CHAPTER

16

Therapeutic Applications

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OUTLINE

Adverse Effects of Engineered Nanomaterials
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INTRODUCTION

Nanomedicine is the application of nanotechnology in medicine and is one of the most extensive and promising subdisciplines of research and development efforts at the nanoscale. Innovative engineered nanomaterials (ENM) constitute an essential component of this emerging field. Well-designed ENM have indeed the potential to overcome the limitations of current medicines (e.g. efficiency, specificity) and, thus, advance disease diagnosis and treatment.

In this chapter, we do not aim to offer an extensive survey of all the ENM developed for therapeutic applications so far, but hope to provide some pertinent examples of nanomaterials that are currently in use in the clinic or are in clinical trials, and others that have demonstrated promise for various biomedical applications but are still under preclinical development. The therapeutic potential and challenges offered by different types of nanomaterials, including nanoparticles, polymer-conjugates, polymerosomes, dendrimers and carbon nanotubes are therefore presented in the following sections.

One of the main and transversal applications of the selected ENM is their use as drug nanovectors in cancer intervention, with the aim to provide a more efficient and controllable delivery of chemotherapeutics compared to more common drug formulations. As knowledge and control of the different physical, chemical and biological properties of these nanoscale materials becomes more advanced, their promise as future therapeutic and diagnostic opportunities to fight cancer or other diseases becomes increasingly more realistic.

NANOMATERIALS FOR THERAPEUTIC APPLICATIONS

Nanoparticles

Nanoparticles display unique physical and chemical properties due to their size. Also within this range are antibodies, receptors, nucleic acids, proteins and other biological macromolecules. Biomimetic features as well as an extraordinary surface to volume ratio and the easy manipulation of properties are attractive tools for the field of medicine, particularly for imaging, diagnosis and therapy [1,2]. In the nano-sized range, agents which are only slightly soluble or even unstable can access cells by transportation [3]. Therefore, a multitude of different macromolecular agents, including antitumor drugs, radiotherapeutic nuclides, genetic material and antibodies, can use the nano-sized vehicle for focused delivery (Figure 16.1) [4]. Thus, substances with a high therapeutic potential can be utilized that

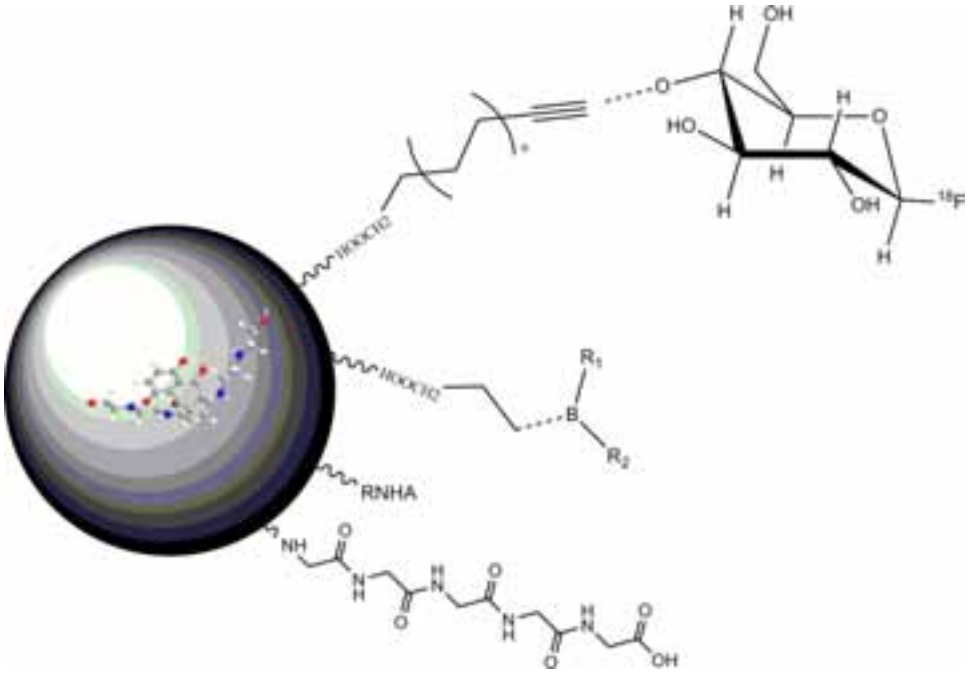

FIGURE 16.1 Schematic drawing of a therapeutic nanoparticle. Ideal model of therapeutic magnetic nanoparticles with multifunctional surface coating. As displayed there are imaging agents, substances for neutron capture therapy, antibodies and aptameres.

may have been disregarded previously because of their high systemic toxicity or because of metabolic barriers. For oncology purposes, this would mean a maximum level of antineoplastic agent reaching the tumor area with minimal levels in healthy tissue.

Nanoparticle Drug Delivery System Design

Nanoparticle systems were first used as pure vehicles for carrying drugs effectively over metabolic barriers. Current research focuses on nanoparticle systems able to release the agents in a controlled way in the targeted area. The release occurs by alteration of the physical and chemical parameters in the area of interest. Transportation and controlled release of therapeutic agents using nano- and micro-carrier systems in the target area of interest is a promising approach in current medicine [4,5]. For example, the specific attributes of diseased tissues can be exploited [6,7]: inflamed tissue can be differentiated from healthy tissue by the lower pH-value, increased temperature and a higher permeability of vessels [8]. These basic facts can be used for "targeting" with temperature- and pH-responsive nanoparticles [9,10].

Nanoparticle Drug Delivery

Most of the thermo-responsive systems are based on polymer-coated, drug-loaded nanoparticles and aggregates of them. Shen et al. developed temperature-sensitive

polymer-conjugated albumine nanospheres, which were co-polymerized with a poly(N-isopropylacrylamid) moiety [11]. In this case, the release occurs by the increased temperature rupturing the interspaces of the polymer network on the outside of the nanoparticles. As a consequence, the nanospheres can be attacked and hydrolyzed easily by trypsin. Poly(N-isopropylacrylamid) is well known for facile alterations of conformation at a temperature level of about 32°C in aqueous media and is, therefore, often used as co-block polymer for thermolabile surfaces [12]. Defined profiles for release on demand can be achieved by connecting alternative polymers featuring different conditions for switching their conformation. Due to the multivalent properties of the surrounding polymer network temperature- and pH-responsive release systems are possible [10,13]. Nevertheless, the thermosensitive polymer system lacked sufficiently differentiated thresholds so the difference in the rate of drug release was only marginal. A different approach was undertaken by Stover et al. in which ceramides triggering apoptosis were used as effective anti-cancer agents [14]. Usually, ceramides barely penetrate the cell membrane and can be easily degraded. Nanoparticles with an incorporated thermoresponsive poly(N-isopropylacrylamid) moiety changed the whole polymer conformation above a threshold temperature. Due to the increased hydrophobicity, the particles could then cross the plasma membrane, become hydrolyzed and deliver the ceramide. Another concept under development is the use of light absorption to trigger drug release: the absorption of light induces heat, which leads to the bond between the drug and coating substance breaking [15]. This type of defined bond breaking enables an abrupt release of relatively large amounts of therapeutic substances and offers a novel way to progress drug release systems.

Theranostic Applications of Magnetic Nanoparticles

Magnetic iron oxide nanoparticles have already been introduced into medical fields. For diagnostic purposes, nanoparticles are used for magnetic cell separation whereby antibodies featuring a specific affinity are attached to particle surfaces. Hence, cells, DNA or bacteria can be separated magnetically. This technique is useful to verify the therapeutic effect after chemotherapeutic treatment [16]. Colloidal magnetic iron oxide particles (ferrofluid) are already used as contrast agents for magnetic resonance imaging (MRI) [17,18]. Commercially available contrast media are in use such as Resovist®, Endorem®, Sinerem® and Combidex®. Magnetite and maghemite particles are most commonly used in medicine and are generally well tolerated [19]. (Magnetic nanoparticles in clinical use and development for imaging, diagnosis and therapy are discussed also in Chapter 8 [see Table 8.1] and Chapter 15.) Their magnetic properties are activated during magnetic field application only.

An important parameter for *in vivo* application is the size of the particles. The magnetic force affecting the particles strongly depends on the radius ($\propto r^3$). Particles of a size of about 100 nm appear to be particularly useful since larger particles can be easily engulfed by MPS cells (mononuclear phagocyte system) and accumulate in the liver and spleen, especially following systemic (intravenous) application [20]. Furthermore, larger particles have a greater tendency to lead to vascular obliterations than smaller particles [21]. Alksne et al. reported one of the first clinical trials of magnetic particles in 1966 based on the use of ferromagnetic iron to close intracranial aneurysms [22]. Furthermore, magnetic albumine-microspheres have been used as a vehicle to carry the cytostatic drug doxorubicin to cause tumor remissions in rats [23]. Carbon-coated iron oxide particles carrying doxorubicin have also been developed for local chemotherapy. These particles (MTC-Dox, Ferex, USA) were applied intravascularly

followed by the application of an external magnetic field in a phase I/II multicenter study on patients with hepatocellular carcinoma [24]. Previously, Lübbe et al. performed a novel human trial (phase I/II) with starch-coated magnetic nanoparticles whereby epirubicin was ionically bound to the particles. The formulation was applied intravenously and was well tolerated [25]. For magnetic drug targeting by intra-arterial administration, a sufficient particle size (80–150 nm) is required to attract the magnetic nanoparticles by an external magnetic field [26,27]. This type of application is advantageous as the particles accumulate in the desired area and by-pass the metabolic barriers of the MPS. Besides the particle size, the charge and chemical features are relevant. For *in vivo* applications, nanoparticles are usually coated for colloidal stability and to prevent segregation into particles and carrier medium [20,28]. A collapse of the colloidal stability would result in a thrombus formation. Figure 16.2 exhibits the principle of magnetic drug targeting (MDT).

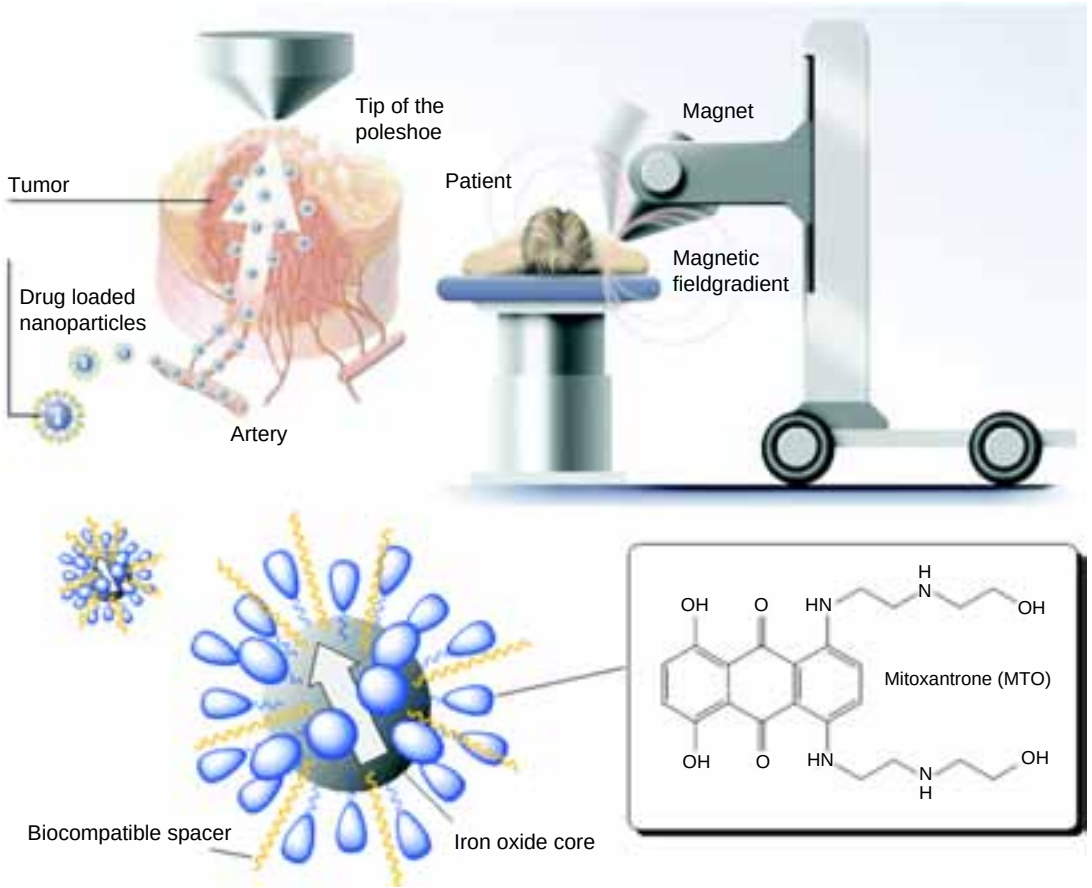

FIGURE 16.2 Principle of magnetic drug targeting. Magnetic particles that are functionalized with therapeutic agents are injected intra-arterially close to the tumor. An electron magnet positioned at the tumoral area directs and attracts the iron oxide particles in the tumor vascular system.

In animal studies, ferrofluids were injected intra-arterially into the tumor-supplying vessel (arteria femoralis) and a strong external magnetic field was applied simultaneously over a VX2-squamous cell carcinoma placed at the hind limb. An accumulation of ferrofluids could be demonstrated by histology (Figure 16.3), MRI and computed-micro-tomography (μCT)[29–32], without pathological alterations to the liver, kidneys, spleen, lung and brain. Metabolic decomposition of the ferrofluids proceeds in the liver and spleen in analogy to the physiological iron metabolism. In these organs, ferrofluids could be detected histologically for 3 months post-application and radiotracer studies using ^{59}Fe showed that iron arising from ferrofluids is embedded into the hemoglobin biosynthesis. Furthermore, these studies produced quantitative results showing a 114-fold higher enrichment of iron oxide nanoparticles per gram tissue due to the application of an external magnetic field (versus no external magnetic field) [26,33]. In a therapeutic study, the intra-arterial application into the arteria femoralis of rabbits transplanted with a VX2-squamous cell carcinoma was performed. Therefore, ferrofluids loaded with the chemotherapeutic agent mitoxantrone were used. It was shown that 20% of the systemic dose (regular systemic dose=10 mg/m^2 equates to 100%) was sufficient for complete tumor remission without any side effects and the cellular uptake of iron oxide particles was also shown [34,35]. Furthermore, electron microscopy

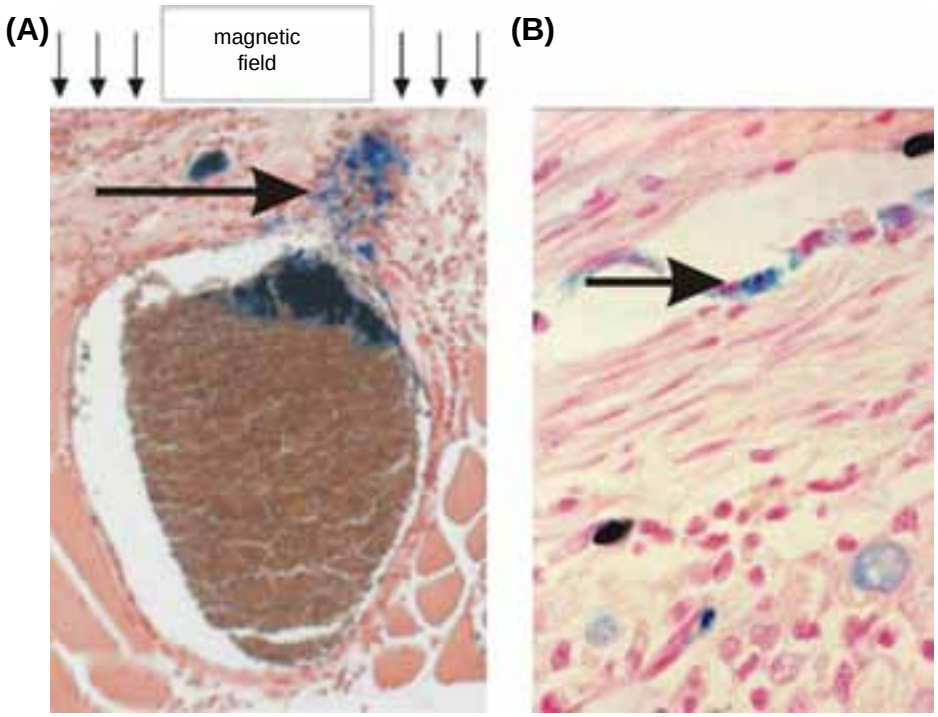

FIGURE 16.3 Particle concentration and infiltration into tumor tissue after MDT. (A) Tumor vessel and extravasation of the particles into the tissue (Magnification: 400×, Prussian blue staining), iron oxide appears dark (arrow). (B) Histological cross-sections of tumor tissue after MDT (Magnification: 1000×, Prussian blue staining), dark-colored iron oxide particle is labeled by an arrow [39].

verified by EDX analysis could visualize the iron oxide spots inside tumor cells, which is evident for the therapeutic effect [35].

Further Development of Nanoparticle Systems in Nanomedicine

By employing a combination of different approaches, different substances (classic drugs as well as non-classic agents like nucleic acids, antibodies etc.) and varying particle surfaces, multifunctional particles can be achieved. Thus, nanoparticles not only offer a platform for therapeutic agents (for a detailed discussion, see Chapter 15), but by binding a linker for functional imaging purposes (^{18}F-PET), multifunctional "theranostic" particles can be put on the stage [36–38]. Magnetic drug targeting and hyperthermia are emerging as promising and innovative applications in oncology [39] and, in future, could be utilized to achieve focused tumor therapy, increasing the success of a low drug dose and, therefore, reducing side effects.

Polymer Nanoparticles

Nanoparticle therapeutics are based on multifunctional particles, which are capable of extending circulation *in vivo*, targeting specific diseased cells, and releasing the therapeutic payload in a stimuli-responsive manner [40–43]. Each of these functions can often be engineered into the particle independently from other particle attributes. This section details some of the important design parameters in polymer nanoparticle engineering, progress made in both targeting specific cells and encapsulation/release of therapeutics, and demonstrates the promise of these materials in the fight against disease.

Design Parameters

One of the major advantages of polymeric nanoparticles in the targeted delivery of therapeutics is the ability to optimize particle size, modulus, shape, and *in vivo* stability independent of other particle attributes [40]. For example, a common misconception is that the ideal particle size for a drug delivery vector is $\approx$5–150 nm [40], but recent efforts in particle engineering clearly demonstrate that these defined limits apply only to highly rigid, spherical particles [44,45]. In one example, filamentous micelles (filomicelles) with single dimensions as long as 18 μm were shown to circulate for longer than 5 days [44]. In another example, particles designed to mimic red blood cells were fabricated where the particle modulus was varied over a physiologically relevant range [45]. The particles, which were discoid shapes 5.2–5.9 μm in diameter and 1.2–1.5 μm tall, showed widely differing biodistribution and circulation times as the particle modulus was varied from 7.8 to 63.9 kPa. As great as a 30-fold increase in elimination half-life was observed as the particle modulus was lowered and elimination half-life was extended to 93 h.

A variety of matrices can be used in particle fabrication with the most commonly employed being poly(lactic-co-glycolic acid) (PLGA) and biopolymers, such as chitosan and dextran. The choice of matrix determines much of the *in vivo* stability of the particle, as well as the degradation products that ultimately must be cleared from the body [46]. The polymers listed above are generally acknowledged to possess good biocompatibility/biodegradation profiles (although there is an ongoing debate over the definition of biocompatible [47]); however, a recent example of biodegradable acrylate-based particles illustrates the potential of acrylate-based materials in targeted drug delivery [48].

Targeted Drug Delivery

One of the most promising aspects of targeted drug delivery is the ability to target diseased cells specifically in order to reduce the exposure of toxic chemotherapeutics to healthy cells. In cancer applications, passive targeting can be achieved by exploiting the enhanced permeability and retention (EPR) effect [49,50]. Active targeting strategies are also routinely employed in targeted drug delivery and can be classified as either selective or non-selective. Selective targeting strategies rely on surface modification of the particle with ligands that recognize various biological receptors [51,52]. Non-selective targeting strategies target cell surface receptors that are expressed ubiquitously, but that are over-expressed on cancer cells. For example, cancer cells proliferate rapidly and as a result routinely express higher levels of the transferrin and folate receptors. As a result, many targeting strategies employ folate, transferrin, or antibodies to these receptors selectively to target rapidly proliferating cancer cells [53–56]. These receptors are also expressed to a lesser degree on many healthy cells, but selective targeting strategies utilize ligands capable of binding to receptors on targeted cells that are not expressed by other cell types [57].

Encapsulation and Release of Therapeutics

Several methods have been used to achieve encapsulation and release of therapeutics from drug delivery vectors. The majority of methods incorporate stimuli-responsive linkages designed to be cleaved once the delivery vector encounters a specified environment [58–60]. The stimuli-responsive linkages are designed either to destabilize the carrier, causing degradation and release of encapsulated therapeutic, or to cleave covalent bonds linking the therapeutic to the particle, facilitating release. Perhaps the most common approach exploits the pH gradient that occurs as a particle is internalized along an endosomal pathway where the pH drops from $\approx$7.4 to $\approx$5.5. A number of acetal and hydrazone linkages have been successfully employed in this regard in both nanoparticle therapeutics as well as polymer-drug conjugates. Some particle internalization pathways do not involve trafficking through an endosome, so other strategies must be developed for those situations. An approach that has received considerably less attention is the use of the reducing nature of the cytosol to break reductively labile bonds. The incorporation of disulfide cross-linkers has been particularly useful in this regard [61,62].

Targeted Delivery of Cisplatin

The potential benefits of utilizing targeted nanoparticles to deliver therapeutics is exemplified in a recent report detailing the performance of aptamer-targeted, cisplatin prodrug-loaded, poly(D,L-lactic-*co*-glycolic acid)-*b*-poly(ethylene glycol) nanoparticles (Pt-PLGA-*b*-PEG-Apt-NP) in a prostate cancer model [63]. Cisplatin was liberated from the prodrug via the intracellular reduction of the platinum complex (Figure 16.4). The particles were targeted by adding an aptamer targeting ligand (A10) to the surface, which recognizes the extracellular domain of PMSA.

The maximum tolerated dose (MTD) of un-targeted Pt-PLGA-*b*-PEG-NPs was tested in male Sprague Dawley rats and in Swiss Albino mice. In rats, the MTD of platinum contained in Pt-PLGA-*b*-PEG-NPs was twice that of cisplatin administered conventionally (MTDs of Pt-PLGA-*b*-PEG-NPs and cisplatin were 40 and 20 mg/kg, respectively). Similarly, the MTD of Pt contained in Pt-PLGA-*b*-PEG-NPs was higher than that for cisplatin in Swiss Albino mice.

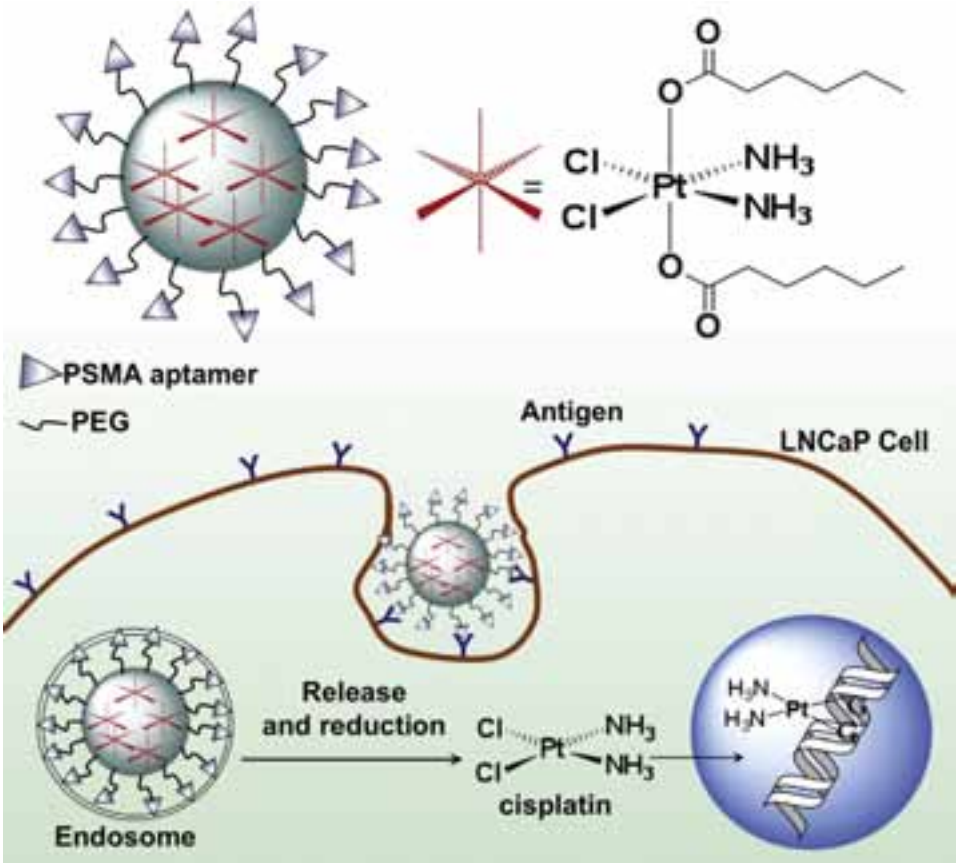

FIGURE 16.4 Schematic representation of the Pt-PLGA-b-PEG-Apt-NP construct. Chemical structure of the Pt(IV) prodrug 1 and intracellular reduction for the release of active cisplatin in PSMA expressing human prostate cancer LNCaP cells after receptor mediated endocytosis of Pt-PLGA-b-PEG-Apt-NP. *(Reproduced with permission from [63].)*

These combined results demonstrate a reduction in the toxicity of cisplatin when incorporated into a prodrug nanoparticle formulation, even in the absence of targeting ligands. It has also been shown that the circulation time of the prodrug is significantly enhanced: cisplatin was cleared rapidly from circulation with only 1.5% of the injected dose remaining after 1 h [64], whereas for the encapsulated cisplatin prodrug greater than 75% of the injected dose remained in the circulation at 1 h. Furthermore, total platinum excretion after 24 h was 17 times less for encapsulated cisplatin prodrug compared to dosing with free cisplatin. Thus, the increased circulation combined with reduced excretion leads to sustained exposure to the active therapeutic at a low dose.

Aptamer targeted Pt-PLGA-*b*-PEG-NPs (Pt-PLGA-*b*-PEG-Apt-NPs) were evaluated for efficacy and tolerability in a LNCaP subcutaneous xenograft mouse model of PCa. Pt-PLGA-*b*-PEG-Apt-NPs were more efficacious than cisplatin over the first 14 days but,

at 28 days, Pt-PLGA-*b*-PEG-Apt-NPs and cisplatin tumor volumes were roughly equivalent. Even though Pt-PLGA-*b*-PEG-Apt-NPs and free cisplatin were comparable in efficacy after 28 days, the amount of cisplatin prodrug contained in Pt-PLGA-*b*-PEG-Apt-NPs was one third that of freely dosed cisplatin. Given the increased efficacy of Pt-PLGA-*b*-PEG-Apt-NPs compared to conventionally dosed cisplatin in a human prostate cancer model, these particles clearly demonstrate the potential of drug delivery vectors.

Hybrid Polymer Vectors—Targeted Nanogels

A recent study combining polymeric materials with liposomes and protein-based particles exploited the favorable properties of each of these vectors [65]. Targeted nanogels combine the ease of synthesis of liposomes and the molecular binding properties of proteins with the stability that can be achieved in polymer cross-linked nanoparticles to produce a stable drug delivery vector. These nanogels employ a cross-linked polymer/protein core for stable drug release with a lipid bilayer surface that can be functionalized with targeting ligands. A variety of therapeutics including natural products, kinase inhibitors, proteasome inhibitors, and chaperone inhibitors could be loaded. Human serum albumin and α1-acid glycoprotein were used in the polymer/protein core because of their known binding properties toward cancer therapeutics in the blood stream. Acrylate-based polymers were used to cross-link the protein core.

Synthesis of nanogels was achieved by adding the desired monomers, proteins, drug molecules, and photo initiator to a lipid film, followed by sonication and then extrusion (Figure 16.5). The protein core was then cross-linked via photo-polymerization of the encapsulated acrylate monomer. The nanogels were then targeted to $\alpha_V\beta3$ integrin by conjugating the cyclic peptide cRGDfK to the surface of the particle. The stability of the nanogels was investigated by measuring the release of the therapeutic in mouse plasma where over 60% of the encapsulated dose of docetaxel was retained in the cross-linked nanogel compared to ≈40% retention in the non-cross-linked analogue. *In vitro* experiments in M21 cells using targeted nanogels showed increased potency of each nanogel composition compared to dosing of the corresponding free drug. In fact, a 20-minute exposure to drug-loaded nanogels was comparable to a 72 h exposure to the respective free drug. The effect of targeting was also investigated *in vitro*, and a 13-fold enhancement in potency was reported for the targeted versus untargeted nanogels.

Taxane-loaded nanogel efficacy was later compared with the FDA-approved taxane formulation of Abraxane in an orthotopic breast cancer model. Abraxane at 1 mg/kg dose showed no tumor suppression at 30 days and was comparable to saline controls in terms of tumor volume (830 mm^3). Targeted nanogels showed significantly slowed tumor growth with a tumor volume of 190 mm^3 at 30 days. In a dose response experiment with Abraxane, a 1 mg/kg dosing of targeted nanogels induced the same level of tumor suppression as a 15 mg/kg dose of Abraxane. Similar improvements in efficacy of nanogels compared to Abraxane were reported in an orthotopic pancreatic cancer model. Furthermore, nanogels showed a 15-fold improvement in reducing metastasis to the hepatic hilar node compared to Abraxane. The efficacy and versatility of this drug delivery platform will undoubtedly lead to the application of nanogels in a variety of other cancer models.

The future is bright for polymer-based drug delivery vectors. Some of the benefits of targeted drug delivery, including increased efficacy and reduced non-specific toxicity, are beginning to be realized. Based on the success of using aptamer-targeted nanoparticles and targeted

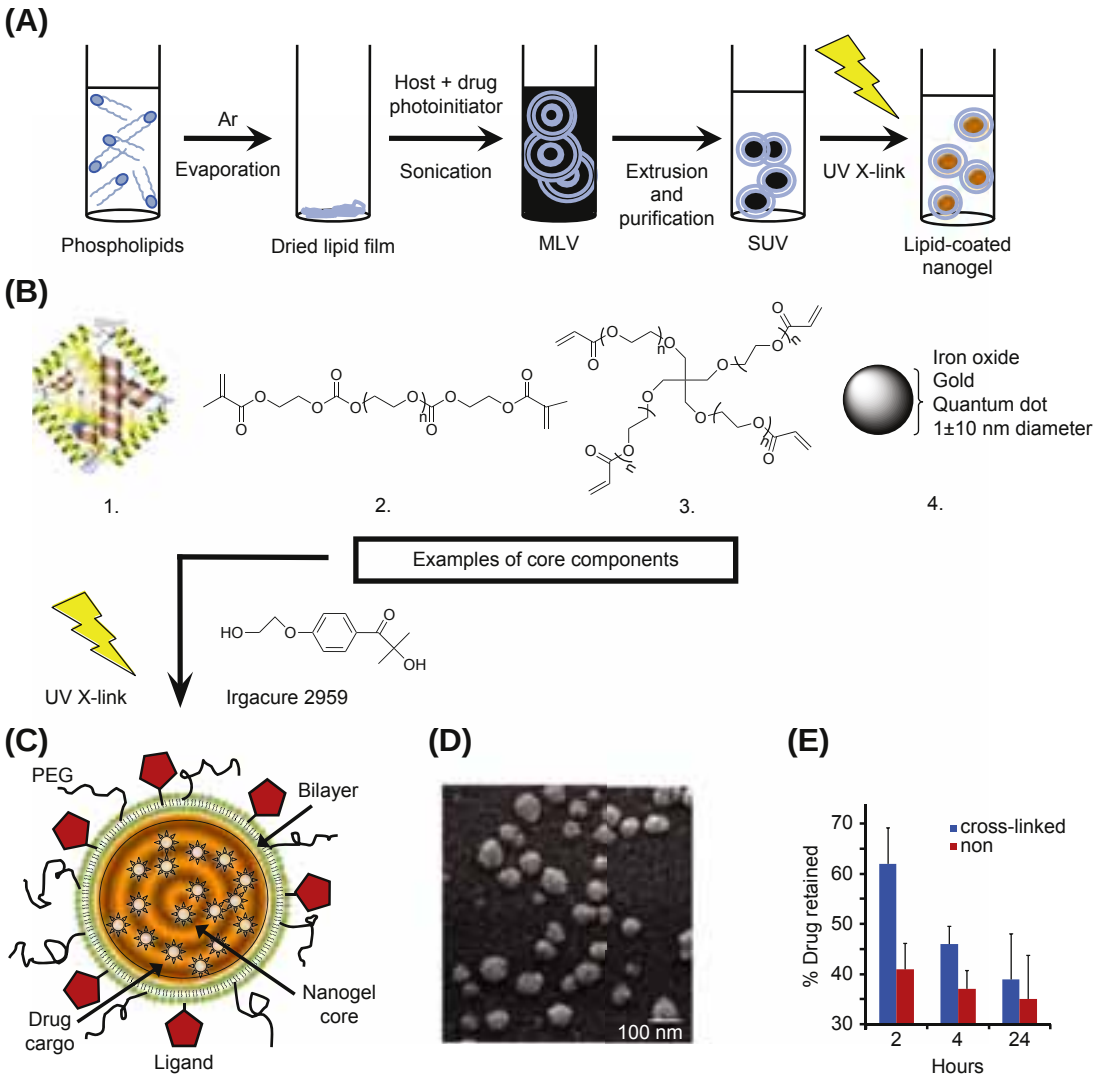

FIGURE 16.5 Formation of lipid-coated nanogels. (A) The method for controlled photo-cross-linking and formation of the nanogel core. (B) Examples of various monomeric inputs and imaging agents that can be used to form the nanogel core are shown. (C) A schematic representation of the final nanogel product with a lipid bilayer, presenting targeting ligands and polymeric coatings surrounding the gel core containing drug cargoes. (D) Scanning electron micrograph of a lipid-coated nanogel. (E) Nanogels with RGD peptide on the surface and docetaxel-loaded HSA in the cross-linked core were incubated in the presence of 20% plasma for the indicated time points. *(Adapted and reprinted by permission from the American Association for Cancer Research: Murphy, E.A., Majeti, B.K., Mukthavaram, R., Acevedo, L.M., Barnes, L., Cheresh, D.A. Targeted nanogels: a versatile platform for drug delivery to tumors*, Molecular Cancer Therapeutics, *2011, 10, 972–982.)*

nanogels loaded with therapeutics in preclinical studies, the potential impact of polymer-based vectors in the pharmaceutical industry is likely to be profound. One of the major obstacles in drug development has long been the poor pharmacokinetics of many small molecule therapeutics. The use of drug delivery vectors, which can themselves be designed to possess the appropriate biodistribution profile, facilitates the decoupling of therapeutic activity with pharmacokinetics. Thus, there will be a significant opportunity to re-examine a variety of potential drug candidates that have failed for reasons of poor pharmacokinetics. Additionally, if it is possible to guide a vector to the desired location *in vivo* and thereby concentrate its payload in target cells, the delivery of cargo that might otherwise be considered benign, such as essential transitions metals like Cu and Fe, may be capable of eliciting unexpected biological responses including targeted cell death via induced oxidative stress [66].

Dendrimers

Because of the precise structure and the extensive possibilities for designing dendrimers with ligands in exact positions in the structure, much work has been focused on applications in biomedical research [67,68]. Dendrimers offer specific advantages over many other classes of nanomaterials for therapeutic applications, such as controlled solubility, many end-groups, exact placement of functional groups, and control over size, shape, and molecular weight of the structure and, therefore, show great potential for the development of therapeutic applications.

Design Principles

Dendrimers represent a very special architectural type of polymeric materials. Dendrimers are synthesized from branched monomers of the type AB_x were x is >2 or higher. These monomer units are layered around a core molecule to build up an onion-like structure where each layer is called a generation. Typically, the synthesis is conducted either from the core and outwards (divergent method) or from the end groups and inwards (convergent method) (Figure 16.6). These growth steps are performed in a controlled manner, often utilizing organic protection/activation chemistry to yield spherically shaped dendrimers with almost perfect structure, and exact molecular weight and size. In this respect, dendrimers are similar to proteins, with controlled exact molecular weights (virtually no polydispersity [PDI] if produced correctly) and globular shapes. The size of the constructs is most often less than 5 nm in diameter, as it is difficult to control the synthesis of dendrimers of higher generations than 5 or 6. Because of the tedious synthetic preparation and purification necessary for generating high molecular weight dendrimers of high quality, they are typically only prepared in small quantities and the commercial prices are set thereafter. In addition to the dendrimer architecture, related cousins have been prepared that stem from the branched structure and the dendritic family now includes subclasses, i.e. dendrimers, dendrons, hyperbranched polymers, dendrigraft polymers and dendritic-linear polymers.

There are numerous examples of dendrimers synthesized in literature, made from virtually any AB_x monomer commercially available. To name a few, Poly(propyleneimine) dendrimers (PPI), Poly(amidoamine) dendrimers (PAMAM), Poly 2,2-bis(methylol)propionic acid (PBisMPA), Poly(benzyl ether) dendrimers (PBzE), poly(lysine) dendrimers (PLL), and polymelamine (triazine) dendrimers. Of these dendrimers the PAMAM, PPI, and

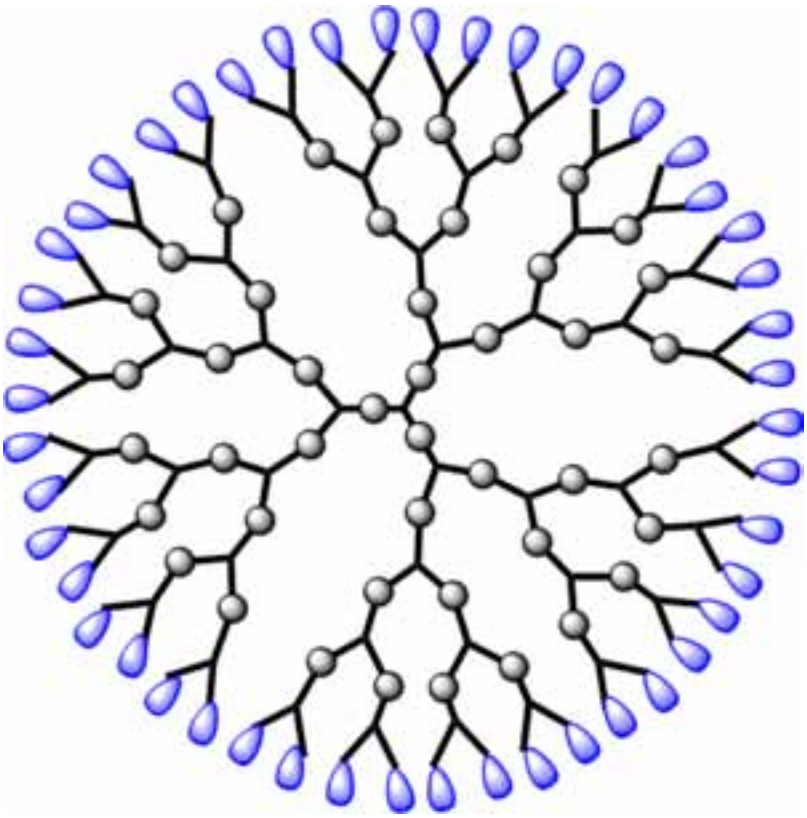

FIGURE 16.6 Schematic structure of a dendrimer.

PBisMPA dendrimers are readily available from large commercial vendors (some others are also produced on demand from other small companies). Here we will focus our attention on the most commonly used dendrimers in therapeutic applications, such as the PAMAM, PBisMPA, PPI and to some extent Poly(lysine) class, that have been evaluated in detail in animal experiments (Figure 16.7). Figure 16.7 depicts, from left a PAMAM dendrimer, PPI dendrimer and to the right a PBisMPA dendrimer.

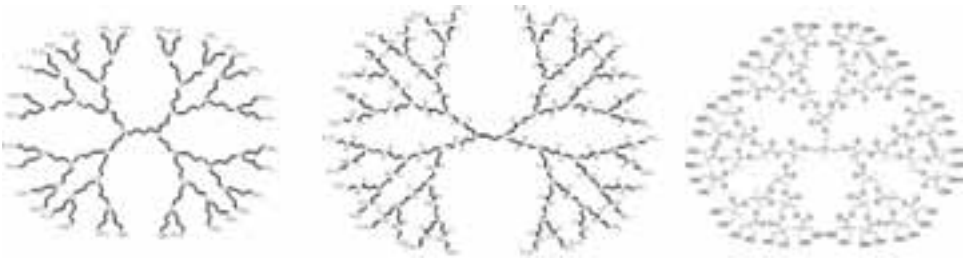

FIGURE 16.7 Commonly used dendrimers in biomedical applications.

Drug Delivery with Dendrimers

Up to now, most therapeutic applications of dendrimers have been focused on two topics, imaging and drug delivery. The examples in this chapter will focus on the drug delivery applications of dendrimers. Dendrimers are exact nano-sized molecules, therefore, they can be systematically controlled by size, surface and conformation, resulting in biodistribution and pharmacokinetic properties that can be precisely evaluated [69]. Similarly, the reproducibility and extremely low size distribution of these constructs in comparison to self-assembled systems, such as polymer micelles, can be a regulatory advantage [69]. Other factors that contribute to this interest is the high functional group density at the dendrimer surface that can be utilized for targeting applications and/or multivalent interactions, and the ability of dendrimer structures to be designed for controlled degradation. Moreover, unlike polymer micelles and liposomes that are assembled via hydrophobic interactions, dendrimers do not have a critical micelle concentration (CMC). This means that dendrimers used in systemic circulation applications cannot disassemble upon dilution. This avoids the burst release effect (very rapid release of drug cargo) often encountered with liposomes or polymer micelles which can be a major drawback [70–72].

However, dendrimers have other drawbacks such as their very small size. Dendrimers are typically less than 10 nm in diameter, and this small size often results in their very rapid renal excretion. This counteracts the effect of reaching a therapeutic concentration in blood, so, like most nanoparticles, dendrimers are usually equipped with polyethylene glycol chains to increase their size and therefore enhance their blood circulation time and reduce RES activation by providing a stealth surface. The other major drawback with dendrimers in therapeutic applications is related to the cost and scalability of manufacturing high generation dendrimers. So far, most studies have been conducted on a sufficiently small scale and this factor has not become an issue, but as the research progresses towards clinical testing this will be major challenge.

As with other nanoscale drug delivery agents, dendrimers have been explored in two major directions when it comes to drug loading: as passive drug delivery agents where the drug is encapsulated within the dendrimers framework via favorable drug–dendrimer interactions, or as covalent drug carriers with the pharmaceutical linked to the dendritic scaffold. Dendritic encapsulation or "dendritic box" was first demonstrated in 1994 [73], and since then a range of therapeutics including chemotherapeutics have been explored for dendrimer encapsulation. Most chemotherapeutics have hydrophobic structures and can, therefore, interact with the often hydrophobic interior of dendrimers, increasing the solubility of the pharmaceutical and using the dendritic exterior to shield the drug from its surroundings, which can reduce premature degradation. Further modifications, such as PEGylation of the dendrimers' end groups can extend and alter the pharmacokinetics of the drug. To generate successfully passive dendrimer-based drug carriers one must consider some issues that are general for passive drug delivery agents, mainly stability and premature release, concentration variations between batches, low drug-loading capability, which can result in high volumes of carrier, and rapid burst release under physiological conditions.

Dendrimer Encapsulated Drug Formulations

PAMAM and PPI dendrimers are commonly used for dendritic encapsulation studies based on their ability to be easily functionalized on the exterior, due to the presence of surface

amine groups. Both classes of dendrimers are not degradable and are often found to evoke toxic or hemolytic responses in their original form [74,75]. Hence, it is necessary to shield the highly cationic amine exterior with less "provocative" groups, such as OH, COOH or PEG. In terms of chemotherapeutics, PAMAM have been explored for the formulation of several drugs including: Anastrozole [76], 6-mercaptopurine [77], 5-fluorouracil [78], Adriamycin [79], methotrexate [79] and camptothecin with some degree of success [80].

Polyester-based dendrimers, such as the class developed by Grinstaff et al., are constructed from glycerol and succinic acid and are degradable. They have been used to encapsulate topoisomerase inhibitors, such as 10-hydroxycamptothecin and 7-butyl-10-aminocamptothecin, with very promising results against a panel of breast, lung, colorectal and glioblastoma cell lines [81,82]. Similarly, poly-glycerol dendrimers have been shown to increase the solubility of paclitaxel by orders of magnitude compared to the traditional excipient Cremofor EL which can cause toxic side effects [81].

Covalent Dendrimer-Drug Carrier Formulations

Covalent drug carrier formulations can overcome some of the limitations that are often found for the sequestered passively loaded drug carriers. By covalent attachment via cleavable linkages that are activated by changes in such as pH, enzyme levels, reductive environment or temperature, the release of the pharmaceutical can be controlled circumventing burst release effects. In the case of the dendrimers, covalent attachment of drugs can also minimize batch-to-batch variations in drug loading, as the structure is monodisperse, and often higher drug concentrations can be accommodated by the carriers when the drug is covalently attached. Duncan et al. showed an early example of polymer-drug conjugation with a dendritic scaffold by utilizing PAMAM dendrimers carrying carboxylic acid end groups [83]. These COOH groups were used for attaching cisplatin in an effort to increase the effective solubility of the drug and minimize toxicity. The reaction of cisplatin caused intermolecular cross-linking of dendrimers generating larger dendrimer clusters and not discrete nanoparticles of one single dendritic molecule. However, as a model for drug delivery, this system demonstrated that the covalent attachment enabled 25 wt% of cisplatin to be conjugated with a 10-fold increase in solubility and significantly reduced toxicity as determined *in vitro*. *In vivo* investigations indicated that the blood clearance of these constructs was significantly reduced and that intratumoral concentration of platinum was enhanced compared to the free drug. This seminal work has since inspired a range of studies on both PAMAM dendrimers and many others. In terms of cancer therapy, Table 16.1 lists a representative number of dendrimers and the drugs investigated and below is a selected example.

Doxorubicin and PBisMPA Dendrimers

The use of polyester-based dendrimers for therapeutic applications has been well investigated by the group of Fréchet et al. These dendrimers are biocompatible with non-toxic and non-immunogenic properties which is highly desirable for pharmaceutical applications [98,99]. The research has evolved from simple dendrimer structures to more complex linear-dendritic constructs for chemotherapeutic delivery. In initial studies [37], the drug doxorubicin (DOX) was conjugated to the surface via acid-sensitive linkage that would allow

TABLE 16.1 Examples of Dendrimers and Drugs as Covalent Drug Carrier Constructs

Dendrimer	Drug	Reference(s)
PAMAM	Doxorubicin	[84–86]
PAMAM	Paclitaxel	[87–89]
PAMAM	Platinates	[83,90]
PAMAM	5-Fluorouracil	[91–96]
PBisMPA	Doxorubicin	[7–99]
Poly(melamine)	Paclitaxel	[100]
Poly(triazine)	Paclitaxel	[101]
Poly(triazine)	Camptothecin	[102]
PBzE	Methotrexate	[103]

drug release after cellular uptake. These structures depicted much higher IC50 values than the free drug, with no preferential organ uptake *in vivo* but a significantly increased half-life in blood.

The next generation of dendritic structures that was developed was coined "bow-tie" structures (Figure 16.8): dendrimers carrying long PEG chains on one side and reactive handles on the opposite side for drug attachment. These structures enable the number of PEG branches and drug density necessary for optimization of the PK and BioD characteristics to be tailored.

These carriers were shown to be highly effective drug carriers *in vivo*, with similar survivability as Doxil in BALB/c mice bearing subcutaneous C-26 tumors, far exceeding the effect

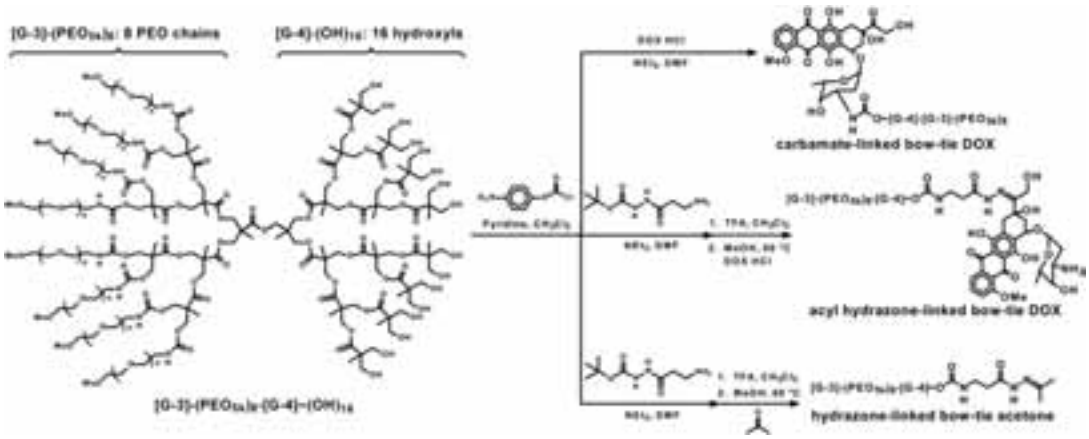

FIGURE 16.8 Bow-tie dendrimer structure. *(Reproduced with permission from [104]. Copyright 2006 Proceedings of the National Academy of Sciences of the United States of America.)*

of the free drug at the same concentrations. These carriers also have better stability and can potentially be developed into generic drug carriers for other types of chemotherapeutics as the half-life in blood can be varied from minutes to hours. The IC_{50} of the construct was determined to be 20 times higher than for the free drug, and the tumor uptake of the dendritic carrier was increased ninefold. The maximum tolerated dose was also increased on a DOX equivalent basis (Figure 16.9).

Perspectives Concerning Dendrimers

Research employing dendrimers in therapeutic applications is still in its infancy. Many opportunities have been demonstrated in the literature but several challenges exist. For these applications dendrimers must be designed for use in a biological system. Thus, dendrimers need to be non-toxic, degradable, equipped with non-charged groups and devoid of organ-specific accumulation. Currently, the number of dendrimers undergoing phase testing for therapeutic applications is limited, perhaps reflecting the complexity of using nanomedicine in reality or an effect of the complexity required to construct these structures.

Carbon Nanotubes (CNTs)

As for other ENM, the biomedical potential of CNTs is determined by their various physical, chemical and biological properties. CNTs are either single-walled (SWCNT) or multi-walled (MWCNT) depending on the number of concentric carbon cylinders they are made of. Their unique combination of intrinsic features (i.e. mechanical, electrical, thermal, optical and spectroscopic) along with a high surface tunability might indeed suit applications in the detection, imaging, monitoring and also the treatment of many diseases, spanning from cancer to neurodegenerative disorders. Furthermore, *in vitro* studies have demonstrated that CNTs present a key advantage due to their specific cylinder shape: they can be easily internalized by cells. They could, therefore, offer a unique solution for the intracellular delivery of small therapeutic molecules as well as macromolecules that do not easily cross the cell membrane barrier.

CNT Design

Pristine CNTs (as produced) are insoluble in most organic or aqueous solvents, and particularly in the high salt milieu that constitutes most biological environments. To overcome this issue and to advance their use for therapeutic purposes, modification of CNTs is required. Two main strategies have been developed, both of which are based on the modification of the nanotube surface, either by non-covalent physisorption of amphiphilic molecules or by covalent grafting of different kinds of chemical groups on the nanotube carbon backbone [105,106] (Figure 16.10). Both approaches result in improved solubility and dispersibility under physiological conditions and seem, according to many studies, to be less toxic when compared to their non-modified forms [107].

The hydrophobic and polyaromatic carbon surface of the nanotube offers a unique surface on which amphiphilic and aromatic molecules, such as polymers (e.g. glycodendrimer, pyrene-polyethylene glycol-lactose, and polysaccharide) or lipids, can easily bind by non-covalent interactions [108]. For instance, to improve the dispersibility of CNTs in aqueous solution PEGylated phospholipids have been used to functionalize SWCNTs which can be

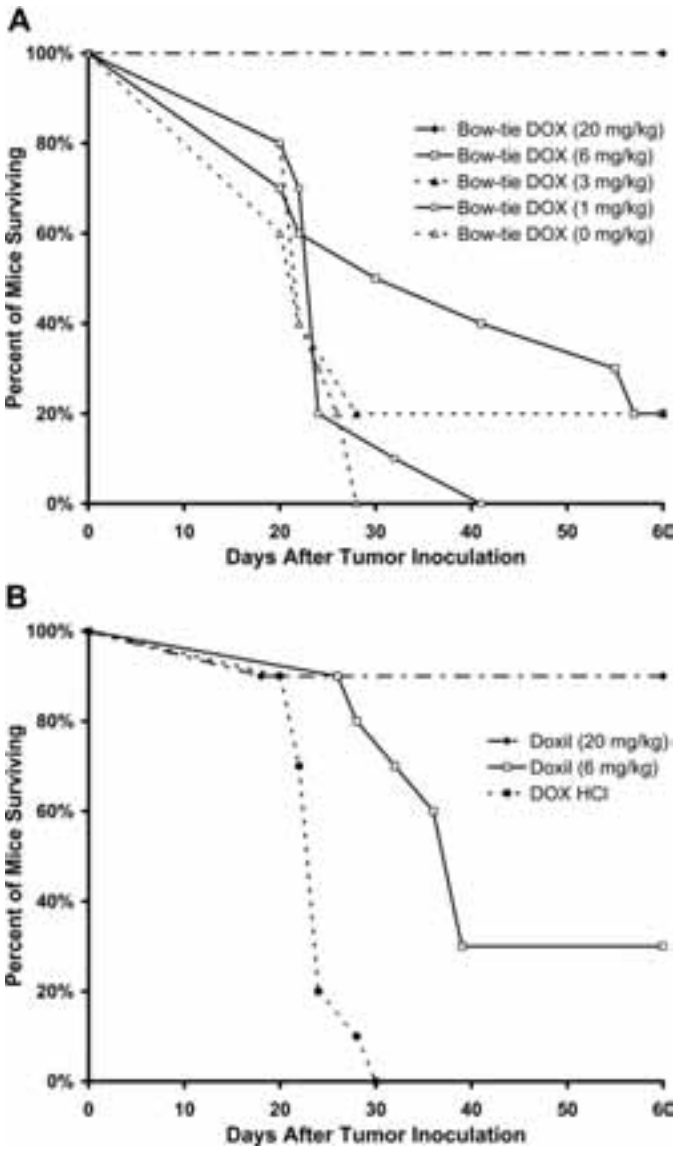

FIGURE 16.9 *In vivo* evaluation of PBisMPA bow-ties as doxorubicin carriers against c26 tumors. Survival versus time for BALB/c mice bearing s.c. c26 tumors. (A) Treatment consisted of a single i.v. dose of hydrazone-linked bow-tie DOX given 8 days after tumor implantation. (B) Treatment consisted of a single i.v. injection of either free DOX or Doxil given 8 days after tumor implantation. *(Reproduced with permission from [104]. Copyright 2006 Proceedings of the National Academy of Sciences of the United States of America.)*

then assessed for their therapeutic potential. PEGylated lipids offer multiple advantages. They improve CNT solubility and dispersibility while being more biocompatible than commonly used surfactants, such as sodium dodecylbenzene sulfonate or the triblock non-ionic copolymer Pluronic F127. In addition, terminal amino groups present on the PEGylated moieties coating the CNT have also been exploited to load therapeutic molecules [109]. Another recently developed method to aid the dispersion of CNTs in physiological media is to wrap the CNT backbone with biocompatible DNA oligos. For compounds containing

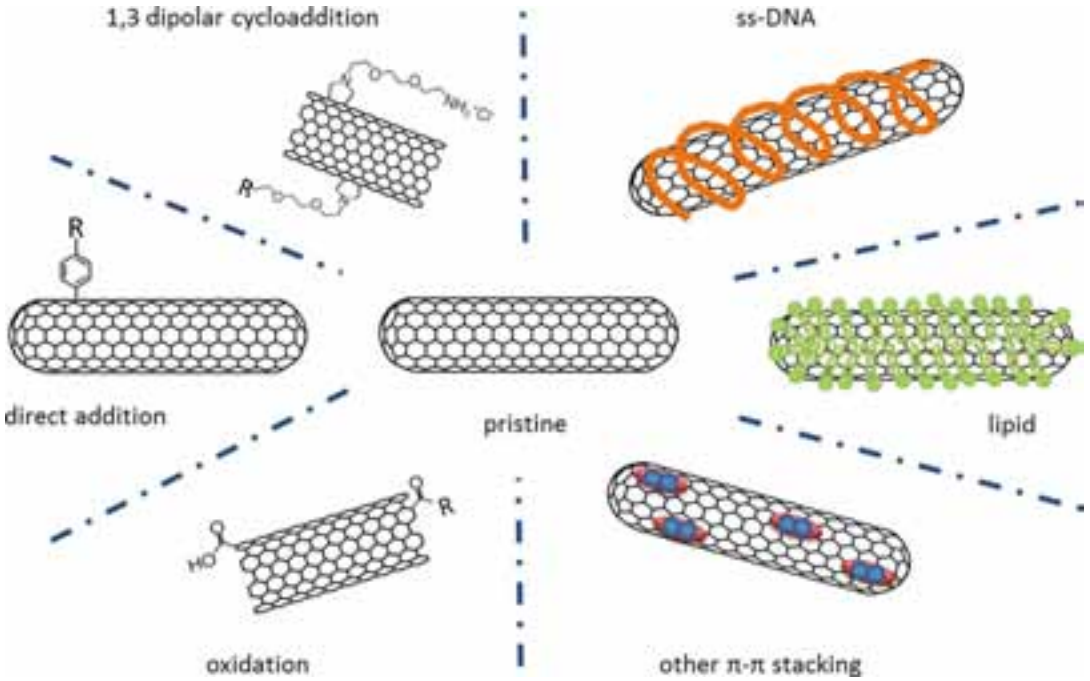

FIGURE 16.10 Different kinds of CNT surface modifications.

aromatic moieties, the supramolecular π-π stacking that is used to coat the surface of the nanotubes can also be exploited to load therapeutic molecules directly onto the CNT. For instance, following this procedure, doxorubicin has been successfully loaded onto CNT [110,111].

Alternatively, the covalent functionalization of CNT consists of the chemical modification of the carbon backbone. One of the most common methodologies is to heat under reflux a strong acid solution containing the CNTs. It is generally used as a first step to create defects on the nanotube sidewall (i.e. carboxyl groups) that can be utilized as mounting sites for further chemical modifications or to load the therapeutic molecule of interest. This oxidation process is also used for purification and shortening since impurities and length have been described as key parameters that may increase toxicological side effects. Alternatively, direct addition of chemical groups can be used for covalent functionalization. A popular strategy uses the 1,3-dipolar cycloaddition reaction of azomethine ylides by which terminal amino groups are used to conjugate different molecules either for imaging or therapeutic purposes or both [112,113].

Compared to other ENM, CNTs have a high surface area due to their specific cylinder shape. This characteristic can be exploited for a highly efficient loading of multiple molecules via different linkers (i.e. a multifunctional system) along the length of the CNT sidewalls. The greater external surface of MWCNT provides more possibilities for conjugation than SWCNT

and, because of a larger diameter, encapsulation of therapeutic molecules is possible in the cavity of the internal cylinder.

Despite the early stage of the field in general, the range and adaptability of CNT properties have already enabled potential applications to be successfully developed for drug delivery systems and therapies for applications spanning from cancer to neurological disorders.

CNT Drug Delivery

ANTICANCER THERAPIES

One of the most studied biomedical applications for CNT is the treatment of cancer by chemical, immunological, or genetic therapies. Due to their ability to penetrate cells, functionalized CNTs have been used to deliver a variety of cytotoxic drugs, such as doxorubicin [110,111], methotrexate [114], paclitaxel [115] and cisplatin [116] into tumor cells. Similarly, an antitumor immune response can be induced by delivering tumor lysate proteins [117]. Finally, chemically functionalized CNTs have shown high efficiency for the delivery of DNA plasmid or siRNA (mainly *in vitro* for DNA [118] and *in vivo* for siRNA). It has even been demonstrated that CNT-siRNA conjugates are more effective in prolonging the survival of tumor-bearing animals than liposomal vector systems [119,120].

IMMUNOTHERAPY

The efficient intracellular delivery of molecules by CNTs has been also explored in the immunotherapeutic field either to stimulate the immune response by delivering antigenic proteins [120,121] or reduce the immune response in immune-related diseases by delivering siRNA into immune-competent cells [122].

Therapeutic Applications

CANCER

SWCNT have distinctive intrinsic optical properties with a strong optical absorption in the near-infrared (NIR) wavelength and can, therefore, also be utilized for photothermal therapy. The strategy is based on the uptake of CNTs by tumor cells, which will then be heated by NIR light and selectively induce the death of the cell that contains them [123].

NEUROLOGICAL DISORDERS

In the last few years, the potential of CNT utilization has been extended to brain diseases. In a rat stroke model, pretreatment with plasma aminated SWCNT has been demonstrated to reduce anatomic (i.e. neuronal loss) and functional (i.e. motor damage) sequelae after induced ischemic damage [124]. At the same time, non-ischemic animals injected with the same material show neither brain damage 3 weeks after injection, nor motor dysfunctions after 6 weeks. Compared to raw or carboxyl-functionalized SWCNT, these amine modified SWCNTs have been shown to be neuroprotective to the injured brains in the absence of drug-loading, possibly because of their surface properties (i.e. positive charges, surface energy) that provide a favorable environment for the neural tissue. It is one of the first *in vivo* studies to demonstrate that a chemically functionalized CNT (without any therapeutic agent) could be considered as a therapy on its own, and not only as a nanocarrier or nanodevice. In another study, using a rat stroke model, Al-Jamal et al. reported that localized administration of chemically functionalized MWCNTs carrying siRNA against caspase-3

can improve the behavioral performance of ischemia-induced animals [125]. More studies exploring the therapeutic capacity of CNTs against CNS diseases will surely appear.

Regenerative Medicine

Due to their size and shape, CNTs seem to influence cell adhesion behavior in such a manner that CNT-coated surfaces could be used to induce differentiation of stem cells [126,127] or to stimulate nerve [128], bone [129,130] or cartilage [131] regeneration. Moreover, interaction of CNT-based surface with neuronal tissue has been shown to favor neuronal electrical signaling [132]. Researchers are, therefore, now exploring the advantages of using CNT-coated electrodes for electric deep brain stimulation, which is one of the most efficient and spectacular treatments developed to reduce Parkinsonian symptoms (i.e. tremor, slow movement, rigidity, and gait disturbances) in an early stage of the disease.

Multimodal Applications

In addition to their intrinsic physical properties, different surface chemical modifications can be performed on the same CNT, leading to a platform that could carry multiple functionalities simultaneously, such as targeting, detecting and treating [133] (Figure 16.11). For instance, using carboxyl-modified CNTs to conjugate both cisplatin and epidermal growth factor, researchers have been able to target tumor cells overexpressing epidermal growth factor receptor specifically to kill them with the carried cytotoxic drug [134]. In another *in vitro* study, Wang et al. used an alternative strategy to kill neuroblastoma cells. They conjugated oxidized (carboxylated) SWCNTs with antibodies directed toward a tumor growth antigen (disialoganglioside, GD2) to target the tumor cells, and then used the intrinsic properties of the CNTs (optical absorbance in the near-infrared region) to generate heat and selectively kill the targeted cells by photothermal therapy [135]. Another *in vitro* work similarly used SWCNT coated with PEGylated phospholipids to target folate receptor-overexpressing cells. Once the SWCNT reached the target cells, their optical absorbance in the near-infrared region either to release intracellularly oligonucleotides (pulsed NIR irradiation), or to induce hyperthermia to kill the cells (continuous NIR irradiation) were demonstrated [123]. The same group also demonstrated that NIR irradiation could also be used for *in vivo* photoluminescence imaging of the PEGylated lipid-coated SWCNTs [136] and using another intrinsic property of the construct, the strong Raman signature of the SWCNT, they were also able to detect the presence of CNTs in physiological fluids (blood, urine, feces) [137]. In conclusion, clearly there is great potential to combine monitoring, imaging, targeting and multimodal treatments in the same CNT-based construct.

Health Monitoring

The high electrical conductivity and high electrochemical potential of the CNT surface have been exploited for biosensing. For instance, CNTs have been proposed as sensitive glucose detectors in the design of new *ex vivo* or implanted devices for monitoring glucose levels in diabetic patients [138]. Similarly, by attaching enzymes on drug-carrying CNT, it is expected that therapeutic biosensors will be generated that would be able not only to detect and measure biological molecules, but also subsequently to release their cargo to prevent or treat morbid processes in which these molecules take part (e.g. prevention of stroke in the case of detection of thrombin or other coagulation factors).

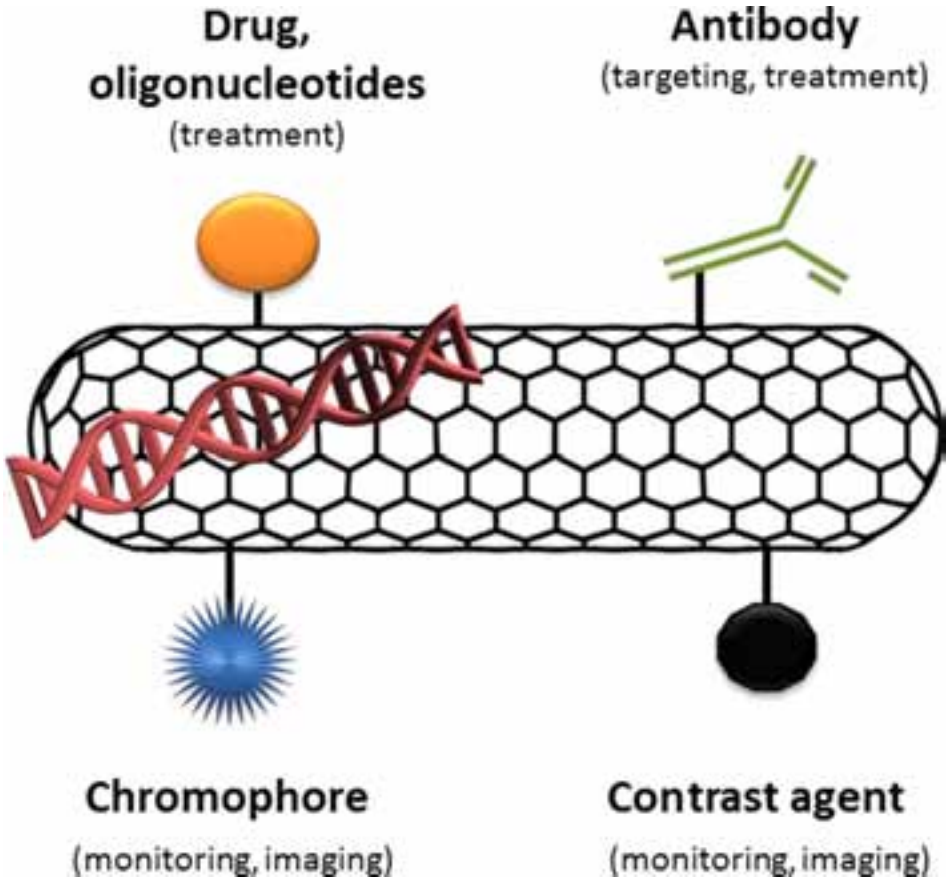

FIGURE 16.11 CNT-based multimodality platform.

Further Challenges for CNTs

Numerous questions remain unanswered before these promising nanomaterials reach the clinic [139]. In particular, three challenges must be overcome. First, CNT are not 100% pure "*nanotube*" material, but also contain different nanostructured impurities (amorphous carbon, multishell carbon balls, metal catalyst residues) that would affect their biological profile. Heterogeneity of the product (variation in length, diameter, number of walls, or graphitic structure) is similarly an important concern because it could impact both their toxicological profile and their behavior in biological systems (i.e. pharmacokinetics, biodistribution [140]). Control of CNT synthesis, purification and functionalization are thus imperatively needed to be overcome to allow clinical translation.

Another issue is the need to avoid the rapid elimination by macrophages and other phagocytic cells (i.e. cells of the reticuloendothelial system), because of the preferential uptake of CNTs by these sentinel cells. This type of cellular uptake is a problem for delivering the

effective dose to a targeted tissue or cell population, and also for unwanted delivery of medicinal molecules to these immunocompetent cells. Further investigations that focus on escape from macrophage recognition, probably via improved surface functionalization or improved targeting, are therefore needed.

Finally, during the last few years there have been numerous reports regarding the potential toxicological profile of CNTs. Hence, many studies have demonstrated that pristine CNTs may exert various adverse biological effects leading to toxicity (see Chapter 7 for a detailed discussion). At the same time, there are various other studies demonstrating that chemical functionalization of the CNT backbone can significantly reduce or abolish such apparent immunological or toxicological responses [104,141,142]. Moreover, the long-term fate (i.e. biopersistence or clearance, biotransformation, and biodegradation) of CNT-based constructs for therapeutic applications have to be further documented. In the case of long-term biopersistence, CNTs could be either inert or induce reaction by the tissue(s) in which they reside. Also, if any biotransformation occurs within tissues, the products could potentially be more reactive and harmful than the original nanotube constructs. Even though biodegradation of CNTs can be seen as beneficial for their safety profile, partial degradation may also lead to questions about the inertness of such by-products. All of these questions have to be addressed by pharmacologists working in close collaboration with toxicologists to allow clinical translation of these promising materials.

TAKE-HOME MESSAGES

1. The field of ENM has the potential to fundamentally renovate pharmacological science and industry by proposing more efficient solutions for therapy and diagnosis.
2. The full potential and limits of ENM for different biomedical applications are still being explored by scientists working in nanomedicine and nanoscience.
3. Biomedical researchers face the grand challenge of translating ENM into a clinical reality.

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