signals *in vivo*.
8. Nanoscale endoscopy has been accomplished via the construction of a hyperlens that takes advantage of evanescent waves and can beat the diffraction limit of a sample.
9. The Singularity, a marrying of man and computer, is theorized to occur in the fifth out of six Epochs of evolution as technology progresses and the future of man and machine become unpredictable.
10. The Transcendent Man represents a time in the future when aging and illness will be reversed and even death will become a solvable problem.

KEY TERMS

- Nanomotor
- Kinesin
- Optomechanical Energy Conversion
- Rotaxane
- Nucleic Acid-Based Nanomotor
- Nanotube-Based Nanomotor
- Nanowire-Based Nanomotor
- Nanogear
- Molecular Planetary Gear
- Nanocomputer
- Electronic Nanocomputer
- Molecular Shuttle Switch
- Refined Molecular Relay
- Atom Relay Switch
- Mechanical Nanocomputer
- Rod Logic
- Chemical Nanocomputer
- Hamiltonian Path
- DNA Computing
- Quantum Nanocomputer
- Wave Interference
- Qubit
- Nanorobot
- Nanobot
- Respirocyte
- Hemostasis
- Clottocyte
- Phagocyte
- Phagocytosis
- Microbivore
- Down's Syndrome
- Chromallocyte
- Outmessaging
- Proboscis
- Theranostics
- Plasmonic Nanobubble
- Whole-Genome Diagnostics
- Sanger Method
- Nanonephrology
- Nephron
- Glomerulus
- Renal Tubule
- Nanoneural Interface
- SU-8
- Sciatic Nerve
- Endoscopy
- Hyperlens
- Diffraction Limit
- Artificial Intelligence
- Singularity Theory
- Transcendent Man

REVIEW QUESTIONS

(Answers to the review questions can be found at **www.understandingnano. org.**)

1. What are the three primary components of an effective therapeutic nanorobot?
2. Cite an example of a naturally occurring nanomotor.

3. What are the five basic requirements behind the construction of a biologically functioning nanomotor?
4. Describe the functional basis behind J. Fraser Stoddart's rotaxane nanomotor and list at least three features of the system.
5. How does light energy get converted to kinetic energy by Weihong Tan's nucleic acid nanomotor?
6. How could one improve electron transfer and thus increase acceleration in a nanowire-based nanomotor?
7. What are the components of the "molecular planetary gear" and how does it work?
8. What are the four types of nanocomputers?
9. Cite at least four primary technologies currently under study for the replacement of conventional transistors for nanocomputing.
10. Which type of electronic nanocomputing technology would your use to achieve high speeds while retaining sub-nanometer size?
11. Diagram an atom relay switch.
12. What is the technological concept behind mechanical nanocomputers?
13. How does Leonard Adelman's DNA-based nanocomputer work?
14. What is the difference between bits and qubits?
15. Describe the design and concept behind a respirocyte.
16. How does a clottocyte trap red blood cells to create a netting to halt bleeding?
17. Describe the digestion mechanism of microbivores.
18. Describe the design and concept of Robert Freitas' conceptual cell repair nanorobot, the chromallocyte.
19. How do Dmitri Lapotko's plasmonic nanobubbles both diagnose and eradicate cancer cells?
20. Why might nanopores be superior to the Sanger dideoxy method of DNA sequencing?
21. Diagram the Biophiltre artificial human nephron.
22. What are two functions of the grooves in the Hughes/Lee neuro-microprobe?
23. Describe the components of a hyperlens and how it works in optical imaging.
24. Cite your opinion on the future realistic possibility of achieving Ray Kurzweil's Singularity in Epoch 5 and the evolution of the "transcendent man."

Appendixes

GLOSSARY

21st Century Nanotechnology Research and Development Act U.S. Public Law 108–153 authorizing $3.63 billion over four years for nanoscience, nanoengineering and nanotechnology research.

Acrylation conversion of a molecule (drug) to a salt or ester of an acrylic acid.

Acticoat a nanosilver-based wound dressing that speeds healing and reduces scar tissue formation developed by Nucryst Pharmaceuticals, Inc.

Active Targeted Drug Delivery the delivery of a drug to a particular tissue or cell type through specific and precise binding of the drug to the target tissues or cells.

Aliphatic carbon atoms linked in open chains.

Alliance for Nanohealth (ANH) founded in 2005, the first Texas-based collaborative research endeavor that uses nanotechnology to bridge gaps between medicine, biology, materials science, computer technology and public policy.

Alliance for Nanomedical Technologies a 2001 partnership between Cornell University, the University of Rochester, the Wadsworth Center and

Tompkins Courtland Community College to help nucleate and invest in nanomedical research programs in the state of New York.

Alpha methyl tryptophan (AMT) a tryptophan derivative used as a positron emission tomography (PET) ligand to identify epileptogenic tissues in several epilepsy conditions.

Alzheimer's Disease the most common form of dementia characterized by memory loss, confusion, irritability, mood swings and language breakdown.

American Recovery and Reinvestment Act (ARRA) a $787 billion stimulus package announced by the U.S. Federal government in 2009 for job creation, promotion of economic activity and the fostering of accountability and transparency in government spending.

Amidization covalent tethering by carbodiimide-mediated condensation.

Amorphous lacking definite form.

Amphiphile a molecule containing a polar water-soluble head attached to a hydrophobic carbon tail and it is the amphiphilic nature of this system that promotes both self-assembly and biological compatibility.

Amphiphilic a molecule having a polar, water-soluble group attached to a non-polar, water-insoluble hydrocarbon chain.

Analgesia a deadening or absence of the sense of pain without loss of consciousness.

Angiogenesis a physiological process involving the growth of new blood vessels from existing ones.

Antigen a substance that prompts the generation of antibodies which specifically bind to it.

Apparent Permeability permeability of a cell measured as a function of solute concentration comparison on the basolateral vs. apical sides of the cellular membrane.

Apolipoprotein E (ApoE) a lipoprotein that binds specific receptors primarily expressed on the surface of liver and endothelial cells.

Apoptosis programmed cell death.

Aptamers oligonucleic acid or peptide molecules that bind to a specific target molecule.

Arc Discharge the passage of a high electrical current between two graphite electrodes in an inert gaseous environment.

Articular Cartilage cartilage that covers the surface of bones at the location of the joints.

Artificial Intelligence the capacity for abstract thought, reasoning, planning, problem solving, communication and learning by machines and/or computers.

Association Constant K a mathematical constant that defines the affinity between two molecules at equilibrium.

Astrocytes star-shaped glial cells that perform many functions including metabolic support of the endothelial cells lining the blood-brain barrier and providing nutrients to CNS tissue.

Atom Relay Switch a nanoscale computational switch composed of precisely patterned lines of atoms on a substrate in a cross formation with a mobile atom providing the switching capabilities.

Atomic Force Microscope (AFM) a high-resolution scanning microscope consisting of a cantilever-based probe that scans the surface of the specimen.

Atomic Layer Deposition (ALD) a process that allows for the efficient depositing of thin film layers of a thickness equal to that of a single atom.

Atomic Layer Epitaxy see "Atomic Layer Deposition."

Atomically Precise Manufacturing (APM) the ability to manufacture materials and structures at the atomic (or at least molecular) scale with controlled precision.

Autologous replacement therapy the transplant of cells or tissues from one part of the body to another in the same individual.

Ballistic Conduction the unimpeded flow of particles carrying a charge or specific energy across long distances.

Basement Membrane the basic substrata for cellular structures throughout the body.

Bending Force, *F* the force exerted upon a carbon nanofiber by interactions with a cell to result in its physical alteration.

β-pleated Sheets a type of secondary protein structure consisting of strands of amino acids interconnected by five or more hydrogen bonds forming a twisted pleated sheet of protein.

Binding Affinity the strength of an antibody's binding to its antigen epitopes.

Bioactivation activation of an inert drug for use in a biological setting.

Biocavity Laser Chip a microfluidic chip for the detection of single cells encompassing a gallium-arsenide laser emitting in the near IR range.

Biocompatibility the capability of co-existence with livings tissues without causing harm.

Biocomposite a material that contains natural fiber reinforcements.

Bioengineering Nanotechnology Initiative a 1999 funding opportunity announcement intended to stimulate Small Business Technology Transfer (STTR) grant applications that employ nanotechnology to enable the development of diagnostics and interventions for treating diseases.

Biomimicry the process of utilizing the way nature produces something in order to create a manmade material.

Biomimetic man-made object or material which mimics what occurs naturally in biology.

Bionanoscience a field of research focusing on the nanoscale physical and chemical properties of naturally occurring biological or at least biomicking structures and materials.

Bionanotechnology the use of biomolecules for applications in nanotechnology.

BioNEMS nanoelectromechanical systems, made up of components between 1 and 100 nm in size, that integrate electrical and mechanical functionality at the nanoscale for use in biological applications.

Blood-brain barrier (BBB) a layer of tightly packed cells that make up the walls of brain capillaries and prevent substances in the blood from diffusing freely into the brain.

Bottom-Up Approach the nanoscale assembly of a material, object or device from individual components.

Brachytherapy radiotherapy in which the source of radiation is placed in or close to the area being treated.

Bradykinesia a slowing of physical movement.

Buckysomes self-assembled, spherical nanostructures composed of the amphiphilic fullerene AF-1.

Burst Effect a large drug volume is quickly released from a complex.

Campus Biometropolis a center for medical research and development integrated with the National Autonomous University of Mexico.

Carbon Nanofiber cylindrical nanostructures with graphene layers arranged as stacked cones, cups or plates.

Carbon Nanotube an allotrope of carbon with a cylindrical nanostructure.

Caspase a family of cysteine protease enzymes which play crucial roles in the activation of apoptosis, in necrosis and inflammation.

Catalase a common enzyme found in nearly all living organisms which are exposed to oxygen that typically functions to catalyze the decomposition of hydrogen peroxide.

Cell Culture the process by which cells are grown under controlled conditions.

Cellularization the infiltration of cells into a particular environment.

Center for Environmental Implication of Nanotechnology (CEINT) a center created by the U.S. EPA and NSF to monitor interactions between nanomaterials, the environment, plant and animal life.

Ceramic inorganic, non-metallic solids prepared by the actions of heat and subsequent cooling.

Cetuximab (Erbitux) a chimeric mouse/human monoclonal antibody designed to target and inhibit the epidermal growth factor receptor (EGFR).

Chemical Nanocomputer computation-capable devices at the nanoscale that store and process information in terms of chemical structures and chemical interactions.

Chemical Vapor Deposition (CVD) a chemically induced reaction in which two process gases and a carbon-containing gas are bled at high temperatures (700 degrees C) into a reaction chamber containing a substrate layer of metal catalyst particles.

Chiral Vector for a carbon nanotube, this is represented as two indices, n and m, which are unique to the chirality or achirality of the molecule.

Chitosan a deacetylated derivative of chitin, a tough, protective semitransparent nitrogen-containing polysaccharide that is the primary component in the exoskeletons of arthropods as well as in certain fungal cell walls.

Chromallocytes futuristic hypothetical cell repair nanorobots capable of performing chromosomal replacement therapy.

Circulatory System an organ system designed to deliver nutrients, hormones and gases to cells within the body.

Class 977 a new classification for nanotechnology patents created by the USPTO in 2004 to provide a cross-reference for examiners and others to search prior art.

Click Chemistry a chemical discipline which describes the design of chemical reactions which are tailored to drive the rapid and efficient formation of substances from the joining of small subunits.

Clottocytes theoretical artificial mechanical blood platelets that may have the ability to promote hemostasis in as little as one second.

Cluster Science the study of small clusters of atoms no greater than $3*10^7$ in number.

Coercivity the intensity of a magnetic field to drive the magnetization of a particular material to zero after saturation.

Coherent Light light composed of in-step waves of identical phase and frequency.

Collagen the fibrous constituent of cartilage, tendons, bone and other connective tissue present within the body.

Colloids a chemical mixture in which one substance is evenly dispersed throughout another.

Composite Nanodevice (CND) a positively charged and soluble poly $^{198}Au^0$ dendrimer radioactive nanocomposite.

Computed Tomography (CT) a medical imaging method employing *in vivo* sectional imaging by computer processing.

Convection Enhanced Delivery (CED) the continuous injection under positive pressure of a fluid containing a therapeutic agent.

Convergent Synthesis the synthesis of a dendrimer by an outward-in approach with pre-synthesized dendrons attached to the core as the final step.

Covalent Bond a type of chemical bond in which atoms shares electron pairs with one another or with additional covalent bonds.

Critical Micelle Concentration (CMC) the concentration of surfactant monomers above which spontaneous micelle formation occurs.

Crystallinity the degree of structural order in a solid, and bond strength when compared to more conventional coatings.

Cytomorphometry the measurement of morphological changes at the cellular level.

Debye Length the distance over which significant charge separation can occur.

Dendrimer synthetic polymers exhibiting branched-like configurations that achieve structural perfection.

Density of States (DOS) the number of available states that an electron may occupy at each energy level.

Dermis the layer between the epidermis and subcutaneous tissue.

Desorption substance release from or through a surface.

Deterministic nanotechnology the handling of individual atoms and molecules.

Diffraction Limit the minimum angular separation of two sources that can be distinguished by a scopic instrument.

Dissociation Constant, *Kd* a mathematical representation of the propensity for two bound objects to reversibly dissociate from one another.

Divergent Synthesis synthesis of dendrimer molecules from the internal core outward.

DNA Computing computation based on the use of DNA, biochemistry and molecular biology instead of traditional silicon-based technologies.

DNA Origami two- and three-dimensional DNA-based structures generated by the manipulation of nucleic acid Watson-Crick base-pairing properties.

Donnan Potential the distribution of an ion species between two ionic solutions separated by a semi-permeable membrane.

Down's Syndrome a congenital disorder caused by having an extra 21st chromosome.

Doxorubicin a drug used in cancer therapy that works by intercalating within the DNA of cancer cells to prevent their survival through inhibition of macromolecular biosynthesis.

Drug Delivery the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals.

Ductile capable of being hammered out as thin metals, malleable.

Electro-blowing a technique combining electrospinning with air flow to yield hyaluronic acid nanofibers consisting of certain desired physical properties.

Electrohydrodynamic atomization (EHDA) a process of filtration and spray drying synthesis that includes a stir/freeze dry procedure employed to keep nanoparticle polydispersity to a minimum.

Electronegativity the ability of an atom to attract electrons in the formation of a covalent bond.

Electronic Nanocomputer computation-capable devices at the nanoscale that use electrical signals to store and process information.

Electrospinning the use of an electrical charge to draw extremely fine fibers from a liquid.

Electrospraying the dispersion of a liquid into an aerosol using electricity as the catalyst.

Elliptivity, *e* the measure of the elliptic nature of an object or shape.

Embryoid Body 3D aggregates of embryonic stem cells.

Encapsulation solubilization of a hydrophobic substance or agent, such as a drug, within a protective outer dendrimer shell.

Endoscopy internal explorations of the body for medical reasons using an endoscope.

Enhanced Permeability and Retention Effect (EPR) a preferential accumulation of certain sizes of molecules in tumor tissues.

Endocytosis the transport of solid matter or liquid into a cell by means of a coated vacuole or vesicle.

Epidermis the outer, nonvascular, non-sensitive layer of the skin containing stratified squamous epithelium and keratinocytes.

Epitope antigenic determinant present on an antigen through which actual binding occurs.

Ester Product a compound produced by the reaction between an acid and an alcohol with the elimination of a molecule of water.

Esterification a chemical reaction resulting in at least one **ester** product.

Estrogen Response Element (ERE) a nucleotide sequence bound by the estrogen receptor during the activation of gene transcription.

Euronanomed a 2009 initiative announced by the European Research Area to support trans-national collaborations of academia, clinical/public health communities and small to medium-sized companies for nanomedicine-related research and technology development.

European Technology Platform (ETP) in Nanomedicine an effort launched in 2005 by the European Commission to establish a clear strategic vision, decrease fragmentation, mobilize funding and identify priorities in nanomedical research.

Excitotoxicity the pathological process by which nerve cells are damaged due to over-excitation, more often a result of high amounts of the neurotransmitter glutamate.

Exfoliation mechanical shearing, chemical modification or a combination of the two to reduce attractive forces between individual carbon nanotubes for dispersion and solubilization purposes.

Ex Vivo an artificial environment outside a living organism.

Failure Analysis the process of collecting and analyzing data to determine the cause of integrated circuitry failure and how to prevent it.

Fibroin the primary protein in silk, it is composed of anti-parallel layers of beta sheets.

Fowler Process a two -step procedure using Cobalt-Fluorine derivatives as reactants to yield a perfluorocarbon.

Fullerene a cage-like hollow pentagonal or hexagonal molecule composed of carbon atoms.

Fullerenol Caged fullerene oxide.

Functionalized Fullerenes those fullerenes that have been modified by various chemical and supramolecular approaches to enhance solubility or targeting agent/drug attachment for therapeutics applications.

Gain Medium source of optical gain within a laser.

Gelatin a protein manufactured from the partial hydrolysis of collagen.

Gelation solidification by freezing.

GEM[4] a 2005 broad-based global effort undertaken to launch an international collaboration to promote the development and use of nanotechnology for global health and medical research.

Glioma a tumor in the brain or spine that arises from glial cells dividing out of control.

Glomerulus a capillary tuft that performs the first step in filtering blood to form urine.

Glucose Consumption Rate (GCR) the rate of glucose consumption and metabolism by a particular cell.

"Golden" Carbon Nanotubes (GNTs) gold-coated carbon nanotubes that act as multimodal photoacoustic and photothermal high-contrast molecular agents.

Gp120 Viral Glycoprotein a glycoprotein expressed on the surface of HIV particles that plays a crucial role in viral binding to and entry into a cell.

Hamiltonian Path a path in an undirected graph which visits each vertex exactly once.

Hanging Drop Cell Culture inverted suspension cell culture.

Hardness the property of a material in the solid phase that gives it resistance to shape change when force is applied. Reported in units of pressure (Pascals).

Hemostasis a complex process which results in the inhibition of bleeding and involves both blood clotting and coagulation, primarily through the activation of platelets.

Hirsh-Bingel Chemistry the use of malonate derivatives to introduce side chains onto fullerenes.

Hooke's Law the extension of a spring is in direct proportion to the load added to it.

Hyaluronic Acid a gel-like polysaccharide primarily located in the extracellular matrix of the synovial fluid of movable joints as well as in the eye humors and in connective tissue.

Hybridoma hybrid cell line of antibody-producing and myeloma B cells.

Hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$, a naturally occurring mineral that is the principle storage form of calcium and phosphorous in bone. A ceramic version has been used in various medical applications.

Hyperlens a nanoprecise lens consisting of multiple layers of silver and aluminum oxide coated along the cavity of a quartz cylinder, for nanoscale optical imaging.

Hyperthermia temperatures above 40 degrees Celsius.

Hypoxia a deficiency in the amount of oxygen that reaches a tissue.

Idiopathic having no known cause.

Immunoconjugates antibodies linked to a second molecule such as a toxin, radioisotope or label.

Implant an artificial object placed inside the body in order to replace, correct or repair a damaged bodily function.

Institute for Bioengineering and Nanotechnology (IBN) an institute created by the Singaporean agency A*STAR to provide international leadership, conduct innovative research and foster the training of students in bioengineering and nanotechnology.

Integrins dimeric cell surface receptors that mediate the attachment of a cell to the tissues surrounding it or the extracellular matrix.

International Council on Nanotechnology (ICON) a global partnership formed in 2004 between the nanotechnology industry, government, academia and other select organizations.

Intracellular Nanosurgery the manipulation of organelles within a cell by manual and instrumental means.

Intravenous Injection use of the circulatory system, often veins, to introduce a therapeutic agent as a suspension into the body.

Ion Beam-Assisted Deposition (IBAD) a materials engineering technique which combines ion implantation with simultaneous sputtering or another physical vapor deposition technique.

Ion Gelification formation of a gel catalyzed by the presence of ions.

Ionization (laser) the formation of or separation of ions by heat.

In situ in a natural biological environment.

Intrabiliary Infusion drug or complex administration by introduction into the bile duct.

IV Curve a graphical plot of current vs. voltage generated during integrated circuit failure analyses.

Kanzius RF Therapy a cancer therapy using a combination of carbon nanotubes and radiofrequency waves to thermally ablate cancer cells.

Kaplan-Meier Survival Curve graph of percent survival (y-axis) to time (x-axis) after initiation of drug delivery.

Keratinocyte An epidermal cell that produces keratin.

Kinesin a naturally occurring class of motor protein dimers found in biological cells capable of transporting cellular cargo.

Kirby-Bauer Antibiotic Testing (KB Test) a classical test using antibiotic-impregnated wafers to discern bacterial killing efficiency.

Krafft Temperature the minimum temperature of a system at which spontaneous micelle formation occurs.

Laser (Light Amplification by Stimulated Emission of Radiation) an instrument that employs a mechanism for emitting electromagnetic radiation, typically light via a process of stimulated emission.

Laser Ablation a process for removing a material from a solid or liquid by irradiating it with a laser beam. Often performed for the generation of fullerenes from graphite.

Laser-assisted Nanosuturing the application of a laser upon a material to create nanosized sutures.

Laser Doppler Vibrometer (LDV) a scientific instrument that is designed to make non-contact vibration measurements of a surface.

Laser Nanosurgery the use of ultrafast laser pulses to permit the precise ablation of cellular and subcellular structures.

Layer-by-layer (LbL) Assembly a technique for surface modification which works on the principle of electrostatic attractions between oppositely charged particles.

Ligaments fibrous connective tissue that attaches bones to other bones and are primarily responsible for joint movement and stability.

Ligand a molecule that binds to a receptor.

Liposome derivatives of micelles which contain dual opposing layers and most closely resemble the structure of the cell membrane.

Localized Injection direct introduction of a therapeutic agent as a suspension at the site of disease.

Lower Critical Solution Temperature (LCST) the critical temperature below which a mixture is miscible in all proportions.

Lymphoscintigraphy a diagnostic technique in which a two-dimensional picture of the lymphatic system is produced through the detection

of radiation emitted by a radioactive substance administered into the body.

Macrophage a large white blood cell that ingests foreign particles and substrates via phagocytosis.

Magnetic Fluid-loaded Liposome (MFL) monodisperse maghemite anionic nanocrystals with an average diameter of 8 nm combined with liposomes labeled with the fluorescent dye rhodamine.

Magnetic Hysteresis a property of ferro- and ferrimagnetic nanoparticles in which atomic dipoles become aligned with the magnetic field, no matter the direction.

Magnetism-Engineered Iron Oxide (MEIO) Nanoparticles metal-doped nanoparticles engineered to possess exceptionally high and tunable nanomagnetism.

Matrigel™ a cell culture matrix derived from basement membrane.

Maxwell's Demon a mythical tiny entity capable of handling and manipulating individual atoms.

Mechanical Nanocomputer a computation-capable device at the nanoscale that uses nanosized gears to store and process information.

Metal Chelators agents that bind metal ions and render them neutral and unavailable, eventually allowing for their removal from the brain via the circulatory system.

Metalloceramics materials which contain both metallic and ceramic substances, the combination of which provides unique physical and structural properties.

Methotrexate an anti-metabolite and anti-folate drug used in the treatment of cancers and autoimmune diseases.

Micelle a three-dimensional spherical accumulation of surfactant molecules.

Microarray a collection of miniaturized test sites.

Microbivores theoretical nanorobotic devices that could provide eradication of blood-borne pathogens in a manner similar to that of phagocytes.

Microfluidics a scientific discipline that deals with the behavior, precise control and manipulation of fluids that are geometrically constrained to a small, sub-millimeter scale.

Microglia resident macrophages of the brain and spinal cord that act as the first defense against foreign materials.

Micrometastases the spread of cancer cells from a primary site and the formation of microscopic tumors at secondary sites.

Micropore a microsized gap between microfibers that often ranges in size from 10 μm to 200 μm across, similar to the size of a single cell.

Mitochondria a membrane-based enclosed organelles that generates most of the cell's supply of ATP.

Molecular Beam Epitaxy a process for the deposition of single crystals under high vacuum.

Molecular Planetary Gear a theoretical nanoscale mechanical instrument composed of silicon and sulfur that would convert shaft power from one molecular angular frequency to another.

Molecular Sentinels (MS) nanoprobes comprised of a metal nanoparticle conjugated to a stem-loop DNA molecule which is Raman label tagged.

Molecular Shuttle Switch a nanoscale computational switch based on a ring-shaped molecule which encircles and slides along a secondary chain molecule composed of biphenol and benzidine stations between which the shuttle moves.

Monoclonal Antibodies identical antibodies due to the fact that they are produced by one type of immune cell.

Moore's Law the number of transistors that can be placed on an integrated circuit doubles approximately every two years.

Multi-valent having several sites of attachment.

Nano ancient Greek term meaning "dwarf."

Nano-1 a 29-amino acid peptide made by solid phase peptide synthesis procedures designed to form an amphiphilic μ-helix for solubilizing carbon nanotubes.

Nano2Life a European Network of Excellence supported by the 6th Framework Programme which seeks to keep Europe competitive with the United States and Asia in the field of nanobiotechnology.

Nanoaxotomy the process of surgically severing an axon at the nanoscale.

Nanobiochips miniaturized laboratories that can in some cases perform hundreds or thousands of biochemical reactions for the identification of a particular molecular signature unique to the diagnosis in question.

NanobioFab a New York-based state-of-the-art fabrication facility for handling biomaterials.

Nanobiosensor a device that combines a biological indicator with an electrical, mechanical or chemical sensing system on the nanoscale.

Nanobiotechnology the development and use of nanotechnological devices for use in biotechnology.

Nanobot see "nanorobot."

Nanocoating the coating of a surface with a nanomaterial.

Nanocomputer a computation-capable device which uses fundamental parts not larger than a few nanometers or for which the total size is measured on the nanoscale.

Nanocrystals see "nanoparticle."

Nanodiagnostics the application of nanotechnology for the diagnosis of a physiological anomaly or disease in humans or animals.

Nanofibers fibers with diameters on the order of 100 nm or less.

Nanofluidics the study of the behavior, manipulation, and control of fluids that are confined to structures of nanometer (typically 1–100 nm) characteristic dimensions.

Nanogear a mechanical instrument for transmitting motion that is nanoscale in size.

Nanogel any mixture of nano-sized particles or fibers with a gel, typically one that is protein-based in the case of therapeutic or medicinal applications.

Nanohealth understanding and addressing the molecular origins of diseases that originate within a human cell and applying nanotechnology's power to control individual molecules for the detection, diagnosis, and treatment of these debilitating and incurable illnesses.

Nanolaser Scanning Confocal Spectroscopy a precise system for the laser-based spectroscopic measurement of subcellular organelles.

Nanologue a 21-month project commissioned and funded by the E.U. in 2006 aimed at establishing a common understanding concerning social, ethical and legal aspects of nanotechnological applications and facilitating a European-wide dialogues among science, business and civil society about its benefits and impacts.

Nanomaterial material having unique physical properties derived from the inherent nanoscale dimensions of the material.

Nanomedicine the medical application of nanotechnology.

Nanometer (nm) one billionth of a meter, or 10^{-9} meters.

Nanomotor a device, nanoscale in size, often made up of individual molecules, capable of converting energy into movement.

Nanoneedle An AFM probe tip etched using a focused ion beam and utilized for the introduction of exogenous genetic material attached to the sides of the needle into living cells.

Nanonephrology a broad, futuristic category of nanomedicine that can be defined as the study of kidney structures at the atomic level and the development of nanotechnologies for the diagnosis or treatment of renal disorders.

Nanoneural Interface a biocompatible substance that interacts and intermingles with neurons for either the enhancement or transmission of neuronal signaling.

Nanoparticle an object less than 100 nm in diameter that behaves as a whole in terms of its transport and properties.

Nanopolymer Scaffolds three-dimensional microstructures that are composed primarily of nanopolymers.

Nanopore a small electrically insulated hole that can be used as a single-molecule detector upon passage of that molecule through the pore.

Nanoprecipitation the formation of nanoparticles by precipitation of a water-insoluble polymer dissolved in a water-miscible organic solvent upon addition to water.

Nanoproteomics the application of nanobiotechnology to proteomics which can enable the detection of a single molecule of protein in a sample.

Nanopulse a brief, nanosecond pulse of an external field directed at unwanted cells and/or tissues.

Nanorobot a robotic structure, nanoscale in size or utilizing nanoscale parts, which performs complicated, often repetitive tasks at the nanoscopic level.

Nanoscale Science, Engineering and Technology Subcommittee of the NSTC an interagency body responsible for coordination of the National Nanotechnology Initiative.

Nanoscience see "Nanotechnology."

Nanoscopic Scale the size at which the expected fluctuations of particle properties, including motion and behavior, can no longer be reduced to below a desirable threshold.

Nanostructuring the creation of nanosized physical properties throughout a material that can be accomplished by a number of methods including simply using nanoparticles themselves, e-beam evaporation, chemical etching, or lithography.

Nanoshell spherical multilayered particles, typically less than 100 nm in diameter, that contain a dielectric (non-conducting) glass core internalized by a metallic (often gold) sphere or shell.

Nanosphere a spherical particle having diameters of less than 100 nm.

Nanotechnology the study of the control of matter on a molecular or atomic scale.

Nanotechnology Characterization Laboratory (NCL) a testing facility formed by the FDA and NCI in 2005 to standardize and perform the pre-clinical characterization of nanomaterials intended for cancer therapeutics and diagnostics developed by researchers from academia, government, and industry.

Nanotechnology Engagement Group (NEG) a group founded in 2005 by the U.K.'s Office of Science and Innovation to document the learning from a series of groundbreaking attempts to involve members of the public in discussions about the development and governance of nanotechnologies.

Nanotechnology Victoria, Ltd a primary organization created in 2003 that promotes nanotechnological advancements in the Victorian region of Australia.

Nanotechnology Working Group of the EPA a group created in 2004 to examine the potential environmental implications of nanotechnology.

Nanotool an instrument that allows for the observation, fabrication or manipulation of materials and particles with nanometer precision.

Nanotree a branched nanorod structure used in the creation of a detection reactor.

Nanotube-Based Nanomotor a nanoscale motors made of single-walled or multi-walled carbon nanotubes.

Nanowire-Based Nanomotor a motor made of a wire with a diameter no larger than 100 nm.

National Enabling Technologies Strategy a 2010 effort implemented by the Australian government to provide funding and a framework for developing enabling technologies, most notably of which is nanotechnology.

National Nanotechnology Initiative (NNI) an initiative started by the U.S. Federal government in fiscal year 2001 to help shape a major research effort at developing new nanotechnology-based tools to improve human health.

National Nanotechnology Strategy Task Force (NNST) a task force created by the Australian government to compile a formal strategic review and recommendation for the country with respect to nanotechnology.

NCI Alliance for Nanotechnology in Cancer a 5-year initiative announced by the U.S. National Cancer Institute to accelerate the application of the best capabilities of nanotechnology to cancer.

Nephron the basic structural and functional unit of the kidney.

Nitric Oxide a gas well-known to counter inflammatory responses and kill parasites.

Non-Covalent Bond a type of chemical bond which does not involve the sharing of electron pairs and is composed of more dispersed variations of electromagnetic interactions.

Nucleic Acid Nanotechnology the use of inherent nucleic acid inter- and intra-molecular recognition properties (Watson Crick base-pairing) to build two-dimensional and three-dimensional structures.

Neuroprotection strategies and mechanisms for the prevention or reduction of neuronal apoptosis or degeneration.

Neurodegenerative Disease a disorder in which brain and/or spinal cord cells are lost.

Neuroscience the field of study encompassing the various scientific disciplines dealing with the structure, development, function, chemistry, pharmacology, and pathology of the nervous system.

nProber an instrument developed by Zyvex Corporation for performing integrated circuit failure analysis below 100 nm consisting of eight positioners and corresponding probes.

Nucleic Acid-Based Nanomotor a nanoscale motor made of nucleic acids such as DNA or RNA.

Nude Mice mice derived from a genetic mutation that results in a deteriorated or absent thymus.

OECD's Working Party on Nanotechnology a group recommended by the OECD in 2006 to consider how best to organize future international activities to manage and characterize nanomaterials for impacts on the environment, health and safety.

Optical Lithography the process of etching away at a substrate to produce a desired, precise geometric pattern using light and photo-resistant chemicals.

Optomechanical Energy Conversion the conversion of optical light to mechanical energy.

Osteoconductivity the promotion of bone formation.

Outmessaging communication from an *in vivo* nanorobot to the attending physician.

Output Coupler a partially transparent lens or mirror present within a laser through which the beam is emitted.

Oxidative Stress a condition of increased oxidant production in cells characterized by the release of free radicals and resulting in cellular degeneration.

Parallel Assembly a process in which objects are manufactured along many pathways simultaneously.

Parkinson's Disease a neurodegenerative disorder of the central nervous system that often impairs the individual's motor skills and speech.

Parchment Model the resemblance of a graphene sheet rolled back upon itself several times over by multi-walled carbon nanotubes.

Pascal one Newton per square meter (N/m^2 or $kg \cdot m^{-1} \cdot s^{-2}$).

Passive Targeted Drug Delivery a mechanism and process by which certain sizes of molecules tend to preferentially accumulate in tumor tissues and is also known as the enhanced permeability retention effect (EPR).

PC12 Cell a neuronal precursor cell derived from a tumor originating in the rat adrenal medulla.

PCR Amplicon polymerase chain reaction amplification products.

Pegylation the process of covalent attachment of polyethylene glycol polymer to another molecule.

Peptides short oligomers synthesized from the joining of amino acids via peptide bonds.

Perfluorocarbon (PFC) nanoparticles consisting of carbon backbones surrounded by fluorine atoms.

P-glycoprotein (P-gp) a membrane-associated protein expressed in the capillary endothelial cells.

Phagocytes white blood cells that protect the body by ingesting harmful foreign particles such as bacteria and dead or dying cells (phagocytosis).

Phagocytosis the ingesting of harmful foreign particles such as bacteria and dead or dying cells by phagocytes.

Phase separation a technique by which nanofibrous foams are produced that are very similar in morphology to naturally occurring collagen present within the ECM.

Phonons quantum vibration effects.

Phosphodiester Bond a dual ester bond including a phosphate atom which links nucleotides in a strand of DNA.

Pi Electron Stacking a stacked arrangement of often aromatic molecules due to inter-atomic interactions between pi electrons.

Plasmonic Nanobubbles gold nanoparticles that generate transient photothermal vapor.

Pluripotency a stem cell's ability to differentiate into many different lineages.

Pluronic P85 a di-functional block copolymer surfactant terminating in primary hydroxyl groups that is a nonionic and 100% reactive.

Poly(amidoamine) (PAMAM) the most common class of dendrimers consisting of an alkyl-diamine core and tertiary amine branches.

Polychelating Amphiphilic Polymers (PAPs) macromolecules containing both hydrophobic and hydrophilic domains that sequester metal ions at multiple sites on each molecule.

Polydispersity Index (PDI) a measure of the distribution of molecular mass in a given polymer sample.

Polyion complex micelles micelles formed through the reaction of hydrophilic block copolymers containing both ionic and non-ionic blocks with macromolecules of opposite charge like DNA or proteins that allow for the incorporation of charged particles within their internal cores.

Poly(lactic-co-glycolic acid) (PLGA) a copolymer that has been used in a variety of FDA-approved therapeutic devices owing to its biodegradability and biocompatibility.

Polymeric Nanofiber nanofibers which contain and are organized into repeating structural units.

Polymersome a polymer vesicle composed of amphiphilic diblock copolymers, using either optical tweezers or a micropipette to create nanotubes that have an aqueous core connected to an aqueous interior.

Polysorbate 80 (Tween 80) a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid that is commonly used in the food industry.

Porosity e, the ratio of the volume of the pores or interstices of a substance.

Proboscis a large axially positioned manipulator that collects old chromatin and transfers new chromatin in its place.

Project on Emerging Nanotechnologies (PEN) a project formed in 2005 as a joint effort between the Woodrow Wilson International Center and the Pew Charitable Trusts to ensure that as nanotechnologies advance, possible risks are minimized, public and consumer engagement remains strong, and the potential benefits of these new technologies are realized.

Proposal for Regulatory Reform of Nanomaterials a public consultation on a nanomaterials regulatory strategy commissioned in 2009 by the Australian government Department of Health and Ageing that addresses the current regulatory efforts for "nanoforms" and proposes an approach for the regulation of industrial nanomaterials.

Protamine a small, positively charged arginine-rich nuclear protein that has been previously used to enhance nucleic acid delivery.

Protein Adsorption the ability of a solid to attract and hold proteins on its surface.

Pulse Intensity energy per area per unit time, the total number of pulses administered and the repetition rate of a laser.

Pumping (Laser) supply of energy to a laser.

Qubits units of quantum information analogous to the classical bit.

Quantitative Nanoproteomics (QNanoPX) the quantitative application of nanobiotechnology to proteomics.

Quantum Nanocomputer computers at the nanoscale that apply quantum mechanical phenomena, such as superposition and entanglement, to store information.

Quantum Plasma Oscillation the collective oscillation of electrons.

Quantum Size Effect unusual properties of extremely small crystals that arise from confinement of electrons to small regions of space in one, two, or three dimensions.

Quantum Tunneling the travel of electrons across the space between a probe and a semiconducting material surface during scanning tunneling microscopy.

Raman Shift the difference in energy between an incident photon and a scattered photon.

Reactive Oxygen Species (ROS) highly reactive oxygen free radicals that can damage cellular structures.

Redox a chemical reaction between two substances in which one substance is oxidized and the other reduced.

Refined Molecular Relay a nanoscale computational relay switch that relies on atom movement with the rotational aspects of a molecular group affecting the electrical current of the system.

Regenerative Medicine and Nanomedicine Initiative (RMNI) an effort launched in 2003 to support the development of new and emerging areas of integrative biomedical research including the study of stem cells, tissue engineering, rehabilitation sciences and nanomedicine.

Renal Tubule the portion of the nephron containing the tubular fluid filtered through the glomerulus.

Resonant Frequency the tendency of a system to oscillate at larger amplitudes for certain frequencies.

Respirocytes hypothetical artificial red blood cells, roughly 1 micron in diameter, that can supplement or perhaps even replace the function of much of the human body's normal respiratory system.

Responsible Nano Code an outline of 7 principles formulated by the U.K.'s Royal Society and the Nanotechnology Industries Association in 2006 primarily directed at business entities on how to minimize risks to human health and the environment as well as to reduce negative social or ethical implications of nanotechnology development and usage.

Restenosis a narrowing or constriction of a blood vessel, often due to inflammation induced after stenting.

Reticuloendothelial system (RES) a component of the immune system including monocytes and macrophages that is capable of clearing conventionally designed liposomes from the circulation.

RGD Peptide a peptide that is known to bind specifically to integrins and provide cellular attachment to the extracellular matrix.

Ribozymes nucleic acid-based enzymes.

Roadmap on Nanomedicine Initiative an effort by the NIH undertaken in 2005 to establish a national network of eight nanomedicine development centers.

Rod Logic displacement of solid rods to represent the digital signal in mechanical nanocomputers.

Rose Bengal 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein, a collagen fiber-based dye used in laser-assisted nanosuturing.

Rotaxane a mechanically interlocked molecular compound consisting of a dumbbell-shaped molecule threaded through a ring.

Russian Doll Model concentric circles of graphene sheets, the equivalent of single-walled carbon nanotubes, arranged within each other.

Sanger Method developed by Frederick Sanger in 1975, is a DNA sequencing procedure involving chain termination using dideoxynucleotide triphosphates.

Scanning Tunneling Microscope (STM) an instrument that allows for viewing the surface of a wide variety of substances at the atomic level by applying an electrical current to probe the density of electronic states, which correspond to the density of the material being probed.

Scatchard Equation an equation for calculating the affinity constant of a ligand with a protein.

Schiff Base Linkage a linkage formed by the condensation of an aldehyde and a ketone.

Sciatic Nerve the largest nerve in the vertebrate body, it is a sensory and motor nerve originating in the sacral plexus and running through the pelvis and upper leg.

Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) a committee formed in 2004 by the European Commission to provide the Commission with unambiguous scientific advice on the safety of issues and technology which require a comprehensive assessment of risks.

SELEX systematic evolution of ligands by exponential enrichment, a technology that allows for very rapid *in vitro* selection of aptamers that tightly bind anything from proteins to small molecules to even other nucleic acids.

Self-assembly the formation of an organized structure or pattern from pre-existing disorganized components as a result of specific local interactions and without external direction.

Sepsis an inflammatory state due to a known or suspected infection. It is a major concern for surgeons following a surgical procedure and can wreak havoc on other types of open wounds such as lacerations.

Singularity Theory a marrying of man and computer driven by smaller node sizes and increased computation efficiencies allowing for the advancement of both human and artificial intelligence.

Small Molecules low molecular weight organic molecules that are not considered to be polymers.

Solid-Phase Peptide Synthesis a method for the synthesis of peptides using a deprotection and washing procedure.

Solvent Displacement Method a procedure for the large-scale preparation of nanoparticle dispersions in which one solvent gradually displaces the presence of another.

Specific Absorption Rate (SAR) the power dissipated in the form of heat in a hysteresis loop.

Spin-Lattice Relaxation Time, T1 a time constant that characterizes the rate at which the longitudinal Mz component of magnetization recovers.

St. Louis Institute of Nanomedicine Working Group a 2009 St. Louis-based regional effort to apply advances in nanotechnology for the treatment of human diseases by fostering collaborative efforts and joint research projects.

Starburst® PAMAM dendrimers a dendrimer subclass used in a wide variety of biomedical applications consisting of multiple linear polymer arms attached to a central core.

Stochastic Nanotechnology the manipulation and handling of atoms and molecules in a chemical or bulk fashion.

Strain a geometrical measure of deformation representing the relative displacement between particles in the material body.

Stress a measurement of the amount of force exerted per unit area of a surface within a deformable body on which internal forces act.

Structure Factor a mathematical description of how a material scatters incident radiation as it pertains to matter physics and crystallography.

SU-8 an epoxy-novolac resin and a well-established negative photoresistor for microfabrication and microengineering.

Superparamagnetic Iron Oxide (SPIO) Nanoparticles nanosized magnetic chemical compounds composed of iron and oxygen.

Superparamagnetism random flipping of nanoparticle magnetization direction due to the influence of temperature.

Surface Enhanced Raman Scattering (SERS) a technique that results in the enhancement of molecular photon scattering effects on rough metal surfaces.

Surface Plasmon a free electromagnetic wave.

Surface Plasmon Resonance (SPR) the oscillation of free electron's along a particle's surface upon exposure to an external field.

Surface Plasmons coherent electron oscillations that exist at the interface between any two materials where the dielectric function changes sign across the interface.

Surfactant a substance that, when dissolved in water or an aqueous solution, reduces its surface tension or the interfacial tension between it and another liquid.

Surgery dealing with the treatment of injury, deformity or disease by both manual and instrumental means.

Suture a medical device that is employed to hold skin, internal organs and other tissues of the body together or in place often following a laceration or surgical procedure.

Synapses junctions through which neurons signal to each other and to non-neuronal cells.

Targeted Drug Delivery a method of delivering medication to a patient in a manner that increases its concentration in some parts of the body or to certain cell types relative to others.

Taylor cone the exact point of liquid emergence from a droplet acted upon by electrostatic repulsion counteracting surface tension.

Theranostics the simultaneous diagnosis and treatment of a disease or disorder.

Thermal Spraying the spraying of a melted or heated material onto a particular surface.

Tissue Culture the growth of tissues and/or cells separate from an organism.

Top-Down Approach the use of microfabrication machinery to externally control the atomically or molecularly precise synthesis of a desired material.

Transcendent Man a cyborg-like entity created as a result of the merging of man and machine in Epoch 5.

Transcytosis a mechanism for transcellular transport in which a cell encloses extracellular material in an invagination of the cell membrane to form a vesicle, then moves the vesicle across the cell to eject the material through the opposite cell membrane by the reverse process.

Transfection the process of introducing nucleic acids into cells by non-viral methods.

Transferrin a blood plasma protein involved in iron ion delivery to cells through transferrin receptor binding.

Translocation Event Signal changes in the ionic current of an electrolyte solution containing the molecules in question which results in a change in electrical current.

Tropoelastin a 70 kDa water-soluble protein composed of multiple monomers covalently linked to one another to form the three-dimensional protein known as elastin.

Tumor-Associated Antigen an antigen expressed by both normal and cancer cells but which tends to be higher in level in cancer cells.

Tumor-Specific Antigen an antigen which is unique to, or much more abundant in, cancer cells than normal cells.

Ultrafast Laser Nanosurgery a process that uses very brief picoseconds and femtosecond laser pulses to ablate unwanted tissue.

Ultramicroscope a system of illumination for viewing tiny particles based on light scattering and not light reflection.

Ultra-Web® a synthetic polyamide matrix that resembles the ECM/basement membrane through nanofibrillar 3D organization.

University of Houston Nanofabrication Facility a state-of-the-art cleanroom equipped with an extensive toolset for nano/micro-device prototype development and characterization.

U.S. Food and Drug Administration Nanotechnology Task Force an effort initiated in 2006 to determine regulatory approaches that encourage

the continued development of innovative, safe, and effective FDA-regulated products that use nanotechnology materials.

van der Waals Attractive Forces relatively weak attractive forces between molecules other than those due to covalent bonds or ionic bonding.

Van Hove Singularities sharp spikes in the density of states exhibited by carbon nanotubes.

Watson-Crick Base-Pairing the specific connection of two nucleotides on opposite complimentary strands of DNA or RNA through hydrogen bonding.

Wave Interference the interaction of particle waves utilized to calculate correct outputs by constructive interference eliminating incorrect outputs through wave-canceling destructive interference.

Wet Chemistry Synthesis the synthesis of nanoparticles utilizing a reducing agent in the presence of a stabilizing agent.

Whole-Genome Diagnostics the comprehensive sequencing and analysis of an individual's entire genome for purposes of determining that individual's genetic predisposition to disease.

SUGGESTED READINGS

Books and Compilations

Bushko, R. G. (Editor). *Future of Health Technology*. 2002. IOS Press, Amsterdam, The Netherlands.

Drexler, K. E. *Nanosystems: Molecular Machinery, Manufacturing, and Computation.* 1992. John Wiley & Sons, New York, New York.

Dunning, B. *Timeline: Nanotechnology Policy and Regulation in Canada, Australia, the European Union, the United Kingdom and the United States.* 2010. Regulatory Governance Initiative. Carleton University.

Freitas, R. A. *Nanomedicine, Volume I: Basic Capabilities.* 1999. Landis Bioscience. Georgetown, Texas.

Jain, K. K. *The Handbook of Nanomedicine.* 2008. Humana Press. Totowa, New Jersey.

Kurzweil, R. *The Singularity is Near: When Humans Transcend Biology.* 2005. Viking Press, New York, New York.

Lamprecht, A. *Nanotherapeutics: Drug Delivery Concepts in Nanoscience.* 2009. Pan Stanford Publishing Pte Ltd., Singapore.

Nicolini, C. *Nanobiotechnology and Nanobiosciences.* 2009. Pan Stanford Publishing Pte Ltd., Singapore.

Shong, C. W., *et al. Science at the Nanoscale: An Introductory Textbook.* 2010. Pan Stanford Publishing Pte Ltd., Singapore.

Zhang, M. and Xi, N. *Nanomedicine: A Systems Engineering Approach.* 2009. Pan Stanford Publishing Pte Ltd., Singapore.

Selected Scientific Articles

Adleman, L. M. Molecular computation of solutions to combinatorial problems. *Science.* 1994 Nov; 266: 1021–24.

Akhtari, M., *et al.* Functionalized magnetonanoparticles for MRI diagnosis and localization in epilepsy. *Epilepsia.* 2008 Aug; 49(8): 1419–30.

Alyautdin, R. N., *et al.* Delivery of loperamide across the blood-brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. *Pharm Res.* 1997 Mar; 14(3): 325–8.

Ausuwan, N., *et al.* Antibacterial surfaces on expanded polytetrafluoroethylene; penicillin attachment. *Biomacromolecules.* 2007 Feb; 8(2): 713–8.

Baker, B. M., *et al.* The potential to improve cell infiltration in composite fiber-aligned electrospun scaffolds by the selective removal of sacrificial fibers. *Biomaterials.* 2008 May; 29(15): 2348–58.

Baker, B. M., *et al*. New directions in nanofibrous scaffolds for soft tissue engineering and regeneration. *Expert Rev Med Devices.* 2009 Sep; 6(5): 515–32.

Balzani, V., *et al*. Autonomous artificial nanomotor powered by sunlight. *Proc Natl Acad Sci USA.* 2006 Jan 31; 103(5): 1178–83.

Batrakova, E. V., *et al*. A macrophage-nanozyme delivery system for Parkinson's disease. *Bioconjug Chem.* 2007 Sep–Oct; 18(5): 1498–506.

Basso, A. S., *et al*. Reversal of axonal loss and disability in a mouse model of progressive multiple sclerosis. *J Clin Invest.* 2008 Apr; 118(4): 1532–43.

Ben-Yakar, A., *et al*. Ultrafast laser nanosurgery in microfluidics for genome-wide screenings. *Curr Opin Biotechnol.* 2009 Feb; 20(1): 100–5.

Berndt, P., *et al*. Synthetic lipidation of peptides and amino acids: mono-layer structure and properties. *J Am Chem Soc.* 1995; 117: 9515–22.

Bisht, S., *et al*. *In vivo* characterization of a polymeric nanoparticle platform with potential oral drug delivery capabilities. *Mol Cancer Ther.* 2008 Dec; 7(12): 3878–88.

Bhirde, A. A., *et al*. Targeted killing of cancer cells *in vivo* and *in vitro* with EGF-directed carbon nanotube-based drug delivery. *ACS Nano.* 2009 Feb 24; 3(2): 307–16.

Blanco, E., *et al*. Beta-lapachone-containing PEG-PLA polymer micelles as novel nanotherapeutics against NQO1-overexpressing tumor cells. *J Control Release.* 2007 Oct 8; 122(3): 365–74.

Bourges, J. L., *et al*. Ocular drug delivery targeting the retina and retinal pigment epithelium using polylactide nanoparticles. *Invest Ophthalmol Vis Sci.* 2003 Aug; 44(8): 3562–9.

Bourquin, C., *et al*. Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient anti-tumoral immunity. *J Immunol.* 2008 Sep 1; 181(5): 2990–8.

Cardinal, J., *et al*. Noninvasive radiofrequency ablation of cancer targeted by gold nanoparticles. *Surgery.* 2008 Aug; 144(2): 125–32.

Catledge, S. A., *et al*. Nanostructured ceramics for biomedical implants. *J Nanosci Nanotechnol.* 2002 Jun-Aug; 2(3–4): 293–312.

Cellot, G., *et al*. Carbon nanotubes might improve neuronal performance by favouring electrical shortcuts. *Nat Nanotechnol.* 2009 Feb; 4(2): 126–33.

Cerdan, C., *et al*. Formation and hematopoietic differentiation of human embryoid bodies by suspension and hanging drop cultures. *Curr Protoc Stem Cell Biol.* 2007 Oct; Chapter 1: Unit 1D.2.

Chakravarty, P., *et al*. Thermal ablation of tumor cells with antibody-functionalized single-walled carbon nanotubes. *Proc Natl Acad Sci USA.* 2008; 105(25): 8697–702.

Chen, B. A., *et al*. Daunorubicin-loaded magnetic nanoparticles of Fe_3O_4 overcome multidrug resistance and induce apoptosis of K562-n/VCR cells *in vivo. Int J Nanomedicine.* 2009; 4: 201–8.

Cheng, P. C., *et al*. Quantitative nanoproteomics for protein complexes (QNanoPX) related to estrogen transcriptional action. *Mol Cell Proteomics.* 2010 Feb; 9(2): 209–24.

Cho, S. H., *et al*. Biocompatible SU-8-based microprobes for recording neural spike signals from regenerated peripheral nerve fibers. *IEEE Sens J.* 2008; 8(11): 1830–6.

Choi, H. W., *et al*. Structuring electrospun polycaprolactone nanofiber tissue scaffolds by femtosecond laser ablation. *J Laser Appl.* 2007; 19(4): 225–31.

Constantinidis, I., *et al*. Use of magnetic nanoparticles to monitor alginate-encapsulated betaTC-tet cells. *Magn Reson Med.* 2009 Feb; 61(2): 282–90.

Dai, H., *et al*. Chitosan-DNA nanoparticles delivered by intrabiliary infusion enhance liver-targeted gene delivery. *Int J Nanomedicine.* 2006; 1(4): 507–22.

Damascelli, B., *et al.* A novel intraarterial chemotherapy using paclitaxel in albumin nanoparticles to treat advanced squamous cell carcinoma of the tongue: preliminary findings. *AJR Am J Roentgenol.* 2003 Jul; 181(1): 253–60.

Dang, J. M., *et al.* Myogenic induction of aligned mesenchymal stem cell sheets by culture on thermally responsive electrospun nanofibers. *Adv Mater Deerfield.* 2007; 19(19): 2775–9.

Das, H., *et al. Ex vivo* nanofiber expansion and genetic modification of human cord blood-derived progenitor/stem cells enhances vasculogenesis. *Cell Transplant.* 2009; 18(3): 305–18.

Davis, M. E., *et al.* Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells. *Circulation.* 2005 Feb 1; 111(4): 442–50.

Davis, M. E., *et al.* Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature.* 2010 Apr 15; 464(7291): 1067–70.

De Asis, E. D., *et al.* High efficient electrical stimulation of hippocampal slices with vertically aligned carbon nanofiber microbrush array. *Biomed Microdevices.* 2009 Aug; 11(4): 801–8.

De la Fuente, M., *et al.* Novel hyaluronic acid-chitosan nanoparticles for ocular gene therapy. *Invest Ophthalmol Vis Sci.* 2008 May; 49(5): 2016–24.

Destache, C. J., *et al.* Combination antiretroviral drugs in PLGA nanoparticle for HIV-1. *BMC Infect Dis.* 2009 Dec 9; 9: 198.

Devalapally, H., *et al.* Modulation of drug resistance in ovarian adenocarcinoma by enhancing intracellular ceramide using tamoxifen-loaded biodegradable polymeric nanoparticles. *Clin Cancer Res.* 2008 May 15; 14(10): 3193–203.

Dhar, S., *et al.* Targeted delivery of cisplatin to prostate cancer cells by aptamer functionalized Pt(IV) prodrug-PLGA-PEG nanoparticles. *Proc Natl Acad Sci USA.* 2008 Nov 11; 105(45): 17356–61.

Ding, J. J., *et al. In vivo* expression of green fluorescent protein gene and immunogenicity of ES312 vaccine both mediated by starburst polyamidoamine dendrimers. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao.* 2005 Aug; 27(4): 499–503.

Dion, I., *et al.* Diamond: the biomaterial of the 21st century? *Int J Artif Organs.* 1993 Sep; 16: 623–7.

Dong, W., *et al.* Biocompatible nanofiber scaffolds on metal for controlled release and cell colonization. *Nanomedicine.* 2006 Dec; 2(4): 248–52.

Drexler, K. E. Rod logic and thermal noise in the mechanical nanocomputer. Proceedings of the third international symposium on molecular electronics devices. 1989. Elsevier Science Publishers B.V., North Holland.

Drexler, K. E. Nanosystems: molecular machinery, manufacturing, and computation. 1992. John Wiley & Sons, New York.

Dugan, L. L., *et al.* Buckminsterfullerenol free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons. *Neurobiol Dis.* 1996 Apr; 3(2): 129–35.

Dugan, L. L., *et al.* Fullerene-based antioxidants and neurodegenerative disorders. *Parkinsonism Relat Disord.* 2001 Jul; 7(3): 243–6.

Elechiguerra, J. L., *et al.* Interaction of silver nanoparticles with HIV-1. *J Nanobiotechnology.* 2005 Jun 29; 3: 6.

Fabel, K., *et al.* Long-term stabilization in patients with malignant glioma after treatment with liposomal doxorubicin. *Cancer.* 2001 Oct 1; 92(7): 1936–42.

Fan, R., *et al.* DNA translocation in inorganic nanotubes. *Nano Lett.* 2005 Sep; 5(9): 1633–7.

Farokhzad, O. C., *et al.* Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy *in vivo. Proc Natl Acad Sci USA.* 2006 Apr 18; 103(16): 6315–20.

Fennimore, A. M., *et al.* Rotational actuators based on carbon nanotubes. *Nature.* 2003 Jul 24; 424(6947): 408–10.

Freitas, R. A. Exploratory design in medical nanotechnology: a mechanical artificial red cell. *Artif Cells Blood Substit Immobil Biotechnol.* 1998 Jul; 26(4): 411–30.

Freitas, R. A. The ideal gene delivery vector: chromallocytes, cell repair nanorobots for chromosome replacement therapy. *J Evol Technol.* 2007 Jun; 16(1): 1–97.

Ghaghada, K. B., *et al.* New dual mode gadolinium nanoparticle contrast agent for magnetic resonance imaging. *PLoS One.* 2009 Oct 29; 4(10): e7628.

Galanzha, E. I., *et al. In vivo* magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells. *Nat Nanotechnol.* 2009 Dec; 4(12): 855–60.

Gandhi, M., *et al.* Mechanistic examination of protein release from polymer nanofibers. *Mol Pharm.* 2009 Mar–Apr; 6(2): 641–7.

Gannon, C. J., *et al.* Carbon nanotube-enhanced thermal destruction of cancer cells in a noninvasive radiofrequency field. *Cancer.* 2007; 110(12): 2654–65.

Gorantla, S., *et al.* Quantitative magnetic resonance and SPECT imaging for macrophage tissue migration and nanoformulated drug delivery. *J Leukoc Biol.* 2006 Nov; 80(5): 1165–74.

Gourley, P. L., *et al.* Ultrafast nanolaser flow device for detecting cancer in single cells. *Biomed Microdevices.* 2005 Dec; 7(4): 331–9.

Gourley, P. L., *et al.* Mitochondrial correlation microscopy and nanolaser spectroscopy new tools for biophotonic detection of cancer in single cells. *Technol Cancer Res Treat.* 2005 Dec; 4(6): 585–92.

Guo, S. X., *et al.* Femtosecond laser nanoaxotomy lab-on-a-chip for *in vivo* nerve regeneration studies. *Nat Methods.* 2008 Jun; 5(6): 531–3.

Gupta, A.K., *et al*. Anomalous resonance in a nanomechanical biosensor. *Proc Natl Acad Sci USA*. 2006 Sep 5; 103(36): 13362–7.

Hahnel, R. and Twaddle, E. Estimation of the association constant for the estrogen-receptor complex in human breast cancer. *Cancer Res*. 1973; 33: 559–66.

Han, S., *et al*. Gene expression using an ultrathin needle enabling accurate displacement and low invasiveness. *Biochem Biophys Res Commun.* 2005 Jul 8; 332(3): 633–9.

Harada-Shiba, M., *et al*. Polyion complex micelles as vectors in gene therapy--pharmacokinetics and *in vivo* gene transfer. *Gene Ther.* 2002 Mar; 9(6): 407–14.

Harrison, R. The outgrowth of the nerve fiber as a mode of protoplasmic movement. *J Exp Zool*. 1910; 9: 787–846.

Hartgerink, J. D., *et al*. Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science*. 2001; 294: 1684–8.

Hartgerink, J. D., *et al*. Peptide-amphiphile nanofibers: A versatile scaffold for the preparation of self-assembling materials. *Proc Natl Acad Sci USA*. 2002; 99 (8): 5133–38.

Holden, C. A., *et al*. Surface engineering of macrophages with nanoparticles to generate a cell-nanoparticle hybrid vehicle for hypoxia-targeted drug delivery. *Int J Nanomedicine.* 2010 Feb 2; 5: 25–36.

Horii, A., *et al*. Biological designer self-assembling Peptide nanofiber scaffolds significantly enhance osteoblast proliferation, differentiation and 3-D migration. *PLoS One.* 2007 Feb 7; 2(2): e190.

Hu, H., *et al*. Chemically functionalized carbon nanotubes as substrates for neuronal growth. *Nano Lett.* 2004; 4(3): 507–11.

Huang, R. Q., *et al*. Efficient gene delivery targeted to the brain using a transferrin-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. *FASEB J.* 2007 Apr; 21(4): 1117–25. Epub 2007 Jan 11.

Iqbal, S. M., *et al.* Solid-state nanopore channels with DNA selectivity. *Nat Nanotechnol.* 2007 Apr; 2(4): 243–8.

Ito, A., *et al.* Construction of heterotypic cell sheets by magnetic force-based 3-D coculture of HepG2 and NIH3T3 cells. *J Biosci Bioeng.* 2007 Nov; 104(5): 371–8.

Ito, A., *et al.* Magnetic force-based cell patterning using Arg-Gly-Asp (RGD) peptide-conjugated magnetite cationic liposomes. *J Biosci Bioeng.* 2007 Oct; 104(4): 288–93.

Ito, Y., *et al.* A composite of hydroxyapatite with electrospun biodegradable nanofibers as a tissue engineering material. *J Biosci Bioeng.* 2005 Jul; 100(1): 43–9.

Jayasinghe, S. N., *et al.* Cell electrospinning highly concentrated cellular suspensions containing primary living organisms into cell-bearing threads and scaffolds. *Nanomedicine (Lond).* 2007 Aug; 2(4): 555–67.

Jin, H., *et al.* Polyhydroxylated C(60), fullerenols, as glutamate receptor antagonists and neuroprotective agents. *J Neurosci Res.* 2000 Nov 15; 62(4): 600–7.

Jokerst, J. V., *et al.* Integration of semiconductor quantum dots into nano-bio-chip systems for enumeration of CD4+ T cell counts at the point-of-need. *Lab Chip.* 2008 Dec; 8(12): 2079–90.

Joner, M., *et al.* Site-specific targeting of nanoparticle prednisolone reduces in-stent restenosis in a rabbit model of established atheroma. *Arterioscler Thromb Vasc Biol.* 2008 Nov; 28(11): 1960–6.

Kam, N. W., *et al.* Electrical stimulation of neural stem cells mediated by humanized carbon nanotube composite made with extracellular matrix protein. *Nano Lett.* 2009 Jan; 9(1): 273–8.

Kamegaya, Y., *et al.* Evaluation of photochemical tissue bonding for closure of skin incisions and excisions. *Lasers Surg Med.* 2005 Oct; 37(4): 264–70.

Kang, H., *et al*. Single-DNA molecule nanomotor regulated by photons. *Nano Lett.* 2009 Jul; 9(7): 2690–6.

Khan, M. K., *et al*. Fabrication of {198Au0} radioactive composite nanodevices and their use for nanobrachytherapy. *Nanomedicine.* 2008 Mar; 4(1): 57–69.

Khang, D., *et al*. Selective adhesion and mineral deposition by osteoblasts on carbon nanofiber patterns. *Int J Nanomedicine.* 2006; 1(1): 65–72.

Khil, M. S., *et al*. Electrospun nanofibrous polyurethane membrane as wound dressing. *J Biomed Mater Res B Appl Biomater.* 2003 Nov 15; 67(2): 675–9.

Kim, J. K., *et al*. Self-assembling peptide amphiphile-based nanofiber gel for bioresponsive cisplatin delivery. *Mol Pharm.* 2009 May–Jun; 6(3): 978–85.

Kim, J. W., *et al*. Golden carbon nanotubes as multimodal photoacoustic and photothermal high-contrast molecular agents. *Nat Nanotechnol.* 2009 Oct; 4(10): 688–94.

Kisiday, J., *et al*. Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair. *Proc Natl Acad Sci USA.* 2002 Jul 23; 99(15): 9996–10001.

Kitchens, K. M., *et al*. Transepithelial and endothelial transport of poly (amidoamine) dendrimers. *Adv Drug Deliv Rev.* 2005 Dec 14; 57(15): 2163–76.

Kohli, V., *et al*. An alternative method for delivering exogenous material into developing zebrafish embryos., *Biotechnol Bioeng.* 2007; 98(6): 1230–41.

Kommireddy, D. S., *et al*. Stem cell attachment to layer-by-layer assembled TiO_2 nanoparticle thin films. *Biomaterials.* 2006 Aug; 27(24): 4296–303.

Kukowska-Latallo, J. F., *et al*. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. *Cancer Res.* 2005 Jun 15; 65(12): 5317–24.

Kumber, S. G., *et al*. Electrospun poly(lactic acid-co-glycolic acid) scaffolds for skin tissue engineering. *Biomaterials.* 2008 Oct; 29(30): 4100–7.

Kunnavakkam, M. V., *et al*. Low-cost, low-loss microlens arrays fabricated by soft-lithography replication process. *Appl Phy Lett*. 2003 Feb 24; 82(8): 1152–54.

Laocharoensuk, R., *et al*. Carbon-nanotube-induced acceleration of catalytic nanomotors. *ACS Nano.* 2008 May; 2(5): 1069–75.

Lee, C. H., *et al*. Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast. *Biomaterials.* 2005 Apr; 26(11): 1261–70.

Lee, J. H., *et al*. Artificially engineered magnetic nanoparticles for ultra-sensitive molecular imaging. *Nat Med.* 2007 Jan; 13(1): 95–9.

Lee, J. H., *et al*. Enhanced stem cell tracking via electrostatically assembled fluorescent SPION-peptide complexes. *Nanotechnology.* 2009 Sep 2; 20(35): 355102.

Li, M., *et al*. Electrospun protein fibers as matrices for tissue engineering. *Biomaterials*. 2005; 26: 5999–6008.

Li, X., *et al*. Novel patterning of nano-bioceramics: template-assisted electrohydrodynamic atomization spraying. *J R Soc Interface.* 2008 Feb 6; 5(19): 253–7.

Electrohydrodynamic atomization spraying. *J R Soc Interface.* 2008 Feb 6; 5(19): 253–7.

Liu, G., *et al*. Nanoparticle iron chelators: a new therapeutic approach in Alzheimer disease and other neurologic disorders associated with trace metal imbalance. *Neurosci Lett.* 2006 Oct 9; 406(3): 189–93.

Liu, G., *et al*. Metal chelators coupled with nanoparticles as potential therapeutic agents for Alzheimer's disease. *J Nanoneurosci.* 2009 Jun 1; 1(1): 42–55.

Liu, H., *et al*. Nanomedicine for implants: A review of studies and necessary experimental tools. *Biomaterials.* 2006 Oct 12; 28(2): 354–69.

Liu, L., *et al*. Self-assembled cationic peptide nanoparticles as an efficient antimicrobial agent. *Nat Nanotechnol.* 2009 Jul; 4(7): 457–63.

Liu, X., *et al*. Engineering therapeutic monoclonal antibodies. *Immun Rev*. 2008; 222: 9–27.

Liu, Z., *et al*. Drug delivery with carbon nanotubes for *in vivo* cancer treatment. *Cancer Res.* 2008 Aug 15; 68(16): 6652–60.

Lovat, V., *et al*. Carbon nanotube substrates boost neuronal electrical signaling. *Nano Lett.* 2005 Jun; 5(6): 1107–10.

Lukianova-Hleb, E.Y., *et al*. Tunable plasmonic nanobubbles for cell theranostics. *Nanotechnology.* 2010 Feb 26; 21(8): 85102.

Ma. P. and Zhang, R. Synthetic nano-scale fibrous extracellular matrix. *Blood Mat Res.* 1999; 46: 60–72.

Magadal, P., *et al*. Epidermal growth factor receptor-targeted gelatin-based engineered nanocarriers for DNA delivery and transfection in human pancreatic cancer cells. *AAPS J.* 2008 Dec; 10(4): 565–76.

Mahmoud, W., *et al*. Emerging applications of fluorescent nanocrystals and quantum dots for micrometastases detection. *Proteomics.* 2010 Feb; 10(4): 700–16.

Maruccio, G., *et al*. A nanobiosensor to detect single hybridization events. *Analyst.* 2009 Dec; 134(12): 2458–61.

McDevitt, M. R., *et al*. Tumor targeting with antibody-functionalized, radiolabeled carbon nanotubes. *J Nucl Med*. 2007; 48: 1180–9.

Michaelis, K., *et al*. Covalent linkage of apolipoprotein E to albumin nanoparticles strongly enhances drug transport into the brain. *J Pharmacol Exp Ther.* 2006 Jun; 317(3): 1246–53.

Min, B.M., *et al*. Electrospinning of silk fibroin nanofibers and its effect on the adhesion and spreading of normal human keratinocytes and fibroblasts *in vitro*. *Biomaterials*. 2004. Mar–Apr; 25(7–8): 1289–97.

Misra, N., *et al*. Bioelectronic silicon nanowire devices using functional membrane proteins. *Proc Natl Acad Sci USA*. 2009 Aug 18; 106(33): 13780–4.

Morawski, A.M., *et al*. Quantitative "magnetic resonance immunohistochemistry" with ligand-targeted (19)F nanoparticles. *Magn Reson Med*. 2004 Dec; 52(6): 1255–62.

Mutusuga, M., *et al*. Development of novel drug-eluting biodegradable nano-fiber for prevention of postoperative pulmonary venous obstruction. *Interact Cardiovasc Thorac Surg*. 2009 Apr; 8(4): 402–6; discussion 406–7. Epub 2009 Jan 12.

Nam, J., *et al*. Improved cellular infiltration in electrospun fiber via engineered porosity. *Tissue Eng*. 2007 Sep; 13(9): 2249–57.

Nguyen-Vu, *et al*. Vertically aligned carbon nanofiber architecture as a multifunctional 3-D neural electrical interface. *IEEE Trans Biomed Eng*. 2007 Jun; 54(6 Pt 1): 1121–8.

Nissenson, A. R., *et al*. The human nephron filter: toward a continuously functioning, implantable artificial nephron system. *Blood Purif*. 2005; 23(4): 269–74.

Nuccitelli, R., *et al*. Nanosecond pulsed electric fields cause melanomas to self-destruct. *Biochem Biophys Res Commun*. 2006 May 5; 343(2): 351–60.

Nur-E-Kamal, A., *et al*. Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells. *Stem Cells*. 2006 Feb; 24(2): 426–33.

O'Neal, D. P., *et al*. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Lett*. 2004 Jun 25; 209(2): 171–6.

Oroudjev, E., *et al.* Segmented nanofibers of spider dragline silk: atomic force microscopy and single-molecule force spectroscopy. *Proc Natl Acad Sci USA.* 2002 Apr 30; 99 Suppl 2: 6460–5.

Partha, R., *et al.* Biomedical applications of functionalized fullerene-based nanomaterials. *Int J Nanomedicine.* 2009; 4: 261–75.

Patel, H. M., *et al.* Assessment of the potential uses of liposomes for lymphoscintigraphy and lymphatic drug delivery. Failure of 99 m-technetium marker to represent intact liposomes in lymph nodes. *Biochim Biophys Acta.* 1984 Sep 7; 801(1): 76–86.

Peer, D., *et al.* Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science.* 2008 Feb 1; 319(5863): 627–30.

Pham, Q. P., *et al.* Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules.* 2006 Oct; 7(10): 2796–805.

Pinteala, M., *et al.* Binding fullerenol C(60)(OH)(24) to dsDNA. *Int J Nanomedicine.* 2009; 4: 193–9.

Popovtzer, R., *et al.* Targeted gold nanoparticles enable molecular CT imaging of cancer. *Nano Lett.* 2008 Dec; 8(12): 4593–6.

Puckett, S., *et al.* Nano rough micron patterned titanium for directing osteoblast morphology and adhesion. *Int J Nanomedicine.* 2008; 3(2): 229–41.

Puvanakrishnan, P., *et al.* Near-infrared narrow-band imaging of gold/silica nanoshells in tumors. *J Biomed Opt.* 2009 Mar–Apr; 14(2): 024044.

Ramay, H. R., *et al.* Biphasic calcium phosphate nanocomposite porous scaffolds for load-bearing bone tissue engineering. *Biomaterials.* 2004 Sep; 25(21): 5171–80.

Reiner, J. E., *et al.* Stable and robust polymer nanotubes stretched from polymersomes. *Proc Natl Acad Sci USA.* 2006 Jan 31; 103(5): 1173–7.

Richardson–Burns, S. M., *et al.* Electrochemical polymerization of conducting polymers in living neural tissue. *J Neural Eng.* 2007 Jun; 4(2): L6–L13.

Risveden, K., *et al.* Branched nanotrees with immobilized acetylcholine esterase for nanobiosensor applications. *Nanotechnology.* 2010 Feb 5; 21(5): 055102.

Riviere, C., *et al.* Magnetic targeting of nanometric magnetic fluid loaded liposomes to specific brain intravascular areas: a dynamic imaging study in mice. *Radiology.* 2007 Aug; 244(2): 439–48.

Rothemund, P. W. Folding DNA to create nanoscale shapes and patterns. *Nature.* 2006 Mar 16; 440(7082): 297–302.

Saito, R., *et al.* Distribution of liposomes into brain and rat brain tumor models by convection-enhanced delivery monitored with magnetic resonance imaging. *Cancer Res.* 2004 Apr 1; 64(7): 2572–9.

Sarin, H., *et al.* Effective transvascular delivery of nanoparticles across the blood-brain tumor barrier into malignant glioma cells. *J Transl Med.* 2008 Dec 18; 6: 80.

Schneider, A., *et al.* Self-Assembling Peptide Nanofiber Scaffolds Accelerate Wound Healing. *PLOS One.* 2008; 1: e1410.

Schubert, D., *et al.* Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem Biophys Res Commun.* 2006 Mar 31; 342(1): 86–91.

Shi, N. and Pardridge, W. M. Noninvasive gene targeting to the brain. *Proc Natl Acad Sci USA.* 2000 Jun 20; 97(13): 7567–72.

Shih, Y. R., *et al.* Growth of mesenchymal stem cells on electrospun type I collagen nanofibers. *Stem Cells.* 2006; 24: 2391–7.

Shin, M., *et al. In vivo* bone tissue engineering using mesenchymal stem cells on a novel electrospun nanofibrous scaffold. *Tissue Eng.* 2004; 10: 33–41.

Shin, H.J., *et al*. Electrospun PLGA nanofiber scaffolds for articular cartilage reconstruction: mechanical stability, degradation and cellular responses under mechanical stimulation *in vitro. J Biomater Sci Polym Ed.* 2006; 17(1–2): 103–19.

Sitharaman, B., *et al*. Water-soluble fullerene (C60) derivatives as nonviral gene-delivery vectors. *Mol Pharm.* 2008 Jul–Aug; 5(4): 567–78.

Soni, G. V., *et al*. Progress toward ultrafast DNA sequencing using solid-state nanopores. *Clin Chem.* 2007 Nov; 53(11): 1996–2001.

Sun, C., *et al. In vivo* MRI detection of gliomas by chlorotoxin-conjugated superparamagnetic nanoprobes. *Small.* 2008 Mar; 4(3): 372–9.

Sun, L., *et al*. Preparation and properties of nano-sized calcium fluoride for dental applications. *Dent Mater.* 2008 Jan; 24(1): 111–6.

Tang, X., *et al*. Direct protein detection with a nano-interdigitated array gate MOSFET. *Biosens Bioelectron.* 2009 Aug 15; 24(12): 3531–7.

Theisen, C., *et al*. Influence of nanofibers on growth and gene expression of human tendon derived fibroblasts. *Biomed Eng Online.* 2010 Feb 17; 9(1): 9.

Torchilin, V. P. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. *AAPS J.* 2007 May 11; 9(2): E128–47.

Tripp, R. A., *et al*. Bioconjugated nanoparticle detection of respiratory syncytial virus infection. *Int J Nanomedicine.* 2007; 2(1): 117–24.

Trubetskoy, V. S., *et al*. Stable polymeric micelles: lymphangiographic contrast media for gamma scintigraphy and magnetic resonance imaging. *Acad Radiol.* 1996 Mar; 3(3): 232–8.

Tu, X., *et al*. DNA sequence motifs for structure-specific recognition and separation of carbon nanotubes. *Nature.* 2009 Jul 9; 460(7252): 250–3.

Turos, E., *et al*. Penicillin-bound polyacrylate nanoparticles: restoring the activity of beta-lactam antibiotics against MRSA. *Bioorg Med Chem Lett.* 2007 Jun 15; 17(12): 3468–72.

Unger, E. C., *et al.* Hepatic metastases: liposomal Gd-DTPA-enhanced MR imaging. *Radiology.* 1989 Apr; 171(1): 81–5.

Vasita, R. and Katti, D.S. Nanofibers and their applications in tissue engineering. *Int J Nanomed.* 2006; 1(1): 15–30.

Venugopal, J. R., *et al.* Nanobioengineered electrospun composite nanofibers and osteoblasts for bone regeneration. *Artif Organs.* 2008 May; 32(5): 388–97.

Vinogradov, S. V., *et al.* Nanogels for oligonucleotide delivery to the brain. *Bioconjug Chem.* 2004 Jan–Feb; 15(1): 50–60.

Wabuyele, M. B., *et al.* Detection of human immunodeficiency virus type 1 DNA sequence using plasmonics nanoprobes. *Anal Chem.* 2005 Dec 1; 77(23): 7810–5.

Waters, E. A., *et al.* Detection and quantification of angiogenesis in experimental valve disease with integrin-targeted nanoparticles and 19-fluorine MRI/MRS. *J Cardiovasc Magn Reson.* 2008 Sep 25; 10(1): 43.

Wei, G., *et al.* The enhancement of osteogenesis by nano-fibrous scaffolds incorporating rhBMP-7 nanospheres. *Biomaterials.* 2007 Apr; 28(12): 2087–96.

Weigum, S. E., *et al.* Nano-bio-chip sensor platform for examination of oral exfoliative cytology. *Cancer Prev Res (Phila Pa).* 2010 Mar 23.

Wissner–Gross, A. D., *et al.* Diamond stabilization of ice multilayers at human body temperature. *Phys Rev E.* 76, 020501(R) (2007).

Witt, K. A., *et al.* Pluronic p85 block copolymer enhances opioid peptide analgesia. *J Pharmacol Exp Ther.* 2002 Nov; 303(2): 760–7.

Wu, G., *et al.* Targeted delivery of methotrexate to epidermal growth factor receptor-positive brain tumors by means of cetuximab (IMC-C225) dendrimer bioconjugates. *Mol Cancer Ther.* 2006 Jan; 5(1): 52–9.

Yeh, J. M., *et al.* Noncovalent interaction between gold nanoparticles and multiwalled carbon nanotubes via an intermediatory. *J Nanotech*. 2009; Article ID 217469: 1–7.

Yoshimoto, H., *et al.* A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials*. 2003; 24: 2077–82.

Zhang, D. Y., *et al.* Preparation of chitosan-polyaspartic acid-5-fluorouracil nanoparticles and its anti-carcinoma effect on tumor growth in nude mice. *World J Gastroenterol*. 2008 Jun 14; 14(22): 3554–62.

Zhang, H., *et al.* Dynamic imaging of arginine-rich heart-targeted vehicles in a mouse model. *Biomaterials*. 2008 Apr; 29(12): 1976–88.

Zhang, S., *et al.* Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures. *Semin Cancer Biol*. 2005; 15: 413–420.

Zhang, X., *et al. In vitro* evaluation of electrospun silk fibroin scaffolds for vascular cell growth. *Biomaterials*. 2008 May; 29(14): 2217–27.

Zhang, Y. Z., *et al.* Enhanced biomineralization in osteoblasts on a novel electrospun biocomposite nanofibrous substrate of hydroxyapatite/collagen/chitosan. *Tissue Eng Part A*. 2010 Jan 20.

Zorbas, V., *et al.* Preparation and characterization of individual peptide-wrapped single-walled carbon nanotubes. *J Am Chem Soc*. 2004 Jun 16; 126(23): 7222–7.

This page intentionally left blank

Figure and Table Acknowledgments

The author would like to sincerely thank all the researchers who generously allowed for their data, figures and tables to be reproduced and reprinted in this book. It is your kind generosity that allows for the free dissemination of invaluable findings in the area of nanomedicine that will drive young scientists to dedicate their lives to this exciting field.

This page intentionally left blank

About the Author

Rob Burgess is a scientist, entrepreneur, businessman, and author who switched scientific disciplines in 2006 at the age of 38 to take a chance on nanotechnology. It changed his life forever. He has held numerous academic and industry-related positions, including research fellow at the University of California, San Diego; founding scientist at Lexicon Genetics Inc.; co-founder and president at Genome Biosciences Inc.; vice president, research and development, at Zyvex Corporation; and vice president, business development, at Stem Cell Sciences, LLC. Dr. Burgess is currently co-founder and chairman of the board at Medical Nanotechnologies Inc. and an adjunct professor in the Department of Molecular and Cell Biology at the University of Texas, Dallas. He holds a bachelor's degree in biochemistry from the University of Texas, Austin, and a PhD in molecular biology from the University of Texas M. D. Anderson Cancer Center, Houston. He grew up in Silsbee, Texas, and currently resides in the Dallas area with his wife, Jane, daughter, Zoie, and seventeen-year-old dog Carmy.

www.understandingnano.org
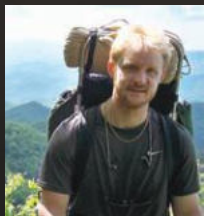
This page intentionally left blank

The scientific field of nanotechnology is rapidly evolving and will soon significantly impact virtually every aspect of our lives. While its greatest effect has been on the materials, semiconductor, and instrumentation industries, nanotechnology's promise in bettering the health of human beings is showing significant signs of maturation. This book comprehensively addresses the latest findings and discoveries in nanomedicine and conscientiously attempts to make up for the dearth of introductory material tailored specifically for students in this exciting field. Methodical and scrupulous, it covers a broad range of therapeutic and diagnostic applications of nanotechnology and explains these fields in such a way that the reader gets a thoroughly engaging as well as expansive view of cutting-edge discoveries along with historical perspectives. As lucid as it is fresh, vibrant, and topical, this is a magic carpet of a book that sweeps from the basic principles of nanotechnology to the most thrilling findings in medical science as they apply to current and future society.

Rob Burgess is a scientist, entrepreneur, businessman, and author who switched scientific disciplines in 2006 at the age of 38 to take a chance on nanotechnology. It changed his life forever. He has held numerous academic and industry-related positions, including research fellow at the University of California, San Diego; founding scientist at Lexicon Genetics Inc.; co-founder and president at Genome Biosciences Inc.; vice president, research and development, at Zyvex Corporation; and vice president, business development, at Stem Cell Sciences, LLC. Dr. Burgess is currently co-founder and chairman of the board at Medical Nanotechnologies Inc. and an adjunct professor in the Department of Molecular and Cell Biology at the University of Texas, Dallas. He holds a bachelor's degree in biochemistry from the University of Texas, Austin, and a PhD in molecular biology from the University of Texas M. D. Anderson Cancer Center, Houston.