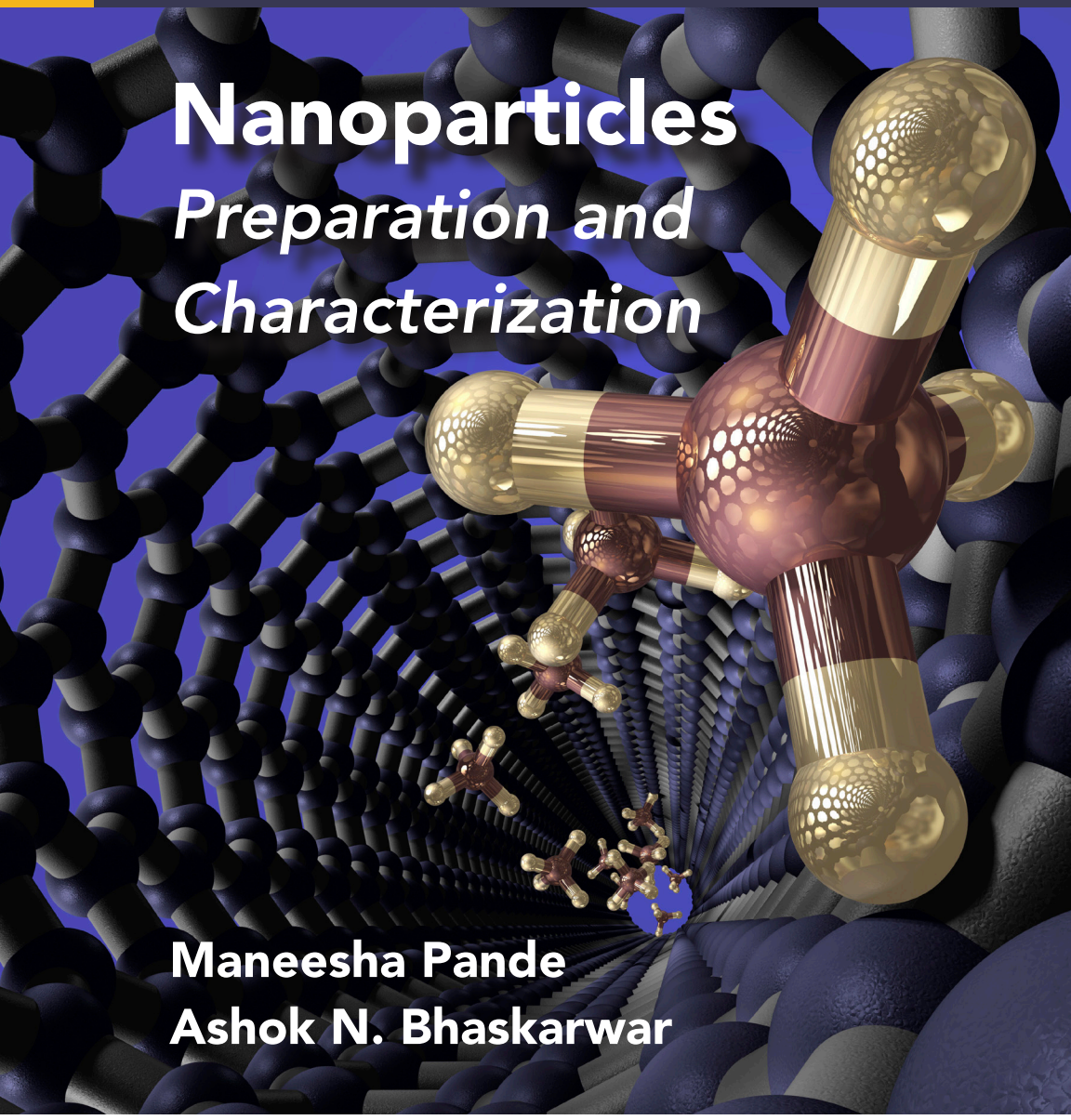
NANOTECHNOLOGY COLLECTION

Nanoparticles

Preparation and Characterization



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MOMENTUM PRESS
ENGINEERING

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Abstract

Nanotechnology and nanoparticles have emerged as an important tool towards improving cancer therapeutics and diagnostics. Recognizing the indispensable role of nanoparticles specifically in targeted delivery of chemotherapeutic and other anti-cancer agents to tumors, this book provides a comprehensive account of the different methods used for the preparation of nanoparticles, including the mechanism behind each method, for a beginner in the field. The commonly used methods of physical post-synthesis characterization, have also been described. The toxicity aspects of nanoparticles have been highlighted, particularly the effect of nanoparticles on different systems of the human body. Appreciating the interdisciplinary nature of nanotechnology applications in cancer drug delivery, a brief description of the genesis and growth of a tumor has also been included in the book.

KEYWORDS

microscopy, nanoparticle characterization, nanoparticle preparation methods, nanoparticle toxicity, nanoparticles, nanotechnology, targeted drug delivery, tumor

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Preface

Cancer has remained among the top five diseases in terms of morbidity for several decades now. It has unfortunately defied a cure in spite of intense research. The most common treatment therapy for cancer, that is, chemotherapy, is much dreaded for its adverse effects. Nanotechnology, a completely man-made and much-awaited curative, has come to the rescue of chemotherapy, or rather the patients, by way of enabling the targeting of chemotherapeutic agents specifically to the cancer cells. Nanotechnology based cancer "theranostics" (a portmanteau of "therapeutics" and "diagnostics," combined into a single drug delivery system) has lately gained popular acceptance. The present collection of volumes, in the sub-area "Nanotechnology Platforms in Cancer Diagnostics, Therapeutics and Imaging," aims at bringing together the important developments as well as addresses the major topics in the field.

The first volume in this collection, "Nanoparticles: Preparation and Characterization," describes the different methods used to prepare nanoparticles for drug delivery in a readable account. As the field of "Nanomedicine," that is, the application of nanotechnology to medicine, is highly interdisciplinary in nature, this book addresses the students/researchers from different academic backgrounds desirous of entering into research in this area and also help encourage every thinking mind to contribute to cancer research in whatever way possible to quicken the pace of progress for overcoming the sizable and apparently insurmountable challenge of this dreaded disease. The topics covered provide the newcomer in this field with an idea of the basic concepts involved so that the contents offering an in-depth treatment of the topics later are understood with a greater clarity.

The first chapter of this book is an introduction to nanotechnology and includes the overall developments in the field since Richard Feynman first introduced this concept to the world together with the unlimited possibilities made available by adopting the nanometer scale. It also introduces the reader to the relevance of nanoparticles in drug delivery,

specifically cancer drug delivery, and the various types of nanoparticles that have come into common practice in the field of nanomedicine.

This being the first volume in the sub-area "Nanotechnology Platforms in Cancer Diagnostics, Therapeutics and Imaging," some relevant aspects of the genesis of cancer and the characteristics that cancer cells acquire while developing into a tumor are described briefly in Chapter 2. These components are important in developing a targeted drug delivery system for cancer, in understanding it as well as for diagnostics and imaging.

The third chapter deals with the different methods of preparation of nanoparticles for drug delivery applications. The salient features of each method are first explained, followed by examples of nanoparticles prepared using the method. The aim of this chapter is to expose the reader to the fundamental principle(s) behind each method so that it may be manipulated to obtain the desired product characteristics.

The fourth chapter deals with the methods used for characterizing nanoparticles. Only the most commonly used characterization methods have been described. In this chapter too the fundamental principles are described so that the reader is able to critically select the method relevant to the case in hand.

The fifth chapter provides an overview of the toxicity aspects of nanoparticles as these are extremely relevant for the person handling the nanomaterials and also for the intended user of the nanomaterial. The effects of nanomaterials on the different organs/systems of the human body are briefly described. Besides, the environmental and ecological impacts of the nanomaterials have also been addressed in brief.

The sixth (and the last) chapter of the book deals with the current developments taking place and the future directions anticipated in this area.

We thank the Indian Institute of Technology, Delhi, India, for providing all the facilities and the students and faculty for creating the intellectual environment conducive to the writing of this book.

Acknowledgments

We thank the Indian Institute of Technology, Delhi, India, for providing all the facilities, all the students, particularly the students in our research group, and all faculty members of the Department of Chemical Engineering at IIT-Delhi for creating the intellectual environment conducive to the writing of this book collection. Special thanks are due to Mr. Gajanan Limkar and Ms. Madhura Pande for helping in the creation of some of the illustrations used in this book.

CHAPTER 1

Introduction to Nanotechnology

Nanoscale materials have been present in nature ever since the beginnings of life on earth. Organisms such as mollusks construct strong shells around them by arranging calcium carbonate into compact nanostructured bricks held together by a carbohydrate-protein mixture that is secreted by the organism and acts as a cementing material. There are numerous examples from ancient times where nanostructures or nanoparticles have been used for various purposes. The varieties of beautiful colors of windows of medieval cathedrals have been attributed to the presence of metal oxide nanoparticles in glass. As far back as the 4th century AD, Roman glass makers were exploiting the exceptional properties attained by particles in the nano range by fabricating glass containing nanosized metal particles. A classic example of this is the Lycurgus Cup present in the British Museum in London. This cup is made of soda-lime glass that contains gold and silver nanoparticles distributed in it in a specific arrangement. The normally opaque green cup appears translucent deep red in color when light is shone through it (Poole and Owens 2003). The Damascus-steel swords made and used between 300 and 1700 AD in the Middle East are yet another example of the use of nanoparticles in ancient times. It has now been confirmed by high resolution transmission electron microscopy that the material with which these swords were made of (i.e., Damascus steel) contains oriented cementite nanowires and carbon nanotubes, which give them their characteristic extraordinary strength and an exceptionally sharp cutting edge (Reibold et al. 2006).

The present nanotechnology has its origins in the famous after-dinner talk "There's plenty of room at the bottom" given by Sir Richard Feynman in an American Physical Society meeting at the California Institute of Technology (Caltech) on December 29, 1959. This historical talk was first published in Caltech's magazine in 1960 (Feynman 1960). In this

lecture, Feynman talked of the limitless possibilities that could arise if one could go down to the nanoscale and manipulate matter at a molecular level. He referred to building small nanoscale "molecular machines" that could make complex molecules by physically building them up atom by atom with utmost precision. Realization of this ability would literally change the dimensions of libraries the world over as one could have volumes of books such as the *Encyclopaedia Britannica* on the head of a pin! He spoke of being inspired by the biological system where these phenomena are already taking place at this scale. The DNA present in the tiny cells not only contains "information" for making different substances such as enzymes, but these are actually being synthesized in the cells and that too with utmost precision. In order to motivate young researchers toward realizing this vision, the Foresight Institute (a think tank and public interest organization created to support transformative technologies) announced the "Feynman Prize in Nanotechnology" in the year 1993 for exceptional contributions toward building such "molecular machines" capable of atomically precise manufacturing. These awards are now being given annually in two categories, for theoretical work and experimental work, by the Foresight Institute (n.d.). Table 1.1 provides a chronological overview of the landmarks and milestones achieved in nanotechnology since Sir Richard Feynman's revolutionary talk.

The Feynman Prizes in Nanotechnology awarded over the years demonstrate how we are gradually reaching toward attaining the vision of "molecular machines." Way back in 1986, Eric Drexler, in his book *Engines of Creation: The Coming Era of Nanotechnology* laid out foundations for practically materializing these "molecular machines" (Drexler 1986). A strong proponent of this technology (using the "top-down" approach in which larger machines are used to make smaller objects), he had predicted the vast possibilities that such machines could achieve. These were based upon sound fundamental principles of science. He had also cautioned about the dangers of such a technology. On the other hand, Prof. Richard Smalley, known for his discovery of fullerenes, rejected such "molecular assemblers" as something that was not physically possible. Though Smalley also believed in the limitless potential of nanotechnology, he was of the opinion that:

> "you cannot make precise chemistry occur as desired between two molecular objects with simple mechanical motion along a few degrees of freedom in the assembler-fixed frame of reference …. You need to guide the reactants down a particular reaction coordinate, and this coordinate treads through a many-dimensional hyperspace." (http://pubs.acs.org/cen/coverstory/8148/8148counterpoint.html)

Table 1.1. Overview of landmarks and milestones achieved in nanotechnology

Year	Milestone achieved	Brief description
1959	First visionary lecture that started the present unhampered progress in nanotechnology	Sir Richard Feynman gave the historical lecture "There's plenty of room at the bottom," emphasizing the limitless possibilities of nanoscale applications.
1974	First use of the term "nano-technology"	Prof. Norio Taniguchi used the term "nano-technology" in a conference paper titled "On the Basic Concept of Nano-technology" in an International Conference on Production Engineering, Tokyo.
1977	Initiation of molecular nano-technology concepts at MIT	Eric Drexler originated the concept at MIT, which culminated in the first doctoral degree in molecular nanotechnology in 1991.
1981	First technical paper on nano-technology	Eric Drexler published the first technical paper titled "Molecular Engineering: An Approach to the Development of General Capabilities for Molecular Manipulation" in *PNAS*, USA.
	Scanning tunneling microscope (STM) invented	STM is an instrument used for imaging surfaces at the atomic level (this led to the discovery of fullerenes in 1985). STM was invented by Gerd Binnig and Heinrich Rohrer (at IBM Zurich Research Laboratory) who were awarded the Nobel Prize in Physics in 1986 for their invention.
1985	Buckyball (fullerenes) discovered	Harry Kroto, Richard Smalley, and Robert Curl discovered fullerene, which helped them win the Nobel Prize in Chemistry in 1996.

(*Continued*)

Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
1986	First book on nanotechnology published	Authored by Eric Drexler, with a Foreword by Marvin Minsky, the first book on nanotechnology "Engines of Creation: The Coming Era of Nanotechnology," published.
	AFM invented	Atomic force microscopy (AFM), one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale, was invented by Gerd Binnig, Calvin Quate, and Christopher Gerber, and first commercialized in 1989.
	First organization formed	Leading think tank "Foresight Institute" founded with the purpose of promoting transformative future technologies with a mission of discovering and promoting the advantages and avoiding the dangers of such technologies, including nanotechnology.
1987	First university symposium	The symposium entitled "Exploring Nanotechnology" was organized by the MIT Nanotechnology Study Group. It provided a platform for participants to discuss the technical, political, economic, and social aspects of nanotechnology.
1988	First university course	A 10-week course taught by Eric Drexler at the Stanford University attended by 50 students.
1989	IBM logo spelled in individual atoms	IBM researcher Don Eigler manipulated 35 Xenon atoms using STM to spell out the IBM logo.
	First national conference	The "First Foresight Conference on Nanotechnology" was chaired by Eric Drexler and hosted by the Stanford University.

1990	First nanotechnology journal	The journal "Nanotechnology" was published by the Institute of Physics, UK.
	Japan's STA begins funding nanotech projects	Japan's Science and Technology Agency (STA) identified nanotechnology as a "priority target for industrial research" and started funding relevant projects through its program, Exploratory Research for Advanced Technology. Stressed on the "bottom-up" approach.
1991	Japan's MITI announced the bottom-up "atom factory"	Ministry of International Trade and Industry (MITI) recognized the "bottom-up" approach as more promising. Promoted interdisciplinary research in nanotechnology.
	IBM endorsed the "bottom-up" approach	IBM Chief Scientist and Vice President for Science and Technology J. A. Armstrong endorsed the bottom-up approach for making electronic and mechanical devices atom by atom.
	Japan's MITI committed ¥25 billion (about $200 million) toward research in molecular nanotechnology	MITI became an international leader in molecular nanotechnology by launching the first major project—a 10-year program—in the form of a research consortium including 46 companies. Focused on development of higher density computer memory, new materials including new catalysts for environmental clean-up, and gene manipulations.
	Carbon nanotube discovered	Facilitated by the development of STM, carbon nanotubes were discovered independently by Sumio lijima of Japan's NEC and Bethune of IBM.

(*Continued*)

Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
1992	First textbook published	A textbook titled "Nanosystems: Molecular Machinery, Manufacturing, and Computation," authored by K. Eric Drexler, published by John Wiley & Sons, Inc.
	First Congressional testimony	U.S. Senate Committee on Commerce, Science, and Transportation's subcommittee on Science, Technology invited Dr. Eric Drexler to testify on molecular nanotechnology.
1993	First Feynman Prize in Nanotechnology awarded	Awarded to Charles Musgrave of Caltech for his work on modeling a hydrogen abstraction tool useful in nanotechnology.
	First coverage of nanotechnology from the White House	A report from the White House Office of Science and Technology Policy, entitled "Science and Technology: A Report of the President," included coverage of molecular nanotechnology and molecular manufacturing.
	First university nanotechnology center envisaged	"Engines of Creation" book, given to Rice administration, stimulated the first university nanotech center with the Rice University in Houston announcing plans to build a nanotechnology research and teaching laboratory.
1994	"Nanosystems: Molecular Machinery, Manufacturing, and Computation" textbook used in the first university course	The first university course based on the book *Nanosystems: Molecular Machinery, Manufacturing, and Computation* started at the University of Southern California, taught by Prof. Ari Requicha of the Computer Science and Electrical Engineering Departments.

	U.S. Science Advisor advocated nanotechnology	Dr. Jack Gibbons, Director of the White House Office of Science and Technology Policy, which coordinates science and technology policy throughout government, advocated nanotechnology in his address to the National Conference on Manufacturing Needs of U.S. Industry, held at the National Institute of Standards and Technology.
1995	First think tank report	This report titled "The potential of nanotechnology for molecular manufacturing" by Nelson and Shipbaugh provided a framework for understanding the scope of nanotechnology: possible benefits, development risks, and policy options, subject to careful and objective feasibility assessment.
	First industry analysis of military applications	Hughes Aircraft Company foresaw a significant role of nanotechnology in military applications.
	Feynman Prize in Nanotechnology awarded for synthesis of complex three-dimensional structures with DNA molecules	Prize awarded to Nadrian C. Seeman, chemistry professor at the New York University for his pioneering work in synthesizing complex three-dimensional structures with DNA molecules.
1996	Feynman grand prize of $250,000 announced	The prize was announced by "Foresight Institute" a not-for-profit organization, for the first persons to design and build two nanotechnology devices, a nanoscale robotic arm and a computing device, which would demonstrate the feasibility of building a nanotechnology computer.
	First European conference	NanoTech®, a newly formed subsidiary of BioSoft (both Danish firms), was the first to stage a major nanotechnology conference in Europe. The purpose of the conference was to create a new European nanotechnology initiative.

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Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
	NASA began work on computational nanotechnology	Inspired by Eric Drexler's book, "Nanosystems: Molecular Machinery, Manufacturing, and Computation," scientists at NASA recognized the potential of nanotechnology in improving aerospace and computer systems. Particularly crucial for this was the role of computational nanotechnology in the design and simulation of programmable molecular machines.
	First nanobio conference	The International Business Communications Conference titled "Biological Approaches and Novel Applications for Molecular Nanotechnology," attended by researchers and business leaders, focused on the following areas of nanosystem technology: (i) fabrication, (ii) characterization, (iii) connections to the outside world, and (iv) near-term applications, particularly in the areas of sensors, electronic devices, and photodevices.
1997	First company "Zyvex" founded	Founded by Jim Von Her with the aim of going beyond simulations and actually starting "Engines of Creation"—nanomanufacturing plants that could manufacture bulk materials or arbitrary structures with atomic precision, getting nearly every atom in the desired place.
	First design of nanorobotic system	This design—a modification of the original "Stewart platform" proposed in the book *Nanosystems*—was proposed by Eric Drexler.
	Feynman Prize in Nanotechnology awarded for work in computational nanotechnology using scanning probe microscopes to manipulate molecules	For the first time, the prize was divided into one prize for experimental work and another prize for theoretical work. The award for experimental work went to teams at IBM Zurich (for using scanning probe microscopes for molecular manipulations) and the award for theoretical work went to teams at NASA Ames (for computational nanotechnology).

1998	First NSF forum held in conjunction with Foresight conference	National Science Foundation (NSF), in conjunction with the Sixth Foresight Conference on Molecular Nanotechnology, sponsored the forum "From Scientific Discovery to the Nanotechnology of Tomorrow" to discuss key issues: Interdependence and synergism between scientific discovery and technology in nanoscale research The path from fundamental discovery of new properties and phenomena to industrial applications in nanotechnology The ways to facilitate and best utilize current and anticipated leap advances in nanotechnology.
	First DNA based nano-mechanical device	Dr. Nadrian C. Seeman and his co-workers at the New York University constructed a controllable molecular mechanical system using synthetic DNA as building material. This system would be capable of making devices using branched DNA molecules. This work was reported in the January 14, 1999 issue of *Nature* (Chengde Mao, Weiqiong Sun, Zhiyong Shen, and Nadrian C. Seeman. "A Nanomechanical Device Based on the B–Z Transition of DNA." *Nature* **397**, 144–46). This could be considered as the first step toward the development of nano-robots that might someday construct individual molecules in molecular-scale factories.

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Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
	Feynman Prize in Nanotechnology awarded for computational modeling of molecular tools for atomically precise chemical reactions and building molecular structures through the use of self-organization	The award for experimental work went to M. Reza Ghadiri of Scripps Research Institute for groundbreaking work in constructing molecular structures through the use of self-organization (the same forces used to assemble the molecular machine systems found in nature). The award for theoretical work went to Ralph Merkle (Xerox Palo Alto Research Center) and Stephen Walch (NASA Ames Research Center) for their computational modeling of molecular tools for atomically precise chemical reactions.
1999	First nanomedicine book published	A book titled "Nanomedicine, Volume I: Basic Capabilities," authored by Robert A. Freitas, was published by Landes Bioscience. This book was envisaged as the first part of three volumes to be published over a course of several years.
	First safety guidelines	Foresight Institute developed "Guidelines for Responsible Nanotechnology Development." These were designed to address the potential positive and negative consequences of nanotechnology with an objective of providing informed policy decisions by citizens and governments. The Guidelines were presented in the active format of self-assessment scorecards for nanotechnology practitioners, industry organizations, and regulatory agencies.
	Congressional hearings on proposed national nanotechnology initiatives	Ralph C. Merkle provided a testimony titled "Nanotechnology: the coming revolution in manufacturing," to the U.S. House of Representatives Committee on Science, Subcommittee on Basic Research.

	Feynman Prize in Nanotechnology awarded for the development of carbon nanotubes for potential computing-device applications and for modeling the operation of molecular machine designs	The award for experimental work went to Phaedon Avouris of IBM for the development of carbon nanotubes for potential computing-device applications and the award for theoretical work was shared by Prof. William A. Goddard III, Dr. Tahir Cagin, and Ms. Yue Qi of Caltech for their work in modeling the operation of molecular machine designs.
2000	President Clinton announced the U.S. National Technology Initiative	An ambitious program to accelerate basic research in the field of nanotechnology announced.
	First state research initiative	A state research grant of $100 million each was announced under the California Institutes for Science and Innovation to help improve the future of the state's economy by focusing on technology challenges in many fields, including nanotechnology.
	Feynman Prize in Nanotechnology awarded for computational materials science for nanostructures and for building a molecular switch	The award for experimental work went to the multidisciplinary team of chemist R. Stanley Williams and computer scientist Philip Kuekes, both from HP Labs in Palo Alto, CA, United States, along with chemist James Heath of University of California, Los Angeles (UCLA) for their work in building a molecular switch (a major step toward their long-term goal of building entire memory chips that are just a hundred nanometers wide, smaller than a bacterium). The award for theoretical work went to Georgia Tech physicist Uzi Landman for his pioneering work in computational materials science for nanostructures.

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Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
2001	First report on nanotech industry	Nanotechnology Opportunity Report was the first one to focus on the near-term business opportunities in nanotechnology.
	United States announces the first center for military applications	Program announced by the U.S. Army to establish an "Institute for Soldier Nanotechnologies (ISN)" and a University Affiliated Research Center, with academic and industry partners, for developing nanometer-scale science and technology solutions that could be incorporated into a soldier's gear.
	Feynman Prize in Nanotechnology awarded for theory of nanometer-scale electronic devices and for synthesis and characterization of carbon nanotubes and nanowires	The award for experimental work went to Prof. Lieber for his pioneering work in molecular nanotechnology, which included significant contributions to the synthesis and characterization of the unique physical properties of carbon nanotubes and nanowires. The award for theoretical work went to Prof. Ratner for his major contributions to the development and success of nanometer-scale electronic devices.
2002	Feynman Prize in Nanotechnology awarded for using DNA to enable the self-assembly of new structures and for advancing our ability to model molecular machine systems	The award for experimental work went to Prof. Chad Mirkin for opening up new possibilities for the fabrication of molecular machine systems by selectively functionalizing nanoparticles and surfaces, particularly with DNA, thus enabling the self-assembly of new structures, moving us closer to the goal of molecular manufacturing. The award for theoretical work went to Prof. Don Brenner for fundamental advances in our ability to model molecular machine systems and for the design and analysis of components likely to be important in future molecular manufacturing systems.

2003	Congressional hearings on societal implications	Testimony on "Molecular Manufacturing: Societal Implications of Advanced Nanotechnology" presented by Christine Peterson at the Committee on Science, U.S. House of Representatives Hearing, to examine the societal implications of nanotechnology and consider H.R. 766, The Nanotechnology Research and Development Act of 2003.
	Call for balancing National Nanotechnology Initiative (NNI) research portfolio	A white paper was submitted to the White House Office of Science and Technology Policy by Neil Jacobstein, Ralph Merkle, and Robert Freitas, which proposed that the portfolio of NNI research and development projects should be balanced periodically to ensure a range of low-, medium-, and long-term projects and those with a wider range of risk.
	Drexler–Smalley debate is published in *Chemical & Engineering News*	An intense debate between Eric Drexler and Richard Smalley on the ultimate possibilities that nanotechnology could offer started with an exchange of open letters between them. Whereas Drexler was a strong proponent of the capacity of "molecular assemblers," that is, nano-machines capable of building atomically precise products (atom by atom assembly) without the need of any solvents or enzymes for desired positioning, Smalley believed that this was not physically possible as one could not make desired and precise chemistry occur between two molecules by simply mechanically bringing them together. http://pubs.acs.org/cen/coverstory/8148/8148counterpoint.html

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Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
	Feynman Prize in Nanotechnology awarded for modeling the molecular and electronic structures of new materials and integrating single-molecule biological motors with nanoscale silicon devices	The award for experimental work went to Dr. Carlo Montemagno of the University of California for his pioneering research into methods of integrating single molecule biological motors with nanoscale silicon devices. The award for theoretical work went jointly to Drs. Marvin L. Cohen and Steven G. Louie of the University of California at Berkeley, United States, for their contributions to the understanding of the behavior of materials. Their models of the molecular and electronic structures of new materials predict and help in the understanding of properties like structure, surface conditions, and interactions with other materials. Many of these predictions have later been confirmed experimentally.
2004	First policy conference on advanced nanotech	First conference on Advanced Nanotechnology: Research, Applications, and Policy held in Washington DC, United States.
	First center for nanomechanical systems	The Center of Integrated Nanomechanical Systems was established with the aim of developing and integrating cutting-edge technologies and promoting advanced research in nanodevices from fundamental building blocks to complete device integration.

	Feynman Prize in Nanotechnology awarded for designing stable protein structures and constructing a novel enzyme with an altered function	The award for experimental work went to Dr. Homme Hellinga of Duke University for groundbreaking work demonstrating the innovative use of "computationally directed protein engineering" techniques to re-engineer an enzyme found in nature into a novel one with different functions. The award for theoretical work went to Dr. David Baker of the University of Washington and Dr. Brian Kuhlman of the University of North Carolina for the development of a computer program called "Rosetta Design," which was used to design the first protein constructed with a backbone fold not found in nature. This structure was tested experimentally and was found to be very stable and matched the predicted structure with atomic level accuracy. Prof. Baker has since made Rosetta Design freely available to the research community.
2005	Feynman Prize in Nanotechnology awarded for designing a wide variety of single molecular functional nano-machines and synthesizing macromolecules of intermediate sizes with designed shapes and functions	The award for experimental work went to Dr. Christian Schafmeister, University of Pittsburgh, for his work in developing a novel technology synthesizing macromolecules of intermediate sizes (between 1,000 and 10,000 Daltons) with designed shapes and functions. Compared with solid-phase peptide synthesis, this technology enabled adjacent monomers to be connected through pairs of bonds (unlike single peptide bonds), thus forming rigid oligomers. The work included computer aided design software, which permitted designing of oligomers with desired shape, functional groups, solubility, and purity. Moreover, these could be assembled using automated equipment. The award for theoretical work went to Dr. Christian Joachim, Centre Nationale de la Recherche Scientifique, France, for developing theoretical tools and establishing the principles for the design of a wide variety of single molecular functional nano-machines.

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Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
2006	U.S. NNI report calls for experimentation toward molecular manufacturing	National Academies' National Research Council stressed the fact that though mechanical molecular assembly had been sufficiently demonstrated for simple devices and materials, its feasibility for more complex materials and devices needed to be established. Engineering of biological systems capable of operating outside the living cell and predicting reliability of such a manufacturing system was also desired to be focused upon.
	Feynman Prize in Nanotechnology awarded for work in molecular computation and algorithmic self-assembly and for producing complex two-dimensional arrays of DNA nanostructures	For the first time, the awards for theoretical as well as experimental work went to a single research team, Drs. Erik Winfree and Paul W. K. Rothemund of Caltech. They demonstrated the methods for universal computation with DNA. They developed the concept of "Algorithmic Self-assembly" in which computations are embedded in the process of crystal growth, thus enabling atomically precise construction. They also experimentally showed that DNA tiles could be designed to form crystalline nanotubes having stiffness greater than actin—the biological protein nano-filament. This pioneering work could lead to production of increasingly complicated two-dimensional arrays of nanostructures. This could be one more step toward "construction of atomically precise products through the use of molecular machine systems."

2007	Feynman Prize in Nanotechnology awarded for construction of molecular machine systems that function in the realm of Brownian motion, and molecular machines based upon two-state mechanically interlocked compounds	The award for experimental work went to Prof. Fraser Stoddart of UCLA for his contribution toward building active molecular machines for the production of practical nanoscale devices such as a functional "molecular muscle," which could amplify and harness molecular mechanical motions. The award for theoretical work went to David Leigh of University of Edinburgh for his work on the design and synthesis of artificial molecular motors and machines from first principles. He constructed molecular machine systems that were capable of functioning in the realm of Brownian motion.
2008	Technology roadmap for productive nanosystems released	Titled "Productive Nanosystems: A Technology Roadmap," Foresight Institute in collaboration with Waitt Family Foundation and Battele proposed a stepwise course of development to take place and milestones to be achieved in order to move from one stage to another, beginning with the then current nanotechnology capabilities to advanced systems. That was the first such attempt of its kind.
	Protein catalysts designed for non-natural chemical reactions	Considered a major milestone in nanotechnology, computational methods enabled the creation of "designer enzymes"—enzymes, which catalyzed reactions for which biological enzymes did not exist in nature.
	Feynman Prize in Nanotechnology awarded for work in molecular electronics and the synthesis of molecular motors and nano-cars and for theoretical contributions to nanofabrication and sensing	The award for experimental work went to James M. Tour of Rice University for the synthesis of nano-cars. This work could prove to be useful in exploring possibilities of bottom-up manufacturing. The award for theoretical work went to George C. Schatz of Northwestern University for his exceptional theoretical contributions to nanofabrication and sensing, particularly for modeling and optimization of the dip pen nanolithography method of nanofabrication and his explanation of plasmon effects in metallic nano-dots.

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Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
2009	An improved walking DNA nano-robot (nanobot)	Researchers S. J. Green, J. Bath, and A. J. Turberfield at Oxford University developed a two-legged molecular machine that could "walk" unaided along a single strand of DNA. The "feet" were made up of a short sequence DNA bases that attached to a complementary sequence on the surface of the DNA molecule. The sequence of bases was designed such that the "feet" had to compete for a "foothold." Thus, as one "foot" stepped down, the other was forced to lift off.
	Structural DNA nanotechnology arrays devices to capture molecular building blocks	Going a step further toward the aim of "molecular assembly," scientists Hongzhou Gu and Nadrian C. Seeman of New York University and Jie Chao and Shou-Jun Xiao of Nanjing University together demonstrated that it was possible to place a specific DNA target species into a selected slot in a "dynamically programmed DNA nanotechnological system." An error-correction system included in this device improved the reliability of the procedure.
	Design "from scratch" of a small protein that performed the function of natural globin proteins	Using basic engineering principles based on study of natural proteins, scientists Ronald L. Koder, J. L. Ross Anderson, Lee A. Solomon, Konda S. Reddy, Christopher C. Moser and P. Leslie Dutton of the University of Pennsylvania designed oxygen-transport proteins (similar to natural neuroglobin), starting from a non-natural helix forming a sequence of just three different amino acids.
	Organizing functional components on addressable DNA scaffolds	Scientists Sherri Rinker, Yonggang Ke, Yan Liu, Rahul Chhabra and Hao Yan of the Arizona State University showed that multiaffinity ligands could be incorporated into DNA nanostructures with precise, nanometer-level spatial control. This work could make it possible to create DNA nano-scaffolds that could engineer more complex and interactive biomolecular networks.

	Feynman Prize in Nanotechnology awarded for experimental demonstrations of mechanosynthesis using AFM to manipulate single atoms and for computational analysis of molecular tools to build complex molecular structures	The award for experimental work went to the team of Yoshiaki Sugimoto, Masayuki Abe (Osaka University, Japan), and Oscar Custance (National Institute for Materials Science, Japan) for their pioneering experimental demonstration of mechanosynthesis. Using noncontact AFM, they showed that it was possible to manipulate single atoms vertically and laterally on semiconductor surfaces at room temperature. The award for theoretical work went to Robert A. Freitas Jr. for his pioneering work in mechanosynthesis in which he proposed specific molecular tools and, starting from fundamental quantum chemistry principles, theoretically validated their ability to build complex molecular structures.
2010	DNA-based "robotic" assembly begins	The first "assembly line" made entirely of "nano-robots" (molecular machines made up of DNA strands) was successfully operated. Using DNA strands that could move with precision on special surfaces and other DNA strands that can "hold" molecules–atoms, a team of Hongzhou Gu, Nadrian C. Seeman (New York University), Jie Chao, and Shou-Jun Xiao (Nanjing University) demonstrated how three gold particles could bind together in different configurations to form eight different products.

(*Continued*)

Table 1.1. Overview of landmarks and milestones achieved in nanotechnology (Continued)

Year	Milestone achieved	Brief description
	Feynman Prize in Nanotechnology awarded for work in single atom manipulations and atomic switches and for the development of quantum mechanical methods for theoretical predictions of molecules and solids	The award for experimental work went to Masakazu Aono (MANA Centre, National Institute for Materials Science, Japan) for his "pioneering and continuing work, including research into the manipulation of atoms, the multi-probe STM and AFM, the atomic switch, and single-molecule-level chemical control including ultra-dense molecular data storage and molecular wiring; and his inspiration of an entire generation of researchers who have made their own ground-breaking contributions to nanotechnology." The award for theoretical work went to Gustavo E. Scuseria (Rice University) for his "development of quantum mechanical methods and computational programs that make it possible to carry out accurate theoretical predictions of molecules and solids, and their application to the chemical and electronic properties of carbon nanostructures." (www.foresight.org/about/2010Feynman.html)

2011	First programmable nanowire circuits for nano-processors	Yan et al. proposed the design, fabrication, and use of programmable, nonvolatile nanowire transistor arrays (Ge–Si core-shell nanowires) that could build fully integrated nano-processors with computing memory and addressing capability.
	DNA molecular robots learn to walk in any direction along a branched track	Scientists Richard A. Muscat , Jonathan Bath, and Andrew J. Turberfield of Oxford University developed programmable molecular robots capable of moving molecules–atoms on branched tracks with precisely controlled motion (before this, only movement along straight path was possible). This work could find applications in drug delivery.
	Mechanical manipulation of silicon dimers on a silicon surface	Physicist Philip Moriarty from University of Nottingham demonstrated mechanical manipulations of silicon dimers on an Si(100) surface and showed that mechanosynthesis could also be done using Si on lines parallel to using a diamond for this purpose.

Source: www.foresight.org/nano/history.html

The strong debate on the two approaches, which ensued between Drexler and Smalley, became the cover story of the magazine CNEAR of the American Chemical Society (Baum 2003). Smalley's approach of getting nanoscale materials is known popularly as the bottom-up approach in which atoms and molecules are brought together via different mechanisms (such as in the presence of a suitable medium) and under specific conditions so that they assemble to form the desired molecules or substances. A variety of methods have been developed to bring about such an assembly. This approach is the focus of this book, and the various methods that have been developed to prepare nanoscale materials using this approach are the subject matter of Chapter 3.

1.1 NANOTECHNOLOGY: DEFINITION

The term "nanotechnology" is used to define any technology that could be used to manipulate matter at the molecular level and create materials, devices, and structures having a dimension of 1 to 100 nm in at least one direction. Though the nanometer size range per se includes all materials in the range of 1 to 1,000 nm, this particular range of 1 to 100 nm, which defines nanotechnology, has a specific significance—the particles within this size range acquire properties that are radically different from those of the bulk material. This happens because of two major factors: (i) increase in the surface area to volume ratio (which increases progressively as the material gets smaller) and (ii) the size of the particle enters the domain where quantum effects predominate. In this range, as the particle size reduces, there is a dominance of the behavior of atoms on the surface of the particle over those in the interior of the particle (as the size reduces, the number of atoms on the surface increases relative to those in the interior). This effect changes the properties of the particles in this size range as well as the interaction of these particles with particles of other materials. This phenomenon is material dependent and may be observed in the case of many materials for size ranges much larger than 100 nm. Figure 1.1 shows the comparative sizes of objects so that the nanometer size range is appreciated. It is noteworthy to mention here that the smallest object that the human eye can see is around 0.1 mm.

Fundamentally, there are different critical lengths that govern the different properties of materials. For example, physical properties such as thermal and electromagnetic properties of materials are governed by the thermal diffusion length and scattering length. The mean free path

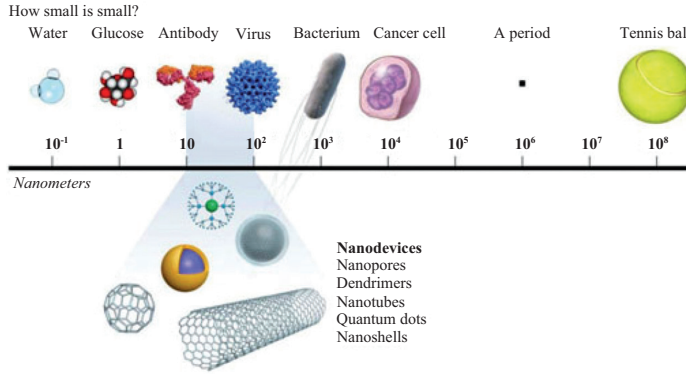

Figure 1.1. Relative sizes in the nanometer range.

Source: http://publications.nigms.nih.gov/chemhealth/cool.htm

of electrons, that is, the distance that electrons travel between collisions, governs the electrical conductivity of the materials. In the case of nanoparticles, as they have a diameter that itself is less than the different critical lengths mentioned earlier, they acquire significantly different thermal, electromagnetic, and electrical properties compared with the bulk materials (Poole and Owens 2003). In the case of polymeric materials and macromolecules, the defining size range of 1 to 100 nm cannot be applied as these molecules are very large. The molecular weight of polymers is usually in the range of 10 kDa to a few hundred kDa, or even higher. Therefore, even a small number of such molecules, say a thousand, coming together to form a nanoparticle would have a size greater than 30 to 50 nm. Hence, as applied to nanomedicine, that is, "drug-delivery systems developed on a nanometer size range having properties that provide exceptionally different medical and pharmaceutical benefits," (Devalapally, Chakilam, and Amiji 2007) the formal size range of 1 to 100 nm of nanotechnology expands to the entire nanometer size range, that is, 1 to 1,000 nm. Particles even much above 100 nm assume a great significance in this field because of the exceptional pharmacokinetic properties they possess compared with larger sized particles. The definition of nanotechnology as applied to nanomedicine could therefore be modified to "the study of materials, devices, and structures that are themselves in the 1 to 1,000 nm size range or have essential components in this entire submicron/sub-cellular size range" (Ferrari 2005).

Advances in nanotechnology, over the years, have steadily promoted a variety of nanostructures with general or specific applications or both in

nanomedicine. The following section includes definitions and descriptions of important nanostructures having applications in cancer diagnostics, therapeutics, and imaging.

Nanocarrier is a general term that describes a nanoparticle in the size range of 1 to 1,000 nm used to carry a single or multiple payloads such as drug, targeting agent, and imaging agent. Usually, nanocarriers are polymeric materials suitably functionalized for carrying the payload.

Nanovector is a term used to describe a multifunctional nanocarrier having a hollow or solid structure that contains a drug, particularly an anticancer agent, along with a targeting or imaging agent or both.

Core-shell nanoparticles is a general term used to describe nanostructures that comprise of a core of one material surrounded by a coating of a different material.

Nanocapsules are core-shell nanostructures having a liquid core coated by a biocompatible polymeric shell.

Nanoshells are core-shell nanoparticles having a core comprising of a dielectric material such as silica with a coating of a metal such as gold.

Nanocrystals are nanometer-sized single crystals.

Nanofibers are fibers having diameters in the nanometer range, usually used in the textile industry, but those made from biocompatible materials have applications in the biomedical field (as scaffold materials in tissue engineering) and drug delivery.

Nanorods are solid nanostructures having each of their dimensions in the 1 to 100 nm range and an aspect ratio (ratio of length to width) of 3 to 5.

Nanosponges are nanostructures comprising of hyper-crosslinked biocompatible and biodegradable polymers having nanosized pores or cavities.

Nanowires are nanostructures having diameters in the nanometer range and an aspect ratio of 1,000 or more and have applications in cancer detection.

Nanococoons are single strands of DNA that self-assemble and coil-up into a cocoonlike structure. The core of this cocoonlike structure contains anticancer drugs such as doxorubicin and a protein DNAse.

Liposomes are phospholipid-based concentric bi-layered vesicles with a hydrophilic aqueous interior and a lipophilic external layer. They resemble the cell membrane of animal cells. Due to their amphiphilic nature, they can be used to encapsulate hydrophilic as well as hydrophobic drugs. Liposomes are the first drug-carrying nanoparticles to reach the clinic (liposomes encapsulating the anticancer drug doxorubicin have already reached cancer clinics).

Polymerosomes are similar to liposomes except for the fact that these are composed of synthetic polymeric amphiphiles such as polylactic-acid based copolymers.

Solid lipid nanoparticles (SLNs) are composed of solid lipid cores surrounded by a phospholipid monolayer unlike liposomes, which are hollow in nature.

Polymeric micelles are spontaneously self-assembling colloidal micellar structures formed from amphiphilic copolymers containing both hydrophobic as well as hydrophilic blocks. A variety of hydrophobic blocks are available, but the most preferred hydrophilic block is poly(ethylene glycol) (PEG) as it is biocompatible, flexible, and gives "stealth" characteristics to the structure, that is, it prevents opsonization of the polymer micelle, thus giving it a longer circulation time in the blood.

Dendrimers are synthetic, symmetrically branched macromolecules having a dendritic structure similar to the dendrons or nerve cells present in our body. Starting from a central core comprising of a monomer, branched polymeric structures are created by repetitive, layer by layer attachment of other monomers to the functional end groups present on the previous layer, leading to a spherical polymeric structure of size usually less than 5 nm. Polyamidoamine dendrimers have shown a great promise in the biomedical field.

Inorganic nanoparticles having applications in nanomedicine are usually metal based, nearly monodisperse particles, useful for targeting, imaging or therapeutic effects or both, such as producing thermal ablation in cancer therapeutics.

Quantum dots are "nanocrystals" in the size range of 2 to 10 nm, composed of only about 10 to 50 atoms of semiconductor material (such as cadmium selenide capped by zinc sulphide (CdSe/ZnS)). Depending on their size, they produce fluorescence in different colors, allowing for their use as probes for high-resolution molecular imaging of cellular components and tracking movements of other bigger nanoparticles injected into the blood stream (accumulation of nanoparticles in various organs).

Nanoemulsions are transparent or translucent, oil-in-water or water-in-oil emulsions in which the size of the droplets constituting the dispersed phase is in the range of 100 to 500 nm. They are also called submicron emulsions. These are kinetically stable emulsions, as against microemulsions that are thermodynamically stable in nature.

Nanosuspensions are surfactant-stabilized colloidal dispersions of nanoparticles in a suitable liquid medium. In nanomedicine, the term nanosuspension is usually used for systems in which nanosized drug particles (distinct from polymeric drug carriers and SLNs) constitute the

dispersed phase. Drugs which are insoluble in both water as well as oil are maintained in a preferred crystalline state of sufficiently small size suitable for intravenous administration.

1.2 NANOMEDICINE

Nanomedicine, as the name itself indicates, is the use of nanotechnology/nanoparticles in the treatment of disease. Though the word "nanoparticles" has been coined only recently, the use of nanoparticles in medicine is not really new. Nanoparticles have been in use from ancient times. Ancient Indian literature mentions the preparation, characterization, and use of "bhasmas" that are none other than nanoparticles (Sarkar and Chaudhary 2010). The methods of their preparation and uses have been elaborately documented in the literature. Besides this, gold nanoparticles have also been used by the Egyptians at least 5,000 years ago for overall mental, physical, and spiritual purification and well-being. The Chinese have been using gold nanoparticles for longevity (Patra et al. 2010). A better understanding of the fundamental mechanisms of biological processes and the molecular basis of the various biosynthetic and metabolic pathways has, however, made it possible to better exploit the various advantages that nanoparticles can offer in relation to the drug delivery, therapeutics, and diagnostics.

Any new chemical/molecular entity (NCE/NME) has to undergo a rigorous development process before it can be approved as a "drug." The complete "new drug development process" is schematically shown in Figure 1.2.

The early screening, which may take anywhere between 1 and 2 years, evaluates the new chemical entity (NCE) for its physical and chemical properties because these properties will determine the extent of absorption and distribution (the bio-pharmaceutics and pharmacokinetics) in the body. At this stage, factors such as solubility, permeability across different biological membranes, lipophilicity, degree of ionization, gastrointestinal metabolism, stability in biological fluids, systemic pharmacokinetics/pharmaco-dynamics, and protein binding are some of the important aspects that need to be considered. A chemical substance that passes the early screening goes to the next stage, that is, preclinical evaluation wherein assessment of its safety or toxicity or both is done. This is done on the basis of pharmacological evaluation in suitable animal models. This phase may take two to three years. Clearing this stage makes NCE fit for investigational new drug application (INDA) submission.

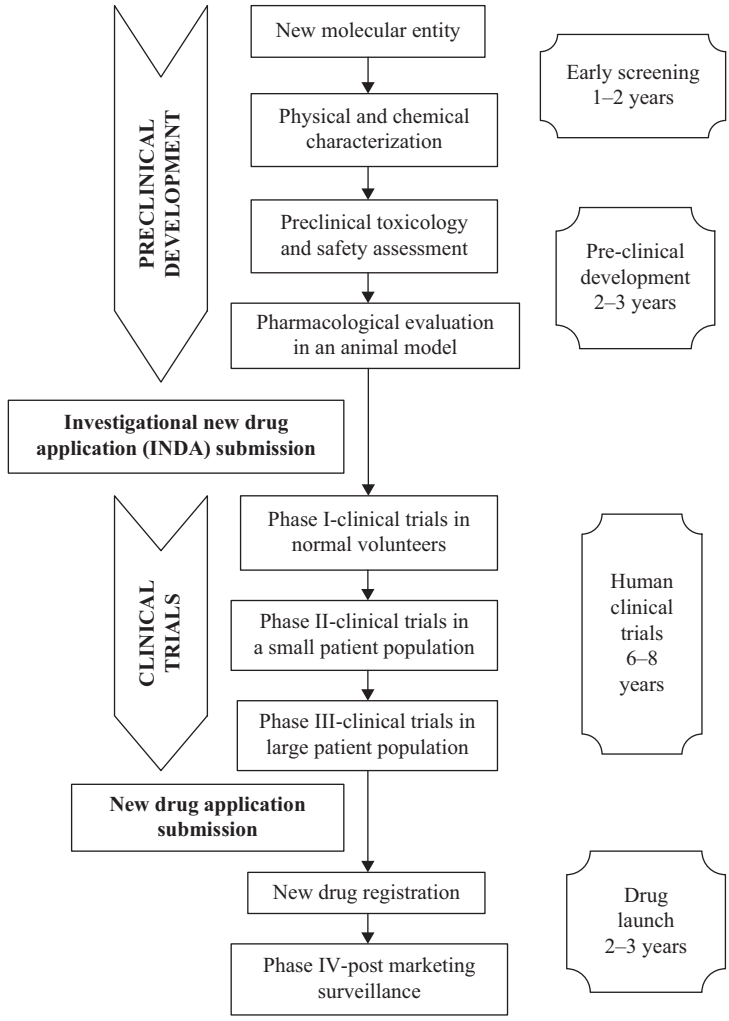

Figure 1.2. New drug development process: Stages and timelines.

Source: Adapted with permission from Devalapally, Chakilam, and Amiji (2007).

Following INDA approval, the human clinical trials can begin. This is the longest stage which takes about six to eight years. After successful completion of the first three phases of these clinical trials, a new drug application submission can be done. This approval clears the stage for launching the new drug candidate in the market. The final phase, that is, phase IV, which takes two to three years, is the postmarketing phase of the

clinical trials. After this, NCE becomes a "Drug." It has been found that about 40 percent of NMEs selected for full-scale development on the basis of preclinical development stage fail to reach the clinical trial stage due to poor biopharmaceutical properties, which are expected to translate into poor systemic absorption and undesirable pharmacokinetic properties. The nanoparticulate form of a drug or an NCE may completely alter its bio-pharmaceutic and pharmacokinetic properties. Thus, a substance with a poor solubility or permeability or both, which may be considered as an unsuitable candidate for development on the basis of its early screening, may find merit as a drug candidate after it is converted into the nanoparticulate form as this form improves its solubility and permeability to a significant extent. On the other hand, an approved drug substance, which has successfully passed all stages of the development process, may prove to be toxic after its conversion to the nanoparticulate form, if given in the same dose due to the significantly altered pharmacokinetic profile that such a change may cause. Thus, a complete reformulation may be required after converting a drug into the nanoparticulate form. Table 1.2 lists examples that demonstrate a favorable influence (in terms of improved systemic pharmacokinetics) when used with different nano-carrier systems (Devalapally, Chakilam, and Amiji 2007). It can be seen from the table that the total amount of drug absorbed, which subsequently reaches the blood stream (proportional to the area under the curve or AUC for a plot of concentration of the drug in the blood/plasma versus time), increases significantly for a nanoparticulate formulation. Simultaneously, in most cases, the drug clearance (CL) is decreased, which results in an increased mean retention time (MRT) and, thus, a greater bioavailability of the drug in the body (given in terms of F_{abs} and F_{rel}). The overall half-life, $t_{1/2}$, is increased whereas the elimination half-life ($t_{1/2\beta}$) decreases.

The importance of geometrical and physicochemical properties such as size, shape, and surface properties, targeting properties, drug loading and release characteristics, and toxicity considerations of nanoparticles in therapeutics has been highlighted by a number of researchers and reviewers (Balogh et al. 2007; Jiang et al. 2008; Kong, Braun, and Dewhirst 2000; Patra et al. 2010), facilitating appropriate modifications to be made to suit specific applications.

The distribution and accumulation of nanoparticles in the body have been shown to be predominantly size dependent. The diameter of the smallest capillaries in the body is around 5 to 6 μm. Hence, the ease in transport of nanometer-sized particles, as compared with micron-sized particles, in an unobstructed manner is quite evident. All nanoparticles less than 20 nm in diameter are able to pass through the blood vessel walls and

Table 1.2. Improvements in systemic pharmacokinetic parameters of drugs incorporated in different nano-carrier systems

Formulation	Pharmacokinetic parameters	Effect/improvement
SLNs		
Palmitate SLNs and PEG coated SLNs containing the drug azidothymidine	Area under the curve (AUC)	6-fold increased
Triglyceride SLNs containing the drug clozapine	$AUC_{0-\infty}$	2.91-fold increased
	CL	2.93-fold decreased
Polymeric nanoparticles		
Cyclic arginine-glycine-aspartic acid tripeptide modified nanoparticles containing doxorubicin	C_{max}	14 times increased
	$t_{1/2\beta}$	18% decreased
	CL	No change
Poly(butylcyanoacrylate) nanoparticles containing doxorubicin	AUC	1.3 and 2.2 times increased with dispersion polymerization (DP) and emulsion polymerization (EP) nanoparticles
	MRT	1.6 times increased
	CL	1.5 and 2 times decreased
	$t_{1/2}$	1.6 times increased
Poly(DL-lactide-co-glycolide) nanoparticles containing insulin	AUC, t_{max}	2 times increased
	MRT	1.5-fold increased
Chitosan nanoparticles containing the drug insulin	AUC	14.9% increased

(*Continued*)

Table 1.2. Improvements in systemic pharmacokinetic parameters of drugs incorporated in different nano-carrier systems (Continued)

Formulation	Pharmacokinetic parameters	Effect/improvement
Poly(DL-lactide-co-glycolide) and alginate nanoparticles containing the drugs clotrimazole/econazole	AUC	42 and 170 times enhanced with PLG nanoparticles and 138- and 500-fold enhanced with alginate nanoparticles
Eudragit-RS, LS nanoparticles containing the drug cyclosporine	AUC, F_{rel}, F_{abs}	4-fold decreased
Poly(N-isopropylacrylamide) and Poly(N-vinylamine) nanoparticles containing salmon calcitonin	AUC	2 times increased
Poly(isobutylcyanoacrylate) nanoparticles containing insulin	AUC	Significantly increased
Poly(ε-caprolactone)-block-poly(ethylene glycol) nanoparticles containing minoxidil	Skin retention	1.8–2.5-fold higher
Poly(DL-lactide-co-glycolide) nanoparticles containing the drugs rifampicin and isoniazide	F_{rel}	12.7-, 32.8-, and 14.7-fold increased
	F_{abs}	6.5-, 19.1-, and 13.4-fold increased

Source: Adapted with permission from Devalapally, Chakilam, and Amiji (2007).

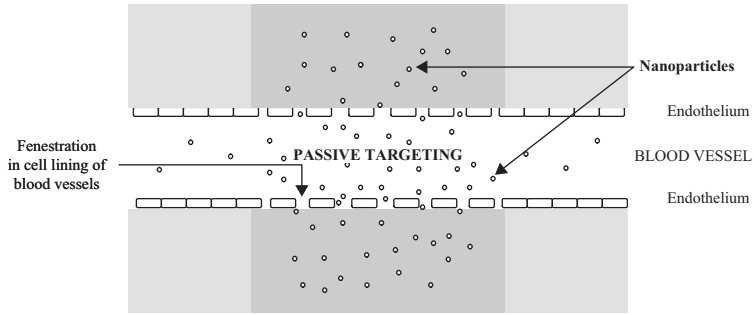

Figure 1.3. Passive targeting of nanoparticles by the EPR effect.

are suitable for intravenous injections, intramuscular, and subcutaneous applications (Praetorius and Mandal 2007). In addition to this, a number of researchers have shown that nanoparticles can be "passively" targeted and accumulated preferentially in tumors. This takes place because of the enhanced permeation and retention (EPR) effect (Figure 1.3). The vasculature supplying blood to tumors is largely abnormal around the tumor and has gaps or fenestration in the cells lining the blood vessels. The size of these gaps is just appropriate to allow nanoparticles smaller than 400 nm to permeate easily into the tumor, but exclude those particles that are larger than 400 nm.

In this manner, preferential accumulation of nanoparticles into tumors is possible. This accumulation can be enhanced if the nanoparticles are made to circulate in the blood stream for longer times by combining them with PEG, which prevents their detection and opsonization by the reticuloendothelial system. It has been shown that 10- to 100-fold higher concentrations of drug can be achieved by exploiting these two effects compared with administration of just the free drug (Kaul and Amiji 2002). The 250 nm particles with and without PEG showed more rapid clearance compared with 70 nm PEG-modified particles and particles without modification with PEG, respectively. In addition to this, size and surface charge of the nanoparticles further affect the clearance/uptake of the particles. Neutral particles show much lower opsonization compared with positively or negatively charged particles. Besides this, hydrophobic particles are more rapidly opsonized due to enhanced adsorption of serum proteins on the surface of these particles (Ferrari 2005). A study of the uptake of 2 to 100 nm particles showed that the nanoparticles in the size range of 40 to 50 nm showed enhanced receptor mediated uptake and internalization. In addition to this, shapes such as disclike, cylindrical, and hemispherical outperformed plain spherical particles with respect to uptake by cells (Jiang et al. 2008).

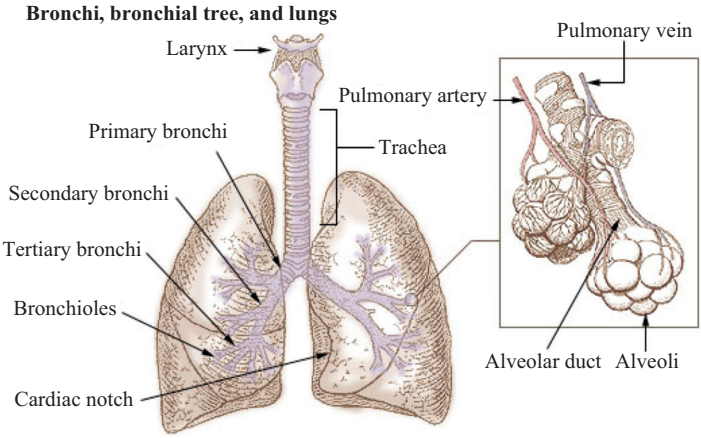

Figure 1.4. The bronchi, bronchial tree, and lungs.

Source: http://commons.wikimedia.org/wiki/File:Illu_bronchi_lungs.jpg#file

Particle size assumes exceptional importance in the case of pulmonary delivery of drugs, particularly for those required to be taken by inhalation. The diameters of bronchi progressively decrease as they successively branch out from trachea to the alveoli (Figure 1.4).

Consequently, it is not surprising that due to such a gradual narrowing down of the bronchioles, together with continuous cycles of inhalation and exhalation, different sized particles get deposited at different locations along the airway. The mechanisms of deposition are also different, with impaction being the main mechanism at the primary bronchi, whereas deposition by sedimentation occurs at the secondary bronchi and bronchioles. At the most distal portion, that is, at the terminal bronchioles, there is only a Brownian motion of the particles. Deposition of particles greater than 5 μm occurs in the proximal part—the upper respiratory tract—whereas those with diameter between 1 and 5 μm are deposited in the smaller airways and bronchioles. Particles less than 0.5 μm in diameter are able to reach up to the most distal part, that is, the terminal bronchioles or the alveolar region (Paranjpe and M**ü**ller-Goymann 2014). Thus, those drugs that are required to be delivered in the alveolar region must necessarily be in the nanometer range. Besides the size, the shape of the particle, surface morphology, and surface properties also play an important role in the deposition of inhaled particles. Nanoparticles because of their large surface areas and high surface energies may tend to form aggregates during their transition through the respiratory tract, and hence in spite of being in the nanoscale size range may not be able to reach the alveolar region, and instead may get

deposited in the proximal region. It has been shown that particles smaller than 260 nm, particularly those with a porous matrix, are able to successfully avoid uptake by alveolar macrophages and able to reach and get deposited at the alveolar level (Azarmi, Roa, and Löbenberg 2008).

The size-dependent uptake of nanoparticles is further modified by temperature. Studies with albumin liposomes for tumor uptake showed that the pore cut-off size for nanoparticles was 7 to 100 nm at 34°C (i.e., particles larger than 100 nm were not able to extravasate into the tumor) whereas this size increased to ~400 nm at 42°C (Kong, Braun, and Dewhirst 2000). Enhanced trans-dermal permeation (Kotyla et al. 2008) and enhanced permeation of nanoparticles across other biological membranes such as gastro-intestinal mucosa (Jia 2005) and blood–brain barrier (Koo et al. 2006) have also been shown.

Besides the passive targeting of nanoparticles to tumors, it has also been shown that selective, size-dependent uptake and accumulation of nanoparticles in certain tissues and organs in the body is possible (Balogh et al. 2007). Even without any targeting agent attached, nanoparticles with appropriate size, shape, and surface charge are able to preferentially accumulate into specific organs in the body. Balogh et al. (2007) carried out a study in which they prepared 5, 11, and 22 nm gold-dendrimer nanoparticles. The 5 nm particles were prepared with positive, negative, and no charge on their surface; the 11 nm particles had a negative charge; and the 22 nm particles had a positive charge on the surface. Studies in mice over a four-day period showed that: (i) all nanoparticles disappeared from the blood circulation rapidly with a half-life of two hours or less; (ii) with 5 nm particles, the positively charged nanoparticles remained in the kidneys for up to four days and were mostly excreted in the urine, whereas the negative and neutral particles remained in the liver and spleen; (iii) with the 22 nm positively charged particles, only low levels were found in the kidneys, whereas most of them reached liver, lungs, and spleen; (iv) none of the nanoparticles reached the brain; and (v) all nanoparticles, irrespective of size and charge, accumulated in the body over time, after repeated exposure, which suggests possible toxicity issues upon long-term exposure.

Thus, there is ample evidence that size and other factors such as shape and surface charge do affect to a great extent the uptake, accumulation, and clearance of nanoparticles in the various organs and tissues in the body. These factors gain exceptional importance, particularly in cancer diagnostics and therapeutics. The following chapter (Chapter 2) explains briefly the genesis of cancer and typical characteristics of cancer cells and tissues so that the role of nanotechnology/nanoparticles in cancer nanomedicine is sufficiently well appreciated.

CHAPTER 2

Nanotechnology in Cancer Diagnostics, Therapeutics, and Imaging

Cancer is a disease characterized by an uncontrolled proliferation of cells. The term "cancer" has been broadly used in literature to include both benign and malignant tumors. "Neoplasm" is a more inclusive term (generally used synonymously with the term "tumor"), which literally means a "new growth." Prior to the 19th century, it was thought that much like microbial infections, tumors are also consequences of some foreign bodies entering and taking root in the body of the afflicted person (Weinberg 2007). However, with advances in technology making possible invention of microscopes capable of high resolution and magnification, the field of histology or histopathology emerged, and it was possible to actually view the different tissues in the body. These examinations using microscopes and comparisons of normal tissues with tumor tissues led to the present knowledge that all tumors, whether benign or malignant, are all composed of the body's own cells that have somehow got modified and, instead of multiplying and functioning in the normal manner, have acquired the capacity of uncontrolled proliferation and abnormal functioning that defy the usual norms set for the tissue. Tumors may be classified as "benign" or "malignant," depending on their degree of invasiveness. Those tumors that are localized and do not infiltrate into the adjacent tissue are termed as benign tumors. These can be surgically removed and, in most cases, will not recur if completely removed. *Malignant* tumors are those that have a tendency to invade the adjacent tissues and whose cells have the capacity to break away from the tumor and relocate to other locations through entering into the blood stream (known as "metastasis"). The word "cancer" is, strictly speaking, associated with

such malignant tumors. Most benign tumors are named by attaching the suffix "-oma" to the cell type constituting the tumor. The terms "sarcoma" or "carcinoma" are used to designate malignant tumors. Robbins (1974) provides details of nomenclature of tumors and a comprehensive classification of all tumors.

The field of cancer research was initially dominated by oncologists—clinical, surgical, or medical—who diagnosed cancer on the basis of clinical or histopathological tests or both and either surgically removed the tumor or used chemotherapy followed by radiotherapy to burn out the tumor. Simultaneously, pharmaceutical researchers were involved in finding new chemotherapeutic agents for fighting different cancers. Mechanisms of action of the different chemotherapeutic agents were sought to be understood and new targets for their action were discovered. This was followed by efforts to develop novel drug delivery systems for improving chemotherapy. Better understanding of the causes and genesis of cancer and the progressive discovery of different cell components and genes involved made it possible to identify specific targets, which could be exploited for improving the treatment of these cancers. Thus, targeted delivery systems have been developed that are able to specifically reach the tumor and kill only the tumor cells, leaving normal cells intact. Simultaneously, in-depth knowledge of the genesis of cancer has now made it possible to identify various markers that can be used as early indicators of the presence of tumors in the body. The importance and advantages of using nanoparticles for targeted delivery of chemotherapeutic agents have been realized. Thus, cancer research is now becoming increasingly interdisciplinary with a wide spectrum of fields having applications in cancer diagnostics, treatment, and imaging. Top-down and bottom-up synthesis and characterization of nanoparticles involving manipulation of the processes to yield desired sizes of particles involve chemists and physicists; targeted drug and gene delivery involves pharmaceutical researchers, microbiologists, and biotechnologists; biomarker research and cancer diagnostics involve biotechnologists and physicists; the study of microfluidics associated with cancer drug delivery involves chemical engineers: this list may involve several other areas and is only indicative of the highly interdisciplinary nature of the area of cancer research. Considering such an interdisciplinary involvement in cancer research, this chapter provides an overview of certain topics such as the genesis and growth of a tumor, aspects of cancer diagnostics and imaging, cancer therapeutics, and the role of nanoparticles in each of these areas so that the synthesis of nanoparticles—the main focus of this book—is done objectively.

2.1 GENESIS OF CANCER

The fully grown human body is a result of innumerable repeated cycles of cell growth and cell divisions (through a process called mitosis) of a single fertilized egg. In other words, a single fertilized egg, as a result of repeated cycles of cell division, results in a multitude of cells that eventually differentiate into a variety of tissues and organs present in the fully grown human body. This is possible because of the fact that most cells carry a complete organismic genome, that is, the complete capacity of developing into any type of cell present in the body. Thus, the individual cell is empowered with an immense autonomy and versatility. Many cells retain this ability long after the complete development of the organism, which enables replacement and maintenance of damaged cells or tissues. However, this same autonomy and versatility pose a grave danger when the genomic sequences get corrupted due to some internal or external factors. This corruption of genomic sequence, called mutation, can lead to the formation of an army of cells that have a completely altered cellular growth agenda. These cells defy all rules governing normal tissue construction and maintenance and are committed to generating more copies of themselves. Such a tissue results in what we call a tumor or neoplasm. The abnormal genes formed as a result of mutations are called "oncogenes." The precursors of these oncogenes are "proto-oncogenes," which are normally present in cells. In the presence of certain triggers, these proto-oncogenes get converted to oncogenes. However, all organisms have such tightly regulated mechanisms favoring normalcy that the activity of these oncogenes is suppressed by "antioncogenes," which are also present in the same cells. Thus, not merely the generation of oncogenes, but simultaneous mutations causing inactivation of antioncogenes is required before a tumor cell can take root in the organism. In addition to this, even the tumor cells have normal feedback controls that prevent their excessive growth. In spite of all this, defying all normal regulatory mechanisms, even if the mutated cancer cells do survive, the very fact that they are mutated forms leads to the formation of abnormal proteins within the cells, which are detected as a "foreign body" by the body's immune system, triggering formation of antibodies that eventually destroy these cells. Still further, different activated oncogenes are required *simultaneously* for the generation of tumor cells. For example, one gene is required to promote rapid proliferation; simultaneously, another gene is required to generate new blood vessels (called "angiogenesis") so that the increased demand for oxygen and nutrients required for cell growth and maintenance is adequately fulfilled (otherwise, the newly generated volume of cells will starve to their death). To facilitate the sprouting of these new blood vessels,

genes are required that generate factors capable of partially degrading the extracellular matrix (ECM). In addition to these factors, a number of other changes in the normal configuration of the cell and cellular processes needs to be effected. As a result, only a minute fraction of cells that mutate have the capacity for developing into a tumor (Guyton 2000).

2.1.1 CAUSES OF MUTATION

As mutations in genes are the starting point in the birth of a tumor, it is important to know the factors that cause such mutations. There are trillions of new cells that are formed in the human body each year. The process of mitosis (i.e., cell division), which is responsible for the generation of these new cells, is essentially preceded by the replication of the existing DNA chromosomal strands in the cell. There is a "proof reading" process that cuts and repairs any abnormal DNA strand formed. The process of mitosis is initiated only after ensuring the formation of normal DNA replicas. Thus, normally, the probability of mutations is very low. However, there are a number of external and internal factors that increase the probability of mutations (Guyton 2000).

1. Ionizing radiation: Radiations such as X-rays, gamma-rays, particle radiations from radioactive substances, and even ultraviolet radiations have a capacity of triggering mutations in normal cells. This is because such radiations are instrumental in the formation of ions or free radicals in tissues. These ions or free radicals, being highly reactive, can rupture the DNA strands, thus causing several mutations. Hence, frequent exposure to such radiations increases the chances of having cancer.
2. Chemical substances: Certain chemical substances such as aniline-based dye derivatives are known to cause cancer. Chemical substances that are capable of causing mutations are called "carcinogens." Tobacco is also a carcinogen. There are scores of other organic and inorganic substances that are carcinogenic in nature. Some of these are listed in Table 2.1.
3. Physical irritants: All types of foods that are capable of causing irritation to the intestinal lining resulting in its abrasion have the potential of causing cancer. This is because the damage caused to the tissue as a result of continuous abrasion of the lining triggers rapid mitotic divisions in an effort to replace the damaged cells. This rapid multiplication of cells can sometimes adversely affect the "proof reading" process prior to mitosis, thus increasing the chances of mutations and, hence, cancer.

Table 2.1. Some cancer causing substances

Type of carcinogen	Some examples
Physical carcinogens	X-rays, gamma-rays, alpha-particles, ultraviolet rays, and radioactive decay
Chemical carcinogens	Reactive oxygen species, nitrous acids, poly-aromatic hydrocarbons, alkylating agents, aromatic amines, sodium azide, benzene, and heavy metals such as arsenic, chromium, cadmium, and nickel
Biological carcinogens	Human papilloma virus, Epstein–Barr virus, hepatitis B and hepatitis C virus, human immunodeficiency virus, human herpes virus 8, human T-lymphotrophic virus-1, and Merkel cell polyomavirus

4. Hereditary tendencies: Mutated genes may be inherited from parents. Such genes already mutated increase the chances of more mutations occurring and hence increase the risk of leading to cancer.
5. Certain types of viruses: There are a number of viruses that have been identified as potential sources for originating mutations in human cells, thus causing cancer. Some of these viruses suspected of causing cancer are included in Table 2.1.

Besides these causes, another important factor that has been found to contribute toward an increased risk of getting cancer is the lifestyle related factor. Smoking and other tobacco related habits have been found to cause an increased risk of lung, bladder, and kidney related cancers. Diet low in vegetables and high in nitrates or salts or both have been found to be responsible for cancers of stomach and esophagus whereas fried foods and diet high in fats and low in fiber have been found to increase the risk of getting bowel related cancers, cancer of the pancreas and prostate, and breast cancer. On the other hand, substances such as anti-oxidant-rich vegetables, fruits, foods containing vitamins E and C, polyphenols, flavanoids and selenium-rich foods have been found to reduce the risk of cancer due to their capacity of offering protection against mutations.

2.1.2 CHARACTERISTICS OF CANCER CELLS

As has already been emphasized, cancer cells are the body's own cells in which the normal regulatory mechanism that governs the growth and multiplication

of cells has gone wrong. The cancer cells typically take over all control mechanisms to facilitate the generation of more copies of themselves. In order to do this, they acquire the following characteristics (Rang et al. 2003).

1. Uncontrolled proliferation:
   As already mentioned earlier, the transformation of proto-oncogenes to oncogenes and simultaneous inactivation of tumor suppressor genes are responsible for the generation of cancer cells. These cells grow and multiply without following the controls that govern the formation of normal cells.
2. Development of resistance to apoptosis:
   New cells are continuously formed by the process of cell division called mitosis. However, as newer cells are formed, it is essential that the older cells get removed from the body. Apoptosis is a process by which such older nonfunctional cells are removed from the body. It is, in a way, a genetically programmed cell death in which a sequence of biochemical events occurs and as a result a cleavage of the cell constituents such as the DNA, cytoskeletal components, enzymes, and so on takes place, and the cell is reduced to a cluster (still bound by the cell membrane), which is eventually phagocytosed by macrophages. This process is routinely involved in the elimination of unwanted cells like in the shedding of the intestinal lining, the regression of mammary gland after lactation, and so on. (This process, in which at no stage do the coagulated cell constituents spill out of the membrane within which they are bound, is markedly different from cell death by necrosis where the damaged cell disintegrates in an unorganized manner, resulting in cell debris that is capable of triggering inflammatory response.) Cancer cells develop resistance to apoptosis through the process of inactivation of the pro-apoptotic factors or activation of anti-apoptotic factors or both.
3. Expression of the enzyme telomerase:
   The chromosomes of all cells have a specialized structure (made up of nucleotides) at their ends. These are called telomeres. These telomeres protect the chromosomes from degradation, rearrangement, and fusion with other chromosomes. Each round of cell division erodes the telomere and this portion, unlike the remaining part of the chromosome, cannot be duplicated by the enzyme DNA polymerase. Eventually, as the entire telomere gets eroded, the cell undergoes senescence and subsequent cell death. An enzyme called telomerase is capable of maintaining and stabilizing the telomeres. This enzyme is present in certain cells like the germline cells, stem

cells, proliferating cells of the gastrointestinal (GI) tract, bone marrow, and so on, but the differentiated somatic cells do not have this enzyme. Hence, such differentiated cells that do not express telomerase have a predefined life span. However, most late stage cancer cells do express telomerase and are successful in achieving an unlimited life span or "immortality."

4. De-differentiation and loss of function:
   The fully grown human body results from the repeated multiplication of the stem cells, which eventually gives rise to different tissues and organs capable of performing specific functions. In other words, the stem cells are de-differentiated cells that can give rise to a complete set of differentiated cells. For example, some stem cells differentiate into muscle cells whereas others into fibroblasts. The muscle cells are capable of contracting and relaxing whereas the fibroblasts are capable of secreting. However, after differentiation, the muscle cells will always create other muscle cells and fibroblasts will create other fibroblasts. The muscle cells will not be able to form fibroblasts and vice versa. Cancer cells acquire the capacity to become de-differentiated to varying degrees. Such poorly differentiated cells can multiply faster and have a poor prognosis.
5. Lowered adhesiveness:
   ECM present between cells is capable of holding the cells together. Cancer cells become capable of secreting the enzyme matrix metalloproteinase (MMP) that breaks down ECM. As a result, cancer cells become less adhesive to each other and can break away from the tissue and infiltrate into the neighboring tissue, or slip through ECM and enter into the blood stream to be transported to distant tissues, leading to metastasis.
6. Invasiveness:
   Normally, cells that happen to break away from the original tissue lose their "survival signals"—the anti-apoptotic factors—and subsequently undergo apoptosis. However, as already mentioned in the earlier section, cancer cells develop resistance to apoptosis by the activation of anti-apoptotic factors and thus survive even after breaking away from their own tissue.
7. Angiogenesis:
   The tumor, when it grows beyond the diffusion limited size (see Section 2.1.3.), is unable to get adequate nutrition from the existing blood vessels. Hence, the cancer cells start producing certain growth factors that initiate the formation of new blood vessels (called angiogenesis).

8. Metastasis:
   As a result of the reduced adhesiveness of cancer cells due to their capacity to disintegrate ECM, the capacity of cancer cells to survive in an environment other than their own tissue, the ability of these cells to slip into the blood vessels, the increased invasiveness, and the capacity of cancer cells to generate new blood vessels, cancer cells are capable of lodging themselves into distant tissue (called metastasis). The result of metastasis is that secondary tumors are developed.

Figure 2.1 provides an outline of the genesis of cancer, starting from the initial trigger, that is, mutations in the gene sequences, to the development of secondary tumors. These characteristics of cancer cells cause changes in and around the cancer tissue as detailed in the following sections.

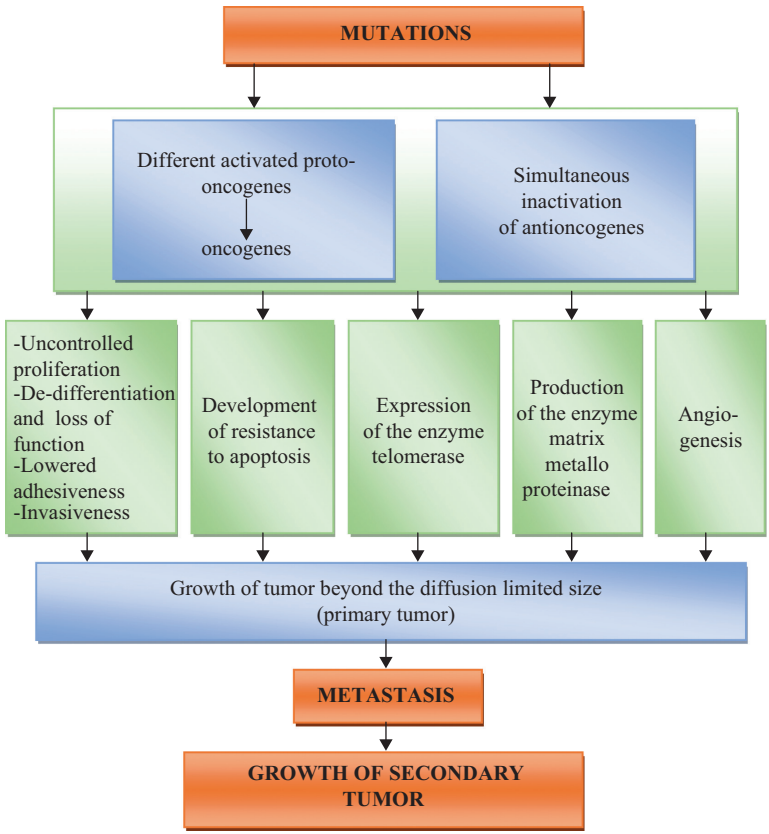

Figure 2.1. Genesis of cancer.

2.1.2.1 Enhanced Permeation and Retention Effect

As angiogenesis takes place in the tumor tissue at a very rapid rate, there are imperfections in the endothelial lining of the blood vessels. These imperfections are in the form of fenestrations or gaps between the cells of the endothelium. Depending on the tumor type and its location, these gaps may vary from ~200 to ~2,000 nm (Bertrand et al. 2014; Hobbs et al. 1998). Thus, nanoparticles, which would otherwise be unable to permeate through the vascular endothelium, can easily permeate such a "leaky" vasculature. Besides this, tumor tissues have a very poor lymphatic drainage. In normal tissue, the extracellular fluid drains into the lymphatic vessels at a mean flow velocity of around 0.1 to 2 μm/s (Bertrand et al. 2014; Swartz and Fleury 2007). This allows for a continuous renewal of the extracellular fluid with unwanted elements being expelled into the fluid for subsequent excretion, and fresh essential and nutritional elements being brought to the tissue from the blood. However, in tumor tissue, this drainage is significantly hampered leading to an accumulation of the material extravasated from the leaky blood vessels. This accumulation of particles in the tumor tissue is termed as the "enhanced permeation and retention (EPR) effect." It is currently being realized that this EPR effect is a more complex process than initially thought (Bertrand et al. 2014). It depends greatly on the tumor biology and the nonhomogeneities in the tumor tissue.

2.1.2.2 Increased Interstitial Fluid Pressure

The impaired lymphatic drainage in tumors causes an increase in the interstitial fluid pressure (IFP), which may be as high as 60 mmHg in some cases (Hassid et al. 2008). As the central core of the tumor comprises of necrosed tissue, in which there is practically no lymphatic drainage, the IFP is maximum at the core of the tumor and progressively lowers toward the periphery. This pressure gradient may reduce the EPR effect to a significant extent as whatever accumulation of drug takes place due to extravasation through the leaky vasculature may not be able to penetrate into the deeper tissue due to the outward diffusion gradient created by the IFP.

2.1.2.3 Up-regulation of Certain Receptors

There are a number of growth factors and growth factor receptors that are up-regulated to facilitate the rapid growth of the tumor. Simultaneously, the factors that serve as check points for uncontrolled growth and

multiplication of cells get down-regulated, and the tumor continues to grow, modifying all the normal cellular processes to its favor.

2.1.3 GROWTH OF A TUMOR

The rate of growth of tumor cells is exponential and may be different in different types of tumors. For some tumors, the doubling time (the time period within which the number of cancer cells become double the initial number present) may be about 24 hours as in the case of Burkitt's lymphoma, whereas for others such as mammary cancer, this period is about three months (Rang et al. 2003; Robbins 1974). A tumor mass of about 2 cm diameter is within the limits of being diagnosed. Thus, the tumor may go unnoticed in most cases for a major period of its existence and when finally diagnosed may be so advanced that it may be very difficult to treat it successfully. However, fortunately, continuous exponential growth of tumor cells does not usually occur. This is because, though most of the cells multiply exponentially initially and gradually displace the normal cells, in the absence of normal apoptosis, the continuous multiplication leads it to a stage when all the cells do not get the nutrients required for cell growth and maintenance because the vasculature is as yet normal. The peripheral cells have access to the limited blood supply whereas the cells toward the center of the mass, not having access to the nutrients supplied by the blood vessels, start undergoing necrosis. In addition to this, not all cells multiply continuously. Hence, the tumor reaches a diffusion limited maximal size (which is usually about 2 mm^3) when a steady state is reached in which the rate at which the cells are dying due to necrosis is nearly equal to the rate of proliferation. This is shown in Figure 2.2.

It is believed that there may be a number of tumors having such a diffusion limited size throughout the body and may remain so for years (Brannon-Peppas and Blanchette 2004). However, in order to survive further, the tumor cells essentially need a greater blood supply, and if these cells are successful in acquiring this by initiating angiogenesis (growth of new blood vessels), then the tumor grows further (Figure 2.2).

Thus, in most fully grown tumors, at any given time beyond angiogenesis, there is a central core of necrosed tissue and a peripheral layer of rapidly proliferating cells. The cells between these two areas (the seminecrotic region) are nonmultiplying dormant cells that can attain the active proliferating state in the presence of certain triggers. Such a typical growth dynamics of a tumor has a special significance. As the center consists of necrosed or dead tissue, and this is combined with an absence of adequate lymphatic drainage (parallel to the lack of vasculature at the

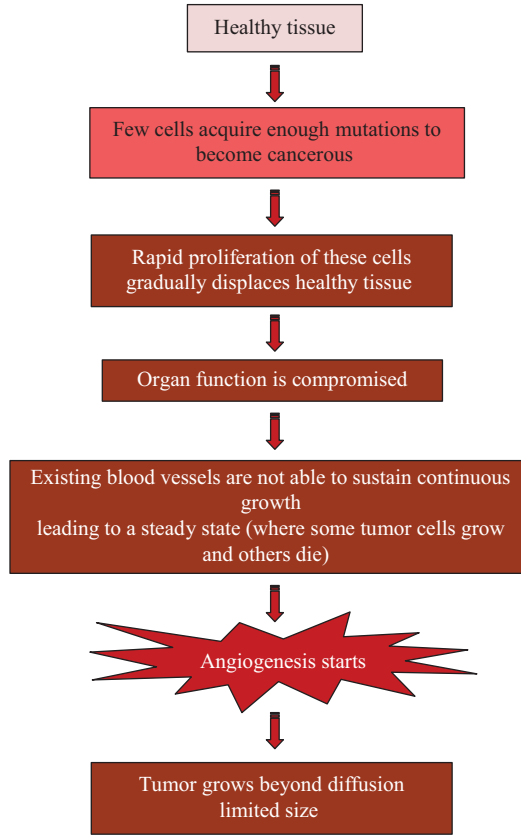

Figure 2.2. Tumor development from healthy tissue to a size beyond the diffusion limited maximal size.

center, there is also a lack of lymphatic vessels that collect waste matter excreted into the extracellular fluid and deliver it into the venous blood flow for excretion from the body), the extracellular fluid accumulates at the central core, increasing the IFP at the center. As against this, the vasculature at the periphery, being normal or increased in many cases, facilitates normal drainage of the interstitial fluid at the periphery. Hence, the IFP is increased at the core whereas it is zero at the periphery. This creates a pressure gradient from the core to periphery; the pressure at the core can be as high as about 60 mmHg in some tumors (Hassid et al. 2008). One major consequence of this is that all drugs, whether conventional or targeted nanoparticles delivered to the tumor surface, experience an outward pressure gradient that prevents them from diffusing into the tumor (Figure 2.3).

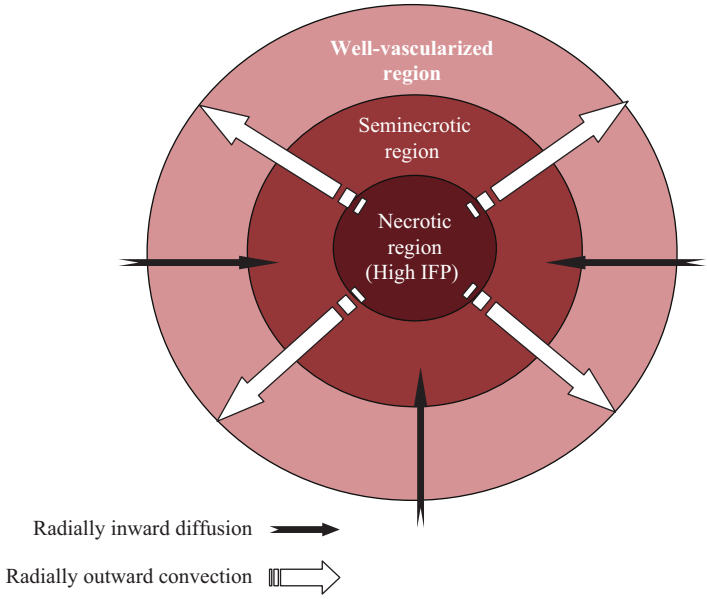

Figure 2.3. Outward pressure gradient created due to the three different regions: the necrotic, the seminecrotic, and the well-vascularized regions in a growing tumor resulting in reduced radially inward diffusion and increased radially outward convection.

Thus, in spite of being delivered specifically to the tumor, the drugs may be ineffective in killing the tumor cells as, not being able to penetrate the tumor, they remain in the blood and are metabolized and eventually eliminated.

The typical characteristics of tumor tissues mentioned earlier are exploited for diagnosis, therapeutics, and imaging of tumors. The following section provides an overview of cancer diagnosis, therapeutics, and imaging, and the advantages that nanoparticles offer therein.

2.2 CANCER DIAGNOSTICS

Diagnosis of cancer is based on physical examination, imaging procedures, laboratory tests, pathology reports, and surgical reports. Imaging procedures such as X-ray scan, computerized tomography (CT) scans, magnetic resonance imaging (MRI), and positron emission tomography (PET) scans show the location of cancer, size of cancer, and whether the cancer has spread or not. Laboratory tests involve analysis of blood, urine, or other fluids or tissues taken from the body. These are analyzed for the

presence or absence of specific molecules (markers) generally present in the case of cancer or typical for a particular tumor. Pathological reports are based on the microscopic examination of cells in specific tissues (biopsy reports) or in body fluids (fine needle aspiration cytology). Based on these examinations, staging of cancer is done that helps in describing the extent or severity of a person's cancer on the basis of which the plan of treatment and an estimate of prognosis can be made. The stage of cancer is decided on the basis of the site and cell type of the primary tumor, the size or extent or both to which the cancer has spread, involvement of regional lymph node, presence or absence of metastasis, number of tumors (primary and metastasized), and tumor grade (how closely cancer cells and tissue resemble normal cells and tissue). The most commonly followed staging system is based on the TNM classification system (www.cancer.gov/cancertopics/factsheet/detection/staging; www.cancer.org). In this system, "T" denotes the nature of primary tumor, "N" denotes the involvement of lymph nodes, and "M" denotes the presence or absence of metastasis. A number is added to each letter to indicate the size or extent or both of the primary tumor and the degree of spread. Table 2.2 describes this classification system.

Table 2.2. TNM classification for tumors

Notation	Characteristics
Primary tumor "T"	
TX	Primary tumor cannot be evaluated
T0	No evidence of primary tumor
Tis	Carcinoma in situ (abnormal cells are present but have not spread to neighboring tissue; i.e., though not cancerous, there is potential for the cells to become cancerous), also sometimes called "preinvasive cancer"
T1, T2, T3, T4	Size or extent of primary tumor
Regional lymphnode involvement "N"	
NX	Regional lymph nodes cannot be evaluated
N0	No involvement of regional lymph nodes
N1, N2, N3	Degree of involvement of regional lymph nodes (Number and location of lymph nodes)
Distant metastasis "M"	
MX	Distant metastasis cannot be evaluated
M0	No distant metastasis
M1	Distant metastasis is present

Table 2.3. Stages of cancer

Stage	Significance
Stage 0	Localized cancer or carcinoma in situ (cis). No spread to either lymph nodes or other tissues
Stage I, II, III	More extensive disease. Higher number means larger tumor size or spread or both of cancer to lymph nodes or tissues or both or organs adjacent to the primary tumor
Stage IV	Presence of metastasis. Cancer has spread to distant tissues or organs

The following example illustrates the staging of cancer:

A particular breast cancer classified as T3 N2 M0 indicates that a large tumor (denoted by T3) has spread outside the breast tissue to nearby lymph nodes (denoted by N2), but has not spread to other parts of the body (denoted by M0). A prostate cancer classified as T2 N0 M0 signifies that the cancer is located inside the prostate only, without the involvement of any lymph nodes or the presence of any metastasis. Cancer is generally classified into five stages as given in Table 2.3. Different TNM combinations correspond to any of the five stages of cancer, and the criteria for the stages differ for different types of cancers. For example, a bladder cancer classified by T3 N0 M0 is stage III, whereas a colon cancer classified as T3 N0 M0 is stage II. For the staging of lymphomas, the Ann Arbor system (Armitage 2005) is used, whereas there is no specific staging system for cancers such as blood cancer or cancer of the bone marrow. (www.cancer.gov/cancertopics/factsheet/detection/staging)

The failure to diagnose cancer in early stage has, since several decades, been a major setback in the successful treatment of cancer. With advances in technology, especially nanotechnology, and a better understanding of tumors in terms of the typical characteristics of tumors, and the pathological, biochemical, and histological changes occurring after the onset of cancer, it is becoming increasingly possible to detect cancer in relatively early stages. There are now a number of biomarkers that have been identified and which can be used for early diagnosis of cancer. The National Cancer Institute defines a biomarker as:

> a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or a condition or a disease. A biomarker may be used to see how well the body responds to a treatment or disease or condition. It is also called molecular marker or signature molecule. (www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=45618)

The nature and levels of tumor biomarkers may also be used to predict chances of recurrence of the cancer and the suitability of targeted therapy for a particular type of cancer. The different biomarkers that have been found useful for detection and treatment of different types of cancers have been listed by the National Cancer Institute on their website (www.cancer.gov) and reviewed by Cho (2007). Fahad Ullah and Aatif have also reviewed different biomarkers such as proteases, tumor-specific antigens and autoantibodies, nucleic acid based biomarkers, and the role of proteomics and nanotechnology in biomarker profiling (Ullah and Aatif 2009). In spite of the significant advances in biomarker research, the presence of tumor biomarkers may not be exclusively relied upon as increased levels of a particular biomarker may be present in the absence of malignance. Hence, tumor biomarkers should always be used in combination with tissue biopsy for accurate diagnosis of cancers. Advances in proteomics (study of protein structure, function, and patterns of expression) and systematic studies in the evaluation of patterns of gene expressions for different cancers are expected to make early cancer diagnosis more specific and accurate (Deng et al. 2005; Pavlou and Diamandis 2010). Concurrently, advances in imaging techniques coupled with advances in nanotechnology are likely to enable accurate assessment of these newer and more specific tumor biomarkers, making it possible to have a simple, noninvasive blood or urine analysis or a whole body scan that will predict the development of cancer. Besides, aptamers have also been used to discover or detect cancer-specific biomarkers or both (Chang, Donovan, and Tan 2013). Development of advanced techniques such hybrid mechanical and opto plasmonic nanosensors for the detection of ultra-low serum concentrations of cancer biomarkers could help in early detection of cancers (Kosaka et al. 2014). Techniques for analysis of most biomarkers, which mainly comprise of proteins, gene sequences, DNA, and RNA, are well established. Circulating microRNA (miRNA) and presence of specific miRNA in tissue samples have been identified for diagnosis, prognosis, and therapy in a number of cancers including lung, breast, prostate, colorectal, gastric, liver, and hematologic cancers (Cheng 2015; Cho 2010; Shen, Stass, and Jiang 2013). miRNA based techniques are noninvasive and offer good sensitivity and specificity. A classification of biomarkers, along with the rationale for their use, and a critical analysis of the present status of clinical relevance of biomarkers have been presented by Saijo (2012).

2.3 CANCER IMAGING

Cancer imaging consists of using noninvasive techniques to obtain detailed pictures of body parts or cancerous tissues or both. Two- or three-dimensional pictures of specific areas in the body are obtained by appropriate

imaging equipments. Cancer imaging is extremely important to assess the location, size and extent of spread of the tumor, response to treatment of anticancer agents, and detecting recurrence of the tumor. To date, there are a number of techniques established, and emerging, which are used for the imaging of cancer (Table 2.4).

The entire electromagnetic radiation spectrum has been exploited in order to develop safer, more effective, and specific methods for imaging of cancer. Figure 2.4 shows the frequency ranges used by different imaging techniques for cancer imaging.

The established and commonly used techniques for imaging include gamma-scintigraphy, MRI, and CT. All these techniques require some signal from the area of interest on the basis of which the desired area is differentiated from the surrounding area. These signals may or may not be enhanced. In cases where relatively large areas or tissues are involved, nonenhanced imaging techniques may suffice. However, where small areas are involved, the signals may need to be enhanced so that the small area of interest is sufficiently differentiated from the surrounding normal area. This signal enhancement is done by contrast agents (also known as tracers) that have an increased capacity to absorb certain types of radiations and hence emit stronger signals compared with the surrounding areas, enabling a clear-cut differentiation of the cancerous tissue from the normal tissue when the appropriate imaging technique is used. The difference in contrast may be obtained due to changes in the physical properties of endogenous substances or factors (substances or factors already present in the body) or with the help of exogenous agents introduced into the body for the purpose. The different endogenous and exogenous factors that can be used to increase contrast in different imaging procedures have been listed by Fass (2008). The contrast agents or tracers may be introduced intravenously, intra-arterially, or via natural orifices such as the GI tract (specifically for imaging locations in the GI tract).

The imaging/contrast agents need to be delivered specifically to the cancerous tissue or cells so that the normal cells do not receive or emit the signals. The different targeting strategies (discussed in Section 2.4.1) may be used to deliver the contrast agents too. Some of the newer imaging techniques are PET and single photon emission computerized tomography. In these techniques, a small amount of radioactive compound (tracer) is administered to the patient and three-dimensional images are acquired with highly sensitive cameras that can detect a small amount of radioactivity. The intensity of signal is proportional to the amount of tracer. Multiple images over time can be obtained to give in vivo dynamic datasets. Different techniques have different sensitivities depending upon the mechanism used for detection and imaging (Fass 2008).

Table 2.4. Different techniques used for cancer imaging

Name of the imaging technique	Description	Use
Gamma-scintigraphy	Gamma-rays emitted by radionucleotides taken internally (injected, ingested, or inhaled) are detected by gamma camera and 2-D images are obtained	Used to get a whole body scan in a single examination
Positron emission tomography (PET) and single photon emission computed tomography (SPECT)	Use highly sensitive cameras to obtain 3-D images of tissue subsequent to administration of small amounts of radioactive tracers. By obtaining multiple images over time, "4-D" in vivo dynamic datasets can be obtained	Routinely used to diagnose and stage malignancy. More sensitive than MRI and X-rays. Capable of estimating tissue glucose utilization (increased glucose metabolism is a common feature in many malignancies). Can also be used to distinguish between viable cancer tissue and residual scar or necrotic tissue after therapy
X-ray scans	Electromagnetic radiation (X-rays) used to take photographs in the body. X-rays are absorbed by dense materials such as cartilage and bone but not by lighter substance like blood	Chest radiographs and mammograms are used for early detection of cancer or to see if the cancer has spread to lungs or other areas of the chest

(*Continued*)

Table 2.4. Different techniques used for cancer imaging (Continued)

Name of the imaging technique	Description	Use
CT scans	A large number of X-ray photos are taken from different angles and put together using a powerful computer to form a 3-D image or a series of pictures of slices through the body	Used for the diagnosis of tumor, detect abnormal growth, determine extent of growth and stage of tumor, guide biopsy procedures, assess response to treatment, and monitor for recurrence
Optical coherence tomography (OCT)	Noninvasive, label-free procedure for imaging of living tissues. Based on the principles of optical interferometry	Emerging imaging technique used for preclinical imaging of cancers in living mouse models
Photo acoustic tomography (PAT) and thermoacoustic tomography (TAT)	Nonionizing laser pulses delivered to biological tissues causing some of the delivered energy to be absorbed and converted to heat leading to transient thermoelastic expansion resulting in wide-band ultrasonic emission. (If radiofrequency energy is used, it is called thermoacoustic imaging.) The generated ultrasonic waves are detected by ultrasonic transducers to form images	Used for the detection of brain lesions, hemodynamic monitoring, breast cancer diagnosis, and in photo acoustic microscopy

Dynamic near infrared optical tomography (DYNOT)	Noninvasive imaging technique in which low energy electromagnetic radiation (~1–2.5 eV) is delivered to one or more desired locations and transmitted or back reflected light intensities or both are measured. The difference in scattering properties of different tissues is used to characterize the tissues	Used to investigate the functional states of vasculature and its interactions with surrounding tissues
Terahertz pulse imaging (TPI)	Low photon energy (0.41–41 meV) used for imaging. Intermolecular vibrations in this region give unique absorption spectra for different biological tissues.	Used to provide complementary information to existing imaging techniques, especially in diagnosis of breast cancer and skin cancers
Ultrasound imaging	1–20 MHz frequency sound waves used to differentiate between normal and cancerous tissue on the basis of nature and intensity of echo produced when these encounter the specific tissues (no echo produced if there is no density difference)	Used for the imaging of breast cancers, uterine cancers; contrast enhanced ultrasound used for the imaging of prostate cancer
MRI	Use radiowaves in presence of a strong magnetic field. Difference in intensity of signals emitted by different tissues used to differentiate cancerous tissue from normal tissue	Used for the detection of brain tumor, primary bone tumors, soft tissue sarcomas, and tumors affecting spinal cord
Immunoscintigraphy	Radio labeling of antibodies is done and the radiation emitted is imaged	Used for radioimmunotherapy and radio diagnostics to obtain a whole body scan in a single examination

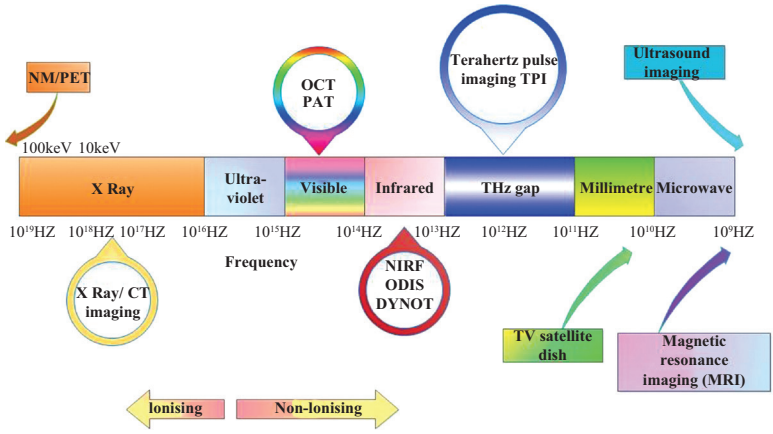

Figure 2.4. Frequency spectrum of electromagnetic radiation used in cancer imaging.

2.3.1 MOLECULAR IMAGING

Molecular imaging is defined by the Society of Nuclear Medicine as: "the visualisation, characterisation, and measurement of biological processes at the molecular and cellular levels in humans and other living systems." Until recently, such molecular level information was obtained solely by procedures such as biopsy and immunohistochemical staining methods. However, with advances in nanotechnology and nuclear medicine, the area of molecular imaging is evolving very rapidly and becoming increasingly popular because, unlike biopsy, it is noninvasive, nondestructive, provides for whole body coverage and whole tumor sampling, and more importantly, evaluates live tissue and not dead fixed tissue. Thus, it provides a more complete and systematic picture of a living tumor, and hence is sometimes referred to as "virtual biopsy." Besides this, the other advantages of molecular imaging techniques over conventional biopsy methods are that these techniques can evaluate blood flow and tumor interactions with its microenvironment. It can also provide real-time dynamic data, and repeated measurements and post-therapy evaluation are possible. Tumor tissue is inherently heterogeneous in nature; therefore, a single biopsy sample taken from a tumor may not represent the entire tumor. Moreover, due to this heterogeneity, repeated biopsies taken with the purpose of assessing response to therapy may not be meaningful. Molecular imaging overcomes this disadvantage as it provides for whole tumor sampling. In spite of all these advantages, presently, it is used only as

a complementary procedure to conventional biopsy or to determine the optimal site for taking the biopsy or both. However, recent advances in this area and the development of novel imaging probes have made it possible to follow molecular pathways and tumor microenvironment. MRI and optical imaging techniques using such probes have been investigated for imaging vascularization, angiogenesis, and metastasis besides image guided drug delivery and molecular targeting (Penet et al. 2010). In the near future, with more and more molecularly targeted therapies getting the Food and Drug Administration (FDA) approvals, molecular imaging techniques will be a necessary tool for assessing the efficacy of such therapies as also the interactions of the targeting agents with the targets.

2.3.2 ROLE OF NANOPARTICLES IN CANCER DIAGNOSTICS AND IMAGING

Most imaging techniques require contrast agents to enhance the contrast between the tumor and the surrounding area. The contrast agents are specific for each imaging modality and it is essential that these contrast agents accumulate in sufficient quantity in the tissue or the location desired to be imaged. Targeting agents in drug therapy are used for targeting contrast agents to the desired location. Nanoparticles, because of their size, are capable of being preferentially accumulated at tumor sites due to the greater permeability of tumor vasculature (known as the "EPR" effect). Surface modified nanoparticles can be used for selective attachment to specific locations. Besides this, modification of nanoparticle surface by polyethylene glycol (PEG) prevents the nanoparticles from being phagocytosed and helps the nanoparticles to remain in circulation for a longer time, thus aiding in preferential accumulation at tumor sites. Nanoparticles can therefore be used to carry therapeutic agents, contrast agents, antibodies, and so on to tumor locations. So far as imaging is concerned, encapsulation of dyes into polymeric nanocapsules/liposomes or incorporating dyes into the matrix of nanospheres increases their photostability by protecting them from the surroundings, and provides signal enhancement due to an increase in the number of dye molecules per nanoparticle. Different types of nanocarriers have been used for concentrating the imaging agents into the cancer tissue (Brigger, Dubernet, and Couvreur 2002; Praetorius and Mandal 2007; Torchilin 2007). The selection of the nanocarrier used for targeting the imaging agent and mode of its administration have to be done on the basis of its capacity to accumulate into the desired tissue. Improved nanoparticle based fluorescent markers have been developed for routine basic research and clinical diagnostic applications. Nanoparticle

based contrast agents can be used for in vitro as well as in vivo applications. For in vitro applications, silica nanoparticles doped with organic based fluorescent dyes have been used for the detection of liver cell malignancy. Fluorescent inorganic biomarkers were found to be more stable and prevented interference from background signals emitted by cells and tissues. Streptavidin coated fluorescent polystyrene nanospheres were found to give brighter images compared with the usual streptavidin–fluorescein conjugates used as biomarkers for the detection of epidermal growth factor receptor (EGFR) on A431 human epidermoid carcinoma cells. Nanoparticles of Rubpy (tris(2,2'-bipyridyl)dichlororuthenium II) doped in silica network with lauryl groups grafted on the surface were found to successfully stain human leukemia cells (in vitro); the hydrophobic lauryl group aids in the penetration of the nanoparticle through the cell membrane (Brigger, Dubernet, and Couvreur 2002). Biomolecules like antibodies can also be attached to detect specific cancer cells. For in vivo applications, nanocarriers such as liposomes and micelles have received special attention. The desired contrast agents can be included in the aqueous interior of the liposome or the outer lipophilic membrane. Membrane bound contrast agents are safer because there are less chances of leakage of the contrast agent. For preparing liposomes with membrane bound metal–chelate complex for gamma or MR imaging or both, the desired metal is chelated into a soluble chelate such as diethylene triamine penta acetate (DTPA) and then simply included in the interior of the liposome, or the metal may be anchored to the liposome surface after attaching it to DTPA or a similar compound that has been suitably derivatized by incorporating a hydrophobic group to it (the hydrophobic group helps in attaching DTPA to the lipophilic outer layer of the liposome). For a better MR signal, all reporter atoms should be freely exposed for interaction with water. Polycyanoacrylate nanoparticles, using DTPA as spacer to fix the isotopes ^{111}In or ^{99m}Tc, have been shown to accumulate in the reticuloendothelial system (RES) of rabbits and humans (Torchilin 2007). Nanoparticles are capable of being phagocytosed by macrophages. Such phagocytosis by macrophages leads to increased concentration of the nanoparticles in the lymph nodes. This phenomenon can be used to concentrate a therapeutic agent, contrast agent, or tracer in lymph nodes enabling imaging of lymph nodes for the detection of cancer. The use of subcutaneously injected iodinated nanoparticles has been done to increase contrast in CT scan procedures for the detection of cancerous lymph nodes in cutaneous melanoma models. The increased contrast permitted detection of more deeply seated normally inaccessible lymph nodes, visualization of internal architecture of the lymph nodes, and elucidation of the lymphatic drainage pattern.

For MRI applications, iron oxide nanoparticles are particularly preferred because iron oxide is considered to be safe as it metabolizes into elemental iron (which can subsequently add up to the normal body stores) and oxygen by hydrolytic enzymes in the body. A variety of modifications of the conventional iron oxide nanoparticles have been developed and are commercially available (Table 2.5).

Superparamagnetic iron oxide nanoparticles (SPIONs) comprising of an inorganic core of iron oxide, maghemite, or other insoluble ferrites, either plain or coated with polymers like dextran, have been used as contrast agents. These are of size greater than 50 nm (including coating) and are now commercially available (e.g., silicon coated iron oxide nanoparticles with a diameter ~300 nm by the name Lumirem and dextran coated magnetite nanoparticles with a diameter ~150 nm by the name Endorem) and have applications similar to conventional nanoparticles. Lumirem nanoparticles have been used for imaging of the GI tract, and Endorem nanoparticles are being used for the detection of liver and spleen diseases. As these dextran coated nanoparticles are massively taken up by kuppfer cells, uptake of these nanoparticles takes place only in the presence of diseased tissue. Absence of these in an MRI indicates that there is no diseased tissue.

Long circulating dextran coated iron oxide nanoparticles (LCDIONs) are dextran coated iron oxide nanoparticles with a diameter less than 50 nm; these have a low diffusivity and are capable of being internalized by endocytosis. They are also taken up by infiltrating macrophages and endothelial cells in areas of active angiogenesis. The combined uptake thus enables clear and distinct signals in an MRI scan. Their concentration at sites of active angiogenesis coupled with their low diffusivities provides a distinct contrast of tumor areas from the surrounding normal tissue. This also offers a distinct advantage over the other commonly used contrast agents such as gadolinium chelate that diffuses into the surrounding tissue causing a blurring of the tumor margins. In addition to this, a positive correlation has been obtained between LCDION uptake by tumor cells and tumor doubling time due to which in vivo tumor growth kinetics can possibly be studied.

Ultra small superparamagnetic nanoparticles (USPIONs), with the trade name Sinerem , due to their small size (~30 nm), also have properties similar to long circulating nanoparticles and permit precise and prolonged delineation of brain tumor margins. These nanoparticles are capable of being selectively recognized by lymph node macrophages while escaping recognition by liver and spleen macrophages; hence, Sinerem nanoparticles can provide adequate concentrations in lymph nodes and be used for lymph

Table 2.5. Commercially available iron oxide nanoparticles for MRI applications

Type of magnetic nanoparticles	Description	Applications
SPIONs		
Lumirem	~300 nm silicon coated iron oxide nanoparticles	Used for GI tract imaging
Endorem	~150 nm dextran coated magnetite nanoparticles	Used for liver and spleen disease detection
Feridex IV	80–150 nm dextran coated iron oxide nanoparticles	Used for RES, liver, and stem cell labeling
Ferumoxsil	~300 nm Si-coated SPIONs	Used for liver disease detection
Banges	~690 nm–1.73 μm SPIONs encapsulated in polystyrene–divinylbenzene	Used for cell labeling
USPIOs or LCDIOs		
Sinerem	~30 nm dextran coated magnetite nanoparticles Have long circulating properties, low diffusivity, and internalize into tumor cells by endocytosis	Used for blood pool and tumor imaging Distinct demarcation of brain tumor Can be used for lymph node disease detection Can be used for studying tumoral growth kinetics
Resovist	~20 nm Carboxydextran coated USPIONs	Used for blood pool and stem cell labeling
Ferumoxytol	~30 nm Carboxymethyldextran coated USPIONs	Used for macrophage and blood pool labeling
Ferucarbotran	~60 nm Carboxydextran coated USPIONs	Used for liver disease detection
Feruglose	~20 nm pegylated starch coated USPIONs	Blood pool labeling

node disease detection after intravenous, subcutaneous, or intramuscular injection (Brigger, Dubernet, and Couvreur 2002). Recently, iron oxide nanoparticles have found application in stem cell labeling. A number of novel SPIONs are being investigated for this purpose (Li et al. 2013).

Thus, superparamagnetic nanoparticles have become indispensable for MRI procedures and could be further modified by coupling them with targeting molecules such as monoclonal antibodies (mAbs), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and so on. Moreover, multifunctional polymeric nanoparticles having appropriate functional groups to attach anticancer agents, targeting agents, and imaging/contrast agents are becoming the subject of intensive research due to the "all-in-one" advantage they offer.

2.3.3 QUANTUM DOTS IN CANCER IMAGING

Quantum dots are nanoparticles made of semiconductor materials ranging from 2 to 10 nm (comprising of merely 10 to 20 atoms) and deserve a special mention in cancer diagnosis and imaging as they have revolutionized the area of optical detection techniques, particularly detection of fluorescent biomarkers, in cancer diagnosis. Fluorescent biomarkers have been routinely used in basic research and clinical diagnostic applications including cancer diagnostics. However, these fluorescent markers require color matched lasers for visualization. For most fluorescent markers, the fluorescence fades rapidly and observations are required to be made rapidly to get useful results. Simultaneous use of multiple dyes is not possible. Radioactive tags used for cancer imaging also have a relatively short half-life. However, fluorescent nanoparticles especially nanocrystals or quantum dots overcome these issues as these can fluoresce for several months in a living animal (Kim 2007). Quantum dots, when excited with ultraviolet light, can fluoresce in different colors depending on their size: larger particles emit light in the red end of the visible spectrum whereas smaller particles emit light in the blue range. The most commonly used quantum dots in cancer diagnosis and imaging are Cd/Se quantum dots. However, plain Cd/Se quantum dots are not biocompatible. Besides, these are also not amenable to surface modification or surface attachment to other molecules or both. For their successful use as imaging agents, they need to be biocompatible and capable of attaching themselves to a variety of biomolecules in vivo or in vitro or both. Hence, Cd/Se quantum dots have been capped with ZnS to improve their biocompatibility. Surface modifications of Cd/Se quantum dots have been performed by

conjugating them with materials such as mercaptoacetic acid (Gerion et al. 2001; Lorenz and Ellis 1998; Rogach et al. 1999); covalent binding to silica has been attempted to enable the formation of a thin layer of silica on the surface of quantum dots (Gerion et al. 2001; Rogach et al. 2000). This increased the stability of the quantum dots. Multiple quantum dots encapsulated within the silica coating have also been done to improve their fluorescence intensity (Katagiri et al. 2002; Rogach et al. 2000). Quantum dots incorporated in polystyrene have been synthesized with a good size control over micro- as well as nano-levels. These polystyrene–quantum dot nanospheres have been further coated with silicon to provide additional surface for further conjugation to biomolecules (Yang and Zhang 2004).

Quantum dots can be used as molecular probes capable of imaging cellular components and tracking their movement inside the body. This property is particularly helpful in detecting metastasis in cancer. Quantum dots can be attached to proteins and receptors and can also be attached to antibodies. As cells are impermeable to quantum dots, they must be coated with special molecules or antibodies to facilitate their uptake. They have also been used as carriers for small interfering RNA (siRNA) (Kim 2007). All these characteristics of quantum dots have been very innovatively exploited for cancer diagnosis and therapeutics by a number of researchers and excellent in-depth reviews regarding the variety of modifications and applications for cancer diagnostics and imaging are available (Biju et al. 2010; Byers and Hitchman 2011; Hild, Breunig, and Goepferich 2008; Liu et al. 2008; Mattoussi, Palui, and Na 2012).

The following example elucidates clearly how the above properties of quantum dots can be used in practice. Zhang, So, and Rao used special peptide ligands consisting of three segments: (1) a "transporter" segment, (2) a "blocker" segment, and (3) a "linker" segment to conjugate with quantum dots (Figure 2.5). This peptide segment was denoted by the sequence R4XPLGVRGE4. The first portion R4 denotes the four arginine residues that serve as "transporters." This portion conjugated with the quantum dots enables transportation of the conjugate into the desired cells. They showed that oligomers of four to nine arginine residues, when conjugated with quantum dots, were able to successfully facilitate the uptake of the quantum dots by cells whereas shorter arginine oligomers were not effective in the internalization of the quantum dots. This quantum dot-attached R4 segment is connected to the amino acid sequence PLGVR that serves as a "linker" and is a substrate for the enzyme matrix metalloproteinase-2 (MMP-2) (this enzyme is responsible for the

degradation of ECM and is overexpressed in the case of cancerous tissue). This portion is further conjugated with four anionic glutamate residues (GE4), which serve as the "blocker." The part "X" (6-aminohexonyl) represents a spacer that is inserted to prevent unfavorable interaction of the conjugate with the enzyme MMP-2. They showed that the cellular uptake of the quantum dots conjugated in this manner was possible only in presence of enzyme MMP-2, as this enzyme, due to its affinity for the substrate, removed the negatively charged groups (the "blocker" part) from the conjugate. Once the "blocker" part was removed, the "transporter" part, still attached to the quantum dots, became available and was able to successfully transport the quantum dots into the cell. In the absence of MMP-2, as the "blocker" part will not be removed, the internalization would not be possible (Zhang, So, and Rao. 2006). Thus, on observation of the cells, fluorescence would only be seen in the case of cancer cells whereas no fluorescence would be seen in absence of cancer (Figure 2.5).

Tan, Jiang, and Zhang showed that quantum dots can also be used for the detection of gene silencing effect by siRNA. The siRNA targeting the gene that encodes for human EGFR-2 was conjugated with quantum dots. This siRNA–quantum dot conjugate got targeted to the breast cancer cells that overexpress the EGFR-2 and subsequently got internalized by receptor mediated endocytosis. The siRNA was able to silence the gene encoding for EGFR-2 resulting in hampered growth of the breast cancer cells. The targeting of the conjugate specifically to the breast cancer cells was seen by the fluorescence due to the internalized quantum dots, and the gene silencing effect of the siRNA was shown by enzyme-linked immunosorbent assay (Tan, Jiang, and Zhang 2007).

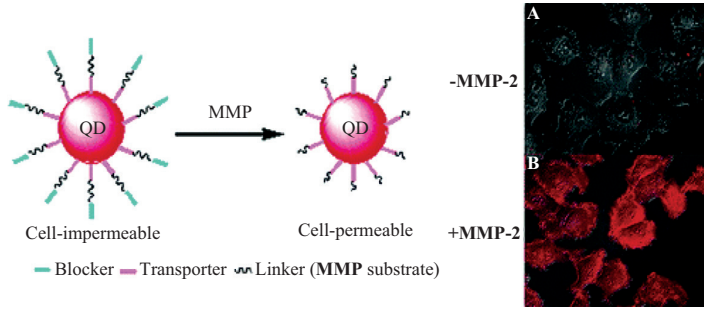

Figure 2.5. Use of quantum dots in cancer diagnosis and therapeutics. *Source*: Reproduced with permission from Zhang, So, and Rao (2006).

2.4 CANCER THERAPEUTICS

Cancer treatment has undergone a radical transformation from the physical surgical removal of the tumors followed by radiotherapy, to different types of therapies such as chemotherapy, immunotherapy, gene therapy, thermotherapy, photodynamic therapy, and the use of focused ultrasound. Table 2.6 provides a brief description of all these therapies currently used in the treatment of cancer.

Table 2.6. Some common therapies used for the treatment of cancer

Type of therapy used	Description
Surgery	Surgical removal of the tumor
Radiotherapy	Use of high energy waves such as X-rays, gamma-rays, and high energy charged particles such as electron or proton beams given from an external source, or systemically or intratumorally given radioactive substances
Chemotherapy	Use of different chemotherapeutic agents for killing the cancer cells
Immunotherapy	Using (mAbs) to trigger the body's immune system to fight cancer
Gene therapy	Inserting specific genes capable of repairing or replacing the genes that are in any way responsible for the generation or growth of tumor or both, such as oncogenes or mutated tumor suppressor genes, into the cancer cells
Hormone therapy	Use of hormones or hormone analogues that have inhibitory actions on tissues to treat cancer involving these tissues
Thermotherapy	Use of thermal ablation methods to burn the cancer cells
Photodynamic therapy	Use of light of an appropriate wavelength, in combination with a specific light-activated substance (photosensitizer), which, on photo-activation, triggers a sequence of biochemical changes that cause irreversible damage to the cancer tissue
Ultrasound treatment	Use of focused ultrasound to kill the cancer cells

Surgical removal of a tumor, followed by radiotherapy, is possible only for localized tumors. In addition to this, the trauma associated with any surgical procedure and the high chances of recurrence in cases in which residual cells remain make this treatment procedure a low priority option, especially when other safer alternatives have now become available.

Chemotherapy uses different chemical agents for killing cancer cells. There are now a large number of chemotherapeutic agents that are capable of successfully rooting out cancer cells with minimal side effects, especially after the development of various targeting strategies that direct the agents exclusively to the cancer cells so that normal cells are not affected. Chemotherapy is usually followed by radiotherapy for minimizing the chances of recurrence.

Immunotherapy uses strategies to augment the body's own immune system by using mAbs to fight cancer cells.

Gene therapy consists of using genes to repair or replace oncogenes or mutated tumor suppressor genes to stifle the growth of cancer cells, eventually destroying them.

Certain cancers require specific hormones for growth. Hormone therapy for cancer uses agents that block the effects of these hormones. This therapy is suitable only for hormone dependent cancers such as breast cancer, prostate cancer, and ovarian cancer.

Thermotherapy works by generating localized, regional, or whole-body hyperthermia, which has been found to be effective by itself, as well as in conjunction with other therapies such as chemotherapy and radiotherapy, to successfully kill cancer cells, even those that have developed resistance to any of the therapies just mentioned. Temperatures from 42°C to 45°C can kill cancer cells by damaging proteins and other structures within cells causing the tumor to shrink. Normal tissues are not significantly affected as they have far greater capacity to dissipate heat compared with the tumor tissue. Studies carried out in human tumor xenografts grown in mouse have shown that hyperthermia increased the pore cut-off size for extravasation from tumor vasculature from 7 to 100 nm at normothermia (34°C) to >400 nm at 42°C (Kong, Braun, and Dewhirst 2000). High intensity focused ultrasound can also be used for creating hyperthermia in tumors, thus shrinking or destroying them or both.

Photodynamic therapy uses a photosensitizer substance that is activated by the light of a specific wavelength (usually in the visible range), triggering a cascade of reactions that result in the generation of a singlet oxygen from molecular oxygen, thereby causing irreversible damage to the target tissue. Neither the photosensitizer nor the light are harmful by

themselves; hence, selective destruction of cancer tissue can be effected by restricting exposure to light, thus protecting the surrounding normal tissue. For deep seated tissues, this can be done by using lasers and fiber optics.

Whatever the therapy used, the focus of all these therapies revolves around the requirement of specificity for cancer cells or tissue or both. In other words, cancer therapeutics now concerns with specifically exploiting the differences between tumor cells and normal cells so that only the cancer cells are affected. Thus, the treatment of cancer is now based on targeting the specific mechanisms involved in the genesis of the cancer with the main objective being to kill the cancer cells while leaving the normal cells intact. However, cancer therapeutics is different from the treatment of other diseases in the sense that the cancer cells that are to be killed are the body's own cells that have become aberrant; hence, it is paramount that while killing cancer cells, normal cells, even those normal cells that are in close proximity to the cancer cells, remain intact. With this objective, a variety of anticancer agents have now been developed that intervene at different stages in the genesis of cancer (Figure 2.6), right from the gene mutation stage to the development of a secondary tumor.

There are gene based agents that act at the very initial stage to eliminate or correct the mutated genes so that the tumor generating characteristics are arrested at the very beginning. Gene therapy, antisense therapy, use of siRNA, and microRNA are examples of such an approach toward selectivity. Agents that act at a molecular level and interfere with or inhibit the synthesis of certain factors such as growth factors responsible for the growth of the concerned cancerous tissue have been developed. A number of these have got FDA approval and are commercially available. Examples include imatinib mesylate (Gleevec), which blocks the activity of an enzyme BCR-Abl (known to cause cancer in GI stromal tumors and in some cases of chronic myelogenous leukemia) essential for the growth of the cells, thus inducing apoptosis leading to cell death. Trastuzimab (Herceptin) is a monoclonal antibody that prevents the generation of the EGF, which stimulates cell proliferation by inhibition of EGFR-2, which is overexpressed in breast cancers. Identification of more such molecular level targets will make cancer treatment more effective and safe. Chemotherapeutic agents are a class of agents that affect the normal cell metabolism at various levels of cell division, thereby causing cell death. Commonly referred to as antimetabolites, these agents are highly cytotoxic and have, as their sites of action, some key metabolic processes such as synthesis of ribo- and deoxy-ribonucleotides, DNA, RNA, certain key proteins and enzymes, or certain cell organelles such as microtubules. Figure 2.6 gives

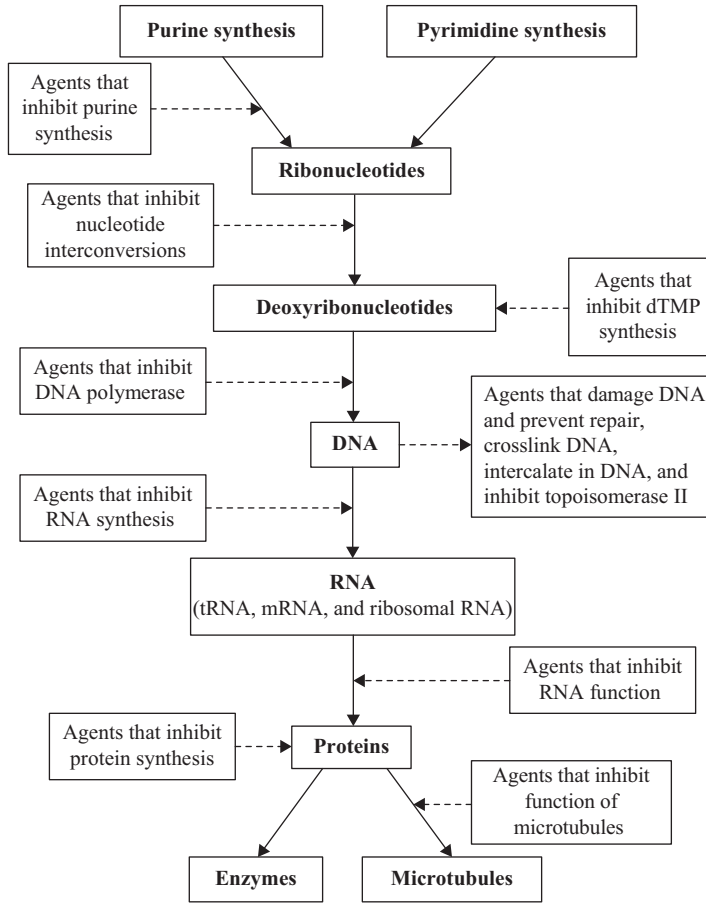

Figure 2.6. Major sites of action for chemotherapeutic agents that disrupt cell division in some manner.
dTMP, deoxythymidine monophosphate
Source: Adapted from Rang et al. (2003).

the major sites or stages or both of cell division that serve as targets for chemotherapeutic agents.

As these sites of action are not exclusive to cancer cells, these agents affect normal cells too. In absence of such specificity, these agents may disrupt the cell functioning in normal tissues and cause irreparable damage to the normal tissues, resulting in some serious side effects. Thus, it is very important that these chemotherapeutic agents be specifically targeted only to the cancerous tissue. Agents that affect some typical property of

cancer cells include those that are able to prevent the formation of new blood vessels (angiogenesis), which leads to inadequate blood supply to the rapidly proliferating cancer cells, thus starving the cells of essential components such as nutrition, oxygen, and so on consequently leading to cell death. There are a number of other such strategies that have been developed over the years some of which directly damage the cell leading to cell death whereas others use a targeting agent that carries the therapeutic agent exclusively to the cancer cells or tissue or both.

2.4.1 CANCER TARGETING

Cancer targeting has a twofold objective: one, to specifically kill the cancer cells and two, to protect the normal cells from the cytotoxic agent. For this to happen, it is essential that the full dose of the cytotoxic agent or therapeutic agent is delivered specifically to the tumor tissue so that minimal damage is done to the normal tissue. Toward achieving this goal, a number of cancer targets and targeting strategies have been developed. As already described in Section 2.4, some of these targets are directly attacked by the anticancer agent effecting cell death whereas others need a targeting agent that carries the therapeutic agent to the site of action subsequently leading to cell death. These targets, which essentially differentiate cancer cells or tissue or both from normal cells or tissue or both, may be a component present inside the cell, on the cell surface, or in the microenvironment surrounding the cells. In many cases, these drug targets may double-up as biomarkers. The different targets and targeting strategies currently used are classified in Figure 2.7.

There are a number of growth factors such as EGF, VEGF, transforming growth factor, and so on, which are essential for the rapid growth of mutated cancer cells. Agents that bind to specific binding sites on these growth factors, or in some manner block the signaling pathways of these growth factors, disrupt the growth of the cancerous cells, thus arresting the growth of the cancerous tissue. A number of such agents, for example, cituximab, panitumumab, trastuzumab, erlotinib, and imatinib, have already been approved for the treatment of cancer. A comprehensive review of all such growth factors that have been identified till now, along with those that are being therapeutically exploited for targeting cancer, is presented by Witsch, Sela, and Yarden (2010).

A significant depletion of growth factors that takes place as the tumor tissue continues to grow accompanied by an inadequate oxygen supply would trigger apoptosis in normal cells. However, cancer cells typically develop resistance to apoptosis by promoting overexpression

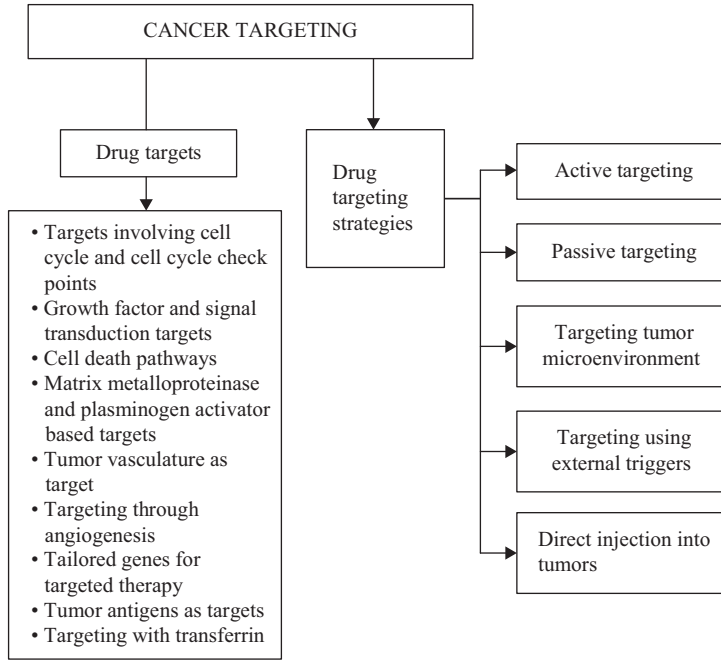

Figure 2.7. Targets and targeting strategies used for the treatment of cancer.

of anti-apoptotic factors such as the inhibitor of apoptosis (IAP) family of proteins. Hence, IAPs and the different stages in the apoptotic pathway have been used as targets to prevent such a suppression of apoptosis (Hunter, LaCasse, and Korneluk 2007). A number of polymer–drug conjugates have been developed, which have the capacity to act as modulators of apoptosis by acting as pro-apoptotic agents by themselves or in conjunction with other anticancer drugs or therapies (Vicent 2007). As the cancer tissue grows rapidly, the existing blood supply proves to be grossly inadequate; hence, cancer cells acquire the capacity to initiate angiogenesis—the capacity to grow new blood vessels. Simultaneously, ECM needs to be partially degraded to allow the sprouting of this neovasculature. Besides this, cancer cells have the tendency to invade the surrounding tissue, which also requires the disruption of ECM. To facilitate this, cancer tissue requires the enzyme MMP that can degrade ECM and allow the growth of new blood vessels and promote metastasis. Thus, MMPs have been used as therapeutic targets to suppress cancer growth and prevent metastasis. These agents are often used in combination with chemotherapeutic agents (Mannello 2006; Zucker, Cao, and Chen 2000). Cell division is a process

by which all tissues in the body maintain their integrity and whereby all damaged cells are replaced or repaired. During the process of cell division, the entire DNA gets replicated and, subsequently, two new cells are formed. It is essential that this replication process takes place precisely and exact copies of the mother cell are generated. With this objective, there are a number of cell cycle check points in the normal cell division process that ensure that no aberrations occur in the process (Funk 2005). In the case of cancer, dysregulation of this process occurs whereby the configuration of proto-oncogenes and tumor suppressor genes changes, resulting in conversion of proto-oncogenes to oncogenes and down regulation of tumor suppressor genes, which, if occurring simultaneously, lead to uncontrolled proliferation of the cells and absence of apoptosis. These cell cycle check points serve as targets for cancer therapeutics, especially gene therapy, which comprises of repairing or replacing these aberrant DNA sequences, thereby maintaining the integrity of the process (Cross and Burmester 2006).

In many cases, the therapeutic agent needs to be directed specifically to the cancer cells or tissue or both so that it acts on its intended target. To achieve this, a number of targeting strategies have been developed (Figure 2.7).

2.4.1.1 Active Targeting Strategies

These strategies are based on specific interactions such as ligand–substrate interactions, ligand–receptor interactions, and antigen–antibody interactions. Cancer drugs are attached by some means to carriers having ligands that specifically bind to some component unique to the cancer cell and not present in significant amounts in normal cells (Figure 2.8). These unique components may be either certain molecules present in the cancer cells, certain receptors present in, or overexpressed in the cancer cell surface, or certain enzymes, proteins (antigens), or other components present inside the cancer cells. The targeting agents used may therefore comprise of antibodies and their fragments, nucleic acids (aptamers), or other receptor ligands (peptides, vitamins, and carbohydrates). All such ligands that are presently being explored for their cancer targeting potential have been reviewed by Bertrand et al. (2014).

Biological agents such as viruses, components of living organisms such as specific antibodies (mAbs), antisense agents, tailored genes, aptamers, microRNA, and siRNA have been used for targeting. Some of these agents themselves have therapeutic effect whereas others can be used

as targeting agents or carriers for other chemotherapeutic agents, imaging agents, and so on. Viral vectors have been successfully used for delivery of genes into the cancer cells in gene therapy. Viruses with surfaces modified with specific proteins that bind selectively to other proteins present in cancer cells have been used to release their genetic material directly into the cancer cells. Viruses such as retrovirus, adenovirus, lentivirus, and herpes simplex virus have been used effectively in gene therapy (Kay, Glorioso, and Naldini 2001). mAbs (e.g., trastuzumab and cituximab) have been used to bind to growth factor receptors, thus adversely affecting cell growth. Alternatively, these antibodies can be used to enhance the body's own immune response to antigenic substances present in and around tumors. Antisense agents are oligonucleotides that bind complementary "sense" mRNA sequences that block the translation of RNA in the DNA synthesis process thereby preventing the replication of faulty DNA. Nonviral vectors such as cell penetrating peptides, nanoparticles, and dendrimers have been used to deliver these agents. Aptamers are small single stranded DNA or RNA oligonucleotides or peptide molecules that have an action similar to antibodies and can bind to targets with high affinity and specificity (Xiang et al. 2015). These are synthesized in vitro by a process known as "systemic evolution of ligands by exponential enrichment" (SELEX) and are not immunogenic. They can be used in the free form or as aptamer–drug conjugate in cancer therapeutics and diagnostics. miRNA and siRNA are small single stranded and double stranded RNA molecules, respectively, which have base sequences complementary to mRNA. They can silence or regulate or both silence and regulate gene expression by either degrading mRNA or interfering in the process of translation of mRNA to synthesize specific proteins.

Carbohydrate based strategies use the lectin–carbohydrate interactions for targeting cytotoxic agents specifically to tumors. Cancer cells show an altered glycosylation pattern due to which their cell surfaces acquire certain glycoprotein and glycolipid conjugates, which are not present in normal cells. Lectins are proteins that can preferentially recognize and bind to these carbohydrate complexes protruding from the glycolipids and glycoproteins present in the cell surface. These interactions are comparable in terms of specificity to the antigen–antibody interactions. Thus, a lectin conjugated drug delivery system can be specifically targeted to the carbohydrate portion on the cancer cell surface leading to adhesion of the drug to the cell surface and subsequent internalization of the drug (Ghazarian, Idoni, and Oppenheimer 2011).

The receptor based targeting strategies use receptor mediated endocytosis to internalize the chemotherapeutic agent into the cancer cell and

destroy it. Certain growth receptors are overexpressed in tumor cells in order to fulfill the extra nutritional or growth factors or both, which is essential for the rapid proliferation of the cancer cells. One such receptor is the folate receptor that is overexpressed in most tumor cells. Folic acid containing chemotherapeutic agents is able to bind to the folate receptors and get internalized into the cell by endocytosis. The potential of these receptors for targeting drugs including anticancer drugs has been reviewed by a number of researchers (Leamon and Reddy 2004; Low, Henne, and Doorneweerd 2008; Lu and Low 2002; Wang and Low 1998). Once inside the cell, the drug is released and available at the site of action. Transferrin receptor is another such receptor that has been used for the targeting of therapeutic agents. Transferrin is a serum glycoprotein that is essential for facilitating the transport of iron into the cells. As increased amounts of iron are required for rapid proliferation of cancer cells, these receptors are present in large amounts (more than 150,000 to 1,000,000) on cancer cells. Anticancer agents such as adriamycin and methotrexate have been attached to transferrin and internalized by receptor mediated endocytosis (Singh 2002). These overexpressed receptors can serve as direct targets for killing cancer cells or as targeting agents that can bind specifically to a moiety present in the drug–nanocarrier complex, thereby directing the drug specifically to the tumor. A variety of such receptors have been discovered and reviewed by Akhtar et al. (2014).

2.4.1.2 Passive Targeting Strategies

This is a random approach for targeting drugs to tumors. Passive targeting strategies take advantage of some typical characteristics of tumor cells or the tissue surrounding the tumor cells (characteristics that are not present in normal cells or tissue). Typically, tumors develop a leaky vasculature so that greater and faster permeation of the essential nutritional requirements is facilitated. This EPR effect is utilized to deliver anticancer agents specifically to the tumors. In addition to this, long circulating characteristics endowed to these agents can build up large concentrations of these agents at the tumor site. Generally, particles having a size <100 nm and a hydrophilic surface (provided by substances such as PEG) are able to preferentially accumulate at the tumor site. In addition to this, it is essential that the anticancer agents used escape uptake by RES so that they are not opsonized. Agents associated with or coated with hydrophilic substances such as polyvinylpyrrolidone (PVP) or PEG are successfully able to avoid such uptake by RES. It has been reported that 35 nm size

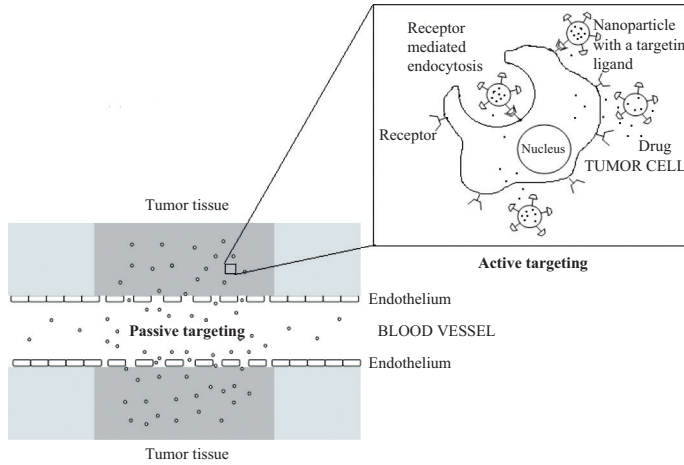

Figure 2.8. Diagrammatic representation of active and passive targeting strategies.

hydrophilic particles (associated with PVP) showed less than 1 percent uptake by liver, and even after 8 hours postinjection, about 5 to 10 percent of these particles were found to persist in the blood stream (Nie 2010). As against this, particles with hydrophobic surfaces were rapidly taken up by the liver, followed by spleen and lungs. The active and passive targeting strategies are described in Figure 2.8. An excellent review of how these strategies have been exploited in cancer drug delivery is presented by Bertrand (2014).

Targeting the tumor microenvironment is yet another nonspecific targeting strategy used to target anticancer agents specifically to the tumors. Tumor-activated prodrug therapy has been proposed for targeting drugs to tumors. Here, the drug is conjugated with some tumor-specific molecule and remains inactive until it reaches the target. Once it reaches the tumor, interaction between the tumor-specific molecule and some factor within the tumor causes the drug to be released. Mansour et al. reported a water soluble maleimide derivative of the anticancer drug doxorubicin containing an MMP-2-specific peptide sequence (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln), which binds rapidly and selectively to the cysteine-34 position of circulating albumin. This albumin–doxorubicin conjugate is cleaved by MMP-2 (overexpressed in most tumors), releasing a doxorubicin tetrapeptide and subsequently doxorubicin (Mansour et al. 2003).

Factors such as pH and temperature have been used as passive targeting strategies to release the anticancer agents specifically to tumors. The tumor pH is slightly acidic (around 6.0) compared with the normal pH 7.4 of the surrounding extracellular fluid. Drugs attached to carriers polymeric via covalent bonds that get selectively cleaved at the acidic pH of 6.0 can be released specifically into the tumor tissue (Bae et al. 2003).

Similarly, tumors have a temperature that is slightly higher (40°C to 42°C) compared with the normal body temperature of 37°C. Anticancer agents encapsulated or entrapped in matrix comprising of a temperature sensitive polymer (a polymer which is a "sol" at normal body temperature of 37°C and gels at the tumor temperature of 40°C) are able to release the drug only into the tumor tissues. Besides this, as already mentioned, such hyperthermia increases the pore cut-off size of extravasation from the normal 100 nm to as high as >400 nm (Kong, Braun, and Dewhirst 2000).

Exploiting tumor hypoxia is yet another method of passive targeting (Brown and Wilson 2004). The central necrotic region of solid tumors is significantly hypoxic (low in oxygen concentration) in nature. The cells surrounding this hypoxic region are resistant to chemotherapy and radiotherapy. However, this hypoxia has been exploited to deliver anticancer prodrugs, which are activated by low oxygen concentrations to convert to the active drug. As only tumors have such hypoxic regions, these agents will act only on tumor cells and not on normal cells.

External triggers such as externally applied magnetic field, focused ultrasound, and externally induced hyperthermia can be used to cause the release of the anticancer agents specifically to tumors. Magnetic nanoparticles are able to accumulate at the tumor site and kill tumor cells by thermal ablation of the cells due to the heat generated by the metallic particles in the presence of alternating magnetic field. Similarly, drug containing lipid encapsulated microbubbles can release the drug when focused ultrasonic waves are applied to the target region causing the lipid bubbles to burst. As mentioned earlier, inducing hyperthermia enhances the penetration of the drug into the tumor tissue. It also makes the tumor cells more sensitive to radiotherapy or chemotherapy or both. Nanoparticles have been shown to have potential applications in photodynamic therapy due to their cancer targeting properties. A number of nanoparticle based delivery systems and photosensitizer–bioconjugates have been explored and reviewed for this purpose (Allen and Sharman 2002). Nanoparticles can be actively (as active participants in photosensitizer excitation) and passively (as biodegradable carriers of photosensitizers) used for photodynamic therapy. Such a functional classification and the challenges to clinical translation

of photodynamic therapy have been reviewed by Chatterjee, Fong, and Zhang (2008).

Direct injection of the chemotherapeutic agent into the tumor can be used for solid tumors, which are easily accessible for this procedure. This strategy avoids the systemic distribution of the agent throughout the body, thus eliminating the possibility of adverse effects and problems of bioavailability. This strategy has been used by a number of researchers for intratumoral delivery of anticancer agents such as radioactive agents, gene therapy, and so on (Bao et al. 2006; Chanda et al. 2010; Nguyen et al. 1995). In situ gelling hydrogels have also been used for intratumoral delivery of various anticancer agents.

2.4.2 ROLE OF NANOPARTICLES IN CANCER THERAPEUTICS

The nanometer size range has distinct advantages in cancer chemotherapy as a number of characteristics of cancer and tumor growth favor the preferential entry of nanoparticles into tumors, which would otherwise have a significantly lower accessibility. The relevance of nanoparticles in some of the cancer therapies and targeting strategies has already been emphasized in Section 2.4.1. In addition, nanoparticles, due to their small size, particularly in the range of 40 to 50 nm, have an inherent cytotoxicity and targetability to some extent, even without any drug or targeting agent incorporated in them (Jiang et al. 2008). The small size (<100 nm) prevents glomerular filtration and consequent excretion of the nanoparticles through the kidneys. This characteristic, combined with EPR of nanoparticles through the leaky vasculature around tumors, contributes significantly toward increased intratumoral concentrations of nanoparticles (Maeda 2001). Besides this, attachment of PEG to the nanoparticle surface gives long circulating characteristics to the nanoparticles, which can help in the accumulation of nanoparticles at the tumor site (Storm et al. 1995). The small size of nanoparticles offers large specific surface areas, improves the solubility of poorly soluble drugs, and also improves the scope for attaching a variety of functional groups to the surface in order to modify the physicochemical and functional properties of the system. Most cancer drugs have poor aqueous solubility, which translate to a very poor bioavailability especially in the case of orally administered drugs (Lipinski et al. 2001). In the case of parenterally administered formulations too, sufficient intracellular concentrations are not achieved due to poor aqueous solubility. Thus, combining anticancer agents to nanoparticulate carriers contributes to a large extent in increasing the solubility

and hence the bioavailability and intracellular concentrations of the drugs. Selective accumulation of nanoparticles can be obtained by manipulating the polymeric composition (type of polymer, size, hydrophobicity, and biodegradation profile). Besides, certain polymeric nanoparticles are able to reverse the problem of multidrug resistance, typical of tumors (Colin de Verdiere et al. 1994; Krishna and Mayer 2000). A comprehensive review of the importance and advantages of nanoparticles/nanocarriers in cancer therapeutics including the various targeting strategies used for their delivery to tumors and the large number of these nanocarriers/nanoparticle–drug conjugates already approved for drug delivery or at different stages of clinical trials has been presented by Wicki et al. (2015).

The rapidly emerging field of nanotechnology offers a very great promise toward the goal of targeted, safe, and optimized delivery of anticancer agents.

CHAPTER 3

Methods of Preparation of Nanoparticles for Drug Delivery

Nanoparticles have been present for a long time now and were being prepared and used for a variety of applications. However, the term "Nanotechnology," followed by "nanoparticles" came into common use after Prof. Feynman's historical lecture "There's plenty of room at the bottom" in 1959 in which he introduced the concept of manipulating materials at the nanometer level to the world, and the unlimited potential of being able to do this (Feynman 1960). The history of nanotechnology and the milestones achieved in this area since Feyman's historical lecture have been summarized in Chapter 1 of this book. As is evident from the sequence of events that have taken place in this field, there have been two approaches toward preparing particles in the nanometer size range. One approach is the "top-down" approach that is followed by Prof. Drexler (1981), which uses small "molecular machines" to manipulate still smaller atoms for preparing new materials including those that would have normally not been possible to make with the conventional wet chemical syntheses methods; the other approach is the "bottom-up" approach followed by Prof. Smalley in which nanoscale materials are prepared by the atoms and molecules coming together in an appropriate medium to form larger molecules and particles, however in the nanometer size range (Baum 2003). The present chapter describes the methods used for the synthesis of nanoparticles by the "bottom-up" approach. A review of literature in this area indicates how brilliantly all the possible physicochemical principles and material properties have been exploited to their fullest potential and, as a result, have culminated in a large variety of methods for preparing nanomaterials of different shapes, sizes, composition, and behavior suited

to a variety of applications. Practically no field has remained untouched by nanotechnology, and the same is true for the field of drug delivery also. One of the greatest advantages of nanoscale materials has been identified as the area of cancer drug delivery in which such a size range has the potential of targeting anticancer agents specifically to tumors thus avoiding all the adverse effects normally associated with these drugs. This tumor targeting capacity of nanoscale materials has also found applications in cancer diagnostics resulting in the possibility of early diagnosis of cancer. The present chapter describes the methods used for the preparation of nanomaterials having applications in the field of drug delivery, especially cancer therapeutics and diagnostics.

Nanoparticles can be fundamentally categorized into two—organic and inorganic. The methods of synthesis for the two differ greatly as organic materials require relatively milder conditions of temperature and pressure and moderate pH, whereas inorganic materials can withstand more extremes of these parameters. Within the category of organic materials, polymeric and nonpolymeric materials again require different treatment methods. Figure 3.1 provides a classification of the various methods that have evolved over the years for the preparation of nanoparticles of these different categories of materials. The selection of a specific method usually depends on the properties of the material used, type of nanoparticles desired and their properties, and the final application for which these will be used. Once the method of preparation is chosen, the effect of the different parameters that could possibly affect the process is studied. Based on these studies, selection of those parameters that give the best possible characteristics for the desired nanoparticles is done. Important characteristics include size, encapsulation efficiency, zeta potential (surface charge), and release characteristics. The technique used, type of polymer selected, and stabilizer used affect the structure and properties of the nanoparticles prepared.

Fundamentally, two approaches are used for synthesis of nanoparticles—bottom-up and top-down. The bottom-up approach involves building of nanostructures atom by atom or molecule by molecule and involves precipitation or condensation of the products or educts dissolved in solvents with subsequent separation of unwanted solvents. The top-down approach consists of size reduction of larger particles using equipment suitable for grinding down to nanosize range. The top-down methods are generally used for the production of inorganic nanoparticles and are not commonly used in the pharmaceutical field or fields in which a high degree of purity is required as the abrasion of the milling element used in these processes causes contamination of the product, which is unacceptable in

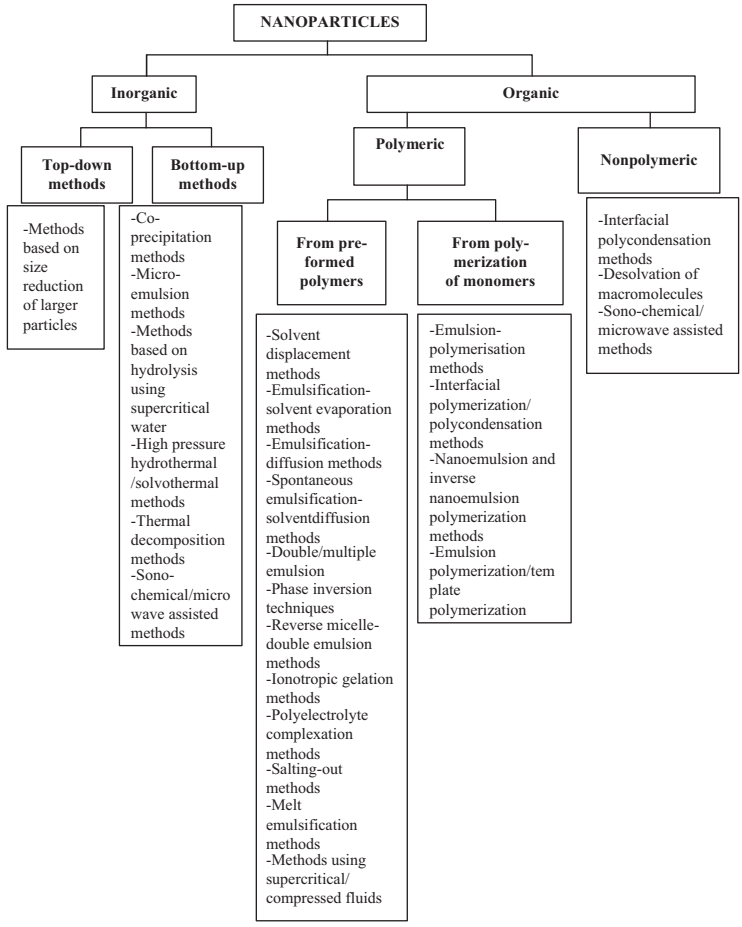

Figure 3.1. Different methods of preparation of nanoparticles for biomedical applications.

these applications. Moreover, these processes are unsuitable for obtaining nanoparticles with a narrow size distribution because the applied mechanical energy in the form of shearing or cavitation used in these processes for decreasing particle size essentially induces agglomeration. In spite of these drawbacks, the top-down method has been successfully used for the preparation of some selected nanoparticles. Because of its overall unsuitability in the pharmaceutical field, this method has not been discussed in this book; the main focus remains the bottom-up approach. As stated, this approach involves a gradual build-up of the nanoparticle from individual atoms or molecules. This can take place either in the liquid or the gas or

vapor phase. The gas or vapor phase synthesis is capable of giving smaller nanoparticles and a better control over the size, but involves relatively extreme conditions of temperature and pressure compared with the liquid phase methods. In the liquid phase methods, the size of the nanoparticles formed depends largely upon the degree of control over the precipitation or phase separation of one of the components from the solution. The precipitation or phase separation may be a result of a chemical reaction or change in solubility (usually a decrease in solubility) of a component due to change in parameters such as pH, temperature, and solvent system. The smaller the space domain in which the chemical reaction or phase separation is made to take place, the smaller the size of the particles separated from the solution. The different methods of preparation of nanoparticles using this approach emerge from the mechanism responsible for phase separation of the desired component. The factors governing phase separation and the individual process parameters will finally affect the nature and size of the nanoparticles formed. Understanding the theoretical aspects involved in phase separation from homogenous and heterogenous systems will help in the selection of an appropriate process and tuning of the physical properties of the nanoparticles to be synthesized. The following section provides an overview of the theoretical aspects relevant to the formation of nanoparticles in general.

3.1 THEORETICAL CONSIDERATIONS BEHIND FORMATION OF NANOPARTICLES

Most methods developed for the synthesis of nanoparticles involve, at some stage, the separation of a solid phase from an initially homogenous system or an initially heterogenous system. Methods such as nanoprecipitation, co-precipitation, solvent displacement, salting out, and desolvation of a macromolecule are examples of the former, that is, formation of nanoparticles from a homogenous solution. The homogenous solution may consist of a soluble solute dissolved in a solvent in which precipitation of the dissolved solute may be a result of change in temperature, composition, or solubility of the solute. The change in temperature may be in the form of sudden cooling (quenching) of an initially hot solution of the solute in the solvent. Based on the initial composition of the solution and the rate of cooling, this may lead the system to either the spinodal or metastable region. A sudden decrease in temperature, taking the system into the spinodal region without entering the metastable region, will result in very fine nanoparticles—the size of quantum dots—because the solute

spontaneously separates into a solid phase from a molecular size. On the other hand, the solute, dissolved in a solvent, may be added to another liquid in which the solvent is soluble but the solute is not. In this case also the solute will precipitate due to the sudden change in solubility of the solute in the new environment. A change in the concentration of solute by way of evaporating the solvent also leads to precipitation of the solute. The size of the precipitate will depend upon the rate of change of parameters such as rate of decrease in temperature, rate of evaporation, or rate of addition of nonsolvent.

It may not always be possible to have a homogenous solution. In the cases in which organic solvents such as acetone, methanol, and carbontetrachloride need to be avoided, it becomes necessary to start with a nonhomogenous solution such as an emulsion. The mechanism of nanoprecipitation may still take place, but the size of particles formed will depend on the size of the dispersed phase. In this case, the dispersed phase that contains the solute is subdivided into micron- or nanosized droplets. Diffusion or evaporation of the solvent may be effected to cause precipitation of the solute. This forms the basis of all the emulsion based methods for the preparation of nanoparticles such as emulsion solvent diffusion method and emulsion solvent evaporation method.

3.1.1 PHASE EQUILIBRIA: FUNDAMENTAL CONSIDERATIONS

Nanoparticles are formed as a result of separation of one component from a mixture of either two or more miscible or partially miscible components. Figure 3.2 shows the phase diagram for such a solution of a soluble solute in a solvent, or a mixture of two partially miscible liquids or both. The inner curve in Figure 3.2a represents the spinodal curve whereas the outer curve represents the binodal curve. The curve shown in Figure 3.2b is the corresponding free energy-composition diagram for the system. Depending on the initial composition of the homogenous mixture, a change in any of the parameters such as temperature or composition or both will result in a phase separation either in the spinodal region (region within the spinodal curve) or metastable region (the region between the spinodal and binodal curves). The mechanisms of separations in both cases differ significantly. Spinodal decomposition is the mechanism whereby a homogenous solution of two or more components separates into distinct phases with distinctly different chemical composition and physical properties. It is spontaneous (there is no thermodynamic barrier for separation) and occurs uniformly throughout the mixture. On the other hand, binodal

decomposition is not spontaneous and there exists a thermodynamic barrier that needs to be overcome for phase separation to take place. Phase separation, in this case, is characterized by the existence of discrete nucleation sites in which small nuclei are first formed, followed by the growth of these nuclei to form crystals, which grow to form particles. The phase separation by both these mechanisms is explained on the basis of the phase and free energy diagrams shown in Figure 3.2. Suppose a mixture with a composition corresponding to X_0 is heated to a temperature T_1 to obtain a homogenous solution (represented by point P in Figure 3.2a); if this solution is suddenly cooled (quenched) to a temperature T_2 (having corresponding free energy G_0), the system will become unstable, and small fluctuations in composition will decrease the free energy of the system (as shown in Figure 3.2b) and, hence, complete separation into an "A"-rich phase and a "B"-rich phase (corresponding to equilibrium compositions X_1 and X_2) can take place. On similar lines, if a mixture with a composition corresponding to X_0' is heated to a temperature T_1 to obtain a homogenous solution (represented by point Q in Figure 3.2a), and if this solution is cooled to a temperature T_2 (having corresponding free energy G_0'), any fluctuation in composition will result in an increase in the free energy of the system (as seen from Figure 3.2b), and the system will become metastable. A decrease in free energy in this case will only be possible if nuclei are formed with a composition different from the original solution. Thus, sufficiently large fluctuations will result in the formation of stable nuclei having a composition different from the initial solution, and these subsequently grow into discrete crystals or particles. Thus, a separation in the metastable region (binodal decomposition) will take place by nucleation and crystal growth, whereas phase separation in the spinodal region will spontaneously result in very fine and uniform nanoparticles (in the case of a solution of solute in solvent) or in two bi-continuous phases with a different composition (in the case of a mixture of two immiscible liquids, as in microemulsions).

Spinodal decomposition has been well defined and characterized; however, a number of in-depth studies on the mechanism of separation by nucleation and phase separation (binodal decomposition) suggest a modification of the classical nucleation theory. The modern nucleation theory suggests that prior to the formation of nuclei, prenucleation clusters (PNCs) are formed which, unlike the nuclei of the classical nucleation theory, are relatively long-lived species. These PNCs, over time, develop into nuclei that subsequently form clusters that either aggregate to form particles or each nucleus grows as more solute from the solution condenses on its surface to form a larger crystal. This crystal then grows at the expense

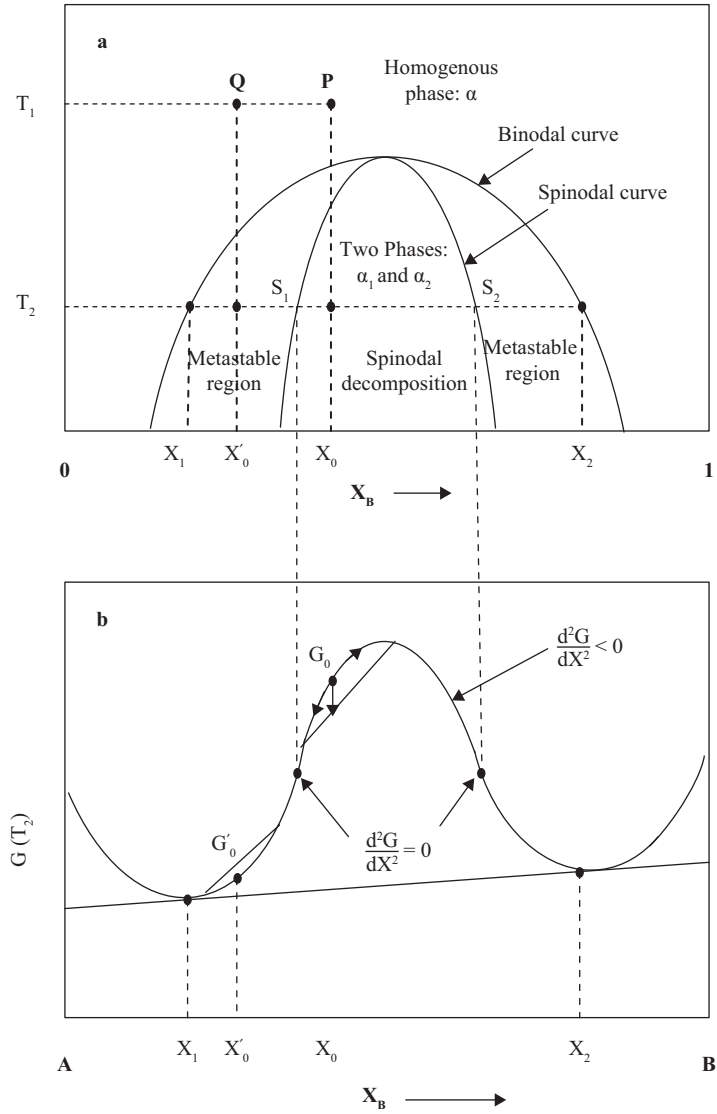

Figure 3.2. Phase and free energy diagrams for a mixture of components A and B.

Source: www.eng.utah.edu (n.d.)

of the solution. There are different theories proposed for nucleation, but for crystal growth the classical theory of nucleation and crystal growth is followed.

3.2 METHODS BASED ON NANOPRECIPITATION FROM SINGLE PHASE SYSTEMS

3.2.1 PRECIPITATION/CO-PRECIPITATION

This is one of the oldest methods for nanoparticle preparation and is most commonly used for the synthesis of inorganic nanoparticles, particularly metallic nanoparticles. As the name itself suggests, it involves precipitation of the product from a homogenous solution. When multiple species are involved in the precipitation process, it is termed as co-precipitation. Precipitation is usually brought about by a chemical reaction wherein two or more reactants react to form a product that has limited solubility in the solvent. The reaction involved may be either a simple addition or combination reaction in which two oppositely charged reactants exchange ions to form a product that precipitates; it may involve oxidation or reduction of a species in which the oxidation or reduction may be brought about by simple chemical, electrochemical, or photo assisted methods; or, it may involve the hydrolysis of a reactant to give a product precipitate. The precipitation may also be brought about by a change in parameters such as temperature, pressure, or solute concentration resulting in reduced solubility of the solute, and effecting its precipitation from the solution. Microwave- and sonication-assisted precipitation/co-precipitation methods have also been used for the generation of nanoparticles. Irrespective of the method used, the precipitation of the product is a result of supersaturation conditions brought about either in the bulk of the solution or localized supersaturation occurring in the immediate vicinity of the reactants or solutes (as in sonication assisted methods). Hence, all such factors that will affect supersaturation, such as the rate at which supersaturation takes place and the degree of supersaturation, will influence the nature of precipitate obtained. Rapid supersaturation will increase the chances of phase separation by spinodal decomposition and result in a large number of small particles, whereas slow supersaturation will result in a small number of large particles. A high degree of supersaturation, such as that observed in the case of low molecular weight substances, will also lead to phase separation by spinodal decomposition, resulting in very fine particles. In the case in which supersaturation conditions favor binodal decomposition, nucleation and crystal growth will control the size and morphology of the particles formed. Thus, effectively, this method of nanoparticle preparation by co-precipitation is controlled by all such factors that influence supersaturation, nucleation, and Ostwald ripening. The fundamentals of particle formation from a homogenous solution and

the effect of solvent and additives on the morphology and the supramolecular structure of the nanoparticle formed have been discussed in-depth, particularly in the context of organic/polymeric nanoparticles by Horn and Rieger (2001), and inorganic nanoparticles by Cushing, Kolesnichenko, and O'Connor (2004).

Considering the most general chemical reaction representing this process:

$$xA^{y+}_{(solution)} + yB^{x-}_{(solution)} \leftrightarrow A_xB_{y\ (solid)}$$

The equilibrium relation between product and reactants is expressed as a solubility product constant K_{sp}:

$$K_{sp} = (a_A)^x\ (a_B)^y$$

where a_A and a_B are the activities of cation A and anion B in the aqueous solution. Consequently, all the factors that reduce the solubility product constant would favor the formation of nanoparticles. The values of K_{sp} are available in literature. When the product contains only one or two elements, the precipitation reactions are relatively straightforward. However, in the case of ternary, quaternary, or higher systems, the process of co-precipitation of more than two species simultaneously becomes more complex. In all cases, the factors such as concentration of cations and counterions, pH and the ionic strength, choice of solvent, temperature, order, and speed of addition are important factors that control the size of nanoparticles. Conditions promoting rapid creation of many nuclei and minimizing subsequent growth give crystals that are very small. Nanoparticles prepared by the co-precipitation method are usually polydisperse in nature. A very stringent control of the reaction conditions is required so that a sudden burst of nucleation takes place simultaneously throughout the medium resulting in a large number of very small nuclei, which subsequently grow by Ostwald ripening to yield a small-sized, monodisperse crop of the product.

Magnetite nanoparticles, often used for magnetically targeted drug delivery systems for cancer, have been commonly prepared by this method. The general reaction involved is:

$$2FeCl_{3(solution)} + FeCl_{2(solution)} + 8BOH \rightarrow Fe_3O_{4(solid)} + 4H_2O + 8BCl$$

where BOH represents a base with B^+ being any suitable cation such as Na^+ or K^+. As mentioned above, the size, shape, and composition of

the nanoparticles formed depend greatly on the type of anion used (e.g., chloride, sulfate, and nitrate); ratio of Fe^{+2} to Fe^{+3}, reaction temperature, and pH (acidic or alkaline); ionic strength of media and other additives such as stabilizers used during reaction; and so on. The effect of all these parameters on the formation of nanoparticles has been independently demonstrated or reviewed by a number of researchers (Chastellain et al. 2004; Jolivet and Tronc 1988; Khaleel 2004; Kim et al. 2003; Liu et al. 2004; Sugimoto and Matijevic 1980; Tartaj et al. 2003, 2005; Tronc et al. 1992). One major difficulty with this method is that the magnetite nanoparticles so formed are not very stable at ambient conditions and tend to agglomerate or oxidize or both to maghemite (Fe_2O_3). Agglomeration is a result of high surface to volume ratio of the nanoparticles. Strong magnetic dipole–dipole attractions between particles arise in an attempt to reduce their surface energy. Besides this, aggregation is also affected by short range forces like van der Waals attraction between two particles. Hence, techniques of stabilization of nanoparticles by preventing their aggregation essentially become an inherent step in the synthesis of nanoparticles by this method.

The most obvious option for keeping particles separate is introducing electrostatic repulsion between particles. This is done by adding anionic surfactants as dispersing agents and thereafter using counterions, pH, or ionic strength to stabilize the charged particles via interactions between the electrical double layers. Factors which compress the double layer such as increased concentration of an inert electrolyte promote coagulation. However, suspensions of nanoparticles stabilized entirely on the basis of electrostatic repulsion are too vulnerable to external conditions such as pH and ionic strength, and hence may not be easily amenable to any surface modifications, which are usually required for drug delivery applications. Coating of particle surfaces with materials such as proteins, starches, or polyelectrolytes serve as a more acceptable alternative to electrostatic repulsion for the prevention of agglomeration of nanoparticles. This strategy allows for the use of much lower electrolyte concentrations and also further surface modification if required. A large variety of substances have been explored for stabilizing and modulating the size, shape, surface, and other properties of the nanoparticles.

To counter the van der Waals attraction between nanoparticles, short range repulsive forces are required to promote stability (Bönnemann and Richards 2001). These can be provided by either electrostatic repulsion between particles or coating the particles with long chain molecules (Chang, Chang, and Chen 2006; Zhi et al. 2006). Magnetite nanoparticles of 4 to 10 nm length have been obtained by using polyvinyl alcohol (PVA)

as stabilizing agent. These nanoparticles formed into chainlike clusters in the presence of small amounts of carboxyl groups (Lee, Isobe, and Senna 1996). Oleic acid has been used as a coating agent for stabilizing dispersions of water based magnetic fluids containing magnetite nanoparticles (Shimoiizaka et al. 1980). Subsequently, it has gained considerable popularity and has been preferred by a number of researchers (Cushing, Kolesnichenko, and O'Connor 2004; Willis, Turro, and O'Brien 2005). Stable aqueous magnetic fluids have also been produced using dodecanoic acid as a dispersing agent (Khalafalla and Reimers 1980). Unlike the dispersions stabilized by oleic acid, the dispersions using dodecanoic acid as a stabilizing agent were found to be stable on dilution. The use of saturated and unsaturated fatty acids to stabilize magnetic fluids has been investigated by Wooding, Kilner, and Lambrick (1991). Besides these, the presence of long chain capping molecules such as phospholipids (egg phosphatidylcholine) during precipitation have also been found to be of use in controlling aggregation of magnetic nanoparticles (Giri et al. 2005).

Stabilization has been done by a variety of agents. Ishikawa, Kataoka, and Kandori have stabilized iron oxide nanoparticles by organic anions, that is, carboxylate and hydroxyl carboxylates (Ishikawa, Kataoka, and Kandori 1993). Phosphate ions have been used by Kandori et al. (1992). Highly stable superparamagnetic iron oxide (SPIO) nanoparticles have been prepared by alginate (SPIO-alginate) (Ma et al. 2007). The high stability is probably because of the binding of carboxyl group of alginate to the iron oxide nuclei.

Stabilization can also be achieved by using organic solvents. However, using organic solvents (organosols) like hexane and decane has a disadvantage of having limited biological applications because of their poor solubility in aqueous solutions and poor biocompatibility and biodegradability. Hence, Zhu et al. used a modified form of the natural polymer chitosan, o-carboxymethyl chitosan (OCMS), for stabilizing iron oxide nanoparticles. The o-hydroxyl group in chitosan when substituted by o-carboxymethyl group gives an amphiphilic nature to the polymer, in addition to blood compatibility and improved membrane penetration (Zhu et al. 2005a, 2005b). They showed that the stability emerges due to tight binding of chitosan or OCMS to the nanoparticle surface and the surface charge provided by binding, which causes repulsion between particles (Zhu, Yuan, and Liao 2008). Eschbach et al. prepared iron iodate ($Fe(IO_3)_3$) nanoparticles in the size range of 20 nm by co-precipitation of iron nitrate ($Fe(NO_3)_3.9H_2O$) with iodic acid (HIO_3) (Eschbach et al. 2007).

Lin, Lee, and Chiu prepared stable ferrofluids containing Fe_3O_4 nanoparticles in which the nanoparticles were prepared by co-precipitation in the presence of poly(acrylic acid) (PAA) oligomer. The PAA oligomers were found to promote the nucleation and inhibited the growth of nanoparticles. Stable nonaggregating nanoparticles with an average diameter less than 10 nm were formed in which PAA oligomers provided electrostatic and steric repulsion against particle aggregation. The stability of the dispersion could be controlled by adjusting the pH (Lin, Lee, and Chiu 2005).

Besides aggregation, another factor contributing to the instability of magnetite nanoparticles prepared by this method is the oxidation of magnetite to maghemite under ambient condition. The oxidized state of iron oxide, that is maghemite, is also ferrimagnetic in nature and as it is stable in acidic as well as alkaline media, and not prone to further oxidation, often magnetite nanoparticles are converted to maghemite after their initial formation (Lu, Salabas, and Schüth 2007). The physical and magnetic properties of the different forms of iron oxide and their stability have been reviewed by Teja and Koh (2009).

Nanoparticles other than iron oxide have also been prepared by co-precipitation. Jiang et al. prepared core-shell nanoparticles of ibuprofen coated with diethyl aminoethyl-dextran by co-precipitation method. They used a complex coacervation process in which initially the drug ibuprofen precipitates from a supersaturated solution as the pH is decreased. This is almost simultaneously accompanied by co-precipitation of the positively charged polysaccharide on the surface of the precipitating ibuprofen resulting in core-shell nanoparticles (Jiang et al. 2005).

3.2.2 MICROEMULSION METHOD

A microemulsion is a thermodynamically stable, isotropic system formed by at least three components, two of which are immiscible with each other and the third is a surfactant having an amphiphilic behavior. In many cases, a fourth component, a cosurfactant, is required. Microemulsions are formed spontaneously over a specific region of the ternary phase diagram that describes the system, that is, under specific conditions of temperature and compositions of the immiscible solvents and surfactants. The proportion of the immiscible solvents and the hydrophilic-lipophilic balance (HLB) value of the surfactant used determine the nature of the dispersed phase and the continuous phase. In any case, the size of the dispersed phase is in the nanometer range (typically <100 nm). If the reactants are present separately

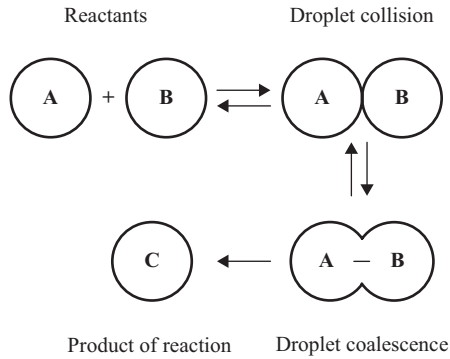

Figure 3.3. Coalescence of droplets (containing reactants A and B, respectively) in a microemulsion to give product C.

in the dissolved state in the dispersed phase droplets, when the droplets transiently collide with each other due to Brownian motion, they act as "nanoreactors," causing the reactants to react for microseconds and in a highly reduced space domain, resulting in the formation of a nanoparticulate product (Figure 3.3). However, as the reactants present in the dispersed phase are simultaneously diffusing in and out of the continuous phase, only under certain critical conditions can the solute molecules be transported from one droplet to another without going through the continuous phase. Thus, nanoparticles in the size range of 5 to 50 nm can be obtained when two different microemulsions, respectively, containing the two reactants are mixed together in specific proportions.

Capek has reviewed this method for the preparation of a number of metal oxides including iron oxide nanoparticles. The kinetics of metallic particle formation, effect of stabilizer type and concentration, type of continuous phase, influence of temperature, incident light, nature of metal salts, and reaction conditions are discussed in detail. The flexibility of the surfactant film also affects the size of the nanoparticles formed (Capek 2004).

There are several ways in which microemulsions can be used for the preparation of nanoparticles. Two identical water-in-oil (w/o) microemulsions containing reactants A and B, respectively, dissolved in the aqueous dispersed phases can be mixed together in which the two reactants react when the dispersed phase droplets collide or coalesce with each other, resulting in a precipitate AB formed in the dispersed phase itself. Alternately, the primary reactant A can be dissolved in the aqueous dispersed

phase of the w/o microemulsion, and the secondary reactant can be added to the microemulsion or bubbled through the microemulsion, as a result of which precipitates are formed in the dispersed phase droplets. In either case, the reaction occurs in the tiny nanosized droplets of the microemulsion, thus limiting the size of the product formed (Pillai et al. 1995).

w/o Microemulsions have been used to synthesize a variety of nanoparticles such as iron oxide, metallic iron, magnetic polymeric iron oxide, and silica-coated iron oxide (Deng et al. 2003; Dresco et al. 1999; Xu et al. 2004; Zhi et al. 2006). Teja and Koh have reviewed all such methods used for the preparation of nanoparticles. A modified reverse micelle-microemulsion method for the preparation of ultrafine magnetite containing spherical silica nanoparticles doped with proteins has been reported. A variety of surfactants such as bis(2-ethylhexyl) sulfosuccinate (AOT), sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide, polyvinylpyrrolidone (PVP), and diethyl sulfosuccinate have been used for the preparation of microemulsions. The main disadvantage of the microemulsion method for the preparation of nanoparticles is that the residual surfactant remaining in the process is undesirable. Besides this, most of these methods are difficult to scale-up (Teja and Koh 2009).

3.2.3 SOLVENT DISPLACEMENT METHOD

This method, also sometimes called the interfacial deposition method, was first described by Fessi et al. (1989) and Fessi, Devissaguet, and Puisieux (1991), and is one of the easiest methods for the preparation of nanoparticles. It is a reproducible, fast, and economic one-step method that usually yields nanospheres from preformed polymers. The essential components of this procedure are a preformed polymer, a suitable solvent for the polymer, and a nonsolvent for the polymer. The drug may be incorporated along with the polymer. A schematic representation of the process along with the salient features of each component involved is given in Figure 3.4.

The polymer solution—with or without the drug—is added gradually, dropwise, with stirring to the nonsolvent that may or may not contain a stabilizer dissolved in it resulting in rapid diffusion of the aqueous nonsolvent into the polymer solvent phase. This displacement of the polymer solvent by the nonsolvent causes almost instantaneous precipitation of the polymer. A surfactant present in the aqueous phase improves the yield of the nanoparticles and the long-term stability of the nanoparticles by preventing particle aggregation in the dispersion. Subsequent to the formation of nanoparticles, the solvent is eliminated by evaporation.

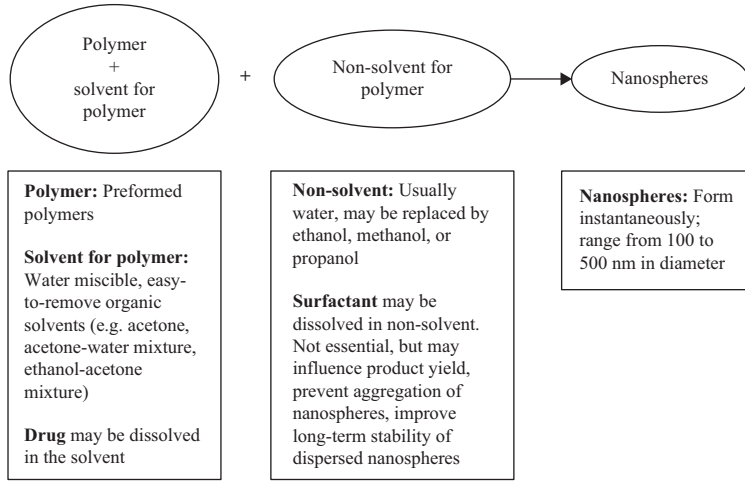

Figure 3.4. Solvent displacement process for the preparation of nanoparticles.

The mechanism of nanoparticle formation by this method has been explicitly described by Quintanar-Guerrero et al. (1997) and Jung, Breitenbach, and Kissel (2000). Jung, Breitenbach, and Kissel studied the formation of nanoparticles of sulfobutylated PVA-graft-poly(lactide-co-glycolide) using this method and proposed that this mechanism of nanoparticle formation and the amphiphilic nature of the polymer are responsible for the core/corona structure of the final nanoparticles obtained. When the solution of a polymer in a water miscible solvent like acetone is introduced dropwise into an external aqueous phase, the solvent starts diffusing rapidly into the aqueous phase because of the fact that the solvent–water interfacial tension is less than the solution (polymer in solvent)–water interfacial tension. The diffusion of this pure solvent into the aqueous phase induces interfacial turbulence between the two phases that is governed by the so-called Marangoni effect (Sternling and Scriven 1959). Due to this interfacial turbulence, the initial droplet is subdivided into smaller and smaller droplets until finally nondivisible polymer aggregates are left behind. This mechanism is described in Figure 3.5.

The decrease in the size of the droplets as a result of diffusion of the solvent into the external aqueous phase is accompanied by an increase in the concentration of the polymer solution in each droplet and, consequently, an increase in osmotic pressure and viscosity of the remaining polymer solution. This reduces the diffusion velocity of the solvent, which finally tends

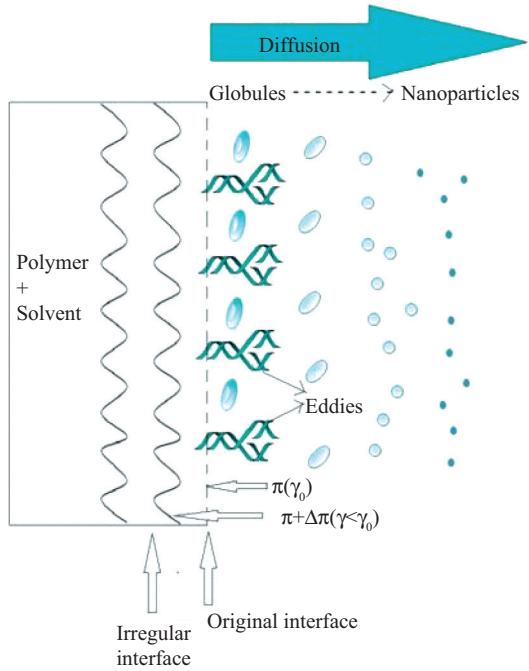

Figure 3.5. Diagrammatic representation of nanoparticle formation by the solvent displacement process based on an interfacial turbulence mechanism.

to reach zero. Thus, factors increasing the diffusion velocity of the solvent, like reduced viscosity, reduced polymer concentration, and increased speed of stirring, reduce the particle size of the final particles. An experimental plan using the central composite design was used to investigate the effect of five factors: (i) polymer concentration in the oil phase, (ii) volume of oil phase, (iii) flow rate, (iv) surfactant, and (v) stirring speed to confirm the above mechanism of nanoparticle formation. A similar mechanism and justification for the formation of poly(Є-capro-lactone) nanoparticles was also proposed by Molpeceres et al. (1996).

Jung, Breitenbach, and Kissel (2000) further prepared nanoparticles of sulfobutylated poly(vinyl alcohol)-g-PLG (SB-PVAL-g-PLGA) loaded with tetanus toxoid on the same lines. Here, they showed that the average diameter of the nanoparticles varied depending on the solvent used in their preparation. Nanoparticles with an average diameter of ~100 nm were obtained when only acetone was used as a solvent; the size of nanoparticles increased to ~500 nm when acetone:ethylacetate (65:35) was used

whereas ethyl acetate:dichloromethane (95:5) yielded microparticles with a diameter of ~1 μm.

Depending on the solvent, polymer type, polymer concentration, and addition of emulsifiers, the particle size of nanoparticles formed by this method can range from 80 to 500 nm or larger (Peltonen et al. 2002; Wehrle, Magenheim, and Benita 1995). The applicability of this method is confined to hydrophobic drugs and polymers. Nanocapsules with an internal oil core can be formed when a small volume of oil is introduced into the organic phase. The oil can be used to dissolve substances with low water solubility and increase encapsulation efficiency. This approach for preparation of nanoparticles is discussed at length in Section 3.3.1.2 (emulsion based processes). Nanoparticles containing β-carotene were produced by this method in which poly(l-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) were the biodegradable polymers dissolved in acetone to later form the dispersed phase. Gelatin or Tween-80 was used as a stabilizing hydrocolloid in continuous phase. β-Carotene was entrapped in the polymer matrix in the absence of any oily core material (Ribeiro et al. 2008). Pinon-Segundo et al. modified the standard solvent displacement method for preparing polycaprolactone (PCL) nanoparticles by using a small recirculating device that recirculates the aqueous phase, providing a continuous flow. The nanoparticles formed when such a recirculation device was used were found to be smaller in size compared with those obtained by the standard solvent displacement method. Whereas the entrapment efficiencies remained unaffected, the yield of nanoparticles was adversely affected by such a modification (Pinon-Segundo et al. 2006). Calvo et al. prepared chitosan coated PCL nanocapsules in which PCL, lecithin, and oil Miglyol-840 were dissolved in acetone, and this mixture was poured with moderate stirring into the aqueous phase containing nonionic surfactant Poloxamer-188 and different amounts of chitosan (Calvo et al. 1997a).

3.3 METHODS BASED ON FORMATION OF NANOPARTICLES FROM HETEROGENOUS SYSTEMS: EMULSION BASED PROCESSES

Emulsion based processes form a major part of the methods used for the preparation of nanoparticles for drug delivery. These processes may comprise of nanoparticles formed from preformed polymers or may involve in situ polymerization of monomers subsequent to the addition of an initiator to the emulsion. The methods based on preparation of nanoparticles

from preformed polymers are two-step processes in which the first step invariably comprises of the formation of an emulsion of two immiscible liquid phases by any appropriate method in which the polymer or the polymer–drug conjugate is dissolved in the dispersed phase; the second step comprises of removal of the solvent by any suitable means such as evaporation, diffusion into the external continuous phase, or physical removal by cross-flow filtration resulting in the precipitation or separation of the polymer/polymer–drug conjugate. Generally, the principle of the second step gives its name to the method. The emulsion formed may be a single oil-in-water (o/w) or water-in-oil (w/o) emulsion or a w/o/w or o/w/o multiple emulsion. Modifications may consist of phase inversion techniques for simple emulsions or reverse micelles double emulsion techniques. The use of miniemulsion, nanoemulsions, and microemulsions has been proposed to show better results as compared with classical emulsions because of their greater stability (Anton, Benoit, and Saulnier 2008; Bouchemal et al. 2004; Weiss, Ziener, and Landfester 2007).

The methods for the formation of emulsions can be divided into two categories.

1. High energy methods and
2. Low energy methods

The high energy methods include homogenization, microfluidization, and ultrasonication in which, generally, a preformed polymer along with a drug is dissolved in an organic solvent that is water immiscible. Microfluidization uses a high pressure positive displacement pump operating at very high pressures, up to 20,000 psi, which forces the emulsion product through microchannels onto an impingement area resulting in very fine emulsion droplets. Homogenization methods include a variety of methods for reducing the droplet size of the dispersed phase. These methods are typically based on mechanical subdivision of larger droplets using high energy emulsification techniques that allow the formation of uniformly sized emulsion droplets and are amenable to scale-up. Colloid mills and other homogenizers based on producing shear stress by employing a rotor stator assembly are used. Ultrasonication methods use ultrasonic energy for the division of larger droplets into smaller ones.

All high energy methods for the preparation of an emulsion affect the subdivision of the dispersed phase by providing shear stress, which is essential for the formation of new surfaces. The size of the dispersed phase droplets is inversely proportional to the shear stress provided. Hence, all factors that affect the shear stress applied to the dispersed phase

will influence the size of the dispersed phase droplets in the emulsion. These factors include:

a. energy density (external energy applied per unit volume) and
b. viscosity of the organic/aqueous phase.

The higher the energy density, the higher the shear stress, and the finer the dispersion formed. However, the increase in surface energy due to formation of new surfaces makes these dispersed phase droplets highly susceptible to agglomeration or coalescence. Hence, efficient stabilizers are required to prevent the coalescence of the dispersed phase droplets in emulsions formed by the high energy methods. Viscous forces in the organic/aqueous phase oppose the shear stress in the system. Hence, reducing the viscosity of either of the phases will reduce the shear stress, consequently increasing the size of the dispersed phase droplets (Kwon et al. 2001).

The low energy methods involve spontaneous emulsification solvent diffusion (SESD) and phase inversion methods. These are discussed separately in the sections that follow.

Other methods are based on extrusion processes in which the dispersed phase is forced to permeate through a microfiltration device by which the fine droplets of dispersed phase are extruded into the continuous phase. Such microfiltration units may take the shape of porous membrane (Charcosset and Fessi 2005), a series of small channels (Freitas, Merkle, and Gander 2005), or a grid perforated with calibrated holes (Charcosset and Fessi 2005; Kobayashi, Mukataka, and Nakajima 2005).

After reduction in the size of the nanodroplets, the removal of the solvent leads to precipitation of the solute (polymer/polymer–drug conjugate) present in the nanodroplets to form nanoparticles.

The nanoparticles formed by high energy methods generally tend to agglomerate over time due to the higher energy content present in the particles whereas those formed by low energy methods are relatively more stable.

3.3.1 METHODS BASED ON HOMOGENIZATION

3.3.1.1 High Pressure Homogenization Methods: Methods for the Preparation of Solid Lipid Nanoparticles

Lipids that are solid at room temperature can be converted into a nanoparticulate form by homogenizing a mixture of the molten lipid and water to form an o/w emulsion comprising of a fine dispersion of the molten lipid

in water. Subsequent cooling of the emulsion results in fine solid lipid particles dispersed uniformly in the aqueous phase. These finely dispersed lipid particles are called solid lipid nanoparticles (SLNs). If the lipid that is used for the preparation of SLNs is physiologically compatible, lipophilic drugs can be easily entrapped in the SLNs. Being both lipids and nanoparticulate in nature, these are very efficiently absorbed through biological membranes. Besides this, SLNs serve as a very good alternative to emulsions and liposomes as they are comparatively much more stable and allow for a controlled release of the drug from the matrix, at the same time, preventing leakage of the drugs from the matrix. In addition to this, SLNs, which use biocompatible lipids, also serve as a superior alternative to polymeric nanoparticles as many polymeric nanoparticles have been shown to have cytotoxic effects after internalization into cells (Müller et al. 1997; Smith and Hunneyball 1986; Wang, Sun, and Zhang 2002).

High pressure homogenization processes are used for the preparation of nanoparticles (Mehnert and Mader 2001). The high pressure homogenization methods involve two approaches: (i) hot homogenization and (ii) cold homogenization.

The hot homogenization method involves processing at temperatures at least 5°C to 10°C above the melting point of the lipid. The lipid is heated above its melting point, and the drug is dissolved in the molten lipid. The aqueous phase containing the emulsifier is also heated to the same temperature as the lipid phase. A pre-emulsion is formed by homogenizing the two phases (at the same temperature), followed by high pressure–high shear homogenization (3–5 homogenization cycles at 500–1500 atm pressure) resulting in the formation of a nanoemulsion. The emulsion temperature increases during homogenization (approximately 10°C for every 500 atm pressure). Whereas higher temperatures lower the particle size of the dispersed phase (because of the decrease in viscosity of the dispersed phase), it may cause degradation of the drug. The number of homogenization cycles may need to be limited because the increased kinetic energy provided may lead to coalescence of the dispersed phase droplets. Subsequent to homogenization, the hot emulsion is cooled to room temperature whereby the dispersed phase solidifies to give SLNs dispersed in the aqueous phase. Besides the possibility of degradation of drug at the high processing temperatures used, another major limitation of this method is that the final stage of cooling of the dispersed phase resulting in solidification of the lipid may be a very slow process due to the presence of emulsifiers in the emulsion. In many instances, a supercooled melt may exist for several months. During the cooling process, formation of lipid crystals takes place. The system, in an effort to form nearly perfect

organized crystal lattices, leaves very limited space for the drug and hence excess drug tends to get expelled from the system during cooling and subsequently during storage.

These limitations of the hot homogenization process have been circumvented, to some extent, in the cold homogenization process. In this process, the drug is dissolved in the hot lipid as in the hot homogenization process; however, the drug containing melt is rapidly cooled at this stage itself whereby the drug is distributed uniformly throughout the lipid matrix while it is solidifying. The solid lipid containing the drug is milled to give microparticles. These microparticles are suspended in a chilled aqueous solution containing an emulsifier and then subjected to high pressure homogenization. In this process, as the cooling is done in the absence of an emulsifier, a supercooled melt is not obtained. However, as high pressure homogenization carried out at the final stage may lead to a rise in temperature, cooling may be required during homogenization.

You et al. prepared SLNs incorporating vinorelbine bitartrate by the cold homogenization method. The drug, along with emulsifier lecithin, and oleic acid were dissolved in a minimum amount of ethanol and added dropwise to the hot lipid (glyceryl monostearate) at 60°C. The mixture was cooled by pouring in liquid nitrogen to remove ethanol. The solid dispersion so obtained was ground to obtain microparticles, which were then suspended in the aqueous phase. This dispersion was homogenized under moderate conditions followed by high pressure homogenization (with simultaneous cooling) at ~1,360 atm to obtain nanoparticles within 150 to 350 nm. The effect of composition of the lipid phase and the duration of homogenization cycles on the size of the particles were studied (You et al. 2007).

Attama et al. prepared SLNs with a single lipid and a mixed lipid core through the hot high pressure homogenization method. They used theobroma oil as the single lipid and a mixture of theobroma oil, goat fat, and a heterolipid phospholipon as the lipid phase. The lipid matrix was heated to 60°C. The aqueous phase containing emulsifier (Polysorbate 80) was separately heated to 60°C and added dropwise to the lipid phase with stirring. This hot mixture was homogenized (at 24,000 rpm) to form a pre-emulsion, which was subsequently subjected to 20 cycles of high pressure homogenization at 1,000 atm pressure and cooled to room temperature to give SLNs. The effect of single lipid and mixed lipid on the growth of nanoparticles over long periods of storage was studied. The main disadvantages with SLNs are that drug loading capacity is limited, the drug may be expelled during storage, and as they are in the form of aqueous dispersions, the water content is high (around 70–95 percent) (Attama et al. 2007).

3.3.1.2 *Melt Emulsification Method: Method for the Preparation of Nanostructured Lipid Carriers*

The melt emulsification method can be considered to be a modification of the high-pressure homogenization methods and is commonly used for the preparation of nanostructured lipid carriers (NLCs), which are in turn a modification of the SLNs. The NLCs were developed to avoid the disadvantages of drug expulsion during cooling and storage, which are characteristic of the SLNs. The NLCs, instead of comprising entirely of solid lipids (like in the case of SLNs), are made up of a blend of solid lipids and liquid lipids (the terms solid and liquid lipids refer to the state in which the lipid exists at room temperature). The liquid lipids (with the drug dissolved in it) exist in small compartments in a solid matrix. The presence of liquid lipid offers higher drug loading capacity and prevents the expulsion of the drug during storage. The nanostructure, where liquid lipid is present in compartments in a solid lipid matrix, is created as a result of phase separation process during the nanoparticle formation; advantage is taken of the miscibility gap that exists between the two lipids. The melted solid lipid is mixed with a high concentration of a liquid lipid (concentration higher than the solubility of the liquid lipid in the solid lipid at room temperature). This hot oil phase is homogenized with the emulsifier containing aqueous phase at the same temperature. When this hot emulsion is cooled to room temperature, the liquid lipid, which was miscible at higher temperatures, separates in the form of small droplets in the matrix of solid lipid. A comprehensive review of the characteristics and mechanisms of formation of different types of NLCs has been presented by Müller et al. (Müller et al. 2007; Müller, Radtke, and Wissing 2002a, b).

The melt emulsification method has been used by Yuan et al. for the preparation of NLCs for the controlled release of progesterone in which they used monostearin and stearic acid (in a proportion of 2:3) as the solid lipid and oleic acid (5–20 percent of the solid lipid) as the liquid lipid. The lipid mix with the drug dispersed in it was heated to 70°C and homogenized (using a mechanical stirrer) with an aqueous solution of Tween-20 (separately heated to 70°C) to form a primary emulsion in which the hot lipid melt (containing the drug) was dispersed uniformly in the aqueous phase. This primary emulsion was further homogenized using an ultrasonic emulsifier to form a miniemulsion, which was then rapidly cooled by immersing into chilled water. Agitation was continued during cooling and a uniform dispersion of NLCs in the aqueous phase was obtained (Yuan et al. 2007). In a similar manner, NLCs containing the drug Cyclosporine-A and a fluorescent dye-fluorescein, respectively, were prepared

by Shen et al. in which the solid lipid used was glyceryl palmitostearate and the liquid lipid was Miglyol-840. Two percent Tween-80 was used as the emulsifier in the aqueous phase. Poly(ethylene glycol) (PEG)-stearate was used for surface modification. NLCs in the range of 50 to 80 nm were obtained (Shen et al. 2010). Melt emulsification followed by high pressure homogenization has been used for the formation of etoposide-incorporated tripalmitin nanoparticles (Reddy et al. 2004).

3.3.2 METHODS BASED ON THE FORMATION OF NANOPARTICLES FROM PREFORMED POLYMERS

3.3.2.1 Emulsification Solvent Evaporation Process

In this method, nanoparticles are prepared by dissolving the polymer and the compound in a water immiscible organic solvent such as methylene chloride or chloroform. An emulsion is prepared by adding water and a surfactant to the polymer solution. The emulsion is homogenized to reduce the size of the dispersed phase droplets subsequent to which the organic solvent is evaporated to obtain solid nanoparticles, which are usually collected by centrifugation and lyophilization. Figure 3.6 provides a schematic representation of the process.

Polyvinyl alcohol (PVA) and albumin have been used as stabilizers. Factors such as polymer concentration, type and concentration of the colloidal stabilizer, and oil to water phase volume ratio affecting the polydispersity of the final nanoparticles obtained by this process have been studied and reported by a number of researchers (Julienne et al. 1992; Scholes et al. 1993; Verrecchia et al. 1993). The aqueous solubility of the drug to be entrapped affects the entrapment efficiency; water soluble drugs cannot be entrapped, whereas drugs with low water solubility can be entrapped

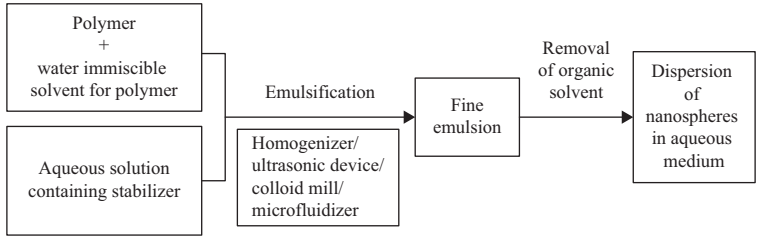

Figure 3.6. Emulsification solvent evaporation method for the preparation of nanoparticles.

successfully by this method (Gurny et al. 1981; Krause, Schwarz, and Rohdewald 1985; Ueda and Kreuter 1997). Budhian, Siegel, and Winey compared the characteristics of particles obtained from sonification with those particles obtained from homogenization and nanoprecipitation under similar conditions. Nanoprecipitation yields a drug content that is too low to be practical and sonification effectively yields particles ~220 nm with a narrow size distribution (Budhian, Siegel, and Winey 2007). This technique has been successful for encapsulating hydrophobic drugs but has had poor results incorporating bioactive agents of a hydrophilic nature (Hans and Lowman 2002).

A typical emulsification solvent evaporation process employed to produce PLGA/PLA nanoparticles showed that the emulsion nanodroplets (containing polymer and drug dissolved in organic solvent) shrink as a result of evaporation of the solvent. The size of the final nanoparticles formed correlates with the size of nanodroplets (Kunii, Onishi, and Machida 2007).

PLA/PEG–PPG–PEG nanoparticles loaded with camptothecin were prepared by o/w emulsification and subsequent evaporation of organic solvent (dichloromethane) at 18°C. Nanoparticles with a diameter of ~230 nm with a fairly high drug content (1.6 percent by weight) were obtained. PLGA nanoparticles loaded with magnetite/maghemite were also prepared with the emulsification evaporation method (Okassa et al. 2007). The surface of iron oxide nanoparticles was coated with oleic acid for better compatibility with organic phase containing polymer. Examples of drug containing polymeric nanospheres prepared by this method including important process parameters and the resultant size of nanoparticles obtained and the drug entrapment efficiencies obtained have been compiled by Quintanar-Guerrero et al. (1998).

3.3.2.2 Emulsification Solvent Diffusion Method

This method evolved as a means of avoiding the use of toxic chlorinated solvents in the emulsion solvent evaporation method. In place of such solvents, this method uses partially water soluble solvents like benzyl alcohol, propylene carbonate, and ethyl acetate. The polymer and bioactive compound are dissolved in the solvent (which is presaturated with water) and emulsified with the aqueous phase (which is presaturated with the solvent) containing the stabilizer. The stabilizer prevents the aggregation of emulsion droplets by adsorbing on the surface of the droplets. Excess water is added to the emulsion, which initiates the diffusion of the solvent into the water. The solution is stirred leading to the nanoprecipitation of

the particles, which can be subsequently collected by centrifugation, or the solvent can be removed by dialysis (Kwon et al. 2001; Takeuchi, Yamamoto, and Kawashima 2001). Quintanar-Guerrero et al. (1997) critically studied the mechanism of PLA nanoparticle formation by this method (Figure 3.7) and compared it with the simple solvent diffusion or solvent displacement method described in Section 3.2.3.

In the emulsion solvent diffusion method, instead of the water miscible solvent (such as acetone) used in the simple solvent displacement method, a partially soluble solvent is used for dissolving the polymer or drug or both. Prior to dissolving the polymer or drug or both in the solvent, it is saturated in water so that it is in thermodynamic equilibrium with water. Due to this, spontaneous emulsification does not take place as in the solvent displacement method; a separate emulsification procedure is required to be carried out before the diffusion step (i.e., dilution with aqueous phase) using suitable homogenization techniques, whereby the partially miscible solvent containing the polymer or drug or both is dispersed uniformly in the aqueous phase containing a suitable stabilizer. Subsequent to the formation of a fine emulsion, when dilution with the aqueous phase is done, the solvent diffuses into the aqueous phase until gradually the polymer or drug or both that was in a dissolved state previous to the dilution step starts getting "stranded," and regions

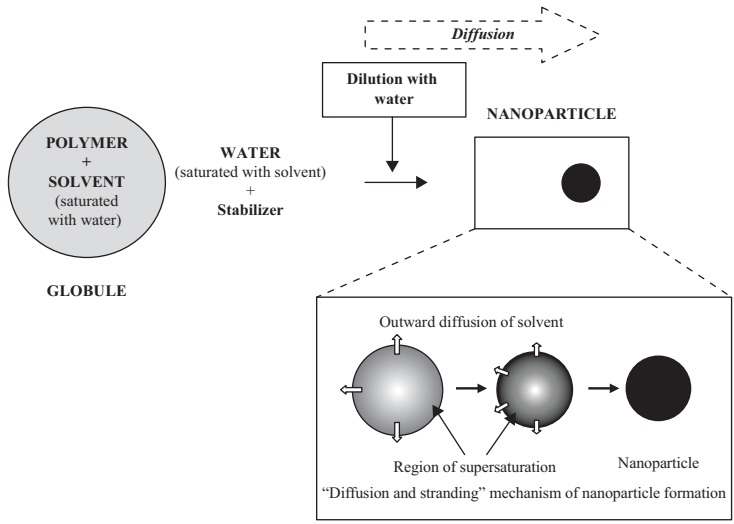

Figure 3.7. Schematic description of the diffusion and stranding mechanism for the formation of nanoparticles using the emulsification solvent diffusion method.

of high supersaturation are created. Finally, the polymer or drug or both precipitate out in the form of nanoparticles in the aqueous phase. The presence of stabilizer in the aqueous phase prevents the agglomeration of the supersaturated globules and subsequently the nanoparticles. This "diffusion and stranding" mechanism was initially proposed by Davies and Rideal (1961) for an oil solvent solution in which they showed that when such a solution was brought in contact with water, diffusion of solvent from the solution forms a three component phase in the immediate vicinity of the interface (spontaneous emulsification), and when more water is added, the entire solvent gradually diffuses into the water until finally only the oil is left behind in the form of droplets. It was shown experimentally that this same phenomenon of "diffusion and stranding" was responsible for the formation of nanoparticles by the emulsion–diffusion method (Quintanar-Guerrero et al. 1997). In many cases, during the dilution step, especially if the drug is hydrophilic in nature, the drug diffuses from the dispersed droplets into the continuous aqueous phase along with the solvent resulting in a significant loss of the drug. However, it has been shown experimentally (for PLGA-quinidine system) that there is no loss of drug once solidification of polymer starts, which typically takes place from the surface to the core (Bodmeier and McGinity 1987). Such a drug loss can be prevented, or at least minimized, by manipulating processing or polymer parameters such as: (i) reducing diffusion time; (ii) increasing diffusional resistance to the drug molecules (by increasing the viscosity of the continuous phase); (iii) reducing drug solubility in the aqueous phase; and (iv) increasing drug–polymer interactions. Modification of the aqueous phase has been done by the use of medium chain triglyceride in place of the aqueous medium; modification of the dispersed phase has been done by addition of surfactant Span 80 to the polymer phase. This has been shown to reduce the leakage of water soluble drugs into the aqueous phase during the solvent step (Takeuchi, Yamamoto, and Kawashima 2001).

Esmaeli et al. prepared rifampicin loaded PLGA nanoparticles by the homogenization solvent diffusion evaporation method using single o/w emulsion. They studied the effect of different variables such as the amount of drug, amount of surfactant, and internal phase volume composition on the characteristics of nanoparticles (Esmaeili et al. 2007). In a similar study, Kwon et al. studied the effect of process variables such as type and concentration of stabilizer, speed of homogenization, and polymer concentration on the size of PLGA nanoparticles. When didodecyl dimethyl ammonium bromide was used as a stabilizer and estrogen was used as a model drug, the size of nanoparticles obtained was smaller than

100 nm as compared with the use of PVA as stabilizer, in which the size of nanoparticles was found to be larger (Kwon et al. 2001). Nanocapsules have also been prepared by this method (Quintanar-Guerrero et al. 1997; Quintanar-Guerrero et al. 1998).

Besides low drug loading due to loss of drug during solvent diffusion, another major disadvantage of this method is that large amounts of water are required to initiate the solvent diffusion process.

3.3.2.3 Spontaneous Emulsification Solvent Diffusion Method

This method is a low energy emulsification process. In this method, the polymer and drug are dissolved in a mixture of a water soluble solvent such as acetone or methanol and a water insoluble organic solvent such as dichloromethane or chloroform (Jaime, Delgado, and William 1998). When water is added to this mixture, spontaneous emulsification takes place. Due to spontaneous diffusion of the water soluble solvent, an interfacial turbulent flow is created between the two phases leading to the formation of nanoparticles. As the concentration of water soluble solvent increases, a considerable decrease in particle size can be achieved (Murakami et al. 1996; Niwa et al. 1993; Wehrle, Magenheim, and Benita 1995). In principle, this method is similar to the nanoprecipitation or solvent displacement method described earlier. The main difference between the two is that in the solvent displacement method, spontaneous emulsification results in the formation of large aggregates or floccules of the dispersed phase and an efficient stabilizer is required to prevent this aggregation; however, in the SESD method, instantaneous formation of very fine, almost unimodal, narrow distribution (in the size range of 50–300 nm) of dispersed phase droplets takes place, even without any stabilizer. This happens due to the "ouzo" effect, which is used to describe the formation of the Greek beverage "ouzo" (called "pastis" in France) in which a mixture of fixed proportion of three components, water (~55 percent), alcohol (~45 percent), and a water insoluble oil transanethol (~0.2 percent), when diluted with water, spontaneously turns milky due to instantaneous desolvation of the oil in the form of stable, very fine globules, uniformly distributed throughout the mixture (Lepeltier, Bourgaux, and Couvreur 2014). Thus, at specific proportions of water, solvent, and solute (polymer or drug or both), spontaneous emulsion formation takes place leading to the formation of nearly monodisperse nanoparticles. This is shown in the ternary phase diagram in Figure 3.8.

Nanosized particles of PLGA or PLA have been prepared by dissolving the polymer in a binary mixture of acetone (water miscible) and

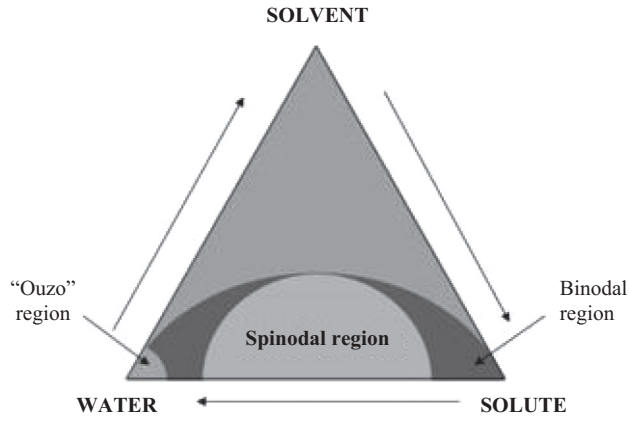

Figure 3.8. Ternary phase diagram of a hydrophobic solute/solvent/water system showing the region where the spontaneous emulsion formation occurs (the "ouzo" region).

dichloromethane (water immiscible) and pouring this into the aqueous phase with moderate stirring. Spontaneous emulsification resulting in a fine dispersion of the organic water immiscible solvent containing the polymer in the aqueous water acetone mixture is obtained in which the evaporation of the organic solvent leaves behind the polymer nanoparticles dispersed in the aqueous phase (Niwa et al. 1993).

However, it has been found that in large-scale production of PLGA nanoparticles by the SESD method, sometimes severe aggregation takes place during the particle formation process when the polymeric concentration is increased to an acceptable range for industrial purposes. Due to this, the need for modification of the SESD method was felt.

The major modification points worked out were: (i) a mixture of two water miscible organic solvents was used as the solvent for the polymeric solution instead of the mixture of water miscible and water immiscible organic solvents and (ii) exclusive low hydrolyzation and polymerization grade of PVA, that is, Poval®-403, was used in this new method as a quasi-emulsifier in the aqueous phase, which closely correlated to the nanoparticle formation mechanism (Murakami et al. 1999). Liu et al. prepared spherical triptolide loaded PLA nanoparticles with a smooth surface using this modified SESD method. They reported nanoparticles with a narrow size distribution (mean size ~150 nm with a polydispersity index of 0.088) and an encapsulation efficiency of 65 percent (Liu et al. 2005).

3.3.2.4 Salting-Out Method

This method is very similar to the solvent displacement method and was first applied to pseudolatexes (Bindschaedler et al. 1988) and later adapted and optimized for drug loaded biodegradable nanospheres by Allémann et al. (1993). This method is based on the separation of water miscible solvent from aqueous solution by a salting-out effect. An o/w emulsion is formed by adding a solution of the polymer and drug in a water miscible solvent, for example, acetone, into an aqueous gel containing a salting-out agent, for example, electrolytes such as sodium chloride, magnesium chloride, calcium chloride, magnesium acetate, and nonelectrolyte sucrose (Allémann, Gurny, and Doelker 1992; Allémann et al. 1993; Ibrahim et al. 1992; Matkovich and Christian 1973; Quintanar-Guerrero et al. 1998), and a colloidal stabilizer (vigorous stirring is required for the formation of an emulsion). Water is added to dilute this mixture until the volume is sufficient to allow for diffusion of the water miscible solvent into the water as a result of which nanoparticles are formed. The solvent and salting-out agents are then removed by cross-flow filtration. Galindi-Rodriguez et al. prepared nanoparticles of methacrylic acid copolymer (Eudragit L 100-55) using PVA as an emulsifying agent. They prepared an aqueous solution of magnesium chloride hexahydrate containing PVAL and added this to an organic phase containing Eudragit L 100-55 in acetone, with mechanical stirring. After mixing, excess water was added to induce diffusion of the organic solvent in water resulting in the formation of nanoparticles. Size ranges from 123 to 710 nm were obtained when the concentration of PVA was changed from 21 percent to 5 percent, respectively (Galindo-Rodriguez et al. 2004). They further compared the physicochemical parameters of the nanoparticles formed by other methods, that is, the nanoprecipitation and emulsion diffusion methods. A major advantage of the salting-out method is that once an appropriate solvent/salting-out agent/stabilizer combination is arrived at, high drug loading capacity and high product yields can be obtained irrespective of the proportions of these components. Besides this, the process is easily amenable to scaling-up. However, this method is suited to only lipophilic drugs (as hydrophilic drugs will come out of the dispersed phase and enter into the aqueous phase during the diffusion of the solvent), and a careful selection of the salting-out agent and the stabilizer is essential. The salting-out agent should enable phase separation without precipitation; similarly, the stabilizer should not aggregate in the saturated aqueous solution in the presence of the solvent (Quintanar-Guerrero et al. 1998).

3.3.2.5 Double or Multiple Emulsion Based Methods

Double or multiple emulsions are either w/o/w or o/w/o emulsions. As the name suggests, these emulsions are made up of minute single emulsion droplets, either w/o or o/w, dispersed in a continuous phase comprising of water or oil, respectively. The w/o/w emulsions are more commonly used in drug delivery. These are usually prepared by a two-step procedure in which first, a w/o emulsion is prepared by emulsifying water with the oil phase containing a surfactant having a low HLB value. In the second step, this primary emulsion is re-emulsified with water containing a surfactant having a high HLB value. In this manner, a w/o/w emulsion is obtained (Zhen et al. 2010). The mechanisms and methods of formation of double or multiple emulsions and their applications have been reviewed in detail by Garti and Bisperink (1998). Hydrophilic drugs can be successfully incorporated in the aqueous phase of the primary emulsion, and subsequent to the formation of the double emulsion in the second step an aqueous dispersion of the drug encapsulated in the oil phase is obtained. Removal of the solvent by any of the usual methods such as solvent evaporation results in the formation of nanoparticles or nanocapsules depending on the nature of the lipophilic substance used. The standard emulsification solvent evaporation technique described in Section 3.3.2.1 is applicable for encapsulating or preparing nanoparticles of hydrophobic drugs and has shown poor results with hydrophilic drugs. The double emulsion method can be successfully used for preparing nanoparticles incorporating hydrophilic drugs. The hydrophilic drug and stabilizer are dissolved in water and a primary w/o emulsion is prepared by dispersing the aqueous phase into a water immiscible organic solvent containing a dissolved polymer. This is then re-emulsified in an outer aqueous phase also containing stabilizers.

Thus, a dispersion of fine droplets of the primary w/o emulsion in a continuous aqueous phase is obtained. Evaporation of the organic solvent leaves behind polymeric nanoparticles containing the hydrophilic drug dispersed uniformly in it (Figure 3.9). Many hydrophilic drugs have been successfully incorporated into nanoparticles by this method (Blanco and Alonso 1997; Lemoine and Preat 1998; Rafati et al. 1997; Vila et al. 2002). Lamprecht et al. have investigated the influence of process parameters on nanoparticle preparation by the double emulsion pressure homogenization technique. They attempted to optimize the homogenization procedure with regard to particle size and monodispersity by studying the influence of homogenization time and amount of polymer and surfactant in the external aqueous phase. They used PLGA 50/50 and poly(ϵ-caprolactone

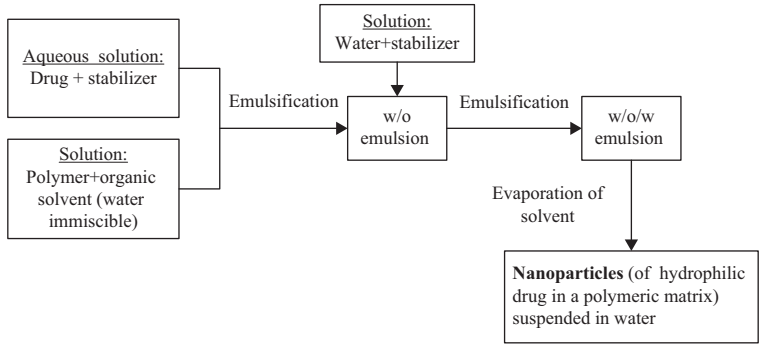

Figure 3.9. Double or multiple emulsion (w/o/w) method for the preparation of nanoparticles.

PCL) for the purpose. They also attempted to study the efficiency of drug loading for protein drugs (they used bovine serum albumin [BSA] as a model protein drug). They found that the drug loading increased by varying the concentration of the protein in the inner aqueous phase (the BSA encapsulation efficiency decreased with a higher protein concentration in the inner aqueous phase). By increasing the protein concentration in the inner aqueous phase, the polydispersity was slightly higher while particle size was not influenced significantly (Lamprecht et al. 2000).

Farokhzad et al. used this method for preparing bioconjugates of PLA-PEG-COOH with aptamers for encapsulating rhodamine labeled dextran for targeted delivery of these nanoparticles to prostate cancer cells (Farokhzad et al. 2006). Zhen et al. have prepared BSA SLNs using 1-monocaprate glycerol as the lipid and Span 80 and Tween-20 as the low and high HLB surfactants, respectively, by this method (Zhen et al. 2010).

3.3.2.6 Reverse Micelles Based Methods

Micelles are aggregates of amphiphilic surfactant molecules dispersed in liquid and are formed at concentrations above the critical micelle concentration (CMC). They typically consist of a hydrophilic head group and a lipophilic tail. In an aqueous solution, the hydrophobic tails form the core of the micelle whereas the hydrophilic head groups form the shell of the micelle. On the other hand, in a lipid solution, the hydrophilic head groups form the core and the lipophilic tails form the shell. These are called inverse micelles or reverse micelles (Figure 3.10).

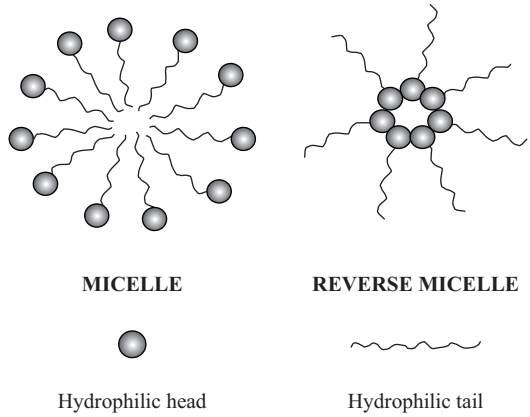

Figure 3.10. The structure of a micelle and a reverse micelle.

At surfactant concentrations above CMC, these are capable of forming either o/w or w/o emulsions. The reverse micelle based methods have been successfully used for the formulation of hydrophilic drugs and water soluble peptides into nanoparticles. Ordinarily, the formulation of such hydrophilic, water soluble drugs and peptides into nanoparticles using the usual homogenization based methods (w/o/w and high pressure homogenization based methods for the preparation of SLNs) poses a problem because the high shear involved is likely to destroy thermolabile drugs and peptides. Besides this, the drug loading is low and the hydrophilic drug tends to migrate into the external aqueous phase during storage. All these limitations are avoided if reverse micelles based methods are used. Cui et al. prepared nanoparticles comprising of an insulin–phospholipid complex, in the range of ~200 nm, with a drug loading of ~90 percent by the reverse micelle solvent evaporation method. Initially, an insulin–soyabean phosphatidylcholine (SPC) complex was prepared to which a solution of polymers (PLA or PLGA) and an appropriate organic solvent were added by gentle agitation. This resulted in a clear micellar solution (in which reverse micelles comprising of the insulin head groups forming the hydrophilic micellar "core" and the lipophilic SPC tail groups forming the micellar "shell" were formed) of the insulin–SPC complex in the organic polymer solution. This solution was poured into an aqueous solution of PVA to form an o/w emulsion. When stirred continuously at atmospheric pressure for 6 hours, the organic solvent evaporated, resulting in the precipitation of the insulin–SPC complex in the nanoparticulate form. Centrifugation of this aqueous colloidal solution followed by freeze drying yielded the desired nanoparticles (Cui et al. 2006).

3.3.2.7 Reverse Micelle Double Emulsion Method

This method is a slight modification of the previous method in which the formation of reverse micelles is followed by the formation of w/o/w double emulsion instead of the single o/w emulsion finally formed in the previous method. As in the previous method, this method too is applicable to the preparation of nanoparticles of peptides and hydrophilic drugs. SLNs incorporating hydrophilic drugs have been prepared by this method (Liu et al. 2007). An aqueous solution of insulin (whose pH was adjusted to 3) was first mixed with sodium cholate (a solubilizer used to improve the liposolubility of insulin) to form reverse micelles with the aqueous insulin solution forming the inner phase. A mixture of stearic acid and palmitic acid were dissolved in ethyl acetate and a solution of this mixture was added to the oily phase comprising of SPC. This oily phase was mixed with the insulin–sodium cholate reverse micelles formed earlier. The resulting mixture was homogenized using an ultrasonic homogenizer to form a w/o primary emulsion. This w/o primary emulsion was mixed with an aqueous solution of Poloxamer-188 and further sonicated to form a w/o/w double emulsion, followed by dilution with more of the aqueous external phase (aqueous solution of Poloxamer-188). The solvent (ethyl acetate) was evaporated in a rotary evaporator to form SLNs (containing insulin) dispersed in water.

The formulation of water soluble peptide drugs into nanoparticles (including SLNs) is considered to be difficult because most methods involve either the use of high temperatures as in the microemulsion based methods in which temperatures in the range of 60°C to 70°C are generally used or high pressures as in the high pressure homogenization methods for the preparation of SLNs in which high shear stress is induced (which is accompanied by the resultant increase in temperature). Besides this, the microemulsion based methods require high concentrations of surfactants and cosurfactants. All these conditions are likely to degrade such peptide/protein based drugs. On the other hand, most methods such as diffusion based methods for the preparation of nanoparticles result in a loss of water soluble drugs because of the diffusion of such drugs into the aqueous phase during the dilution step, whereas the simple w/o/w method yields particles in which the average particle size is in a micrometer range and drug loading is less (Cortesi et al. 2002; Cui et al. 2006; Morel et al. 1998). The reverse micelle double emulsion method, as it uses mild processing conditions and is able to avoid the other disadvantages mentioned earlier, is considered to be a superior method for the preparation of nanoparticles of peptide/protein based drugs or other water soluble or thermolabile drugs or both water soluble and thermolabile drugs.

3.3.2.8 Phase Inversion Temperature Method

This method is an organic solvent-free and low energy method for the preparation of nanoparticles. It is a relatively simple method and can be easily scaled up. This method is based on the initial experiments carried out by Shinoda and Saito in which they showed that for a mixture of water–cyclohexane systems containing a nonionic surfactant, there exists a temperature at which the mutual solubility of oil and water increases markedly in the presence of the nonionic surfactant, and this temperature is closely related to the phase inversion temperature (PIT) (Shinoda and Saito 1968; 1969). They proposed that except for extreme volume fraction ranges for the components, the water phase is the continuous phase at low temperatures; the oil phase is the continuous phase at high temperatures; and the surfactant phase is continuous at medium temperatures near to PIT. This phenomenon has been taken advantage of in the preparation of nanoparticles by the PIT method. Specifically, the PIT method for the preparation of nanoparticles uses nonionic surfactants such as polyethoxylated surfactants that have an ability to modify their affinities for water and oil as a function of temperature. Due to this ability, they are able to cause phase inversion in a nanoemulsion comprising of oil, water, and the nonionic surfactant. In other words, a w/o emulsion changes to an o/w emulsion when temperature is changed. The method typically consists of mixing together the oil phase, water phase, and the surfactant in specific proportions (previously determined by creating phase diagrams for the system and determining the "nanoemulsion feasibility domain"), and heating the mixture to temperatures approximately 10°C to 15°C above PIT to form a w/o nanoemulsion. Subsequently, the nanoemulsion is cooled to a temperature below PIT whereby it crosses PIT and spontaneously converts to an o/w nanoemulsion. If the oil phase comprises a material that solidifies at this lowered temperature, a dispersion of solidified oil phase in an aqueous phase is obtained. If the dispersed phase oil droplets are in the nanometer range, a dispersion of nanoparticles in an aqueous phase is obtained, which can subsequently be collected by centrifugation. The theoretical aspects behind the formation of nanoemulsions and phase inversion therein, such as the factors affecting formation of nanoemulsions and the droplet size, stability of nanoemulsions produced by the PIT method, role of temperature cycling on emulsion phase inversion, and phase behavior in nanoemulsion prepared by the PIT method, have been studied by a number of researchers (Anton et al. 2007; Ee et al. 2008; Fernandez et al. 2004; Izquierdo et al. 2004; Ostertag, Weiss, and McClements 2012). The design and production of nanoparticles from nanoemulsion templates

have been reviewed by Anton, Benoit, and Saulnier (2008). The practical application of the PIT method for the preparation of nanocapsules has been demonstrated by Lamprecht, Bouligand, and Benoit (2002) in which they prepared nanocapsules containing the drug amiodarone by this method. A mixture of all the components—the oil phase (containing the drug amiodarone dissolved in it), the surfactant (polyethylene glycol-660 hydroxystearate), and the aqueous phase—all in specific proportions was heated to a temperature of 85°C (temperature beyond PIT), with continuous magnetic stirring. This mixture was then cooled to 55°C, whereby PIT was crossed again. This cycle was repeated two to three times after which chilled water was added to obtain nanocapsules with size range between 23 and 103 nm and an encapsulation efficiency of 92 to 94 percent. In another instance, lipid nanocapsules were prepared in which the oil phase comprised of caprylic-caproic acid triglyceride and soyabean-lecithin phosphatidylcholine, the surfactant used was a mixture of Poly(ethylene glycol)-660 and Poly(ethylene glycol)-660 hydroxystearate, and the aqueous phase was saline. From the phase diagram for the system, a feasibility zone was identified, and specific proportions of the three components were used. A mixture of all the components was heated to 85°C at a rate of 4°C/minute, with constant stirring. The mixture was then cooled to 60°C at the same rate, followed by addition of chilled water to form nearly monodisperse nanocapsules in the range of 25 to 100 nm (Heurtault et al. 2002).

3.3.3 METHODS BASED ON POLYMERIZATION FROM MONOMERS

3.3.3.1 Emulsion Polymerization/In Situ Polymerization

The methods mentioned in all of the previous sections involve preparation of nanoparticles from preformed polymers. The emulsion polymerization method uses in situ polymerization of monomers to form polymeric nanoparticles in which all polymerization reactions take place in the continuous phase. In this method, a water insoluble monomer is dispersed in an aqueous phase containing a colloidal stabilizer. A chemical initiator, added to the continuous phase, induces polymerization of the monomer. Variations in physical parameters such as pH and radiation may additionally be used to initiate polymerization. Both hydrophilic and lipophilic drugs can be entrapped in the polymeric wall when added to the polymerization medium or adsorbed on preformed particles. The most popular examples for this method of synthesis include the formation

of poly(methyl methacrylates), poly(alkyl cyanoacrylates), and poly-(methylidenemalonates) nanopoarticles (Couvreur 1988). The origins of emulsion polymerization go way back to the 1920s when attempts were made to polymerize isoprene from emulsions stabilized by surface active agents. Since then, the process has been extensively studied and elaborate theories have been proposed for the mechanism of formation of polymer particles and the locus of polymerization in an attempt toward improving and modifying the method to satisfy different material and process requirements suited to a variety of applications. The early development of the process and the theories and mechanisms proposed for the process have been critically studied and reviewed by a number of researchers (Baxendale, Evans, and Park 1946; Brodnyan et al. 1963; Harkins 1947, 1950; Hohenstein and Mark 1946; Khaddazh, Gritskova, and Litvinenko 2012; Smith and Ewart 1948; Yamak 2013). The conventional emulsion polymerization consists of the formation of an o/w emulsion in which the monomer (usually considered to be water insoluble) droplets are dispersed in an aqueous continuous phase. These are stabilized by means of a surfactant or an emulsifier. A fine emulsion is formed by homogenizing the mixture using any of the high energy emulsification methods. Thus, the final emulsion consists of micron-sized monomer droplets stabilized by a layer of surfactant; nanometer-size surfactant micelles, whose core comprises of the hydrocarbon tail groups and the "shell" consists of the hydrophilic head groups; and some free surfactant molecules, all distributed in the aqueous continuous phase. The initiator is usually water soluble and is dissolved in the aqueous phase. When the initiator comes in contact with the monomer molecules, polymerization takes place and polymer particles are formed. However, surprisingly—and it has been confirmed experimentally—the main locus of polymerization is not the monomer droplet as expected. The monomer droplets simply act as a "storehouse" for the polymer molecules. Small amounts of monomer molecules continuously diffuse into the aqueous phase and enter into the micelles. These monomer-swollen micelles are much smaller in size, and hence offer a large surface area, compared with the monomer droplets. Hence, there is more probability that the initiator molecules, present in the continuous phase, collide with and enter into the monomer-swollen micelles rather than the monomer droplets (which are hydrophilic in nature). Besides this, as the initiator molecules are hydrophilic in nature they would have greater affinity toward the micelles than toward hydrophobic monomer droplets. Hence, these monomer containing micelles serve as the main locus for polymerization and initially small polymer molecules are formed. Eventually, as the size of these polymer molecules becomes larger than the micellar cores, they are forced

out of the micelles into the aqueous solution as extremely small polymer particle nuclei. These small polymer particle nuclei move about freely in the aqueous solution in which they capture monomer molecules diffusing out of the monomer droplet and form the polymer monomer particles. The monomer polymerizes as it comes in contact with the initiator molecules as simultaneously more monomer molecules are captured. Hence, the second locus for polymerization is the polymer monomer particle. The third locus—a minor one—for the generation of polymer particle nuclei is the aqueous phase in which the diffusing monomer molecule contacts the initiator molecule to form a polymer nuclei. The effect of diffusion and interfacial phenomena in this context has been studied in detail by Brooks (1971). On the basis of experimental studies, it has been concluded that though both free-radical (initiator) and monomer species are subject to mass transfer resistances in emulsion polymerization and, in most cases, the diffusional processes in the aqueous phase will not significantly affect the course of polymerization; however, diffusional processes occurring within the polymer particle and the interfacial resistance (encountered at the water–polymer particle boundary) can have a significant effect on the polymerization process. Sajjadi and Jahanzad have prepared nanoparticles in the range of 25 nm by a highly diffusion-controlled emulsion polymerization method (Sajjadi and Jahanzad 2006).

The drug can be incorporated into the nanoparticles either noncovalently or by means of covalent attachment to the monomer, which is then subsequently polymerized to form the nanoparticle. Penicillin containing polycyanoacrylate nanoparticles of 25 to 40 nm has been prepared by the latter method (Turos et al. 2007). The procedure used by this research group illustrates how the method of emulsion polymerization can be practically used to obtain an advantage over other methods for preparing drug–polymer nanoparticles: Penicillin is a β-lactam antibiotic that is water soluble in nature. However, it is prone to degradation by certain bacteria (penicillin-resistant bacteria), which acquire the capacity to produce the enzyme β-lactamase. This enzyme hydrolyzes the β-lactam ring of penicillin converting the compound to an open-ring structure that is inactive. To prevent such degradation, penicillin was incorporated into the nanoparticle framework during the emulsion polymerization process. First, penicillin was acrylated with acrylamide monomer and subsequently esterified to produce an esterified acrylated product. This was dissolved in a mixture of butyl acrylate and styrene monomers, and subsequently pre-emulsified with water using SDS as an emulsifier. This mixture was heated to 70°C and stirred vigorously to form an emulsion. Potassium persulfate, a water soluble radical initiator,

was added to the mixture and stirring was continued for 6 hours. Free radical polymerization took place in the mixture to give a product comprising of 25 to 40 nm polymeric nanoparticles in which the drug was covalently bound within the nanoparticle matrix.

One major disadvantage of emulsion polymerization in conventional emulsions, that is, macroemulsions, is that there is a continuous Ostwald ripening effect taking place in the emulsion, that is, relatively larger droplets continually grow at the expense of the smaller droplets. The driving force for such Ostwald ripening is the relatively higher Laplace pressure in the smaller droplets as compared with the lower Laplace pressure in the larger droplets, which causes the smaller droplets to merge with the larger ones when they come close to each other. This eventually leads to the existence of a smaller number of large droplets and consequently to a reduction in the rate of emulsion polymerization. Therefore, the use of kinetically stable miniemulsions or nanoemulsions and thermodynamically stable microemulsions has been found to offer a great advantage for emulsion polymerization as there is almost no Ostwald ripening taking place in these systems. These systems have been extensively studied and reviewed for the preparation of nanoparticles by emulsion polymerization (Anton, Benoit, and Saulnier 2008; Antonietti and Landfester 2002; Asua 2002; Landfester, Willert, and Antonietti 2000).

3.3.3.2 Nanoemulsion Polymerization Methods

Nanoemulsions or miniemulsions are o/w emulsions wherein the particle size of the dispersed phase is in the nanometer range. Unlike the conventional emulsions that can be formed only by high energy methods, nanoemulsions, in addition to the high energy methods, can also be formed spontaneously by low energy methods as has already been described in Section 3.3. For emulsion polymerization in nanoemulsions, as in the case of conventional emulsion polymerization method, the dispersed phase consists of the pure monomer droplets (in nanometer range), stabilized by suitable high concentrations of a surfactant. The continuous phase is water to which the initiator is added either in the beginning itself or when the polymerization is to be started. As the dispersed phase droplets are extremely small and have a large surface area, most of the surfactant is adsorbed onto the surface of the dispersed phase and, hence, there is almost no surfactant remaining for the formation of micelles. Due to this reason, the main locus of polymerization in this case is the monomer droplets themselves (this differs from conventional emulsion

polymerization in which the main locus for polymerization is the monomer-swollen micelles). Polymerization starts in the monomer droplets as the initiator, and when added to the aqueous phase, enters into the monomer droplets. When the initiator is added in the beginning itself, usually the polymerization is triggered by either uv radiation or gamma radiation, or simply by increasing the temperature of the nanoemulsion, or by any other suitable means. In any case, each of the monomer droplet acts as a reaction site where polymerization reaction takes place and polymer nanoparticles are formed. As the monomer droplets are in nanometer size range, the polymer particles formed are also in nanometers, and the number of polymer particles formed is equal to the number of monomer droplets present in the nanoemulsion. Though nanoemulsions are kinetically stable, they are thermodynamically unstable, and Ostwald ripening does take place over time (these time periods are much longer than those for conventional emulsions). Besides this, as already mentioned earlier, in the case of nanoemulsions, polymerization takes place in the monomer droplets itself (in contrast to conventional emulsions in which the monomer diffuses from the monomer droplets and accumulates into the micelles present in the continuous phase); hence, in this case, the diffusion of the monomer from the monomer droplets needs to be prevented. This is done by adding a highly hydrophobic substance into the monomer droplets. The presence of the hydrophobic substance increases the osmotic pressure inside the monomer droplet, thus preventing diffusion of even small amounts of the monomer into the aqueous continuous phase. The principles of formation of nanoemulsions and techniques for formation of nanoparticles using nanoemulsions have been reviewed at length by a number of researchers (Anton, Benoit, and Saulnier 2008; Anton et al. 2007; Ee et al. 2008; Fernandez et al. 2004; Izquierdo et al. 2004; Ostertag, Weiss, and McClements 2012).

As nanoemulsions can also be either o/w or w/o, emulsion polymerization can also be done in both types of emulsions.

3.3.3.3 Inverse Nanoemulsion Polymerization Method

Inverse nanoemulsions are w/o systems in which the monomer is dissolved in the aqueous dispersed phase. A large number of monomer droplets are dispersed with the help of emulsifiers in a suitable oil phase and homogenized to give a w/o nanoemulsion. Polymerization takes place in the dispersed monomer droplets after the addition of an initiator to form polymeric nanoparticles. The mechanism of formation of polymeric nanoparticles is

similar to that of the nanoemulsion polymerization method. Here, as the external phase is the oil phase, surfactants used are usually hydrophobic nonionic surfactants such as Span 80, and partially amphiphilic block copolymers or anionic surfactants such as AOT (sodium bis-2-ethylhexyl sulfosuccinate). As the aqueous phase is the dispersed phase, this method is suitable for polymerization of hydrophilic monomers such as hydroxyethyl methacrylate, (meth)acrylamide, or methacrylic acid, and lyophobes such as NaCl, NaOH, Na_2SO_4, and $MgSO_4$ are used as osmotic agents to prevent Ostwald ripening. These lyophobes suppress molecular diffusion and maintain droplet stability during polymerization. The initiator used to initiate polymerization may be either water soluble such as potassium persulfate and ammonium persulfate, or it may be oil soluble such as benzoyl peroxide and azobisisobutyronitrile (AIBN). The free radicals may be generated either in the dispersed or continuous phase. In any case, polymerization always takes place in the dispersed phase droplets because in the case of nanoemulsions and inverse nanoemulsions, there is almost negligible diffusion of the monomer from the dispersed phase into the continuous phase. This method has been comprehensively reviewed by Capek (2010). Poly(2-hydroxyethyl methacrylate), a biocompatible polymer, has been prepared using this method by the polymerization of the moderately hydrophilic monomer hydroxyethyl methacrylate. Cyclohexane is usually used as the continuous phase and, as an initiator, the monomer soluble initiator PEGA200 or cyclohexane soluble initiator AIBN has been used (Antonietti and Landfester 2002; Landfester, Willert, and Antonietti 2000). Polymeric nanoparticles in the size range of 80 to 160 nm with a narrow size distribution have been obtained. Besides this, polymerization of a number of other hydrophilic monomers such as: acrylamide, to give poly(acrylamide) nanoparticles; N-isopropylacrylamide, to give poly(N-isopropylacrylamide); acrylic acid, to give PAA has been done by the inverse nanoemulsion method. This method has also been used for preparing poly (methylmethacrylate), poly(ethylcyanoacrylate), and poly(butylcyanoacrylate) nanoparticles in which organic solvents such as cyclohexane, n-pentane, and toluene were used as the continuous organic phase. Drugs such as triamcinolone, fluorescein, pilocarpine, and timolol have been encapsulated into these polymeric nanoparticles (Pinto Reis et al. 2006).

3.3.3.4 Emulsion Polymerization/Template Polymerization

In this method, polymerization of the monomer is made to take place in the presence of another polymer or a macromolecule (which serves as a template), such that simultaneously while polymerization is taking place,

an inter-polymer complex forms between the polymer and the template molecule. Thus, the template molecule acts as a "backbone" for the polymerizing monomer, and a desired shape of the polymeric nanoparticle can be formed, depending on the shape of the template. Such a polymerization of monomer and self-assembly between the polymer and the template in which both take place simultaneously has been used to prepare core-shell nanoparticles (Zhang et al. 2007). Nanoparticulate core-shell structures have conventionally been generated by the micellization or self-assembly of amphiphilic block or graft copolymers in their respective solvents. Different types of micellization methods have evolved; however, micellization essentially involves low concentrations of polymers (<5 mg/ml) and, hence, the efficiency of these methods is also low. The emulsion polymerization-template polymerization method is a superior alternative for the preparation of core-shell nanoparticles. Zhang et al. (2005) have used this method to prepare thermosensitive core-shell nanoparticles at a relatively high concentration in which they polymerized N-isopropylacrylamide (NIPAAm) and methylene bis acrylamide (MBA) on the surface of poly(ε-caprolactone) PCL nanoparticles at 76°C by simultaneous polymerization and self-assembly of crosslinked NIPAAm and MBA to form the shell. Instead of PCL, macromolecules could also be used as templates. When gelatin was used as a template and acrylic acid as a monomer, acrylic acid was polymerized in the aqueous gelatin solution to give biocompatible core-shell nanoparticles with a narrow size distribution, where the resultant nanoparticles had a core of insoluble PAA–gelatin interpolymer complexes and shells of soluble gelatin. Their structures were further locked by selectively crosslinking gelatin with glutaraldehyde (GLA) (Zhang et al. 2007). These PAA–gelatin nanoparticles were shown to be temperature sensitive with respect to the size of the nanoparticles: the size increased when temperature was raised above 25°C, until at 40°C an equilibrium size was attained. This size change was found to be reversible provided cooling was done to 4°C. The same core-shell nanoparticles could be made hollow by "locking" the shell structure using GLA as crosslinking agent and switching the pH of the medium from acidic to neutral (Wang et al. 2009). Hydroxyethyl cellulose (HEC) has been used as a template during polymerization of methacrylic acid, resulting in stable poly(methyl methacrylate)-hydroxyethylcellulose (PMAA-HEC) nanoparticles with a size of about 200 nm and a narrow size distribution (Zhang et al. 2010).

3.3.3.5 Interfacial Polycondensation/Polymerization

Interfacial polymerization method is used for the preparation of nanocapsules. In this method, polymerization takes place at the interface

between two immiscible liquids. In cases in which a single monomer polymerizes at the interface between two immiscible liquids, the process is termed as interfacial polymerization. On the other hand, in cases in which two different monomers separately present in two immiscible liquids come together at the interface and polymerization takes place at the interface, the process is termed as interfacial polycondensation. Interfacial polycondensation method originally consists of dissolving two different monomers, usually a diacid and a diamine respectively, in two different immiscible liquids, one of which is preferably water. Polymerization, which is usually very rapid, takes place at the interface resulting in the formation of a thin film at the interface. The mechanism of formation of the thin film has been explained in detail by Mac Ritchie (1969). Both the monomers are present as a mixed monolayer at the interface. As the reaction between the two monomers takes place at a practically two-dimensional interface and is inherently very rapid (almost instantaneous), the equilibrium, which is also established very rapidly, is almost always toward the polymer. As a result, the polymer is formed at the interface and as the interface is almost two-dimensional, the chances that a monomer contacts a polymer is much greater than the chances of it contacting another monomer. Hence, the polymer chain propagates and a high molecular weight polymer is formed, which eventually precipitates in the interface itself resulting in a thin film of the polymer. The thickness of this film is self-limiting as the formation of polymer serves as a barrier toward diffusion of more monomer into the interface. Films of thickness in the range of 10 to 100 nm are usually obtained. If an emulsion of the two immiscible liquids is formed, the dispersed phase comprises of small droplets, and polymerization at the interface results in a peripheral thin film with the dispersed phase liquid encapsulated within the polymer shell. Hence, this method has been successfully used to prepare nanocapsules with a liquid core containing the drug surrounded by a thin polymer envelop. Different polymerization reactions such as anionic polymerization and crosslinking reactions may be used. The most frequently used hydrophilic monomers are diols or diamines and the hydrophobic monomers used are di (acid chlorides) and di isocyanides. The polymers formed are polyesters, polyamides, polymethanes, or polyureas. It is essential that the polymer formed after polymerization has film-forming properties.

Interfacial polymerization method is different from emulsion polymerization in that the polymerization takes place at the interface resulting in the formation of nanocapsules, whereas in emulsion polymerization,

polymerization takes place in the continuous phase resulting in the formation of nanospheres.

Poly(alkylcyanoacrylate) nanoparticles have been prepared by this method (Pinto Reis et al. 2006). The monomer-cyanoacrylate is dissolved in a mixture of oil and absolute ethanol. This mixture is extruded slowly through a needle into an aqueous solution containing the initiator ions and a surfactant. As soon as the oil phase droplet contacts the water phase containing the initiator, polymerization takes place instantaneously at the droplet–water interface resulting in a film of polymer forming around the droplet, which eventually solidifies as the polymer precipitates inside the film. The core of the droplet remains liquid, and nanocapsules with a liquid core are formed: if the dispersed phase is oil, oil-core nanocapsules are formed; and if the dispersed phase is aqueous, water-core nanoparticles are formed. Poly(ethylcyanoacrylate), poly(isobutylcyanoacrylate), and poly(isohexylcyanoacrylate) nanocapsules have been prepared by this process.

Nanocapsules containing insulin have been prepared by interfacial polymerization of isobutyl cyanoacrylate (IBCA) in which insulin was added to the organic phase comprising of Miglyol (oil) and IBCA (monomer) dissolved in absolute ethanol. This mixture was added with stirring to an aqueous solution of Lutrol. This colloidal suspension was evaporated under vacuum to obtain ~250 nm size nanocapsules containing insulin (90 percent loading efficiency). In another method, poly(ethyl-2-cyanoacrylate) nanocapsules, encapsulating insulin, were prepared by interfacial polymerization made to take place in the water–oil interface in an w/o microemulsion (Watnasirichaikul et al. 2000). A microemulsion was first prepared by mixing the oil, a surfactant blend, and an aqueous solution of insulin at 4°C. To this microemulsion, a solution of monomer ethyl-2-cyanoacrylate in a small quantity of chloroform was added slowly, with stirring. Polymerization of the monomer at the interface resulted in the formation of nanocapsules having a diameter of about 150 nm and a polydispersity of 0.101 containing an aqueous solution of insulin at the core. The advantage of using a microemulsion here is that its formation is spontaneous and nanometer size droplets of dispersed phase are obtained without the use of high energy homogenization processes. However, separation of the nanocapsules from the oil phase may pose some problem. Examples of other drugs encapsulated by interfacial polymerization are calcitonin, octreotide, darodipine, indomethacin, and photoactivatable cytotoxic compounds used in photodynamic tumor therapy like phthalocyanines in an injectable vehicle (Pinto Reis et al. 2006). An important advantage of interfacial polymerization method is that high encapsulation

efficiency can be obtained (e.g., insulin with 95 percent). An additional advantage of this method is that as polymerization takes place in situ, the flexibility of the polymer membrane allows it to follow the contours of the inner phase of an o/w or w/o emulsion.

Dhumal, Wagh, and Suresh (2008) have modeled the kinetics and film properties of interfacial polycondensation process in an effort to understand the mechanisms underlying the reaction, phase separation, and film formation so that specific synthesis methods could be designed to suit specific applications. They incorporated, at a fundamental level, all relevant physicochemical processes involved such as: ionic equilibria in the aqueous phase, interfacial reaction, mass transfer resistances involved therein, thermodynamics of phase separation, diffusion through the polymeric film, and formation of a coherent film. This model was tested for experimental data on polyurea microcapsules prepared by this method. Bouchemal et al. (2006) have prepared and optimized the process for preparation of oil core nanocapsules with the oil core comprising of α-tocopherol. Polymeric nanoparticles have also been prepared by the interfacial polycondensation of the lipophilic monomer, such as phthaloyldichloride and the hydrophilic monomer, diethylenetriamine, in the presence and absence of the surfactant. The nanoparticles formed in presence of surfactant Pluronic F 68 were a mix of 150 and 350 nm and those formed without Pluronic F68 were larger in size, around 450 nm (Montasser, Fessi, and Coleman 2002). A modified interfacial polycondensation method was also developed. In this case, polyurethane polymer and poly (ether urethane) copolymers were chosen and successfully applied as drug carriers for a-tocopherol (Bouchemal et al. 2004). Polyurethane and poly(ether urethane) based nanocapsules were synthesized by interfacial reaction between two monomers.

Lambert et al. used the interfacial polymerization method for preparing nanocapsules with an aqueous core for encapsulating oligonucleotides. Encapsulation was done by polymerizing IBCA in a w/o emulsion. Nanocapsules obtained were in the range of 350±100 nm (Lambert et al. 2001). Charcosset and Fessi used a membrane contactor for preparing nanocapsules by interfacial polymerization and studied the effect of process parameters such as pore size of the membrane, flow rate of the aqueous phase, and organic phase pressure on nanoparticle size (Charcosset and Fessi 2006). Torini, Argillier, and Zydowicz (2005) prepared polyurethane oil-core nanocapsules with a mean diameter of 200 nm from o/w miniemulsions. They optimized the process with respect to the concentration of surfactant and choice of surfactant (cationic, anionic, and nonionic). The nanoparticles formed showed a fairly narrow size distribution and were spherical with a smooth external wall. The process was further

used for the incorporation of the drug ibuprofen (Gaudin and Sintes-Zydowicz 2008).

3.4 METHODS BASED ON COMPLEXATION BY ELECTROSTATIC/IONIC INTERACTIONS

These methods involve formation of nanoparticles by ionic or electrostatic interactions taking place between either two oppositely charged polymers (polyelectrolyte complex [PEC]) or a charged polymer and an oppositely charged molecule (crosslinked complex). Figure 3.11 describes these two types of interactions, which result in the formation of nanoparticles.

The interacting polymers could themselves be therapeutically active (e.g., oligonucleotides and plasmid DNA) or the drug could be inluded into the complexing polymers during the complexation interaction. pH sensitive dissolution properties can be obtained by proper selection of the polymers. These methods are simple one-step processes, which take place at room temperature, and are aqueous based processes, which do not involve any harmful organic solvents.

3.4.1 METHODS BASED ON FORMATION OF POLYELECTROLYTE COMPLEX

These methods involve the formation of complexes between two oppositely charged polymers.

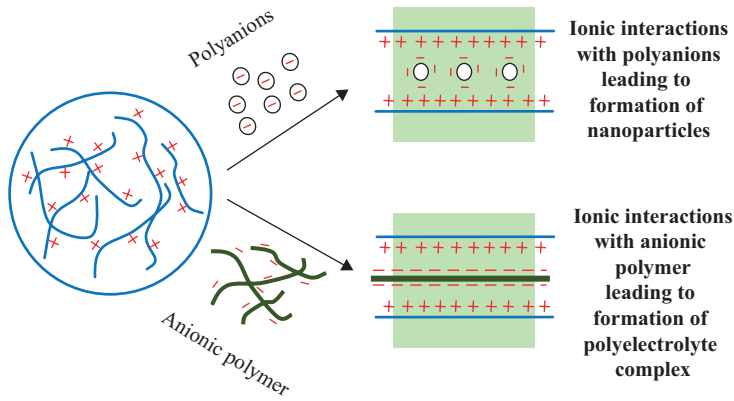

Figure 3.11. Diagrammatic representation of formation of polymeric nanoparticles and PEC by an electrostatic/ionic interaction.

Here, usually, stoichiometric proportions, accompanied by charge compensation of the interacting polymers, are required. Nanoparticles as small as 60 nm were prepared using chitosan as a cationic macromolecule and methacrylic acid as the anionic polymer. The ionotropic gelation method using small molecules with an opposite charge for crosslinking usually produces nanoparticles in the range of 175 to 600 nm (de Moura, Aouada, and Mattoso 2008). Electrically neutral nanoparticles can be obtained if sufficiently high dilutions of polymers are used. However, because of the hydrophobic nature of most polymers, the primarily electrically neutral nanoparticles tend to agglomerate to micron sized particles (Fisher and Glatz 1988).

Chitosan, a natural biocompatible and biodegradable cationic polymer, has been used to form a PEC with a variety of natural and synthetic polymers bearing negatively charged polyions. PECs of chitosan with natural polymers such as alginate, carrageenan, pectin, xanthan gum, hyaluronic acid, gum kondagogu, gelatin, γ-poly(glutamic acid), and maleic-starch half-ester acid and with synthetic polymers such as PAA, polymethacrylate copolymer (Eudragit), and poly alkylenoxide-maleic acid have been reviewed by Hamman (2010) with respect to their preparation, properties, and applications in drug delivery. Nanoparticles containing anticancer drug 5-Fluorouracil in the size range of 85 to 300 nm have been prepared by the formation of PEC between chitosan and poly(aspartic acid) sodium salt (PAsp) (Zheng et al. 2007). The effect of different charge mixing ratios n^+ to n^- for the range of 0.08 to 19.2 on the properties of chitosan–heparin and chitosan–hyaluronan PEC has been determined (Boddohi et al. 2009).

Chitosan–DNA complexes have been prepared by an interaction between cationic polymer chitosan and a negatively charged DNA molecule (Mansouri et al. 2004). In these complexes, the DNA is protected from enzymatic degradation. Besides this, these complexes have a higher transfection efficiency and are useful as effective nonviral vectors for gene therapy. A wide variety of charge bearing polymers can be selected to manufacture composite nanoparticles with varying physicochemical properties (Akiyoshi et al. 1998; Du et al. 2005; General, Rudloff, and Thunemann 2002; Janes, Calvo, and Alonso 2001a; Sang Yoo and Gwan Park 2004; Thunemann and General 2001). Zheng et al. prepared nanoparticles in the size range of 85 to 300 nm based on interactions between chitosan and PAsp. They investigated the physicochemical properties of these complexes formed. They showed that the morphological structure of these nanoparticles could be manipulated and controlled by varying key processing conditions like chitosan to PAsp unit molar ratio, solution pH,

incubation temperature, and ionic strength. Addition of crosslinking agent GLA was found to decrease the size of nanoparticles. This could possibly be due to the tight stacking of the chains. The long-term stability of the nanoparticles also increased from one month in the absence of GLA to six months in the presence of the crosslinking agent (Zheng et al. 2007).

3.4.2 METHODS BASED ON IONIC CROSSLINKING (IONOTROPIC GELATION)

This method was first proposed by Calvo et al. for the preparation of nanoparticles from hydrophilic polymers for the delivery of proteins (Calvo et al. 1997b). They prepared nanoparticles (200–1000 nm) of chitosan and diblock copolymer of polyethylene oxide-polypropylene oxide (CS/PEO-PPO) using sodium tri polyphosphate (TPP) as the crosslinking agent. They showed that as high as 80 percent protein loading was possible using this simple, completely hydrophilic technique based on the mixing of an aqueous solution of cationic polymers with an aqueous solution of TPP at room temperature. Since then, this method has become the most commonly used method for the preparation of chitosan

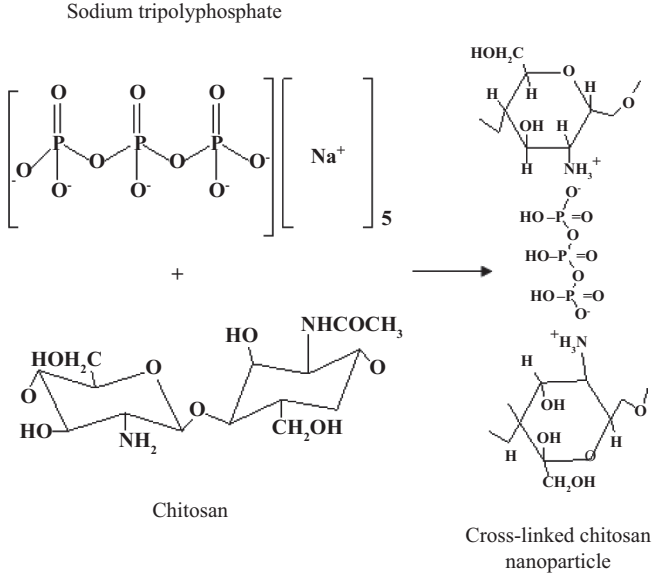

Figure 3.12. Ionic crosslinking of natural polymer chitosan using a small ion, TPP.

nanoparticles. It is based on the fact that charged polysaccharides like chitosan and alginates gel in the presence of small ions of an opposite charge like TPP (Figure 3.12).

This method has been extensively used for the preparation of chitosan nanoparticles incorporating a variety of agents such as dorzolamide and pramiprazole (Papadimitriou et al. 2008), insulin (Pan et al. 2002), doxorubicin (Janes et al. 2001b), levofloxacin (Guan et al. 2011), and a host of other agents. Gan et al. investigated chitosan–TPP nanoparticles for delivering gene or protein molecules. They demonstrated that these nanoparticles can be easily manipulated so far as their size, surface charge, surface morphology, and so on are concerned by simply varying key processing conditions like concentration of polymers, polymer to TPP ratio, and pH of solution (Gan et al. 2005). The effect of input of mechanical energy during crosslinking of chitosan with TPP and the effect of temperature (45°C) on the size and polydispersity of the nanoparticles has been studied by Tsai, Bai, and Chen (2008). Other polyanions such as citrate and sulfate have also been used as crosslinking agents. Trisodium citrate has been found to form compact globular nanoparticles of chitosan (Jin, Feng, and Yu 2011).

Koukaras et al. have investigated the molecular interactions taking place during ionic crosslinking of chitosan with TPP polyanion. They proposed that at close range, proton transfer takes place with maximum interaction energies ranging from 12.3 to 68.3 kcal/mol. The magnitude of this interaction energy depends on the protonation of the TPP and the relative coordination of chitosan with TPP. They also introduced the β-ratio that takes into account the structural details of oligomers involved in the interaction (Koukaras et al. 2012). Zhao et al. prepared folate-conjugated chitosan nanoparticles loaded with anticancer drug 10-hydroxycamptothecin in which they first prepared nanoparticles of the drug by the supercritical antisolvent (SAS) method, and subsequently loaded these nanoparticles into folate conjugated chitosan nanoparticles prepared by the ionotropic gelation method. They studied the effect of four factors: concentration of folate-conjugated chitosan, TPP concentration, drug concentration, and time of crosslinking on two response variables—mean particle size and drug entrapment efficiency. They obtained nanoparticles in the size range of 201.5 to 821.8 nm and drug entrapment efficiency in the range 77.1 to 98.7 percent. Using the response surface methodology, they could optimize the process parameters to obtain nanoparticles of desired size and entrapment efficiency (Zhao et al. 2012).

Nanoparticulate complexes with a core comprising of a stoichiometric complex of poly(amino acid) and dodecanoic acid and a shell formed

by the un-complexed poly(amino acid), in size range of 120 to 200 nm for the inclusion of hydrophobic drugs have been prepared by complexation between poly(amino acids) and dodecanoic acid (General and Thunemann 2001). Separate solutions of poly(amino acid) and dodecanoic acid in water were prepared. The pH of the dodecanoic acid solution was adjusted to 9.5 using a solution of sodium hydroxide in water. The dodecanoic acid solution was then added slowly dropwise to the poly(amino acid) solution (the exact quantities were calculated on the basis of stoichiometries with respect to charge). Nanoparticles were formed immediately with the mixture turning opaque. This suspension was stirred for 1 hour at room temperature (25°C), and filtered using an 800 nm membrane filter after which the pH was adjusted to 6.5 with dilute hydrochloric acid.

3.5 NANOPARTICLES PRODUCED BY DESOLVATION OF MACROMOLECULES

Similar to the "salting-out" method for the preparation of nanoparticles, this method involves the desolvation of macromolecules based on charge and or pH changes or both, or by addition of desolvating agents such as a nonsolvent for the macromolecule or concentrated inorganic salt solutions. This method is applicable at room temperature and is therefore suitable as heat sensitive drugs are used. Ibrahim et al. prepared cellulose acetate phthalate (CAP) nanodispersions in the form of redispersible powder by the desolvation method based on the salting out between two miscible solvents. CAP was dissolved in acetone and appropriate salts were selected and added to the acetone water mixture that caused the salting out of acetone from water resulting in a nanodispersion of CAP in water. The nanodispersion so formed was dried by lyophilization to give dry powder of CAP nanoparticles (Ibrahim et al. 1992). Nanoparticles were prepared using the process of reversible swelling of macromolecules using gelatin, human serum albumin (HSA), BSA, and casein as the macromolecular materials (Marty 1977; Marty, Oppenheim, and Speiser 1978; Weber et al. 2000). Langer et al. prepared HSA nanoparticles by the desolvation of HSA from an aqueous solution, at room temperature, by controlled addition of desolvating agent alcohol. The desolvated HSA was stabilized in the form of nanoparticles by the addition of crosslinking agent GLA. They demonstrated that the particle size of the HSA nanoparticles and the particle size distribution depend on factors such as the amount and rate of addition of the desolvating agent, pH, ionic composition, and concentration of HSA. Nanoparticles with a narrow size distribution in the range of

100 to 300 nm were obtained when the pH of the aqueous solution of HSA was between 7 and 9. A higher pH gave smaller size nanoparticles (Langer et al. 2003). This process offers the advantage of producing nanoparticles directly in aqueous suspension, but the use of potentially toxic compounds such as GLA and desolvating agents requires subsequent purification (Couvreur, Dubernet, and Puisieux 1995). Paclitaxel nanoparticles in the size range of 600 to 1,000 nm were prepared by this method. The size of the nanoparticles was found to be a function of the molecular weight of the polymer (Lu et al. 2004). Variations in nanoparticle production by the desolvation process were described (El-Samaligy and Rohdewald 1983), but unfortunately the yield is comparatively low. Coester et al. used a two-step desolvation process for the preparation of stable nanoparticles with an average size of 60 nm. The first step uses acetone as the desolvating agent. In this step, low molecular weight gelatin nanoparticles (present in the supernant) are removed. In the second step, the sediment from the first step is taken and redissolved in water and, after adjusting the pH to 2.5, desolvation was repeated followed by crosslinking of the desolvated particles (Coester et al. 2000). This two-step desolvation process was used by a number of researchers to prepare gelatin nanoparticles for use as a carrier for DNA and RNA oligonucleotides (Zillies and Coester 2004; Zwiorek et al. 2004).

3.6 TECHNIQUES BASED ON SUPERCRITICAL OR COMPRESSED FLUIDS

The physical state of any substance can be represented by a phase diagram, which relates the temperature and pressure required to get a particular physical state of the substance. Figure 3.13 represents a typical phase diagram showing the relation of temperature and pressure with the physical state of a substance. As seen in the phase diagram, there is a point called the critical point that defines the pressure and temperature limits up to which any substance can exist in a vapor–liquid equilibrium. Beyond the critical point, it attains a state that can be either liquid or gaseous depending on the initial coordinates of the system.

Supercritical fluids are substances above their critical temperature (T_c) and critical pressure (P_c), as shown in Figure 3.13. Under these conditions, the substance acquires certain unique properties that are not seen under subcritical conditions: (i) The viscosity of a supercritical fluid is low (comparable to a gas), but at the same time, its density is high (comparable to a liquid); (ii) The substance, above its critical point, cannot be liquefied

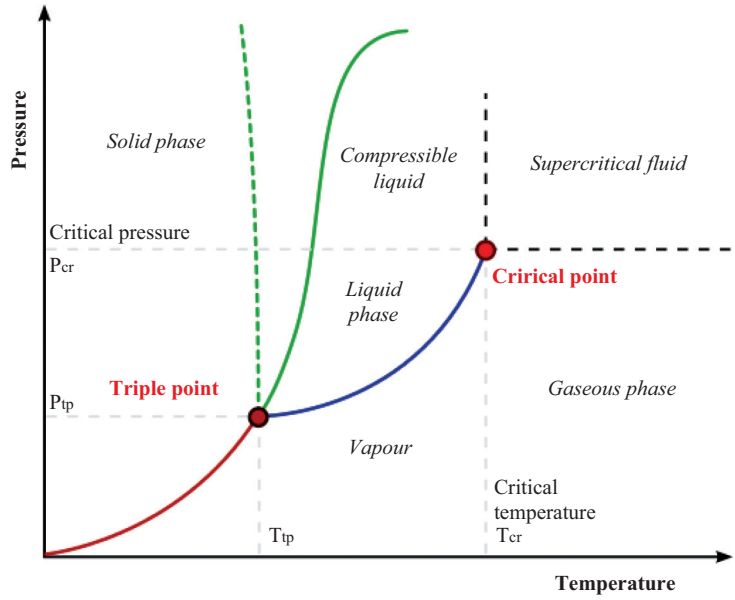

Figure 3.13. A typical phase diagram for a substance showing the relation of temperature and pressure with the physical state of the substance.

Source: http://commons.wikimedia.org/wiki/File:Phase-diag2.svg#/media/File:Phase-diag2.svg (n.d.)

no matter how much pressure is applied; (iii) Supercritical fluids have no surface tension because they do not exhibit any vapor–liquid boundary; (iv) Due to absence of surface tension, a material shows a higher solubility in supercritical fluid compared with a conventional one; (v) Diffusivities of supercritical fluids are much larger compared with the conventional fluids and, hence, mass transfer rates are higher; and (vi) All supercritical fluids are miscible with each other.

These unique properties of supercritical fluids have been appropriately exploited in order to solubilize and subsequently precipitate or crystallize a variety of solutes in the form of micro- or nanoparticles without the need for using toxic organic solvents as in conventional methods of preparing nanoparticles. Fundamentally, two methods have been used for precipitating solutes using supercritical fluids: one method (called rapid expansion of supercritical solution) involves solubilization of the solute in the supercritical fluid, followed by a rapid expansion of the solution through a suitable nozzle, resulting in precipitation of the solute; the second method (called the supercritical antisolvent method) is suitable for

those substances that are not soluble in the supercritical fluid and involves dissolving the solute in a suitable solvent and adding the resulting solution to the supercritical fluid, which is an antisolvent for the solute but not for the solvent in which the substance is dissolved. In the resulting liquid mixture, the supercritical fluid diffuses very rapidly into the solvent and results in rapid supersaturation of the solution followed by precipitation of the solute. As the diffusivities of the supercritical fluids are very high, rapid supersaturation occurs, resulting in nucleation and spinodal decomposition, without crystal growth taking place. Due to this, the particles formed are small (in the nano- or micrometer range) and uniform in size. Carbon dioxide (CO_2) and water (H_2O) are the most commonly used supercritical fluids where the T_c and P_c for CO_2 are 304.1 K and 72.8 atm, respectively, whereas for water, these values are 647.1 K and 217.7 atm, respectively. Supercritical CO_2 has solvation properties comparable to fluorocarbons as well as hydrocarbons; however, for dissolving polar and ionic substances, the use of surfactants or cosolvents or both to form microemulsions is required and formation of microemulsions is usually preferred. Some other solvents used in the supercritical fluid state are propane, acetone, nitrous oxide, trifluoromethane, chlorodifluoromethane, diethylether, fluoroform (CHF_3), and a combination of CO_2 and ethanol.

A number of variants of the two methods mentioned earlier have evolved depending on the properties of solute and solvent, characteristics of the product required, and possible processing conditions. These include: static supercritical fluid process, particles from gas-saturated solutions, precipitation from compressed antisolvent, aerosol solvent extraction system, SEDS, SAS process with enhanced mass transfer, depressurization of an expanded liquid organic solution, supercritical assisted atomization, hydrothermal synthesis under supercritical conditions via flow reactor, hydrothermal synthesis under supercritical conditions via batch reactor, supercritical fluids drying, and supercritical fluid extraction emulsions. All these have been comprehensively reviewed for laboratory as well as manufacturing scale by a number of researchers (Byrappa, Ohara, and Adschiri 2008; Jung and Perrut 2001; Vemavarapu et al. 2005). A large number of pharmaceutical products and substances suitable for biomedical applications, with particle size varying from the nanometer to micrometer scale, have been prepared by these methods (Tservistas et al. 2001). These methods have also been adapted for encapsulation or coating of pharmaceutically and biologically active materials (Wang, Dave, and Pfeffer 2004b). Budesonide has been encapsulated into poly(L-lactic acid) using this technique (Chattopadhyay, Huff, and Shekunov 2006). Protein drugs such as insulin have been encapsulated in PEG/PLA nanoparticles by this

technique (Elvassore, Bertucco, and Caliceti 2001). Polymeric nanoparticles have also been prepared by these techniques (Feng and Chien 2003). Besides these, inorganic particles including magnetic materials, carbon nanotubes, fullerenes, quantum dots, gold nanoshells, and nanocomposites like peptide/hydroxyapatite, which are useful in biomedical applications such as in vivo sensing and imaging and drug delivery, have also been successfully prepared by this technique (Byrappa, Ohara, and Adschiri 2008).

Techniques based on the use of supercritical fluids for the formation of nanoparticles are gaining popularity because of their exceptional advantages over the conventional methods in that these methods do not use toxic organic solvents due to which removal of residual solvent is not required (supercritical fluid based methods produce nanoparticles with solvent levels below 25 ppm). The particles formed by these methods do not need any further processing for solvent removal or separation. High drug loading is possible, morphology of particles can be controlled, and drug degradation and protein denaturation are prevented (Caliceti et al. 2004). In spite of all these advantages, the high initial capital investment required for equipment, costs involved in generating and maintaining the elevated operating pressures and equipment, and cost of recycling the compressed supercritical fluids for economic considerations serve as major impediments in the popularity of these techniques.

3.6.1 HYDROTHERMAL METHODS

Hydrothermal methods used for the synthesis of nanoparticles are those methods in which heterogenous chemical reactions are made to take place in solvents, in a closed system, under moderate to high temperatures and pressures (in which water is used as a solvent, the method is termed as hydrothermal, whereas for solvents other than water, it is known as solvothermal). The temperatures may range from 100°C to 1,000°C and the pressures may range from 1 to 1,000 atm. Thus, processing conditions above the critical point of water may also be used. The advantages of using supercritical fluids have been explained in the previous section. In fact, methods based on the use of supercritical fluids can be said to be a special case of solvothermal methods for the synthesis of nanoparticles.

There are numerous advantages of using high temperature and pressure: (i) the dielectric constant for water decreases to around 10 under supercritical conditions (the value is ~80 at 20°C): this means that water, which is a polar solvent at room temperature, acquires increasingly nonpolar character (similar to organic solvents) at very high temperatures and

pressures and can dissolve substances that usually require toxic organic solvents for dissolution; (ii) as the density, surface tension, and viscosity are lower near the critical point, the solvent properties and diffusivities are considerably enhanced; (iii) vapor pressure and ion-product are higher. These changed properties result in accelerated reaction rates (especially where diffusion is a rate limiting factor) and hydrolysis reactions are intensified. High pressure hydrothermal methods are able to hydrolyze and dehydrate metal salts, and the very low solubility of the resulting metal oxides in water generates rapid supersaturation resulting in the formation of nanoparticles (Eckert, Knutson, and Debenedetti 1996; Hao and Teja 2003). These processes yield very fine crystals as the low solubility of metal hydroxides and oxides enable very high supersaturation levels (Cabanas et al. 2001; Hao and Teja 2003; Lam et al. 2008; Sue et al. 2006). Besides this, as these processes are carried out around the critical point, small changes in temperature and pressure can produce large changes in physical properties such as density and viscosity, which enables a fine control over the nucleation rates and particle size (Burda et al. 2005; Shaw et al. 1991). The process is environment friendly since it does not involve any organic solvents and does not require additional treatments such as calcination (Sue, Kimura, and Arai 2004). Due to these advantages, high pressure hydrothermal processes have been widely investigated for the synthesis of metal oxides as powders, nanoparticles, and single crystals (Adschiri, Hakuta, and Arai 2000; Adschiri et al. 2001; Cabanas et al. 2001; Cote et al. 2002, 2003; Dou et al. 2007; Eckert, Knutson, and Debenedetti 1996; Giri et al. 2005; Hao and Teja 2003; Lian et al. 2004; Sorescu, Diamandescu, and Tarabasanu-Mihaila. 2004; Sue et al. 1999; Sue et al. 2006; Wang et al. 2004a; Yoshimura and Somiya 1999).

As the hydrothermal methods use high temperatures and pressures, they are suitable for the synthesis of metallic nanoparticles. These methods have also been scaled-up to suit continuous synthesis process. Cabanas et al. have used a flow reactor to synthesize a number of metallic nanoparticles including magnetic spinel type oxides such as magnetite by hydrolysis and simultaneous oxidation of the respective metal acetates in near-critical and supercritical water at temperatures of 200°C to 400°C and a pressure of 25 MPa (250 atm). They reported the formation of highly crystalline nanoparticles with a bimodal particle size distribution, with an average particle size of ~10 and ~100 nm (Cabanas et al. 2001; Cabanas and Poliakoff 2001). Another continuous hydrothermal process reported for the synthesis of α-Fe_2O_3 and Co_3O_4 nanoparticles involved a precipitation reaction between the metal salt solution and hydroxide solution under ambient temperature and at an elevated temperature. Though the

average size of particles under both conditions was the same, the elevated temperature conditions yielded more uniform particles. A similar process for the synthesis of $CoFe_2O_4$ by reacting solutions of ferric nitrate and cobalt nitrate with sodium hydroxide solution gave a product with fewer impurities under cold mixing conditions compared with hot mixing conditions. Moreover, the uniformity of the particles was also more for the cold mixing conditions (Cote et al. 2002, 2003). Hematite and magnetite nanoparticles of ~50 nm were prepared without using a strong base such as sodium hydroxide. For the preparation of magnetite nanoparticles, ferric ammonium citrate was used as a precursor. Fe(III) was reduced to Fe(II) by carbon monoxide that was produced by the thermal decomposition of ammonium citrate. The carbon monoxide gas, being miscible with supercritical water, provided a uniform reducing atmosphere throughout the reactor. Ferric ammonium sulfate, ferric nitrate, ferric sulfate, and ferrous chloride were used for the synthesis of hematite nanoparticles (Adschiri, Kanazawa, and Arai 1992; Tadafumi and Kunio 2002; Teja and Koh 2009). The effect of precursor concentration, temperature, and residence time on particle size and morphology of particles produced by the continuous hydrothermal synthesis process has been critically studied (Hao and Teja 2003). Increase in both residence time and precursor concentration has been found to increase the average particle size as well as particle size distribution of the nanoparticles formed. However, the residence time was seen to have a greater effect than the precursor concentration. Monodisperse particles were produced at short residence times.

The continuous hydrothermal method has also been applied to the synthesis of PVA-coated iron oxide nanoparticles. PVA promoted the formation of uniform nanoparticles with a narrow size distribution. The average particle size was found to be inversely proportional to PVA concentration at residence times of the order of 2 seconds and became nearly independent of PVA concentrations at residence times of 10 seconds or higher (Xu, Lee, and Teja 2008; Xu and Teja 2008).

Lester et al. used a special nozzle mixer to improve the mixing between the precursor solution and supercritical water so that reaction time is reduced, and the shorter residence time consequently limits particle growth to give a crop of small uniform particles. This special nozzle mixer is in the form of a vertical, narrow metal tube with provisions for heating around the center of the tube. The supercritical water is introduced from the top of the nozzle mixer (to a point at the middle of the narrow tube), whereas the metal precursor solution is introduced from the bottom. The mixing is almost instantaneous as this arrangement exploits the difference in densities between the two fluids and makes use of “buoyancy

induced eddies" to produce ''ideal'' mixing conditions. The outlet for the reacted mixture is provided near the top of the mixer (at right angle to the main tube). This arrangement leads to very efficient mixing and a very low residence time that limits the size of the particles formed. Many metal oxides in the size range of 6 to 64 nm were synthesized in this nozzle mixer (Lester et al. 2006).

The major drawback of the continuous hydrothermal process is that surface modifications of nanoparticles cannot be done in situ and require postsynthesis treatment. Besides this, as in the case of the methods based on supercritical fluids, another major disadvantage of this method is that a high initial investment in equipment is required and subsequent recurring maintenance costs are involved. Additionally, the high temperatures and pressures used raise serious safety issues, which become all the more important as the progress of the reaction cannot be observed.

3.7 SONOCHEMICAL METHODS

These methods for synthesis of nanoparticles are becoming increasingly popular and involve chemical reactions made to take place in the presence of powerful ultrasonic radiation in the range of 20 kHz to 10 MHz. Ultrasound energy of this magnitude is capable of breaking chemical bonds thereby enabling reactants to react with each other to form the product. It has been proposed (Gedanken 2004) that when reactants dissolved in a suitable medium are subjected to such strong ultrasonic frequency, the formation, growth, and collapse of bubbles—acoustic cavitation—takes place in the medium. The bubbles collapse within nanoseconds, producing localized "hot-spots" where temperatures to the tune of 5,000 to 25,000 K are reached. Such high temperatures are capable of breaking very strong chemical bonds in reactants causing even difficult reactions to take place very rapidly. Subsequent to the collapse of bubbles, the cooling rates are also very high—around 10^{11} K/s. Due to such high cooling rates, very high supersaturation is obtained, resulting in almost an instantaneous formation of nuclei. In the case of volatile substances, the reaction resulting in the formation of product takes place within the bubbles. The exceptionally high cooling rate hinders organization of the molecules and hence results in an amorphous product. On the other hand, in the case of nonvolatile substances, the reaction occurs in a 200 nm ring surrounding the collapsing bubble (i.e., in the liquid phase). Hence, whether the product obtained is amorphous or crystalline depends on the temperature in the ring region (around 2,200K), which is lower than the temperature within the bubble

but much higher than that in the bulk. Irrespective of the nature of the product (crystalline or amorphous), the product obtained is always in the nanometer range. It has been shown that these methods can be used for simultaneous formation and deposition of nanoparticles on substrates to give a uniform, thin nanoparticulate coating on a variety of substrates (Gedanken 2004). This method has a wide range of applications from the synthesis of inorganic metallic nanoparticles, modification of polymeric materials, and polymeric surfaces to biomedical applications including preparation of protein microspheres (Ashokkumar and Grieser 1999; Suslick and Price 1999). The use of this method for the preparation of zinc oxide nanoparticles has been reviewed by Hu, Zhu, and Wang (2004). It is based on acoustic cavitation resulting from the continuous formation, growth, and implosive collapse of bubbles in a liquid. Crystalline nanoporous ZnO spheres and ZnO–PVAL composite have been synthesized by ultrasonic irradiation. Sonochemical synthesis of spherical ZnO nanocrystals has also been reported.

3.8 MICROWAVE ASSISTED METHODS

Microwave based heating for synthesis of nanomaterials is a very rapidly developing research area due to the advantages it offers over conventional heating methods: it makes possible rapid and volumetric heating (i.e., simultaneous and uniform heating over large volumes of liquid); it enables higher reaction rates; it offers higher reaction selectivity and higher product yields; and it permits significant energy saving. Due to rapid heating, especially in the case of aqueous systems, almost simultaneous precipitation takes place, resulting in very small particles with a uniform size distribution. Ag and Au nanoparticles have been prepared by the reduction of Ag^+ ions (from $AgClO_4$ solution) and $AuCl_4^-$ ions (from $HAuCl_4$ solution) with N,N-dimethyl formamide, and stabilized by complexation with PVP. Pastoriza-Santos and Liz-Marzán performed the reduction using both methods: the conventional method of carrying out reduction by refluxing and microwave assisted reduction methods in the presence of PVP. They reported that the conventional method of reduction by refluxing produced nanoparticles with a high degree of polydispersity whereas the microwave method yielded smaller and nearly monodisperse nanoparticles (in the range of 3–4.5 nm, with only about 3 percent of particles in the range of 20–30 nm) (Pastoriza-Santos and Liz-Marzan 2002). Finely divided metal powders (in the micrometer and submicrometer range) of easily reducible metals such as precious metals and Cu and less reducible metals such

as Co, Ni, Cd, and Pb have been prepared conventionally by the polyol process through the precipitation reaction carried out in liquid polyalcohols (Fievet, Lagier, and Figlarz 1989). This process has been modified to prepare metal nanoparticles with a very narrow size distribution by microwaving mixtures of the metal salt and polyalcohols and is now referred to as the microwave-polyol process. Pt nanoparticles in the size range of 2 to 4 nm prepared by microwaving a mixture of poly (N-vinyl-2-pyrrolidone) and H_2PtCl_6, ethylene glycol and NaOH with 2,450 MHz microwaves for 30 seconds have been prepared by the microwave-polyol process (Yu, Tu, and Liu 1999). In a similar manner, PVP stabilized Ni nanoparticles (~6 nm) have been prepared in ethylene glycol (Tsuji, Hashimoto, and Tsuji 2002). The method was further adapted for synthesis of Pt nanoparticles using a continuous process to yield nanoparticles ~1.5 nm (Tu and Liu 2000). CdSe, PbSe, and $Cu_{2-x}Se$ nanoparticles have been prepared by microwave refluxing aqueous solutions of suitable salts of these metals in the presence of an amine based stabilizer. The process yielded CdSe nanoparticles of 4 to 5 nm whereas the other materials formed aggregates (Zhu et al. 2000).

3.9 SEPARATION OF NANOPARTICLES

Nanoparticles prepared by any of the aforesaid methods need to be separated from the medium in which they are prepared for the purpose of characterization and further use. The usual filtration methods are not successful in separating nanoparticles because the pore size of the usual filter media is too large to retain them. Besides this, the RPM of usual centrifuges also is insufficient to cause sedimentation of nanoparticles. Therefore, the routine separation methods need to be modified depending on the size of the nanoparticles. This section describes some of the methods used by researchers to separate the nanoparticles synthesized by various methods.

3.9.1 ULTRACENTRIFUGATION FOLLOWED BY LYOPHILIZATION

This is one of the most commonly used methods for separation of nanoparticles. The nanoparticles prepared by any of the wet chemical methods are centrifuged in an ultracentrifuge at RPMs ranging from 21,000 × g to as high as 145,000 × g for an appropriate time period, and the supernant is removed by decanting. The precipitates are washed with water a number

of times by repeated centrifugation followed by removal of supernant to free the nanoparticles of any residual solvents or reactants. The wet nanoparticles are then freeze dried to remove all traces of water to obtain dry powder of the nanoparticles.

3.9.2 MAGNETIC SEPARATION METHODS

These methods are specific for metallic (magnetically active) nanoparticles especially iron oxide nanoparticles. The container containing the nanoparticulate dispersion is placed on a magnet (the intensity of the magnet used could be changed depending on the type of material and size of nanoparticles). The supernant is discarded and the nanoparticles are washed repeatedly with water. Finally, the residual water is removed by drying or evaporation.

3.9.3 MEMBRANE FILTRATION

Membrane filtration unit equipped with cellulose membrane having a suitable pore size is used to either retain nanoparticles or remove particles larger than the desired size. In the latter case, the filtrate containing the desired size nanoparticles is subjected to ultracentrifugation and freeze drying to obtain a dry powder of the nanoparticles.

3.9.4 CALCINATION

This method is suitable for metallic and other inorganic nanoparticles. The precipitates formed by methods such as co-precipitation are collected by simple filtration methods. These precipitates are then subjected to calcination (heating in a muffle furnace) at high temperatures (usually >350°C) for prolonged periods of time (more than 2 to 3 hours).

CHAPTER 4

CHARACTERIZATION OF NANOPARTICLES

Nanoparticles exhibit properties that are significantly different from the bulk material. This is due to the fact that the ratio of the number of molecules at the surface to those in the interior is much higher for nanoparticles compared to the bulk material of the same substance. The major influence of this factor is seen on some of the important physical and chemical properties of nanomaterials. In addition to this, as surface energy increases with decrease in size of particles, nanomaterials have a melting point or a phase transition temperature that is lower compared to the bulk material. The increased surface energy makes nanoparticles highly reactive. Other properties such as mechanical, electrical, optical, and magnetic properties of nanostructures also vary from those of the bulk material. The mechanical strength of nanomaterials is higher (theoretically, one to two orders of magnitude higher than that of single crystals in bulk form). The optical absorption peaks of semiconductor nanomaterials are seen to shift to lower wavelengths due to an increased band gap in these materials. The color of nanoparticles changes as a function of size, especially in the case of metallic nanoparticles. Some nanomaterials show decreased electrical conductivities compared to their bulk counterparts due to increased surface scattering; however, electrical conductivity may also be enhanced in some cases due to a better ordering of molecules or atoms. Magnetic nanomaterials exhibit superparamagnetism rather than ferromagnetism seen in the bulk material. The differences in properties that arise due to particles going into the nanoscale domain have been discussed in some detail by Cao (2004). These differences in material properties that emerge with the material entering the nanoscale domain make postsynthesis characterization of nanomaterials very important. Thus, the nanoparticles synthesized by either of the methods described in Chapter 3

need to be properly characterized for their physicochemical properties. The type of characterization will depend on the specific application for which the nanoparticles will be used. The typical characterization of nanoparticles comprises: (i) actual visualization of the nanoparticles for their size, shape, and surface morphology, which is done by electron microscopy (there are now a variety of electron microscopes with a wide range of resolutions and a variety of add-on instrumentation capable of determining, recording, and analyzing different essential features of the nanoparticles) and atomic force microscopy (AFM); (ii) determination of the size and surface charge of the nanoparticles, which is done by methods such as dynamic light scattering (DLS) and zeta potential measurement; (iii) determination of the crystal structure of the nanoparticles by X-ray diffraction techniques; (iv) elemental analysis of the nanoparticles or nanostructures by methods such as energy-dispersive X-ray spectroscopy and X-ray photoelectron spectroscopy; (v) spectroscopic techniques such as Fourier transform infrared spectroscopy FTIR, Matrix assisted laser desorption/ionization-Time of flight Mass spectrometry MALDI-TOF MS, UV-visible spectroscopy, and Rutherford back-scattering spectrometry; (vi) dual polarization interferometry; and (vii) nuclear magnetic resonance techniques. Besides these, nanoparticle tracking techniques may be applied to keep track of the Brownian motion, which is characteristic of the nanoparticles because of their small size.

For nanomedicine applications, accurate particle size analysis and distribution are very important because absorption or penetration through biological membranes is very much size dependent. Hence, these methods form a very important part of the characterization of nanoparticles. Particle size determination and distribution can be done on the basis of direct visual observation of the particles under a microscope (electron microscope for nanoparticulate dimensions) or by indirect methods such as estimation of the scattering of light by the particles and relating this to the particle size. This chapter describes briefly the methods that are most commonly used to characterize nanoparticles.

4.1 METHODS BASED ON MICROSCOPY

4.1.1 ELECTRON MICROSCOPY

The fundamental principles behind the working of an electron microscope are similar to those involved in a light or optical microscope; in both, the illumination of the sample is done by a suitable source,

which after magnification by appropriate lenses, produces an image of the sample after suitable magnification. In optical microscopes, illumination is done by either white light or light of a suitable wavelength and magnification is done by glass lenses whereas in the case of electron microscopes, illumination is done by a beam of electrons (electron probe) and magnification is done by the use of electromagnetic lenses. As the wavelength of the high energy electron beam is about 100,000 times smaller than that of light, objects much smaller than those that can be seen under an optical microscope become visible under the electron microscope (IBM Research n.d.). The electron probe may be a stationary beam (parallel or convergent), incident along a fixed direction, or it may be scanned across the specimen. The former is the basis for transmission electron microscopy (TEM), while the latter forms the basis for scanning electron microscopy (SEM) (Williams and Carter 2009). In either case, in order to prevent collisions between electrons of the beam and the gas molecules, a high vacuum is required to be maintained in all functional areas of the electron microscope (at or near atmospheric pressure, the collisions between the electrons and gas would nearly stop the electron beam over a path of the order of a few millimeters). A vacuum of around 10^{-4} torr has been found to be adequate to maintain a mean free path of about 2.5 m, which is considered to be sufficient for most electron microscopes. To date, there are a number of modifications (based on the resolution, voltage applied, electron diffraction pattern, etc.) in each of these fundamental microscopes leading to a large variety of commercially available TEMs and SEMs suited for specific applications. The general instrumentation and components of the electron microscope have been described in detail by Wischnitzer (1989). Very high magnifications and resolutions are now possible with the modern electron microscopes that are commercially available today. However, the high resolutions are at the cost of narrower sampling areas. In other words, the higher the resolution, the narrower the region that can be observed. Hence, the images obtained by electron microscopy may not adequately represent the entire sample of interest. The following sections give brief descriptions of the essential components and procedures involved in electron microscopy.

4.1.1.1 Transmission Electron Microscopy

The conventional TEMs, which use a stationary beam of electrons, are based on the image formation processes in which the image is produced

due to local diffraction phenomena. Absorption contrast plays only a minor role. The major components of a TEM are:

- An illumination system
- A specimen stage
- An objective lens system
- A magnification system
- Data recording system(s)
- Chemical analysis system(s)

A simplified ray diagram showing the working principle and image formation in a TEM is given by Wang (2000).

The *illumination system* comprises a beam of electrons (usually parallel) that is made to be incident on the specimen. This beam is generated by an electron gun that uses either a thermionic emission source such as tungsten or lanthanum hexaboride (LaB_6) or a field emission source. The thermionic electron emission source generates thermionic electrons due to heating of the tungsten or LaB_6 filament, whereas the field electron emission source generates a high density, fine beam of electrons by applying high voltage (several thousand volts) to a "point source"—a tip of a metal needle with a radius <100 nm (ideally 5 to 10 nm) where electrons are extracted from the metal tip due to the high voltage applied. The field emission source gives brighter and higher resolution images than those obtained by the thermionic emission source because the electron current density in the field emission source is much higher (in the range of 10^4 to 10^6 A/cm^2) compared to the thermionic emission source's electron current density of 1 to 10 A/cm^2 (Engineering and Technology History Wiki 2010). Thus, due to the high current density and beam coherence that it provides, the field emission source can be used for lattice imaging, electron holography, and high spatial resolution microanalysis. The illumination system also includes a condenser lens, which is responsible for focusing the electron beam emerging from the electron gun onto the specimen.

The *condenser lens* focuses the electron beam emerging from the electron gun onto the sample. This lens is also electromagnetic in nature and has a focal length of a few centimeters, which is adjustable over a considerable range of values.

The *specimen stage* is the portion where the specimen is placed. It is located either between the condenser and the objective lens or within the objective lens. The specimen is mounted on a specimen holder, which consists of a grid made of copper, molybdenum, gold, or platinum (the grid is around 3 mm in diameter with an inner meshed area of around 2.5 mm

diameter and a thickness of around 100 μm). The specimen mounted on the grid is inserted into the electron microscope in such a manner that there is minimum increase in pressure in other areas of the microscope. The specimen stage is such that it can be spatially manipulated in order to get the region of interest in the sample in line with the electron beam.

The *objective lens system* is the most critical portion of the electron microscope. It forms the initial enlarged image of the illuminated portion of the specimen and determines the limit to which image resolution can be done. This initial image is enlarged further by the projector lens. Since the objective lens of an electron microscope is an electromagnetic lens, its focal length is dependent on the magnitude of the lens current, and the sharpness of the image is dependent on the stability of the lens current. An exceptionally high current stability is required for a resolution capability of around 2 Å.

The *magnification system* of a TEM consists of the projector lens, which magnifies the already enlarged image formed by the objective lens. The image plane of the objective lens serves as the object plane of the projector lens, and the intermediate image formed by the objective lens is further magnified by the projector lens to form the final magnified image that is displayed on the screen. A number of projector lenses can be used to get the desired magnification. A magnification as large as 1.5 million can be obtained in modern TEMs by a suitable assembly of objective lens and projector lenses (Wang 2000).

The *data recording system* consists of a digital charge-coupled device (CCD), which is capable of quantitative analysis of the data.

The *chemical analysis system* usually consists of an energy-dispersive X-ray spectrometer and electron energy loss spectrometer.

4.1.1.1.1 Sample Preparation for TEM

In TEM, the incident electrons from the electron beam essentially travel through the material before falling on the screen, CCD, or photographic plate to finally form the image. Hence, the sample should be "electron transparent," or in other words, the sample should be extremely thin so that the electrons are able to pass through the sample. In the case of nanoparticles, they should be sparsely distributed across the sample grid to get a clear image. This requirement becomes less strict if a higher voltage is used for electron acceleration. TEMs using voltages between 120 and 300 kV are commercially available. Some of the variants of conventional TEM include high resolution TEM (HRTEM), scanning TEM (STEM), high throughput TEM/STEM, and cryo-TEM.

4.1.1.1.2 Conventional TEM for Nanoemulsion Systems

Nanoemulsions usually require cryogenic techniques for optical characterization by electron microscopy. However, conventional TEM can be used in this case to obtain certain basic information. For this purpose, a procedure of negative staining, using staining agents such as phosphotungstic acid (or its salt solutions) or uranyl acetate, needs to be followed. A droplet of the nanoemulsion is placed onto the carbon-coated grid. Subsequently, a drop of an aqueous solution of the staining agent is applied. The sample is then left to dry and, after drying, is observed under a TEM. As the aqueous phase portion contains strongly scattering metal ions and the oil droplets comprise a weakly scattering oil component, the image is seen in reverse contrast, that is, light droplets against a darker background (Brenner and Horne 1959; Klang et al. 2012). Some caution needs to be exercised while interpreting the images obtained using such a negative staining procedure. The staining and drying procedure is likely to affect the structure and morphology of the sample. Shrinkage, collapse of the structure, dimensional modification of the structures, aggregation of the colloidal droplets, and flattening of the sample during drying are some of the phenomena that may give an image that is totally unrelated or distantly related to the original structure (Klang et al. 2012). In the cases where such distortions are likely to take place, cryo-TEM techniques become essential.

4.1.1.2 Scanning Electron Microscopy

SEM uses the same illumination source as TEM (the electron gun) for the generation of an electron beam. However, in this case, the condenser lens and objective lens together work toward focusing a fine electron beam onto the specimen (in TEM, the electron beam is first transmitted through the sample and then passed through the objective lens to form a magnified image). Here, the objective lens determines the final diameter of the electron probe. A simplified diagram showing the different components of an SEM is shown in Figure 4.1.

The fine electron probe, when it impinges onto the sample, causes excitation of the electrons present in the sample, thereby generating various types of signals—attributed to secondary electrons, back-scattered electrons, transmitted electrons, and other electrons—which are detected by suitable detectors to provide a variety of information (Figure 4.2).

Unlike TEM where the electron probe is stationary, in SEM the electron probe scans the entire surface of the sample. This is made possible

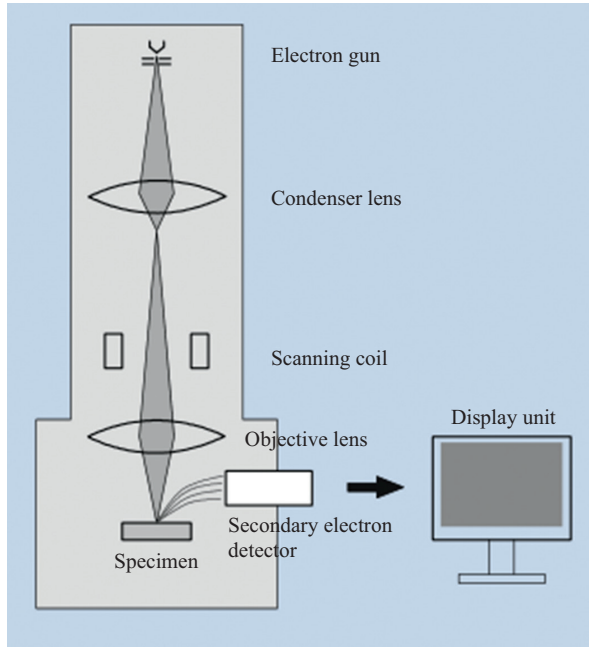

Figure 4.1. Schematic diagram showing the various components of an SEM.

Source: Reproduced with permission from JEOL Ltd. (n.d.)

by the presence of "scanning coils" in the instrument. The scanning coils deflect the electron beam so that it can scan the sample surface along the x- or y-axis. In addition to this, the specimen stage in SEM is such that it can move in x-y-z-plane (horizontal plane in the x- and y-direction and vertical plane in the z-direction), can be tilted at an angle, and can rotate along 360°. Thus, the entire surface of the sample can be scanned. The functional components of an SEM also need to be maintained under high vacuum for the same reasons as for a TEM.

4.1.1.2.1 Signal Generation in SEM

When the electron beam impinges on the specimen, it interacts with the specimen up to a certain depth beneath the surface. The depth to which it can interact depends on the nature of the material, energy of the incident electron beam, and incident angle. There are two types of interactions

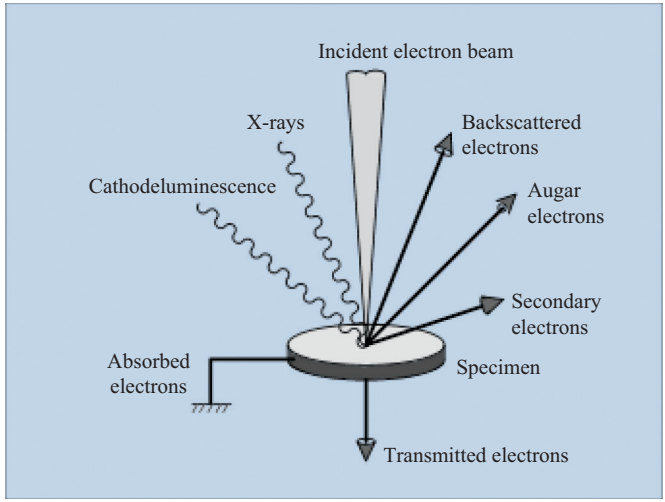

Figure 4.2. The different types of signals emitted by the sample after the electron beam impinges on the sample in an SEM.

Source: Reproduced with permission from JEOL Ltd. (n.d.)

that can occur: elastic scattering and inelastic scattering of electrons. In the elastic scattering process, the electrons retain all of their energy after interaction and travel back to the specimen surface as "back-scattered" electrons, which subsequently escape into the vacuum. On the other hand, in the inelastic scattering process, the electrons lose energy, simultaneously exciting electrons in the specimen. These low energy electrons (with energy less than around 50 eV) are the secondary electrons. Due to their low energy, only those electrons that are near the surface can escape in the surrounding vacuum, whereas those relatively deep inside the specimen cannot escape and are absorbed within the specimen. In contrast to this, the back-scattered electrons, due to their greater energy, can come from greater depths under the specimen surface. In addition to the back-scattered electrons and the secondary electrons, X-rays are emitted during the electron beam–specimen interactions. As shown in Figure 4.2, other signals that can be generated during the electron beam–specimen interactions can be attributed to auger electrons, cathodoluminescence, transmitted electrons, and specimen current. The signals due to the secondary electrons (as these are the ones that are near the surface of the specimen) are collected and suitably amplified to generate the surface information or surface image of the specimen. As these are low energy electrons, their detection is difficult. Hence, SEM uses a complex system to intensify and

multiply the signals so that they become measurable. These signals are further translated appropriately to give an image of the specimen, which is suitably enlarged for observation and recording. The mechanism and principles involved in conversion of the signals generated by the specimen and translation of these to an enlarged image of the specimen are explained in detail by Zhou et al. (2007).

4.1.1.2.2 Sample Preparation for SEM

Most nanoparticles can be observed directly by placing them on a carbon tape. However, materials that have a low atomic number give low emissions of secondary electrons and back-scattered electrons when excited by the electron probe. To increase the signal intensity, coating with a suitable metal is done. Sputter coating with metals such as Au, Ag, Au–Pa, and Pt in Argon atmosphere has been done. However, for high resolution SEM, these metals are not suitable because they tend to build up a metal film during sputtering, giving rise to uneven coatings, resulting in less sharp images. Metals such as Cr, Ti, Ta, Ir, and W form even coatings, resulting in sharper images (Zhou et al. 2007). In addition to low signal intensity, most bio-organic materials are nonconducting and often lead to "charging" phenomena, which is explained in Section 4.1.1.2.3.

4.1.1.2.3 "Charging" of Specimen

This happens mainly in nonconducting samples. Electrons from the electron probe when they impinge on the sample lose some of their energy. If the sample is conducting, these electrons flow through the specimen, whereas if the sample is nonconducting, they are absorbed in the sample, and continued irradiation with the electron probe leads to an accumulation of electrons in the specimen, resulting in a negative charge on the specimen. Eventually, there is a build-up of a negative potential at the point of irradiation. This negative potential causes deflection of the electron probe (due to repulsion between like charges), causing a shift in its position. As a result of this shift, there is a distortion in the image. Subsequently, when discharge occurs and the potential returns to its original value, a normal image is formed, but this seems to be broken away from the previously scanned portion. This phenomenon is called "charging" and the effects it produces on the image are shown in Figure 4.3.

If the charging of the specimen is not very high, and there are only localized or point negative charges present on the specimen, these localized

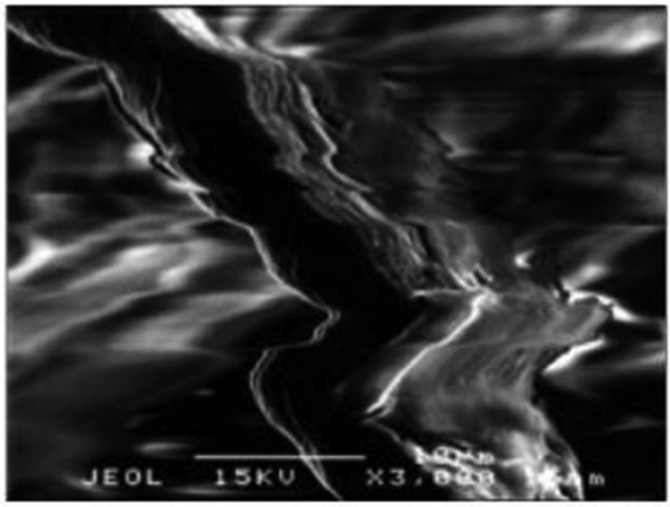

Figure 4.3. Distortion of an SEM image due to "charging" of the sample.

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negative charges change the trajectory of the secondary electrons. Besides this, as the voltage potential between the detector and the charged specimen portion increases due to the localized negative charge build-up, more secondary electrons enter the detector, causing an increase in brightness in the specific portion where charging has occurred. The reverse may also be true, that is, a build-up of a small positive charge at points on the sample may lead to a decrease in the voltage potential and a reduction in secondary electrons reaching the detector, resulting in dark portions in the image. In addition to this, if charging changes the trajectory of the secondary electrons, these may not reach the detector, and thus a part of the image will appear dark. Hence, some portions appear bright, whereas others appear dark, depending on whether a positive or negative voltage potential has been created. This is shown in Figure 4.4.

Hence, knowledge of the properties of the material to be observed is very important in obtaining good images from SEM.

4.1.1.3 Cryogenic Techniques for Electron Microscopy

Solid nanoparticles can be easily observed using the standard electron microscopy techniques described earlier. However, many times, it

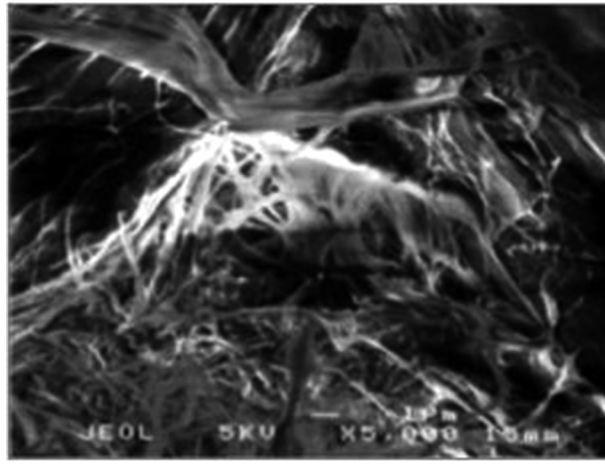

Figure 4.4. Appearance of dark and bright portions in an SEM image due to "charging."

Source: Reproduced with permission from JEOL Ltd. (n.d.)

becomes necessary to observe the dispersed phase in emulsions such as nanoemulsions and microemulsions as these are, in many cases, precursors to nanoparticles, and the size of nanoparticles depends largely on the size of the dispersed phase droplets. Conventional TEM can be used for characterization of nanoemulsions, but as described in Section 4.1.1.1.2, the staining and drying procedure involved in sample preparation is likely to distort the morphology of the sample. Observation of such dispersed phase droplets, where such distortions in the sample are likely, is done by "cryogenic" techniques.

The cryogenic techniques for electron microscopy basically involve two methods of sample preparation: the first is the freeze-fracture technique, where the specimen is rapidly frozen and subsequently fractured along one of the surfaces, after which a metal replica of the fractured surface is obtained, which is then viewed under an SEM or TEM; the second is the cryo-technique, where the sample is not replicated but frozen and immediately transferred to a low temperature stage within the microscope and viewed directly (Belkoura, Stubenrauch, and Strey 2004; Klang et al. 2012). The additional sample preparation step involving coating with a suitable metal enhances the conductance of electrons yielding superior image quality (Klang et al. 2012). The images obtained by the freeze-fracture technique largely depend on the plane of fracture and fracture quality.

Thus, cryogenic techniques, particularly the second technique, provide images of nanoemulsions in their natural hydrated state unlike the images obtained in the dehydrated state by the simple negative staining TEM observations described in Section 4.1.1.1. Cryo-TEM and cryo-SEM can be used effectively for the characterization of nanoemulsion samples. However, cryo-TEM requires an ultra-thin layer of the sample; hence, if the sample is highly viscous, cryo-SEM is preferred. Both these techniques and their different adaptations—done to suit specific sample types—have been described in detail, along with the quality of images that each technique and its adaptation provides, by Klang et al. (2012).

4.1.2 SCANNING TUNNELING MICROSCOPY

This method is used to obtain information about the atomic level surface morphology of samples. When a small metal tip is scanned across the sample surface (at a distance of about 1 nm above the surface), a quantum mechanical tunneling current is produced, and if the tip and the surface are under a small voltage difference, it results in the flow of this current, which can be measured. As this current is strongly dependent on the distance between the tip and the surface, the structural morphology of the surface can be imaged.

4.1.3 ATOMIC FORCE MICROSCOPY

AFM is a versatile instrument used for the physical characterization of nanoparticles. It provides a 3D image of the sample material, thus enabling characterization of spatially oriented features and measurements. AFM can characterize particles much smaller than those that can be characterized by SEM or light scattering techniques (under optimal conditions, even subatomic characterization of samples is possible). Besides this, AFM eliminates many of the limitations associated with SEM. Characterization by AFM can be carried out in air as well as in vacuum (SEM essentially requires high vacuum). Liquid samples can also be characterized, which is not possible with SEM. Conductive as well as nonconductive samples can be characterized (nonconductive samples need to be coated with a thin metallic layer for SEM observation). One disadvantage of AFM over SEM is that AFM scans more slowly compared to SEM. In specific modes, mechanical, electrical, magnetic, and thermal properties can be measured by AFM.

Unlike SEM and TEM, which use an electron probe for imaging, AFM uses a mechanical probe—a cantilever—for scanning the surface of the specimen. The cantilever beam is attached at one end to a piezoelectric displacement actuator and at the other end to a very fine, sharp probe tip, which interacts with the specimen surface. When the probe tip moves on the specimen surface, at close proximity to the surface, the probe experiences a bending moment due to which the cantilever deflects to varying extents depending on the nature of the surface. This deflection is measured using a laser beam and translated to give a 3D image of the specimen. A schematic diagram of the working principle of an AFM is shown in Figure 4.5.

A mixed sample of nanoparticles with a wide size range can be measured by AFM. Besides this, AFM can detect any material inhomogeneities and surface roughness of nanoparticles. An essential requirement for characterization by AFM is that the nanoparticles that are characterized must have a greater affinity for the substrate on which they are placed compared to their affinity for the cantilever probe. If this condition is not satisfied, "streaking" of the image is observed (the image appears to be elongated in one direction). AFM can be used in the "contact mode," "intermittent-contact mode," and "noncontact mode." In the contact mode, the cantilever tip is in contact with the specimen surface to give

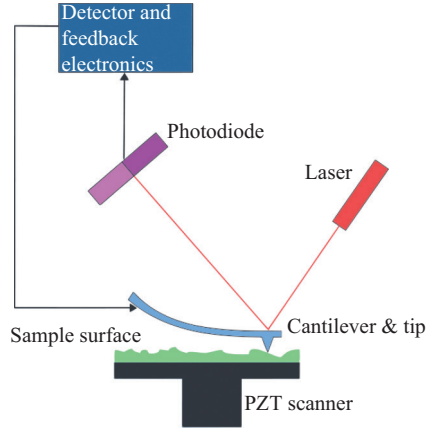

Figure 4.5. Schematic diagram of the working principle of an AFM.

PZT, Lead zirconate titanate.

Source: Wikimedia Commons (2009).

the measurements described earlier, whereas in the noncontact mode, the cantilever moves slightly above the sample surface and interacts with the sample surface via long-range surface force interactions. In the intermittent-contact mode, the cantilever is oscillated in the vertical direction (or perpendicular to the specimen surface), close to its resonance frequency, whereby long-range attractive forces and weak repulsive forces cause a variation in the amplitude of the cantilever oscillations, which are measured. The intermittent-contact mode is ideal for the characterization of soft surfaces or particles that are loosely bound to the substrate. Hence, nanoparticles are preferably characterized by this mode.

Sample preparation for AFM characterization is very critical. The nanoparticles need to be dispersed on flat and smooth surfaces. The surface roughness of the substrate must be much less than the average size of the nanoparticles, particularly if height measurements are required. High quality mica, atomically flat Au(111) deposited on mica, or single crystal silicon substrates can be used to minimize the effect of surface roughness on the measurements. Elaborate procedures for sample preparation, procedures for imaging and size measurement, image analysis, and reporting of particle size distributions have been given in the NIST–NCL Joint Assay Protocol, PCC-6, Version 1.1, prepared by the National Institute of Standards and Technology (NIST), U.S. Department of Commerce (2009).

4.2 METHODS BASED ON SCATTERING OF LIGHT

The nanoparticle characterization methods based on electron microscopy enable size and shape analysis; however, the sample has to be dried completely (as in the case of SEM and TEM) or fixed onto a substrate (in a dried or semidried state, as in the case of AFM) for characterization. Many times, particularly in the case of very small nanoparticles, it becomes difficult to separate the nanoparticles from the liquid in which they are formed. Even if they are successfully separated from the liquid by methods such as ultracentrifugation, they need to be dried further by freeze drying techniques before they can be used for microscopic analysis as mentioned earlier. The procedure employed for drying may cause agglomeration of the particles. The particle size analysis methods based on light scattering techniques allow reliable size distribution analysis in dilute colloidal suspensions of nanoparticles. The nanoparticles in such dilute colloidal suspensions are constantly in Brownian motion. When light is shone through such a colloidal suspension, the colloidal particles scatter the light (they serve as moving scattering centers), resulting in a reduction

in the intensity of the light and a Doppler shift (change in the frequency of the light), which can be detected and used to measure the velocity and size of the scattering centers. The principles and techniques based on light scattering and their applications in particle size measurement have been reviewed by Xu (2015).

4.2.1 DYNAMIC LIGHT SCATTERING

This is also known as photon correlation spectroscopy. It can be used to determine the size distribution profile of small particles in suspension (or macromolecules such as polymers in solution). In this method, a monochromatic light source such as a laser is passed through the sample comprising the colloidal suspension (a very dilute sample is required) where the small colloidal particles present in the sample, which are in constant Brownian motion, act as moving scattering centers and scatter the light incident on them. The scattered light signal is collected with detectors placed at a specific angle (either 90° or 173°) to the laser source. The basis for the measurement of particle size by DLS is the Stokes–Einstein equation, which relates the diffusion motion of particles (resulting from Brownian motion) to the hydrodynamic diameter of the particles:

$$D_h = \frac{k_B T}{3\pi\eta D_t}$$

where: D_h is the hydrodynamic diameter of the particle (to be determined)

k_B is the Boltzmann's constant (value is known)

T is the thermodynamic temperature (value is known)

η is the dynamic viscosity of the medium (value is known)

D_t is the translational diffusion coefficient (determined by the instrument)

In a typical DLS measurement, the dilute colloidal sample is placed in the sample holder (cuvette filled with the sample), which is in the path of a monochromatic laser light. When the light passes through the sample, it is scattered by the colloidal particles present in the sample. As these colloidal particles are in continuous random motion, fluctuating signals are emitted from the sample, which are collected by the detector (usually placed at 90°, or a back-scattering angle of 173°, to the light source). These data are processed in real time with a digital signal processing device known as a correlator to determine the auto-correlation function, which is further used to determine the translational diffusion coefficient D_t. From the value of

D_t, the diameter of the particles is determined. The particle size distribution in the sample can also be determined, from which the average particle size can be found out. The polydispersity index (PDI), which is a measure of the size distribution, can be determined by DLS measurements. A PDI value of 0.1 to 0.25 indicates a narrow size distribution, whereas a PDI > 0.5 indicates a broad size distribution.

It must be noted here that whatever the shape of the particle, the diameter determined by DLS measurements is the hydrodynamic diameter, assuming the particle is a sphere. In addition to this, as seen from the Stokes–Einstein equation, the measurements are dependent on the temperature, which in turn affects the viscosity of the medium, both of which figure in the equation, which forms the basis for particle size determination. Hence, these parameters are important in all the measurements done using a DLS instrument. The major disadvantage of this method is that the presence of a few relatively large particles in the sample may give erroneous results, as the larger particles will scatter more light and may obscure the signals from the majority smaller particles, thus giving false values for the mean particle size of the sample.

4.2.2 STATIC LIGHT SCATTERING

This technique measures the intensity of light scattered by scattering centers (colloidal particles or macromolecules) present in a liquid medium at different angles instead of a single angle as in DLS. A laser light is irradiated on a sample contained in a cuvette. One or many detectors are used to measure the scattering intensity as a factor of the scattering angle θ. From a plot of scattering intensity (I) versus scattering angle θ, information about the particle size, shape, and molar mass is obtained. This method is commonly used for determination of the root mean square radius of particles–macromolecules and average molecular weight of macromolecules such as polymers and proteins. To determine the molecular weight of a polymer or macromolecule, the static light scattering instrument is usually calibrated using a well-known reference material such as toluene whose Rayleigh ratios are available in standard handbooks.

4.2.3 SCATTERING TRACKING ANALYSIS

Also referred to as nanoparticle tracking analysis (NTA), this technique combines laser light scattering by small particles with microscopy and enables visualization and recording of nanoparticles with the help of a CCD

camera. As in DLS, a laser beam is passed through a colloidal suspension of nanoparticles in a suitable liquid, resulting in the light being scattered by the colloidal particles. This scattering is visualized by a microscope (the magnification may be in the range of 20×), mounted with a CCD camera. The camera captures a video file of the light scattering particles moving under the influence of Brownian motion. Many such particles are individually and simultaneously tracked and analyzed for the particle size and size distribution by NTA software based on the Stokes–Einstein equation. Thus, positional changes in individual particles are tracked.

4.2.4 SMALL ANGLE X-RAY SCATTERING

This method uses a focused beam of X-ray radiation instead of light to obtain information such as size, shape, internal structure, and porosity of the sample. Colloidal particles, macromolecules such as proteins, polymers, and other similar particles present in the sample scatter the X-ray beam to produce a 1D scattering intensity pattern, which is obtained by circular averaging of the actual 2D scattering produced by the particles. Data analysis of the 1D scattering pattern gives structural information, including information about the shape and spatial arrangement of the nanoparticles and macromolecules. This method can be used for in situ or online analysis of samples. The samples may comprise aerosols, colloidal suspensions, powders, or thin films. A change in the structure of biomolecules such as proteins can also be identified, which enables detection of certain diseases involving structural changes of proteins. A review of how the scattering data are analyzed to get information about the structure of the particles, particularly nonporous particles, has been presented by Agbabiaka, Wiltfong, and Park (2013).

CHAPTER 5

Toxicity of Nanoparticles

All therapeutic agents that have been approved for medicinal use have a maximum tolerated dose, which is determined on the basis of established protocols for toxicity studies done during the drug development process (see Chapter 1). An important measure of toxicity is the LD_{50} value for the drug substance (a lethal dose that causes 50 percent mortality in the population on which it is tested for the specified test duration). As nanoparticles have properties that are significantly different from those of the bulk material, the toxicity levels (LD_{50} values) for the drug substance in a nanoparticulate form are likely to vary significantly compared with those for the bulk material. The general advantages of nanomedicine and its specific advantages in cancer therapeutics and diagnostics have already been highlighted in Chapter 2. In addition to the immense advantages of nanoparticles and nanoparticulate carriers in drug delivery, their applications in other areas are also increasing and expanding in scope. Hence, the use of nanomaterials in research and subsequently in industry is increasing very rapidly. In light of the altered properties of the material in the nanoparticulate form, the assessment of toxicity needs to be reinvestigated in the case of nanomaterials. Research as well as large scale manufacturing level procedures and operations involving nanoparticles is likely to cause exposure of the researcher/operator to the adverse effects that these materials may have on the human body as well as the environment. Besides this, if appropriate waste treatment protocols are not in place, the disposal of nanoparticulate matter into the drains is likely to contaminate the ground and ground water. Thus, the toxicity of nanoparticles may be a result of adverse effects caused by drug based nanoparticles intentionally taken for therapeutic purpose or due to unintentional exposure to nanoparticles present in the environment (arising from various activities ranging from manufacturing processes to combustion of hydrocarbons and burning of fuel). Whatever the source, in vivo toxicity arises due to inhaled

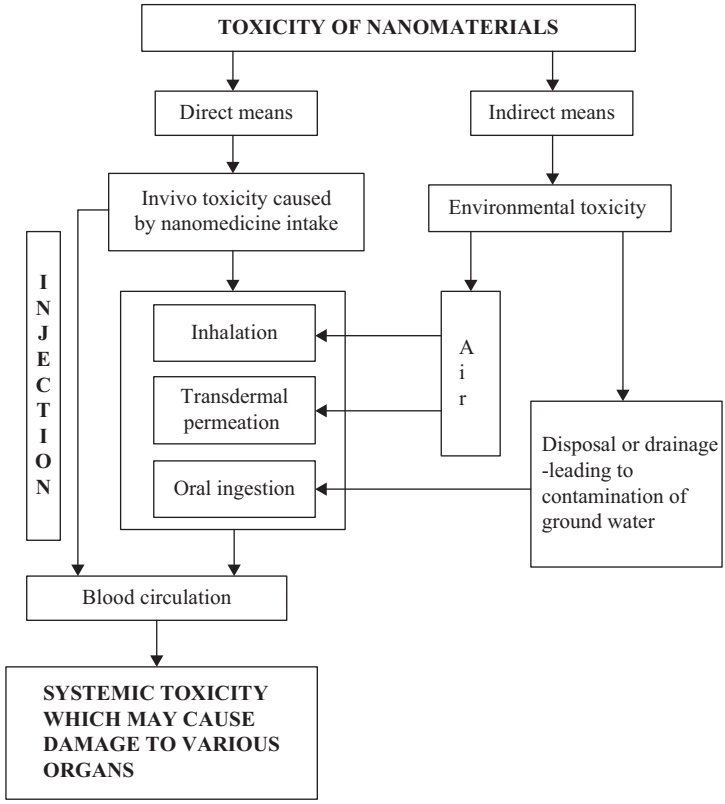

Figure 5.1. Toxicity caused by nanomaterials.

and ingested nanoparticles and nanoparticles permeating through the skin into the systemic circulation. The various modes by which nanoparticle toxicity may be manifested are shown in Figure 5.1.

As more and more nanoparticle based products are being commercialized, issues of occupational hazard for workers engaged in the manufacture of such products are becoming a cause of concern. In the absence of adequate precautionary measures for preventing entrainment of nanoparticles in the air during manufacture or lack of adequate safety measures for workers to prevent inhalation of nanoparticles or both, these are likely to cause severe health related concerns as nanoparticles are known to damage and disrupt the normal functioning of several organs in the human body. This chapter describes the adverse effects that nanoparticles are capable of causing to various organs in the body and some personal and environmental toxicity considerations that must be taken into account while dealing with nanoparticles on a regular basis.

5.1 IN VIVO TOXICITY

Nanoparticle based formulations are becoming increasingly popular not only in the pharmaceutical field (therapeutics and diagnostics), but also in fields such as cosmetics and nutraceuticals where these particles are intentionally taken and directly enter the body via the oral route, through inhalation, or via skin, followed by absorption into the blood stream; or these may be injected directly into the blood in the form of an intravenous injection. Their popularity arises from the fact that these nanosized particles are able to penetrate very easily through the various membrane barriers that protect the different organs against entry of any foreign substance. Thus, increased blood levels and increased efficacy can be obtained with a small amount of nanomaterial compared with a micron sized material. Depending on their size, nanomaterials can also be made to preferentially accumulate in certain organs, thus achieving the targeted drug delivery. However, along with the desired advantage of being able to target the drug to specific organs or tissues, accumulation of nanoparticles in other organs or tissues may pose a problem. Combined with the fact that very small nanoparticles are not cleared through the normal glomerular filtration process, these nanoparticles may continue to circulate in the body for long periods of time leading to a build-up of nanoparticles at specific sites, which is likely to cause damage to these sites. Besides this, the researchers and workers involved in research and manufacturing activities are also likely to be chronically exposed to nanomaterials causing accumulation leading to a number of adverse effects. As these nanomaterials are specially designed for specific purposes by manipulating their size, shape, surface charge, surface functionality or reactivity, and so on, the effects that such engineered nanoparticles (ENPs) have on the body may differ from the effects of other naturally occurring nanoparticles or those that arise as a by-product during certain other processes. Fadeel, Pietroiusti, and Shvedova in their book titled "Adverse Effects of Engineered Nanomaterials" draw a distinction between "man-induced and/or environmental nanoparticulates" (arising from diesel exhaust, emerging from welding fume, resulting from combustion of coal, etc.) and nanoparticles that are specifically designed by tailoring their size and surface properties to suit specific applications by terming the latter as "Engineered Nanomaterials" (Fadeel, Pietroiusti, and Shvedova 2012). Such ENPs usually have an enhanced surface reactivity and, hence, when they enter the biological system and come in contact with biological fluids, they attract the biological constituents such as proteins and other molecules and form a "core-corona" structure (Figure 5.2) where the nanoparticle forms the core and

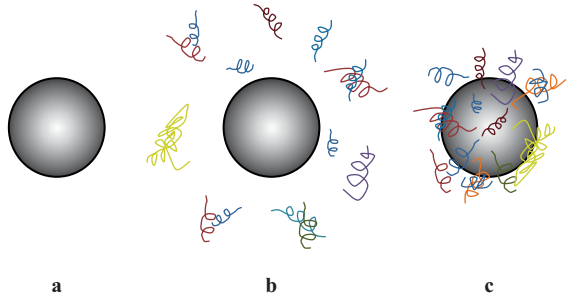

Figure 5.2. Interaction of nanoparticles with proteins in biological fluids. Formation of: (a) bare nanoparticle, (b) nanoparticles surrounded by proteins present in the biological fluid, and (c) corona due to attachment of proteins on the nanoparticle surface.

depending on the nature of the interactions, weak or strong, the biological constituent forms a hard or a soft corona.

Thus, the properties of this corona become important for further assessment of the behavior of the nanoparticles. In many cases, the nanoparticles are taken up and destroyed by the macrophages of the reticulo-endothelial system. To prevent recognition by the macrophages, surface modification by polyethylene glycol is done. Such "pegylated" nanoparticles are able to circulate in the blood for prolonged periods of time to enable absorption of the nanoparticles in the target organs or tissues. Whether the introduction of the nanoparticles into the body is intentional or unintentional, prolonged exposure of the body to nanoparticles is likely to produce a variety of adverse effects depending on the nature of nanoparticles and extent of exposure.

5.1.1 EFFECT OF NANOPARTICLES ON THE RESPIRATORY SYSTEM

This is the most common mode of entry of environmental nanoparticles. The inhaled particles deposit at different locations in the respiratory system depending on their size. Coarse particles (>2.5 μm) mainly deposit in the upper respiratory airway (nasal and oral airway), fine particles (0.1 to 2.5 μm) deposit mainly in the bronchiolar airway, whereas ultrafine nanoparticles (<100 nm) deposit in the alveolar airway (Kolanjiyil and Kleinstreuer 2013). While deposition of larger particles (particles >500 nm) is governed by the aerodynamic diameter (the diameter of a hypothetical sphere having the same unit density and settling velocity as the

particle in question) that of nanoparticles (particles <500 nm) is governed by their thermodynamic diameter (the diameter of a hypothetical sphere having the same unit density and diffusion coefficient as that of the particle in question) (Donaldson and Poland 2012).

5.1.1.1 *Fate of Inhaled Particles*

The fate of inhaled particles is mainly governed by four mechanisms. As larger particles (insoluble particles, size ~6 μm) including nanoparticle agglomerates deposit at the proximal portion of the respiratory tract (upper respiratory airway), they are mainly eliminated by coughing and *mucociliary clearance*. As shown in Figure 5.3, the inner lining of the respiratory airway comprises of a mucus lining, which is supported by ciliated epithelial cells. The movement of the cilia is such that it causes the mucus to move toward the direction of the pharynx. The inhaled particles get stuck in the mucus layer and are carried toward the mouth due to the mucociliary movement. These are then expelled through the mouth (by coughing) or swallowed and eliminated through the gastrointestinal tract. This mode of clearance is very rapid and hence the retention time for

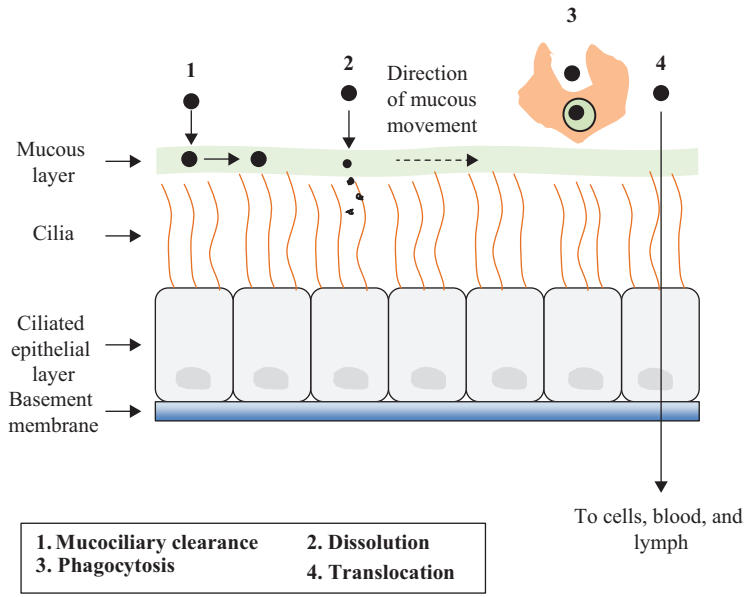

Figure 5.3. Diagrammatic representation of potential pathways for the clearance of inhaled nanoparticles.

large particles is very less. Another mechanism of clearance of particles is by *phagocytosis*. Macrophages engulf the particles and eliminate them by either lymphatic clearance or enzymes such as lysosomes present in the cells. Studies on rodents indicate that clearance by macrophages does not differentiate between microparticles and nanoparticles (Geiser and Kreyling 2010). Some particles undergo *dissolution* due to the presence of pulmonary surfactant present on the lung surface and subsequently get absorbed into the blood. Nanoparticles, which are able to reach the alveolar region, also undergo *translocation* into the blood. Usually, less than 5 percent of nanoparticles are able to translocate systemically. Translocation is via receptor mediated transcytosis through the formation of caveolae.

5.1.2 EFFECT OF NANOPARTICLES ON THE CARDIOVASCULAR SYSTEM

Studies demonstrating the adverse health effects of environmental particulate matter (generally considered to be particles <10 μm), including combustion derived nanoparticles, are available (Barlow et al. 2005; Donaldson et al. 1997; Mills et al. 2007, 2009; Renwick et al. 2004). It has been shown that exposure to ultrafine combustion derived particles (which come in the nano size range) have the capacity of impairing vascular function and causing myocardial ischemia (insufficient flow of blood to heart muscle, leading to chest pain), besides being directly responsible for atherosclerotic plaque (deposits on the inner lining of arteries) and prothrombotic changes in circulation. Though these studies are concerned with ultrafine particles present in the environment, as the size and surface areas involved are comparable, these can be extrapolated to ENPs (Castranova 2011; Eisen et al. 2011; Moller et al. 2010). The mechanism of toxicity generation is believed to be oxidative stress (explained in Section 5.2.1). More studies on humans with collaborative efforts among academic researchers, clinicians, industry, and government are required to obtain an accurate assessment of specific toxicity due to ENPs.

5.1.3 EFFECT OF NANOPARTICLES ON SKIN

The skin is that part of the body most extensively exposed to the environment and, therefore, most prone to penetration by environmental contaminants. Hence, the structure of the skin is such that there are multiple layers tightly bound to each other, the outermost being epidermis, comprising of the stratum corneum, followed by the dermis comprising of a number of

strata made up of connective tissue, and hypodermis that connects the skin layers to the underlying bone and muscles and where the blood vessels and nerves originate. The skin, as a composite structure, forms a very tight barrier to penetration of particulate matter, with a cut-off size of 600 Da (Baroli et al. 2007). A theoretical study done to assess human skin penetration of nanoparticles indicates that nanoparticles are too large to penetrate through intact skin, and only the dissolved portion is able to get absorbed and reach systemic circulation (Watkinson et al. 2013). It has been shown in ex vivo studies using excised human skin that nanoparticles as small as <10 nm were able to penetrate the hair follicle and stratum corneum, but only occasionally reach the viable epidermis (Baroli et al. 2007). Skin penetration studies have been carried out for carbon based nanomaterials, quantum dots, TiO_2 and ZnO nanoparticles, and gold and silver nanoparticles. The penetration assessment for carbon based nanoparticles is important as the applications of these materials in different areas are increasing significantly. The skin penetration of all types of carbon nanotubes (CNTs) and nanowires is significantly restricted because these typically have one dimension in the micrometer size range. However, this is not the case with fullerenes that are spherical with a diameter of <100 nm. These are found to penetrate through all epidermal layers. Studies for the penetration of quantum dots show that these are able to reach only up to the stratum corneum of the intact skin. However, in the case of damaged skin (where most of stratum corneum is removed), they can reach up to the viable epidermal layers (Fadeel, Pietroiusti, and Shvedova 2012; Prow et al. 2012). In vivo studies in mice have shown that quantum dot permeation into skin takes place through intercellular lipid lamellae and UV radiation increases skin permeation of quantum dots (Mortensen et al. 2008). Time related permeation of quantum dots following exposure to UV radiation has also been studied by Mortensen et al. (2010). Biodistribution studies (after injection into blood or entry into systemic circulation through any route) show that once in the blood, these are able to migrate out of the capillaries and enter the surrounding tissues or reach various organs such as liver or penetrate both tissues and organs (Fadeel, Pietroiusti, and Shvedova 2012; Gopee et al. 2007; Lee et al. 2007). Skin penetration studies for TiO_2 and ZnO nanoparticles (size <100 nm) gain importance as these are the most commonly used ingredients of sunscreen and other skin care products. Fortunately, it has been found that no penetration of these occurs through intact skin. However, damaged skin has been found to be slightly permeable to these ingredients. Whereas silver nanoparticles have been found to permeate only up to the stratum corneum, gold nanoparticles (particularly those in the range of ~10 nm) have been found to accumulate in the deeper layers of the skin (Fadeel, Pietroiusti, and Shvedova 2012).

As most of the studies for the assessment of penetration through the skin have been done on excised skin or on small animals, the actual toxic potential of nanoparticles through skin penetration in humans can only be realized after more human long-term skin exposure studies.

5.1.4 EFFECT OF NANOPARTICLES ON THE GASTROINTESTINAL TRACT

Gastrointestinal exposure to nanoparticles can occur mainly through the ingestion of nanoparticle based medicinal products and nutraceuticals and nanoparticle containing food products. High doses of ENPs such as metal and metal oxide nanoparticles and quantum dots have been found to cause significant toxicity in animal and cell culture models. However, the potential toxicity at normal exposure levels needs to be studied to a greater extent before any conclusions regarding the toxicity of ENPs through gastrointestinal route can be drawn. Moreover, the in vivo–in vitro correlation of the studies already carried out needs to be established in order to comment on the actual toxicity that these nanoparticles can cause.

5.1.5 EFFECT OF NANOPARTICLES ON THE REPRODUCTIVE SYSTEM

Reproductive toxicity may manifest as developmental toxicity, which may be considered as an interference in the normal development before as well as after birth. This may be embryotoxicity, which refers to the impaired growth of the embryo, resulting in reduced weight of the fetus, retarded growth of any of the organs resulting in structural defects after birth, or in the extreme the death of the fetus resulting in abortion. Toxicity may also manifest itself up to a few years after birth in the form of abnormal development at any stage of growth, usually up to puberty (Fadeel, Pietroiusti, and Shvedova 2012). Most studies concerned with the assessment of reproductive toxicity carried out on mammalian and nonmammalian models indicate that most ENPs can interfere with normal reproductive functions and normal development of the fetus. However, corresponding human studies are not available.

5.1.6 EFFECT OF NANOPARTICLES ON THE IMMUNE SYSTEM

Nanoparticles have the capacity to either stimulate (adjuvant effect) or suppress the immune system. The immunomodulatory effects of

environmental nanoparticles such as diesel exhaust particles have been known for several years. These particles have been known to enhance allergic inflammatory response leading to increased incidence of asthma and allergic rhinitis. This happens due to increased immunoglobulin E production as a result of activation of macrophages and enhanced production of cytokines and chemokines in response to the nanoparticulate matter (Nel et al. 1998). The adjuvant effects and autoimmune responses of such environmental ultra fine particles (size equivalent to ENPs) on the immune system have been reviewed by Chang (2010).

5.1.7 EFFECT OF NANOPARTICLES ON NEUROLOGICAL SYSTEM: BRAIN AND THE BLOOD-BRAIN BARRIER

Nanoparticle exposure and its possible neurological effects is a major cause of concern because, as already mentioned earlier, the main route of entry of solid particles is through inhalation. Consequently, the olfactory mucosa of the nose is exposed to a relatively higher concentration of nanoparticles than any other parts of the respiratory system. As the olfactory nerve has access to the central nervous system and the brain, the nanoparticles can easily reach the brain by this route. Studies in rats have suggested this possibility (Oberdorster et al. 2004); however, no extensive studies in humans are available that associate the exposure to nanoparticulate matter in air with chronic brain disease.

Normally, those substances that reach the blood are transported to different organs and tissues by the systemic circulation. However, entry to one of the most important organs of our body, the brain, is protected by a tight barrier called the blood–brain barrier (BBB). Thus, even those substances that are able to reach the blood are still not able to enter the brain because the epithelial cells of blood capillaries supplying blood to the brain have very tight junctions with absence of the usual fenestrations, which are present in epithelial cells supplying blood to the other parts of the body (due to which nanoparticles are able to enter other organs and tissues); other transport mechanisms such as pinocytosis are also absent. Usually, lipophilicity helps in the transport of substances through body membranes and tissues; however, even for lipophilic substances, a molecular cut-off of 400 Da exists. In addition to this, a very high electrical resistance (in the range of 1,500 to 2,000 Ω cm^2) is generated by pericytes and glial cells, which prevents ions from entering the brain (Grabrucker et al. 2014). Due to all these reasons, there is little evidence of environmental nanoparticles being able to cause brain toxicity.

Due to the difficulty in crossing the BBB, for specific targeting of therapeutic agents to the brain, surface modification of nanoparticles using specific ligands has been done. Polymeric nanoparticles such as poly(n-butylcyanoacrylate), methoxypoly(ethyleneglycol)-polylactide or poly(lactide co-glycolide), liposomes, and certain inorganic nanosystems are able to cross the BBB (Grabrucker et al. 2014). The neurotoxicity of such ENPs designed to cross the BBB has been reviewed by Hu and Gao (2010). However, most studies are based on animal models and cannot be completely extrapolated to humans.

5.1.8 NANOPARTICLES AND GENOTOXICITY

Certain nanoparticles have the capacity to enter the nuclei and may directly intercalate with the DNA to cause damage or may cause damage via physicochemical or electrochemical interactions. Generation of reactive oxygen species (ROS) is also one of the mechanisms that can cause genotoxicity. This can be either due to a direct effect or indirectly by generation of oxidative stress that damages the cellular components.

5.2 MECHANISMS OF IN VIVO TOXICITY OF NANOPARTICLES

The toxicity due to nanoparticles results from the effects that the nanoparticles have at the interface between the nanoparticles and biological membranes (the nano–biointerface), including the cell membrane at the cellular level. The interactions of the nanoparticles at the nano-biointerface may be either chemical or physical in nature. The chemical interactions principally involve the formation of a reactive oxygen species (ROS), leading to secondary processes that ultimately cause cell damage or cell death. The other chemical interactions include dissolution and release of toxic ions, disturbance of the ion transport activity at the membrane level, oxidative damage due to catalysis, and lipid peroxidation, leading to the disruption of the membrane. The physical mechanisms involve disruption of membrane at the nano–biointerface, leading to abnormal membrane activity and transport processes, causing changes in protein conformation and folding, and causing protein aggregation and fibrillation (Elsaesser and Howard 2012). Certain multiwalled carbon nanotubes (MWCNTs), due to their high aspect ratio lead to a phenomenon called “frustrated phagocytosis” in which phagocytosis is attempted but fails in eliminating the foreign body, which may remain in the body for years. Nanomaterials such

as fullerenes, CNTs, TiO_2, polystyrene, and silver nanoparticles have the capacity to affect mitochondria and mitochondrial function, which may ultimately lead to apoptosis of the cell. Certain nanoparticles also have the capacity to enter the nuclei via diffusion through the tiny pores present in the nuclear membrane or through receptor mediated endocytosis (see Figure 2.8) and cause genotoxicity.

5.2.1 GENERATION OF REACTIVE OXYGEN SPECIES

The most important mechanism of generation of toxic effects by nanoparticles is the formation of ROS by nanoparticles, subsequently leading to oxidative stress, which finally results in either damage to the cells or cell apoptosis. This mechanism, especially relevant in the case of inhaled nanoparticles, has been discussed at length with special reference to the toxic potential of transition metals and CNTs by Manke, Wang, and Rojanasakul (2013). ROS comprises of a pool of oxidative species including free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl). Nanoparticle characteristics such as size, surface charge, and chemical composition are the key parameters that influence the generation of ROS. Due to the high surface to volume ratio of nanoparticles and structural defects present in nanoparticles, reactive groups are created on the surface of the nanoparticles, which may have altered electronic properties. Some sites may act as electron donor while others as electron acceptor sites (may be physically or chemically activated), which may react with molecular oxygen present in the body. Electron capture by molecular oxygen results in the formation of superoxide radical $O_2^{\cdot -}$. This superoxide radical can initiate the generation of more such radicals by dismutation (a biological process involving simultaneous oxidation and reduction) or by Fenton chemistry (where a $OH^{\cdot -}$ radical is generated that reacts with biomolecules to form a series of additional free radicals such as thiyl radical (RS); carbon centered radical $(R_3)C^{\cdot}$; and peroxyl radical $(R_3)OO^{\cdot}$. The generation of such ROS causes oxidative stress in the body. Under normal conditions (in the absence of nanoparticle exposure) in which small numbers of such free radicals are generated resulting in a mild oxidative stress, they are neutralized by the cell's antioxidant defense mechanism via molecules such as glutathione (GSH) and other similar antioxidant enzymes (Nel et al. 2006). In the case of an intermediate level of oxidative stress, a depletion of GSH occurs with a simultaneous accumulation of oxidized GSH (GSSG). The reduced GSH to GSSG ratio results in activation of the body's normal inflammatory mechanism, leading to the formation of

interstitial fibrosis. In an extreme case of oxidative stress (as in long term chronic exposure to nanoparticles), damage of the mitochondrial membrane occurs along with electron chain dysfunction leading to cell death. The overall effects of oxidative stress may result in DNA damage, lipid peroxidation, loss of cell growth, fibrosis, and carcinogenesis (Figure 5.4).

Thus, the main factors responsible for inducing oxidative stress by nanoparticles are: pro-oxidant functional groups on reactive surface of nanoparticles, presence of active redox cycling on the surface of nanoparticles (mainly seen in the case of transition metal based nanoparticles), and particle–cell interactions (general factor for all types of nanoparticles). The most common nanoparticles that can induce oxidative stress are fullerenes, CNTs, and metal oxides (Manke, Wang, and Rojanasakul 2013). Whereas metal based nanoparticles generate free radical mediated toxicity via Fenton type reaction, CNTs cause ROS mediated toxicity due to mitochondrial damage. Thus, it must be noted that the mechanism of ROS generation may be different for different types of nanoparticles. In addition to this, it must also be noted that ROS generation is not an essential prerequisite for nanoparticle toxicity (nanoparticle toxicity may also result from direct interaction with cells or cell components). Besides nanoparticles themselves being

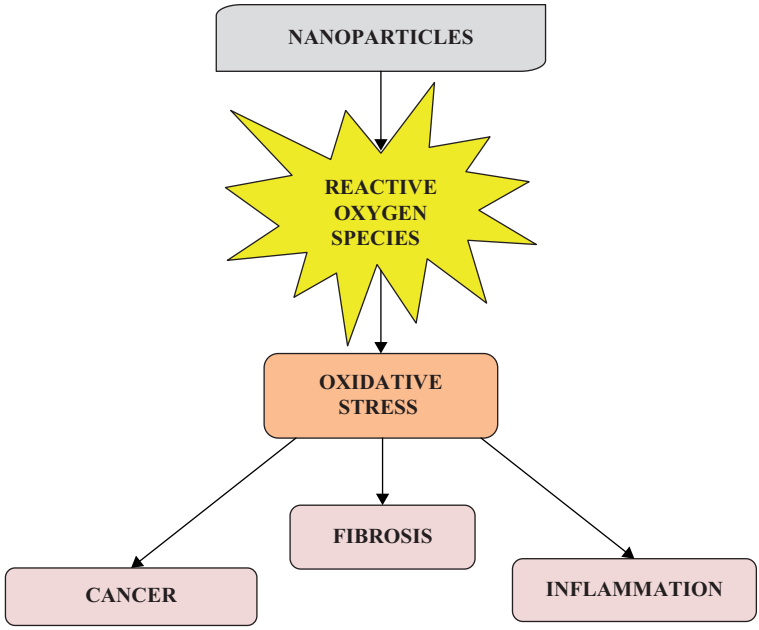

Figure 5.4. The effects of oxidative stress resulting from excessive generation of ROS triggered by nanoparticles.

responsible for the generation of oxidative stress, surface bound radicals present on the nanoparticle surface (in the form of impurities) may also induce formation of $OH^{\cdot -}$ and $O_2^{\cdot -}$. Atmospheric gases such as NO_2 and ozone adsorbed on particle surface are also capable of generating oxidative stress.

5.2.2 FRUSTRATED PHAGOCYTOSIS

This mechanism of toxicity generation by nanostructures is particularly relevant to nanostructures having a high aspect ratio (length to diameter ratio), such as nanowires, nanofibers, and nanotubes (single walled CNTs and MWNTs). These types of nanostructures are increasingly finding applications in a variety of areas leading to increased chances of environmental toxicity caused tract. Fiber toxicity, particularly with reference to asbestos fibers, has been known since many years. It has been identified that the toxicity of fibrous materials depends on three parameters: (i) thinness of fibers, which determines the ease by which the fibers can travel beyond the ciliated respiratory tract to reach the lungs; (ii) biopersistence of the fibers, which means the ability of fibers to remain intact without breaking or degrading; those fibers that have soluble components that can dissolve, break into short fragments, which are usually not pathogenic and are cleared by the process of normal phagocytosis; and (iii) length of fibers, which determines whether the fiber can undergo normal phagocytosis or not.

Phagocytosis is the body's normal defense mechanism that is responsible for eliminating foreign substances such as pathogenic bacteria, viruses, and foreign particles. This is carried out by macrophages, which are specialized cells that engulf the foreign body and internalize it into phagosomes, and which combine together with lysosomes present in the macrophages to form a phagolysosome. The foreign material is destroyed by various enzymes present in the lysosome and the debris is thrown out in the extracellular fluid for clearance by the lymphatic fluid. Small particles or small length fibers can be completely engulfed by the macrophages and internalized into the phagosome for further clearance. However, in the case of thin and long fibers, because of their thinness, they are able to navigate up to the alveolar region, but the alveolar macrophages are not able to completely engulf and internalize the fiber, resulting in an unclosed phagosome and a rupture of the phagocyte membrane, causing the cell contents to spill out of the cell. This is termed as "frustrated phagocytosis," which leads to an inflammatory response followed by fibrosis. The two scenarios are represented diagrammatically in Figure 5.5.

Schinwald and Donaldson determined the cut-off length for frustrated phagocytosis. They found that in in vitro and in vivo studies, frustrated

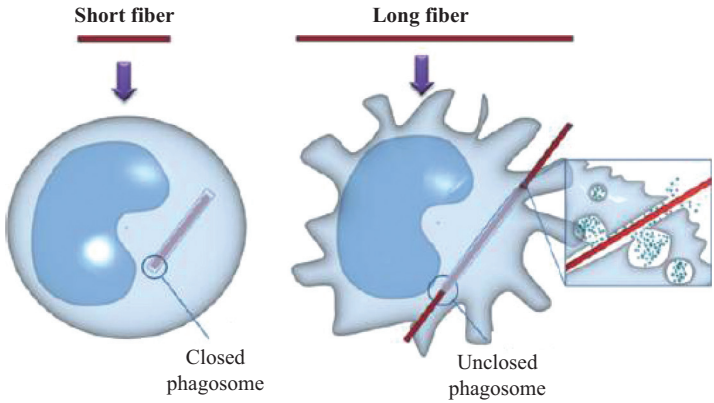

Figure 5.5. Diagrammatic representation of a complete phagocytosis of short fibers and incomplete or frustrated phagocytosis of long fibers leading to rupture of cell membrane and consequent leakage of cell contents.

Source: Reproduced from Schinwald and Donaldson (2012).

phagocytosis occurred at lengths of ≥14 and ≥10 μm, respectively. When injected into the pleural space in mice, only those nanotubes ≥5 μm were able to show inflammatory response whereas those between 5 and 10 μm were able to get engulfed completely into the phagocytes resulting in complete phagocytosis (Schinwald and Donaldson 2012).

It has been found experimentally that silver nanowires and MWNTs are able to compartmentalize into the pleural and subpleural tissue or fluid after inhalation (Ryman-Rasmussen et al. 2009; Schinwald and Donaldson 2012). Studies in mice show that inhaled MWNTs cause suppression of systemic immune system in the animal—the lungs exposed to MWNTs send signals to the spleen to suppress the immune function in the mice (Mitchell et al. 2009).

It must be noted here that most studies for the assessment of toxicity of nanoparticles have been carried out either in vitro or ex vivo, or if done in vivo, they have been done on animal models. Hence, extrapolation of the results to humans needs to be done cautiously. Besides this, it is well known that toxicity is all about the dose of the substance under question. Substances that are toxic only at very high concentrations need not necessarily be toxic at low concentrations or at the concentrations that are encountered in daily routine. Almost all the drugs used for medicinal purpose are toxic at high concentrations, but at the prescribed levels can cure diseases. Therefore, as applied to nanoparticle toxicity, in spite of the nanomaterial exhibiting toxicity in the toxicity studies done on it (which usually use very high doses

of the test material), the normal extent of exposure to the material must also be taken into account in order to declare it toxic or harmful. Toxicity aspects with respect to occupational exposure to nanomaterials (in which chronic exposure may result in accumulation of the nanomaterial in the body) must be treated separately and adequate safety precautions must be prescribed. At present, sufficient information about the toxic effects of different types of nanomaterials on various organs by different modes of entry is lacking; hence, extra precautions need to be taken to prevent exposure to environmental nanoparticles as well as ENPs.

5.3 ECOTOXICITY OF NANOPARTICLES

With an ever increasing impact of nanotechnology over a wide spectrum of applications, there are presently over 1,600 nanotechnology based consumer products (such as self-cleaning windows, stain resistant clothing, bacteria resistant socks, and sunscreen products to name a few) in the global market (www.nanotechproject.org/), and this number is expected to increase at an even faster rate in the near future. Simultaneously, with the realization of toxic effects of some nanomaterials such as CNTs and fullerenes on animals and humans, concern regarding the safety, risks, hazards, and environmental and ecological impact of engineered nanomaterials is also increasing. Dedicated conferences raising these issues are being held the world over, and terms such as "nanoecotoxicology" and "green nanotechnology" are gaining importance. It has already been shown that silver nanoparticles embedded in commercially available socks (to prevent foul smell and bacterial growth) can enter into domestic waste water during washing. This waste water, when treated in waste water treatment plants for reuse, may cause further contamination (Benn and Westerhoff 2008). Besides, in many research facilities all over the world, nanomaterials are simply disposed through the drain in absence of systematic waste disposal policies, which may again be responsible for the contamination of ground water or other water bodies, thus entering different flora and fauna.

Environmental nanomaterials, because of their high surface reactivity, are likely to adsorb synthetic and organic matter present in the environment (Behra and Krug 2008), besides being themselves adsorbed on the surfaces of leaves and other parts of plants. These nanomaterials may enter and subsequently move up the food chain when ingested by insects and animals. The toxic effects of engineered nanomaterials on vertebrates have been well documented and studied; however, their toxicity to aquatic invertebrates also needs to be investigated. Baun et al. have reviewed in

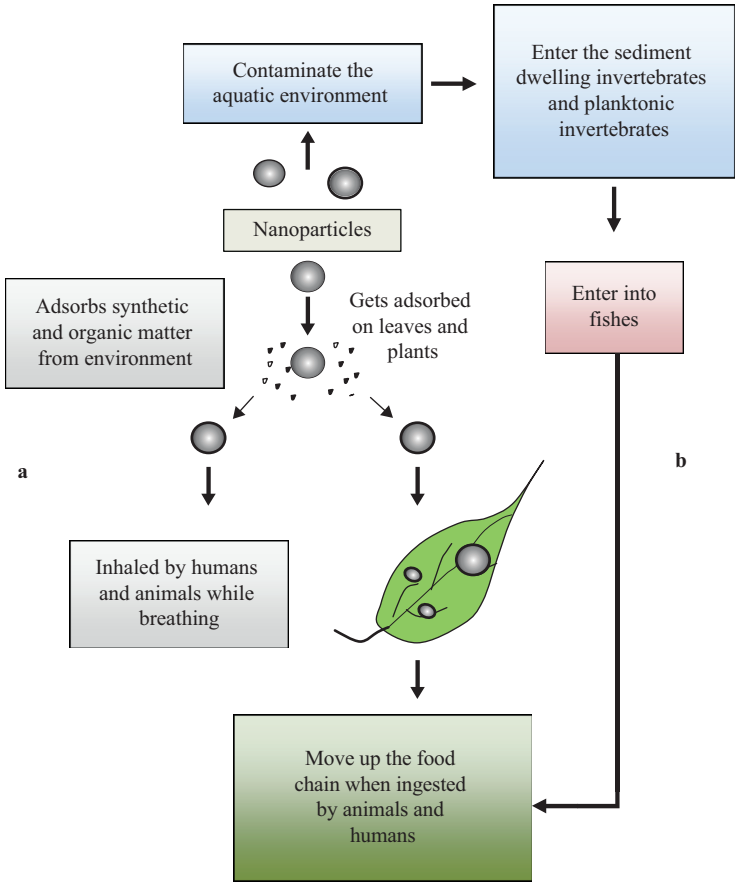

Figure 5.6. Toxic effects of environmental nanomaterials and engineered nanomaterials: (a) directly to humans and animals and (b) indirectly through plants and aquatic animals.

detail the ecotoxicity of such engineered nanomaterials on invertebrates and recommended the methods of toxicity testing in invertebrates (Baun et al. 2008). Figure 5.6 illustrates how invertebrate species can take up ENPs and initiate contamination in other aquatic species, subsequently causing toxicity to humans and other vertebrates.

Toxicity studies in soil environment have been carried out specifically to study the effect of metal oxide based nanoparticles on terrestrial plants and earthworms (Lee et al. 2010). Ecotoxicity of nanomaterials such as fullerenes, CNTs, metal based nanoparticles, and nanocomposites have been reviewed by Rana and Kalaichelvan (2013).

CHAPTER 6

Current Status and Future Scope

Cancer, even today, in spite of the several advancements on various fronts, is still categorized as a disease that defies a satisfactory solution. The focus of research on understanding the causes of cancer, the early indications of the presence of cancer in the body, the pathophysiology of each cancer, the possibility of metastasis, finding simple noninvasive methods for the diagnosis of cancer, and several such aspects is taking us more and more closer toward finding a satisfactory cure for the disease besides reducing the misery associated with the disease. In the case of cancer, it has been realized that for a treatment or therapy to be successful, early and accurate diagnosis of cancer is a prerequisite. Toward this objective, biomarkers that can indicate the onset of cancer at very early stages are being searched. One such biomarker, the length of the telomeres present at the ends of the DNA, has been recently proposed as a fairly certain indication of the possibility of cancer occurring in the near future.

Besides this, an assessment of the response to treatment is also important so that the treatment strategy can be changed or modified. With this in view, the concept of "cancer theranostics," which is a combination of diagnosis and therapy in a single system, is gaining importance. Advances in the field of cancer biomarkers combined with nanotechnology based therapies are likely to improve the treatment of the disease.

The treatment strategies for cancer are moving from a generalized chemotherapy and radiotherapy based approach to more personalized biotechnology based solutions for cancer such as finding cellular level RNA (microRNA [miRNA] and small interfering RNA [siRNA]) based targets in an effort to get at the root cause of cancer; that is, addressing mutations at the cellular level. Efforts are also ongoing to find a "vaccine" for cancer, which will be able to prevent the occurrence of mutations leading to

cancer. There are reports of the cell derived nanoparticles that could be used to mimic many natural properties displayed by their parent cells in order to gain antigenic information of the cancerous tissue. This chapter provides a brief overview regarding the current research efforts, particularly those based on nanotechnology, which are ongoing globally toward improving the prognosis and treatment of cancer.

6.1 CANCER THERANOSTICS

Cancer is a disease that is heterogenous in nature. Different types of cancers are characterized by different features and varying rates of growth and morbidity. A standard therapeutic regimen may not suit all populations and all types of cancer. Even within a particular type of cancer, the manifestation may differ in different people. Hence, it is important that the success of a particular therapy be assessed intermittently during the therapy so that it can be dynamically changed at the earliest indication of its ineffectiveness. In other words, a personalized treatment approach is required. The theranostic approach serves this purpose excellently and, at present, there are a number of agents that have been prepared and tested for theranostic applications.

Polymer based theranostics, quantum dot based theranostics, liposome based theranostics, theranostic micelles, carbon based theranostic contrast agents, iron oxide based theranostic agents, gold nanoparticle based theranostic agents, and silica based theranostic agents are the categories of materials that are currently being explored. The multifunctional nanoparticle approach is used in which the therapeutic and the diagnostic/imaging agents are both attached to the same nanoparticle. Recent advances in this area have been reviewed by Fan et al. (2014) and Chen and Wong (2014).

RNA based theranostics is gaining importance after the potential of miRNA and siRNA in cancer theranostics has been realized. The main bottleneck in exploiting the full potential of RNA based theranostics is their successful delivery into the cytosol. Recently, Zhang, Wang, and Gemeinhart have reviewed the progress in the delivery of miRNA (Zhang, Wang, and Gemeinhart 2013). With newer nanomaterials being developed, coupled with newer methods of their synthesis, it is likely that the field of RNA based theranostics will see exceptional development in the near future. Conde, Edelman, and Artzi have listed the different miRNA based nanosensors and nanocarriers for the delivery of miRNA that have been recently developed and have stated some important requirements

that any potential RNA based cancer theranostic agent must fulfill in order to be successful (Conde, Edelman, and Artzi 2015).

6.2 CELL DERIVED NANOPARTICLES

As cancer cells are abnormal cells, they typically have certain antigenic components on their cell surface, which are not present on normal cell surfaces. This feature of cancer cells has been exploited to enhance the immune response of the body so that the cancer cells can be identified and eliminated by the cells of the body's own immune system. Researchers have coated polymeric nanoparticles by the cell membrane of the cancer cells. By this, the antigenic information (carried by the cell membrane) is retained, and the cancer cell membrane coated core-shell nanoparticles can be used to mimic several natural properties displayed by their source cells. In this manner, the polymeric nanoparticles are "functionalized" by the antigens present on the cell membrane of the source cancer cells. If the antigen is a tumor associated antigen, it can be used for generating an immune response to the cancer, whereas if the antigen is a homotypic binding antigen, it can be used for targeting the polymeric nanoparticle to the tumor. This strategy can also be used for preparing a tumor specific vaccine (Fang et al. 2014).

6.3 UPCONVERTING NANOPARTICLES

Upconversion refers to the optical process in which there is a sequential absorption of two or more photons of a particular wavelength leading to an emission of light of a wavelength shorter than the excitation wavelength. This process has been recently used in the field of cancer therapeutics as a modification of photodynamic therapy (PDT) as well as in cancer theranostics as a new multifunctional platform for the combined treatment and imaging of cancers. In simple PDT, a biocompatible, light sensitive substance (drug) is administered with or without tumor targeting agents. When light (usually visible light) is shone in such a manner that it is focused only on the tumor, the excitation of the light sensitive substance produces a highly reactive form of oxygen called the singlet oxygen that kills the malignant cancer cells. However, as the visible light has a limited capacity to penetrate the tissue (the maximum penetration being that of red light of 620 to 680 nm), only skin cancers or tumors present just below the skin or external body surface can be treated, whereas deep-seated tumors (those >1 cm deep) do not come under the scope of this treatment. To overcome this

limitation, recently, biocompatible, upconverting nanoparticles (UCNPs) have been synthesized. Near infrared (IR) light, which can penetrate deep into tissues, can be converted into visible light by these nanoparticles. Thus, when these UCNPs are administered along with the photosensitive drug, the deep penetrating near IR, when shone over the tumor area, is converted into visible light (which is needed for activation of the light sensitive drug used for killing cancer cells in PDT), thereby killing cancer cells that would not have been otherwise accessible to the light. Punjabi et al. used a conventional PDT prodrug 5-aminolevulinic acid (ALA) covalently combined with UCNPs, which were capable of treating deep-seated tumors (~1.2 cm deep). Deep penetrating near IR light of around 980 nm wavelength was converted to visible red light by the UCNPs. This visible light was able to activate the prodrug ALA to the active photosensitizer protoporphyrin IX, which subsequently converts triplet oxygen to singlet oxygen, consequently causing cell death (Punjabi et al. 2014). A schematic representation of such a mechanism is shown in Figure 6.1.

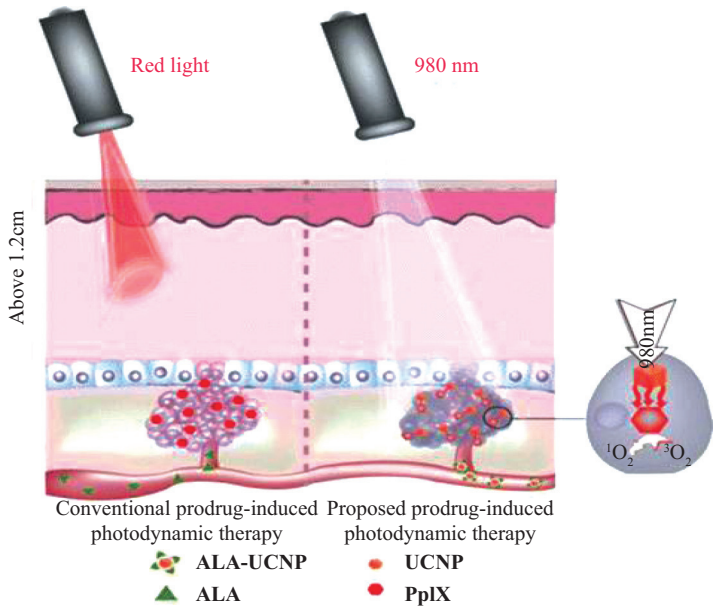

Figure 6.1. Diagrammatic representation of the use of UCNPs in PDT for the treatment of deep-seated tumors.

ALA, 5-aminolevulinic acid; PpIX, protoporphyrin IX; UCNP, upconverting nanoparticles.

Source: Reproduced from Punjabi et al. (2014).

Besides such UCNPs, near IR light responsive nanoparticles such as AuNps, carbon nanotubes, and graphene oxide nanoparticles that show near IR light responsive behavior are also being developed that will enable "on-demand" control of drug release or molecular imaging; that is, they will become functional only when near IR light is shone, thus making it possible to precisely adjust the therapy to the desired site and time (Kim et al. 2015).

The different approved and marketed drug conjugates and nanocarriers for cancer therapy and the various polymeric nanocarriers, polymer–drug conjugates, lipid based nanocarriers, and inorganic nanoparticles that are presently undergoing clinical trials have been listed by Wicki et al. (2015).

6.4 NEED FOR REGULATION

There is no doubt whatsoever that nanotechnology is here to stay. There is not a single area in science and technology that has remained untouched by nanotechnology. The number of consumer products based on nanotechnology is invariably going to increase over time. However, in most cases, nanomaterials are usually disposed in the same manner as any other chemical. In such a scenario, it is important that there should be proper regulation in place as far as the safe manufacture, use, and disposal of these products are concerned. As emphasized in Chapter 5, most of the toxicity studies, especially in the case of nanomedicines, have been carried out on animal models. With more and more nanomedicines being approved by different regulatory authorities, systematic long-term data need to be collected wherever the use of nanoparticles is involved to assess the adverse effects of such materials. Besides this, consequent to increasing approvals being given to nanotechnology based products, it is expected that manufacturing level issues regarding chronic exposure of manufacturing personnel to these materials will also need to be addressed. Efforts in this direction have already begun with a number of governmental agencies becoming involved in the assessment of the environmental and ecological toxicity of nanotechnology based products. A large number of experts have performed in-depth analyses of the environmental and safety issues related to nanotechnology. Financial implications and risks related to occupational hazards posed by nanotechnology and precautionary measures that need to be taken in nanotechnology manufacturing facilities have also been addressed on the basis of actual case studies (Hull and Bowman 2010; Sargent 2011; Schmidt 2009; Youtie et al. 2011).

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Nanoparticles

Preparation and Characterization

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Nanotechnology and nanoparticles have emerged as an important tool towards improving cancer therapeutics and diagnostics. Recognizing the indispensable role of nanoparticles, specifically in targeted delivery of chemotherapeutic and other anti-cancer agents to tumors, this book provides a comprehensive account of the different methods used for the preparation of nanoparticles, including the mechanism behind each method, for a beginner in the field.

The authors describe the commonly used methods of physical post-synthesis characterization, as well as the toxicity aspects of nanoparticles, particularly the effect of nanoparticles on different systems of the human body. Appreciating the interdisciplinary nature of nanotechnology applications in cancer drug delivery, a brief description of the genesis and growth of a tumor has also been included in the book.

Maneesha Pande has a master's degree in pharmaceutics and pharmaceutical technology from Gujarat University, Ahmedabad, India. After having worked in the pharmaceutical industry and academics for about 12 years, she is presently pursuing her doctoral research involving the development of targeted drug delivery for cancer, at the Chemical Engineering Department at Indian Institute of Technology, Delhi, India. She has a book, a book chapter, a review article, and a conference paper to her credit.

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