Nanomaterials Science and Applications

edited by

Deborah Kane Adam Micolich Peter Roger



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Preface

This book reports up-to-the-minute research on nanoparticles for drug delivery and applications in nanomedicine, nanoelectronics and microelectromechanical systems (MEMS) for biosensors, melanin as a nano-based future material, nanostructured materials for solar cell applications, the world of quantum dots illustrated by CdSe, and gas transport and transport-based applications of electrospun nanofibers. The research was primarily undertaken within Australia and gives an excellent overview of topics in advanced nanomaterials and structures, and their applications. The reader also gets a tutorial introduction to the computer software used to generate the 3D illustrations that appear throughout the book.

This is the second book from a project developed and supported by the Australian Nanotechnology Network (ANN; formerly the Australian Research Council Nanotechnology Network). To quote from the ANN website [1]:

The Nanotechnology field is one of the fastest growing areas of research and technology. The Australian Nanotechnology Network (ANN) is dedicated to substantially enhancing Australia's research outcomes in this important field by promoting effective collaborations, exposing researchers to alternative and complementary approaches from other fields, encouraging forums for postgraduate students and early career researchers, increasing nanotechnology infrastructure, enhancing awareness of existing infrastructure, and promoting international links.

One of the key aims of the research network is to support professional and research skill development in postgraduate research



Figure 1 Participants in the book-writing workshop and two of the three editors. From left to right: Gino Putrino, Tristan Clemons, Anna Podolska, Tianyu Yang, Bernard Mostert, Fehmida, Kanodarwala, Dahua Shou, Yang Bai, Deb Kane, and Adam Micolich.

students and early career researchers. This book project was designed and implemented as an innovation in postgraduate/ postdoctoral research skills and networking education. The program and process are described in some detail in the preface to the first book from the project [2]. Briefly, it involved workshopping the writing of the chapters within the community of first-named chapter authors and the editors at a book-writing workshop (see Fig. 1). The workshop supporting this book was held from December 4 to 10, 2013, at Macquarie University in Sydney, Australia [3]. Additional support and training on producing high-quality graphics to visualize the research work being reported in the chapters were undertaken. A guide to using Blender [4] for this purpose is included as Chapter 9 of this book, authored by Dr. Iwan Kelaiah. He trained and supported the workshop participants to use this application to produce figures for their individual book chapters. Some examples of the graphics produced appear on the cover and in Figs. 2-4.



Figure 2



Figure 3



Figure 4 Schematic representation of an AlGaN/GaN sensor array; sensing live human cells for diagnosing diseases and developing new treatments.

Chapters 1 and 2 cover topics on nanoparticles for nanomedicine. Chapter 1 gives a review of nanoparticles developed for drug delivery and then discusses the development and characterization of a multifunctional poly(glycidyl methacrylate) (PGMA) nanoparticle system designed for therapeutic delivery. This system has been researched to deliver a therapeutic peptide for effective use in cardiac ischemia-reperfusion injury. The results have broad applicability beyond the treatment of this condition to a range of disease and injury sites that require rapid cellular delivery of a therapeutic agent. Chapter 2 reports on yolk–shell nanoparticles (YSNs), or "nanorattles," which have a distinct core–void–shell structure. These structures are being researched for delivery of therapeutics and for diagnostic purposes in nanomedicine. Different active medical agents can be delivered by YSNs. These include chemotherapeutic drug and gene delivery, molecular imaging by incorporating different magnetic and fluorescent contrast agents, and photothermal therapy by encapsulating various photoheatconverting agents. The safety and potential toxicity of these advanced nanoparticles are also discussed.

Chapters 3 and 4 report on biosensors that use nanotechnology in their device design and in the principle of sensing. In Chapter 3 the principle of a cantilever functionalized to absorb the molecule/nano-object to be sensed is incorporated into a MEMS device, which has an easy-to-view readout. In Chapter 4 top-down nanotechnology devices—AlGaN/GaN transistors—are engineered into a biosensor for living cells. The biocompatibility, sensitivity, and optimized operational design of these biosensors are discussed, and they emerge as having strong potential for further research.

Chapter 5 details advances in the understanding of the charge transport properties of melanin, an amorphous semiconductor. This is supporting the potential of melanin as a bioelectronic material for use in nanoscale devices where it can act as a transducer of ionic signals to electronic signals. The physical properties of melanin include bistable electrical switching, broadband optical absorbance, water-dependent conductivity, and potential protonic conduction, among others. The improving charge transport model is becoming a tool for nanodevice design and evaluation. In Chapter 6 the methods researched to synthesize, and verify, high-quality crystalline CdSe quantum dots are reported. Also, successful decoration of graphene nanosheets with these quantum dots represents an interesting nanocomposite material with potential for applications.

Applications in energy capture, conversion, and/or storage are among the applications for which nanotechnology and nanoscience research is generating the knowledge to meet future needs. Chapter 7 reviews dye-sensitized solar cells (DSSCs), the third generation of solar cells, which are being considered as a promising alternative to expensive conventional silicon-based photovoltaic devices. Original research on improving the dye uptake ability, efficient charge transfer, and enhanced light harvesting of DSSCs using novel nanostructured designs for the photoanode shows real promise for low-cost solar cells. Chapter 8 introduces the applications of electrospun nanofibers in, for example, filtration, protective clothing, tissue engineering, thermal insulation, and fuel cells. Electrospun nanofibers have significant advantages for gas transport–based applications, but the physical basis of this has been poorly understood. New mechanistic models for the main transport phenomena at the nanoscale are detailed, thus advancing the in-depth understanding of the improved characteristics.

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Chapter 1

The Design and Testing of Multifunctional Nanoparticles for Drug Delivery Applications

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Nanotechnology has the potential to revolutionize the medical profession by improving on traditional drug delivery methods and transforming how disease and injury are currently diagnosed, monitored, and treated. The effective delivery of small-molecule drugs, peptides, and proteins to a site of disease or injury has faced considerable barriers in the past. These include premature clearance from the body, off-site toxicity, and poor bioavailability or pharmacokinetics. Nanoparticles can be used to help improve these characteristics by aiding delivery of therapeutics that otherwise show little efficacy without assisted delivery. This chapter contains

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two separate yet highly related sections. The first section will provide a review and introduction to the field of nanoparticles developed for drug delivery. This introduction will cover a range of different nanoparticle formulations and their associated merits and pitfalls. The second portion will provide an insight into some of our research on the development and characterization of a multifunctional poly(glycidyl methacrylate) (PGMA) nanoparticle system designed for therapeutic delivery. Here we show this multifunctional nanoparticle system and its ability to effectively deliver a therapeutic peptide designed to modulate L-type calcium channel activity following cardiac ischemia-reperfusion injury. These results have broad applicability beyond the treatment of this injury into a range of disease and injury sites that require rapid cellular delivery of an appropriate therapeutic payload. This nanoparticle system provides sound proof-of-concept for peptide delivery ex vivo. With further testing it has the potential to change how we currently treat one of the major contributors to cardiac failure.

1.1 Overview of Nanoparticles in Medicine

1.1.1 Nanoparticles in Modern Medicine

Nanotechnology is characterized by the creation and use of engineered materials or devices that have at least one dimension in the range of 1–100 nm in size [1]. It exploits the physical and chemical properties of nanoparticles, which, as a result of their size, are remarkably different from both atomic species and bulk materials [2]. Since the properties depend on the dimensions of the nanostructure, reliable and continual changes can be achieved by changing the size of single particles. The best example of this is with quantum dots, where altering the size of the quantum dot particle can change the optical properties of the material (Fig. 1.1).

Not only is nanotechnology interesting from a synthetic approach, but this scale also mirrors that of many biological targets and systems. Many proteins, viruses, and important biological molecules are in the size range of 1–10 nm. As a result, structures that can be accurately designed on the nanometer scale have the ability



Figure 1.1 (A) Quantum dots possess unique photophysical properties, making them ideal for applications in biological imaging due to the ability to tune the emission color by altering the quantum dot size (particle size increasing from violet emitters on the left to red emitters on the far right). (B) Narrow emission spectra along with efficient light absorption throughout a wide spectrum of wavelengths make quantum dots suitable for a range of applications, especially in biological imaging. Reprinted from Ref. [5], Copyright (2009), with permission from Elsevier.

to interact on the cellular, subcellular, and molecular levels with unique specificity [1, 3]. This specificity can result in explicit interactions within cells and tissues without causing undesirable side effects [3]. A major field of nanotechnology research is the synthesis of nanoparticles for medical applications, including disease diagnosis, imaging, and most importantly treatment through the delivery of therapeutics. It is envisaged that the global market for nanotechnology-related applications in the medical field could increase to between US\$70 and US\$160 billion by the year 2015 [4].

Nanotechnology and nanoparticle drug delivery vehicles provide an exciting prospect for the delivery of therapeutics in the treatment of a range of diseases and injuries in comparison to current clinical methods [6, 7]. Nanoparticles in particular possess a range of advantages as drug delivery vehicles. These include drug protection from clearance and degradation, high levels of drug loading, the potential for multiple therapeutics to be delivered from the same entity, preferential drug release at target tissues, modifiable drug release kinetics, and, finally, ease of nanoparticle modification for the incorporation of imaging probes that target moieties and surface structure functionalities [8]. This review provides insight into some significant breakthroughs and also highlights some of the challenges still facing engineered nanoparticles designed for drug delivery applications. Following this introduction an application of our own nanoparticle system will be presented in its use as a peptide delivery vehicle in a relevant cardiac ischemia-reperfusion injury model.

1.1.1.1 Nanoparticles for drug delivery

In drug discovery it is easy to find a long list of drug candidates that, although possessing high potency, are unsuitable for clinical application due to poor solubility or poor circulation within the body. Often these candidates have been overlooked in preference for drugs possessing lower potency but better solubility and half-lives [4]. Nanotechnology has the potential to change this by rewriting the rules of drug discovery and improving drug characteristics that were previously seen as limiting or significant enough to warrant rejection [4]. Nanoparticle-based drug delivery systems have been developed to ultimately improve the efficiency of delivery and to reduce systemic toxicity of a wide range of therapeutics. The application of nanoparticles and nanocapsules for drug encapsulation has looked to build on this concept down to the nanoscale. The first generation of nanoparticles developed for drug delivery often only provided one function: drug coating and protection to enhance either drug solubility or circulation time. These nanoparticles are now currently being tested in clinical trials, with some gaining recent approval for clinical applications (Table 1.1) [9]. A wide variety of nanoparticle formulations have been used for drug delivery applications, including liposomes, dendrimers, microemulsions, micelles, solid lipid and polymer nanoparticles, and soluble polymers that have a therapeutic attached via biodegradable linkages (Fig. 1.2). Particles already approved for clinical use include those based on liposomes, biodegradable polymeric nanoparticles,

Brand Name	Composition	Indication	Status
	Liposome-based nanoparticle		
Doxil/Caelyx	PEGylated liposomal doxorubicin	Ovarian cancer, Kaposi's sarcoma	Approved
DaunoXome (Galen)	Liposomal daunorubicin	Kaposi's sarcoma	Approved
Myocet (Sopherion)	Non-PEGylated liposomal doxorubicin	Breast cancer	Approved
	Micelle-based nanoparticle		
Genexol-PM	Paclitaxel-loaded PEG-PLA micelle	Breast cancer, lung cancer	Approved
NK911	Doxorubicin-loaded PEG-pAsp micelle	Various cancers	Phase 2
NK012	SN-38-loaded PEG-Pglu (SN-38) micelle	Breast cancer	Phase 2
NC-6004	Cisplatin-loaded PEG-Pglu micelle	Various cancers	Phase 1
SP1049C	Doxorubicin-loaded pluronic micelle	Gastric cancer	Phase 3
NK105	Paclitaxel-loaded PEG-PAA micelle	Breast cancer	Phase 3
	Polymer-drug conjugate-based nanoparticle	0	
OPAXIO (Cell Therapeutics)	Paclitaxel combined with a polyglutamate polymer	Ovarian cancer	Phase 3
IT-101	Camptothecin conjugated to cyclodextrin-based polymer	Various cancers	Phase 1/2
HPMA-DOX (PK1)	Doxurubicin bound to HPMA	Lung cancer, breast cancer	Phase 2
HPMA-DOX-galactosamine (PK2)	Doxorubicin linked to HPMA bearing galactosamine	Hepatocellular carcinoma	Phase 1/2
CT-2106	Camptothecin poly-l-glutamate conjugate	Various cancers	Phase 1/2
	Albumin-based nanoparticle		
Abraxane	Albumin-bound paclitaxel nanoparticles	Metastatic breast cancer	Approved

Table 1.1 Nontargeted nanonarticles that have been annroved for clinical use or undergoing clinical trials [0]

PLA, poly(l-lactide); pAsp, poly(l-aspartic acid); PEG, poly(ethylene glycol) Pglu, polyglutamate; PAA, poly(l-aspartate); HPMA, N-(2-hydroxypropyl)-methacrylamide-copolymer



Figure 1.2 Schematic representation of some of the main classes of nanoparticle formulations currently being applied in drug delivery applications. Nanocapsules and nanospheres are some of the simplest drug delivery vehicles. Nanocapsules provide an internal cavity suitable for the loading of a drug of interest, whereas nanospheres usually provide drug protection through incorporation within the nanosphere structure. Both of these systems provide suitable platforms for drug release to the intended microenvironment, while offering drug protection from potentially harmful surroundings. Micelles are spherical structures produced from amphiphilic molecules such as detergents. In an aqueous environment the hydrophobic groups are internalized and will group together to form a tight spherical structure with the hydrophilic portion of the molecules on the outside. In a nonaqueous environment the reverse micelle structure will occur. Micelles have found application in the encapsulation of non-water-soluble drugs required for intravenous administration. Dendrimers are highly branched polymeric structures where the 3D branching about a central core can be well controlled. These branches can be easily functionalized to improve targeting or drug-loading characteristics. Liposomes are spherical vesicles comprising one or more lipid bilayers around a central aqueous core. Reprinted from Ref. [11], Copyright (2009), with permission from Elsevier.

and poly(ethylene glycol) (PEG) or protein-based nanoparticle drug conjugates (Table 1.1) [9, 10]. The following section will introduce these major classes of nanoparticles currently being investigated for drug delivery applications.

1.1.1.2 Micelles, liposomes, and dendrimers for drug delivery applications

Micellar nanoparticles consist of a hydrophobic core that is surrounded by amphiphilic block copolymers that have assembled around this hydrophobic core to produce a core/shell architecture in aqueous media [12, 13]. The hydrophobic core region of the micelle acts as a reservoir for hydrophobic drugs; the hydrophilic exterior of the micelle allows for nanoparticle stability in aqueous media [13]. Micelles have the ability to encapsulate a range of therapeutic cargoes, including hydrophobic drugs, oligonucleotides, proteins, and imaging agents with high loading levels (up to 30% w/w) [12, 14]. Micelle nanoparticles have shown great promise as delivery systems, with a number currently in phase 3 clinical trials (Table 1.1). Micelles can also be produced from stimuli-responsive block copolymers to allow disassembly in the presence of triggers such as pH, temperature, light, or ultrasound [15]. This allows for targeted release of the therapeutic payload held within the micelle structure.

A 2012 study by Lee et al. encapsulated the photosensitive Protoporphyrin IX (PpIX) within a pH-responsive micelle based on the block copolymer of PEG-poly(β -amino ester) (Fig. 1.3) [15]. The pH of the microenvironment surrounding tumor tissue is lower (pH 6.4–6.8) than that of normal tissue (pH 7.4) [16, 17]. This reduction in pH allows for protonation of the tertiary amines present in the amino ester, resulting in an increase in the hydrophilicity of the polymer [16]. This change results in rapid de-micellization in the regions surrounding the tumor tissue and leads to the release of the encapsulated photosensitizer PpIX. The strong fluorescent signal of PpIX allows its location, surrounding the tumor microenvironment, to be identified (Fig. 1.3B). Furthermore, when irradiated with the appropriate wavelength of light, PpIX produces cytotoxic singlet oxygen (photodynamic therapy), which in turn destroys nearby tumor cells (Fig. 1.3D).



Figure 1.3 Polymeric micelles for optical imaging and photodynamic therapy. (A) Schematic illustration of PpIX-encapsulated pH-responsive polymeric micelles for tumor diagnosis and photodynamic therapy. (B) Fluorescence images after injection of PpIX-encapsulated pH-responsive polymeric micelles. (C) Ex vivo images of organs and tumors. (D) Tumor growth after injection and laser irradiation. Reproduced from Ref. [15], Copyright (2012), with permission of the Royal Society of Chemistry (http://pubs.rsc.org/en/content/articlelanding/2012/cs/c2cs15261d#!divAbstract).

Similar to micelles, liposomes are closed colloidal structures consisting of an aqueous core surrounded by a phospholipid bilayer, with their main application in the delivery of aqueous biomolecules and hydrophilic drugs [12]. Liposomes have the potential to entrap relatively large amounts of hydrophilic drugs within their aqueous core or between the lipid bilayer shell structure if the therapeutic is lipophilic [14]. A major advantage of liposomes is that they form spontaneously in solution and they essentially possess no inherent toxicity due to the presence of the components of liposomes throughout the body in all cell membranes [12]. Liposomes have had great success in the delivery of anthracycline-based chemotherapeutics, including doxorubicin and daunorubicin, for the treatment of metastatic breast cancer [18, 19], ovarian cancer [18], and AIDS-related Kaposi's sarcoma [20]. An interesting application of liposomes for drug delivery is the utilization of liposomes for the encapsulation and aerosol delivery of the vasoactive intestinal peptide (VIP) for the treatment of various lung diseases such as asthma and pulmonary hypertension. A study from 2008 by Hajos et al. found that encapsulation of the VIP within liposomes was successful in allowing the VIP to avoid enzymatic degradation once inhaled and deposited within the bronchi [21]. This study found that loading of the VIP within the liposomes for inhalation therapy improved the pharmacological and biological activity of the VIP treatment in comparison to the delivery of free VIP [21].

Dendrimers are not nanoparticles per se but more strictly defined as a polymeric macromolecule of nanometer dimensions composed of highly branched monomers that emerge radially from a central core, as shown in Fig. 1.2 [13]. Dendrimers can be biodegradable or nonbiodegradable structures. Natural polymers such as glycogen, and some proteoglycans consist of a dendrimerlike structure. However, for drug delivery, the synthetic polymer poly(amidoamine) (PAMAM) is the most extensively studied [13, 22]. PAMAM has been shown to be effective for the binding and subsequent delivery of cisplatin both in vitro and in vivo where it shows improved efficacy in comparison to cisplatin delivered without the dendrimer [23]. Properties that make dendrimers attractive for drug delivery applications include monodispersed size distributions, modifiable surface chemistry, multivalency, water solubility, and an internal cavity available for drug loading [22]. Due to the ease with which dendrimer surface chemistry can be modified, the addition of contrast agents, imaging probes, and targeting ligands can be coupled with a therapeutic for delivery. This combination of imaging and therapy has led to the production of dendrimer-based multifunctional drug delivery systems [22]. Dendrimers can be produced with low cytotoxicity. Surface decoration of the dendritic structure with PEG can prolong its circulation half-life. Although there is significant interest in dendrimers as drug delivery vehicles, few have translated into clinical trials. Vivagel[®] is the most promising candidate, currently in phase 2 clinical trials [24]. Vivagel[®] is an L-lysine dendrimer that contains a polyanionic outer surface, which exhibits antiviral activity against the sexually transmitted herpes simplex virus (HSV) and the human immunodeficiency virus (HIV) [24].

1.1.1.3 Polymeric nanoparticles and nanocapsules as drug delivery vehicles

Polymeric nanoparticles and nanocapsules are solid formulations ranging in size from 10 to 1000 nm in diameter and can be synthesized from natural or artificial polymers. Generally speaking, the major advantage of polymeric nanosystems over other nanodelivery systems is their inherent stability and structural rigidity [12]. These polymeric nanoparticles often incorporate the therapeutic to be delivered via drugs that are adsorbed, dissolved, entrapped, encapsulated, or covalently linked to the nanoparticle [25, 26]. The most commonly used synthetic materials for the synthesis of biodegradable polymeric nanoparticles are poly(lactic acid) (PLA), poly(D-L-glycolide) (PLG), or the copolymer of these synthetic polymers, poly(lactic-co-glyoclic acid) (PLGA). These are adopted due to their low toxicity, biodegradability, Food and Drug Administration (FDA) approval, and tissue compatibility [26, 27]. Biodegradable nanoparticles based on these aforementioned polymers have been used for the delivery of a range of therapeutics in vivo, for the treatment of cancers [28] and neurodegenerative disorders [29], and for the controlled release of contraceptive steroids and fertility control systems [30, 31].

Nanoparticles synthesized from naturally occurring polymers such as chitosan, albumin, and heparin have been popular choices for the delivery of oligonucleotides, proteins, and small-molecule drugs. Despite significant research in the use of polymers for nanoparticle drug delivery systems, only one, Abraxane, has been approved for clinical applications to date [32]. Abraxane is an albumin-based nanoparticle system developed for the delivery of paclitaxel, a proven chemotherapeutic agent to metastatic breast cancers [32]. Furthermore, Abraxane is currently undergoing clinical trials for delivery to a variety of other cancers, including non-small-cell lung cancer (phase II trial) [33] and advanced nonhematologic malignancies (phase 1 and pharmacokinetics trials) [34].

A number of approaches have been developed for the synthesis of polymeric nanoparticles, most of which involve the use of block copolymers consisting of polymer chains of differing solubilities. The more common techniques for polymeric nanoparticle formulations include layer-by-layer (LbL) approaches, nanoprecipitation (sometimes referred to as the solvent displacement method), emulsification, solvent evaporation methods, and the salting-out method. Further to these traditional methods, techniques that make use of microfluidics, supercritical technology, and the premix membrane emulsification method are increasingly favored due to their potential for producing highly monodispersed nanoparticles in high yields [27]. Usually, the choice of nanoparticle formulation method is dictated by the physicochemical properties of the drug, the polymer intended for encapsulation, and particle size requirements [8].

1.1.2 Nanoparticle and Cell Interactions

In addition to accurate synthesis and drug loading, another integral consideration for nanoparticles developed for drug delivery is how they interact with biological systems. For drugs with intracellular targets, often the cell membrane can loom as a formidable barrier. The concept of nanoparticles, which can be tailored to carry these drugs across the cell membrane and to relevant subcellular compartments, provides an attractive means to achieve improved pharmaceutical transport. Proof-of-concept studies in the 1970s have shown that submicron-sized liposomes [35], as well as synthetic polymer nanoparticles [36], were able to deliver and concentrate in cells therapeutics that previously were unable to do so on their own. The plasma membrane is the barrier that protects the cell against unwanted intruders such as pathogens, macromolecules, and even nanoparticles from entering the cell from the extracellular space [37]. It consists of a self-assembled bilayer of lipids where the hydrophobic interior of this layer is responsible for restricting the passage of water-soluble substances into the cell. Although the passage of small molecules, amino acids, and ions



Figure 1.4 Pathways of entry into cells. Large particles can be internalized by phagocytosis, whereas fluid uptake occurs by macropinocytosis. Both processes appear to be triggered by and are dependent on actin-mediated remodeling of the plasma membrane at a large scale. Compared to the other endocytotic pathways, the size of the intracellular vesicles formed by phagocytosis and macropinocytosis is much larger. Numerous cargoes can be endocytosed by mechanisms that are independent of the coat protein clathrin and the fission GTPase, dynamin. Most internalized cargoes are delivered to the early endosome via vesicular (clathrin- or caveolincoated vesicles) or tubular intermediates (known as clathrin- and dynaminindependent carriers [CLIC]) that are derived from the plasma membrane. Some pathways may first traffic to intermediate compartments, such as the caveosome or glycosyl phosphatidylinositol-anchored protein-enriched early endosomal compartments (GEEC), en route to the early endosome. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Ref. [43]), copyright (2007).

occurs through specialized membrane protein pumps and selected ion channels on the cell surface, the majority of nanoparticles must undergo some form of membrane interaction before the process of endocytosis can occur [38]. Endocytosis can occur through a range of mechanisms (Fig. 1.4) that can be broadly categorized into either phagocytosis (cell "eating" for solid particles) or nonphagocytic pathways (cell "drinking" processes) [38, 39]. With reference to nanoparticles, however, these classical references of cell eating and drinking are not as relevant due to the ability of solid nanoparticles to still be internalized through nonphagocytic pathways [40]. It is important to have an understanding of the relevant pathways of cell entry that could act on or affect nanoparticle uptake, as this will have direct effects on the drug physicochemical characteristics as well as the intracellular fate of the nanoparticle carrier and in turn its therapeutic cargo [40].

Phagocytosis for the internalization of macromolecules and indeed most nanoparticles occurs primarily in specialized cells known as phagocytes, which include macrophages, monocytes, neutrophils, astrocytes, and dendritic cells [41]. Phagocytosis can be described as a general three-step process. An important first step is recognition of the nanoparticle by opsonin proteins in the bloodstream to tag the nanoparticle for phagocytosis. Secondly, this signaling triggers the plasma membrane to form an invagination preparing for the nanoparticle to be internalized, and finally the plasma membrane will pinch off from the surrounding plasma membrane to engulf the nanoparticle, producing a discrete package bound by plasma membrane proteins within the cell (Fig. 1.4) [39, 42, 43]. The internalized vesicle, known as a phagosome, is trafficked within the cytoplasm until it becomes accessible to early endosomes. The phagosome then begins to acidify and matures, fusing with late endosomes and finally lysosomes to form a phagolysosome [41]. The speed with which this process occurs is highly dependent on the particle and its surface characteristics, but typically the process can take from minutes to hours [41].

Phagolysosomes become acidified due to the proton pump ATPase located in the membrane of the phagolysosome; the recruitment of an enzyme cocktail to aid in the degradation of the foreign body also occurs at this time [44]. Although a minimum size of 0.5 μ m is often considered the limit for phagocytosis, previous studies have shown nanoparticles ranging from 250 nm to 3 μ m in diameter can undergo in vitro phagocytosis [40]. Careful control of the nanoparticle surface coating and nanoparticle size can play important roles in producing nanoparticles that can avoid phagocytic uptake [45]. It is generally accepted however that the in vivo fate of nanoparticles is to be opsonized, that is, marked for phagocyte removal, and in turn phagocytosed with little discrimination for nanoparticle composition. This occurs unless the particles are very small in size, that is, less than 100 nm, or, more

importantly, possess a specific hydrophilic coating such as PEG to aid in the avoidance of opsonin recognition [40].

Nonphagocytic pathways, normally referred to as pinocytosis, are not restricted to specialized cells and contain processes that are used by almost all cells for the internalization of fluids and solutes alike. Nonphagocytic uptake into cells can occur through four main mechanisms: clathrin-mediated endocytosis, caveolaemediated endocytosis, micropinocytosis, and other clathrin- and caveolae-independent processes (Fig. 1.4) [40, 43]. Clathrinmediated endocytosis, the most common mechanism for uptake, results in trafficking of cargoes into the lysosomal pathway for biodegradation [42]. Conversely, caveolae-mediated uptake has been shown to produce caveolar vesicles that do not contain a degradative enzymatic cocktail, and hence caveolae-dependent uptake is seen as a mechanism that, if targeted, could avoid trafficking of nanoparticles to the degradative lysosomal pathway [45]. A third process known as macropinocytosis is where actinderived protrusions from the cell membrane can engulf cargoes, upon which the protrusions collapse to again fuse with the cell membrane. These macropinosome cavities containing their entrapped cargo will often fuse with lysosomes, which in turn acidify for the degradation of the payload [40]. By having a better understanding of the variety of internalization pathways by which nanoparticles can be internalized, a clearer understanding will be gained as to what kind of environment nanoparticles may be exposed to once they are internalized. This information is important, for example, when developing new nanoparticles with site-specific drug release capabilities or biodegradation qualities or if the nanoparticle vehicle is engineered with specific escape mechanisms to avoid degradation in endosomes [46, 47].

1.1.2.1 Nanoparticle endocytosis

Nanoparticle size, shape, and relative hardness can dictate which endocytosis pathway is activated and utilized for nanoparticle uptake. A study in 2004 by Rejman et al. investigated the internalization of uniform spherical polystyrene nanoparticles of differing sizes in murine melanoma cells (B16-F10) [45]. This study demonstrated that polystyrene spherical nanoparticles with diameters of 50 and 100 nm were rapidly internalized in less than 30 minutes by a clathrin-mediated pathway [45]. In comparison, larger nanoparticles (200 and 500 nm in diameter), also made from polystyrene, were internalized much more slowly (2–3 h) and exhibit an 8–10-fold decrease in internalization when compared to the smaller particles [45].

The shape of nanoparticles has also been recently investigated to see the role it plays on nanoparticle internalization. Gratton et al. in 2008 investigated the internalization of a series of nanoparticles in HeLa cells where the nanoparticles were fabricated to have differing aspect ratios [48]. High-aspect-ratio rod-shaped nanoparticles were internalized in HeLa cells at a greater rate than spherical nanoparticles of a similar internal volume, a phenomenon similar to that of the appreciable increase seen in the uptake of rodshaped bacteria in nonphagocytic cell lines [48]. Even nanoparticle hardness can influence the interactions of nanoparticles with the cell membrane and in turn can have a direct influence over cell internalization. In 2009 a study by Banquy et al. investigated the internalization of similar particles of differing hardness, that is, Young's modulus [49]. This study found that 150 nm hydrogel nanoparticles with an intermediate Young's modulus (35 and 136 kPa) were internalized by a range of different mechanisms in macrophages, whereas softer nanoparticles (150 nm diameter hydrogel nanoparticles, 18 kPa) were preferentially internalized by macropinocytosis and stiffer nanoparticles (150 nm diameter hydrogel nanoparticles, 211 kPa) via clathrin-mediated endocytosis [49]. Further to this, these nanoparticles with intermediate hardness experienced approximately 67% higher internalization than softer nanoparticles and approximately 25% higher internalization in comparison to the harder nanoparticles [49].

It is evident that size, shape, and hardness can affect nanoparticle endocytosis, but another important characteristic is that of nanoparticle surface charge. The surface charge of nanoparticles plays an integral role in determining by which endocytosis pathway nanoparticles are internalized [39]. Positively charged nanoparticles are the most efficient at plasma membrane interactions and in turn internalization as they interact favorably with the negatively charged residues present on the cell surface [39]. Nonetheless, uptake of nanoparticles with negative surface charges has also been observed despite the unfavorable electrostatic interactions that occur between the nanoparticles and the negatively charged cell membrane [39, 50]. For example, a study in 2008 by Harush-Frenkel et al. investigating the internalization of cationic and anionic nanoparticles in epithelial Madin-Darby canine kidney cells found that cationic nanoparticles experienced rapid uptake, while the anionic nanoparticles, although at a slower rate, still experienced effective cellular internalization [50]. The majority of both nanoparticle formulations was targeted mainly to the clathrin-dependent endocytosis pathways, with a small proportion of both formulations experiencing macropinocytosis-dependent uptake [50]. Further studies from the same group investigated a similar effect in HeLa cells, where it was determined that the cationic nanoparticles once again experienced rapid clathrin-dependent uptake compared to the anionic nanoparticles, being internalized more slowly by a different endocytosis pathway [51].

1.1.2.2 Strategies to enhance cellular internalization

Poly(ethyleneimine) (PEI) is a synthetic polycation well known for its long history as a nonviral transfection agent. Studies have used PEI for intracellular delivery of a range of cargoes, including nanoparticles, proteins, and small-molecule drugs [52-55]. PEI is also suitable for the delivery of short interfering RNA (siRNA) and DNA due to the ability of this positively charged polymer to condense around oligonucleotides, thus enabling transfection of the anionic cell membrane [56–59]. PEI can promote and facilitate endosomal escape due to its strong buffering characteristics in what is referred to as the "proton sponge" effect [52, 53]. After endocytosis, the natural acidification within the endosome protonates PEI, inducing chloride ion influx, osmotic swelling, and destabilization of the vesicle, resulting in the nanoparticles being released into the cytoplasm [60, 61]. The major downfall associated with PEI as a nonviral vector is its inherent toxicity, which has been shown to scale with its molecular weight and transfection efficiency [53].

Another method other than cationic polymers that has had success in transfecting therapeutic cargoes and nanoparticles across cellular membranes is the incorporation of cell-penetrating peptides (CPPs). CPPs are short cationic peptide sequences that were first discovered by investigating the ability of the HIV trans-activator of transcription protein to penetrate cells and subsequently effectively deliver the HIV-1-specific genes [62]. There is a broad spectrum of CPPs available, with most consisting of fewer than 20 amino acids, the most common of these being the *trans*-activator of transcription (TAT), or, as it is more commonly known, the TAT peptide [37]. TAT is an 11-residue-long peptide taken from the protein transduction domain of the HIV-1 TAT protein; this domain is responsible for viral transfection [63]. The TAT peptide is rich in arginine and lysine residues, making it a highly positively charged, basic, and hydrophilic peptide suitable for attachment to the anionic cell membrane and in turn subsequent internalization [63]. The TAT peptide sequence has also been widely used to improve the cellular delivery of a plethora of molecular cargoes, from small-molecule drugs to large proteins and nanoparticles [64].

The exact mechanism for TAT transfection is still an area of debate and contradicting theories. However, most studies agree on the importance of direct contact between the TAT peptide and the negative residues on the cell surface as a preliminary requirement for successful transfection to occur [63–66]. Despite this agreement, TAT-mediated therapeutic delivery still has some major drawbacks yet to be fully addressed. These include the nonspecificity of the current TAT sequence, as well as the associated social stigma surrounding its use due to the origins of this sequence in the debilitating and currently incurable HIV infection [67]. Finally, and just as important, is the possible immunogenicity of the TAT delivery system. It has been speculated that TAT, especially through repeated dosing, could produce a significant immunogenic response, thus limiting its clinical applications, an issue yet to be examined further [62].

1.1.3 Multifunctional Nanoparticles

A major downside to the nanoparticles that are currently being investigated for clinical trials (Table 1.1) is their ability to perform



Figure 1.5 Multifunctional nanoparticles for drug delivery. Multifunctional nanocarriers can be developed to include and combine a range of functions. Some of these functions will include targeting agents (usually an antibody or a peptide), imaging agents (such as fluorescent dyes, quantum dots, or magnetic nanoparticles), a cell-penetrating agent to aid in cellular uptake (e.g., the polyArg peptide, TAT), a stimulus-sensitive element to aid in drug release (i.e., pH or a photosensitive polymer), and a stabilizing polymer to ensure biocompatibility (with the most common of these being PEG or PEG derivatives). The above functions combined within a solitary delivery vehicle along with one or multiple therapeutics provide exciting prospects for drug delivery technology. Development of novel strategies for controlled release of drugs as well as timed release will provide nanoparticles with the capability to deliver two or more therapeutic agents on differing time scales. Reprinted from Ref. [68], Copyright (2008), with permission from Elsevier.

only one primary role as delivery agents. More recent work has resulted in the production of multifunctional nanoparticles for drug delivery that aim to achieve combinations including imaging probes, high drug loading, modifiable drug release kinetics, drug release triggers, targeting ligands (such as antibodies, proteins, and peptides), and nanoparticle coatings to improve circulation times. Figure 1.5 provides a schematic of such a nanoparticle, conveying broadly the different aspects that scientists may look to incorporate into a multifunctional nanoparticle system [68]. Research in this field has resulted in a plethora of nanoparticle formulations and combinations of functions being presented within the literature.

A 2012 study by Zhou et al. describes an octafunctional nanoparticle suitable for the delivery of siRNA to tumors for RNA interference (RNAi) [69]. The octafunctional nanoparticle included (1) a biodegradable PLGA polymer matrix for controlled release; the core of the particle contained (2) siRNA for gene knockdown; (3) an agent to facilitate endosomal escape; (4) an agent to enhance siRNA potency, with the nanoparticle surface containing a range of functionalities, including (5) the attachment of a CPP; (6) a peptide to aid in endosomal escape; (7) a tumor homing peptide; and finally (8) PEGylation of the surface to improve circulation time [69]. It is important to realize, however, that the addition of extra functions results in increases in the cost and time associated with production and purification, as well as the complexity of the nanoparticle system. As a result, there is an ongoing battle in evaluating the benefits of added functionalities versus the extra cost of adding those functions in these multicomponent nanoparticle systems. The following sections will address some of the key considerations and functionalities currently being investigated in the application of multifunctional nanoparticles for drug delivery applications, paying special attention to some pivotal examples making use of these additions.

1.1.3.1 Passive vs. targeted nanoparticles: Surface functionalities of multifunctional nanoparticles

Probably the most significant effort, following on from firstgeneration nanoparticles for drug delivery, is that of nanoparticle targeting. Targeting can be achieved by two main avenues. The first is passive targeting, that is, nanoparticle targeting resulting from disease pathophysiology. The second is active targeting, where targeting ligands and moieties are added to the nanoparticles to produce preferential nanoparticle binding and at times cellular uptake in target tissue [70]. Nanoparticles produced for the treatment of malignant tumors and cancers can be considered to be targeting tumor tissue through a passive process known as the enhanced permeability and retention (EPR) effect (Fig. 1.6) [71]. This is an effect directly due to the leakiness of tumor vasculature combined with poor lymphatic drainage and the high fluid flow



Figure 1.6 Schematic representation of nanoparticle active and passive targeting via the EPR effect. The schematic demonstrates the increased "leaky" vasculature consistent with the rich vascular network of a tumor in comparison to that of normal healthy tissue. Reprinted with permission from from Ref. [78]. Copyright © 2010, John Wiley & Sons, Inc.

often seen with many solid tumors [10]. Tumor vasculature enables nanoparticles to accumulate within tumor tissue without the addition of specific targeting moieties to the nanoparticle surface for tumor recognition [72]. Animal studies have shown that a 50-fold increase in nanoparticle accumulation can be achieved through this passive process when compared to healthy tissue [73]. Hence, to optimize uptake due to the passive process of the