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CHAPTER I.2.19 MICROPARTICLES AND NANOPARTICLES

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INTRODUCTION

Microparticles and nanoparticles have had an enormous impact on a wide-range of biomedical applications including drug delivery, imaging, and basic research. Miniaturization of therapeutic devices to the micron (1–1000 μm), sub-micron (100–1000 nm), and nanometer (1–100 nm) scales has facilitated the integration of biomedical devices with therapeutic biomolecules for improved clinical efficacy (George et al., 2005). Despite an extensive database on microparticles and nanoparticles, a clear universal boundary between nano- and microsize does not exist in the literature.

Expert opinions in the micro- and nano-sciences have emphasized that 1–100 nm is the optimum nanoscale range; however, in biotechnology and medicine, the definition of “nano” is less stringent (Ferrari, 2005). Design of any miniaturized system is dependent on the endpoint application. For example, systemic (intravascular) application requires use of particles less than 500 nm in diameter, whereas for intramuscular application or in some cases oral delivery applications, particles greater than 1 micron and less than 125 μm can be easily administered (Jain, 2000). Other than size, another critical parameter that can significantly modulate the function of these particles is shape (Champion et al., 2007); shape can impact cellular uptake by immune cells, release behavior of biomolecules, and cell targeting. A variety of materials have been synthesized as micro- and nanoparticles, mostly for imaging or targeted delivery of therapeutic biomolecules such as hormones, vaccine antigens and adjuvants, peptides, and anti-inflammatory agents (Aukunuru et al., 2003; Kim et al., 2004). In addition, these particles have been used as biosensors and in affinity bioseparations, immunological assays, cell labeling, and cell sorting. Also see Chapters II.5.16.B1 to II.5.16.B9 for applications of various particulate systems in drug delivery. The following discussion focuses on preparation, characterization, and applications of microparticles and nanoparticles.

MICROPARTICLES

Microparticles have been an active area of research in the biomedical field because of their unique advantages: their small sizes (few microns) render them excellent candidates for injectable drug delivery as compared to surgical implants; high surface area allows for surface functionalization to target specific cells, tissues or organs; and the ability to carry fluorescent molecules enables their use in microscale detection of cells and cellular components (e.g., proteins, DNA). Further, magnetic microparticles have been used for cell sorting and hyperthermia applications.

Materials Used for Microparticle Synthesis

The choice of biomaterial for microparticle synthesis is dependent on the intended use, duration of therapy, desired nature of the polymer (for example, hydrophobic or hydrophilic, neutral or charged), bioactive agents to be delivered (for drug delivery applications), and the chemistry needed for further functionalization and modification. The vast majority of current microparticle research has focused on site-specific drug delivery, controlled drug release, and minimizing toxicity and degradation of drugs (Dumitriu, 2002). Table I.2.19.1 lists some of the natural and synthetic materials used in microparticle drug delivery applications. Only a few commonly used polymers are discussed here.

Natural Materials

Chitosan. Chitosan is a cationic polysaccharide exhibiting $\beta(1\rightarrow4)$ linkages, and is obtained by alkaline deacetylation of chitin, a polymer present in the exoskeleton of many insects and marine crustaceans. Its biocompatibility, complete elimination from the body post-degradation, and abundance of primary amines make chitosan a popular material for drug delivery applications (Agnihotri et al., 2004). Primary amines render chitosan soluble in aqueous acidic solutions, thus eliminating the use of organic chemicals during synthesis, and also offer numerous possibilities for ionic attachment of negatively charged biomolecules (e.g., DNA, RNA). Further, chitosan microparticles exhibit enhanced mucosal residence time, increasing mucosal permeability of encapsulated proteins and drugs for nasal drug delivery (van der Lubben et al., 2001). Chitosan microparticles are also taken up by the Peyer's patch of gut-associated lymphoid tissue (GALT), making them appropriate candidates for oral drug delivery (van der Lubben et al., 2001c; Ahire et al., 2007). Chitosan has been used to orally deliver proteins and antigens such as insulin (Ubaidulla et al., 2007), diphtheria (van der Lubben et al., 2003) or tetanus toxoid (Ahire et al., 2007). Chitosan microparticles have also been successfully used for DNA and vaccine delivery, facilitating transfection and preventing DNA degradation by exogenous Dnase (Guliyeva et al., 2006).

Alginate. Alginate is an unbranched copolymer of D-mannuronic and L-guluronic acid linked by $\beta(1\rightarrow4)$ linkages, and is derived from brown seaweed. This polymer has been actively explored in the pharmaceutical sciences because of its biocompatibility, low immunogenicity, and unique property of sol-gel transition in the presence of multivalent cations (e.g., Ca^{++} , Mg^{++}). The preparation of alginate microparticles involves mild reaction conditions, which minimizes or eliminates the use of organic solvents and high temperatures that could otherwise reduce the bioactivity of encapsulated proteins, polypeptides or nucleic acids (Dumitriu, 2002). Alginate microparticles have been used extensively for protein, drug, and cell microencapsulation (Joki et al., 2001; Wang et al., 1997). Protein drugs have been encapsulated in calcium chloride cross-linked alginate microspheres, with release fine-tuned by varying the cross-linking factors (Wheatley et al., 1991). For example, applying polycations to the outer microcapsule surface can reduce the burst effect and produce more sustained release (Wheatley et al., 1991). Drug-loaded alginate microparticles have been primarily studied for nasal and oral delivery (Bowersock et al., 1996; Hari et al., 1996). Cell encapsulation has been used to obtain high concentrations of hybridoma monoclonal antibodies for mammalian cell engineering applications (King et al., 1987), and to encapsulate rat islet cells for diabetes treatment (Lim and Moss, 1981; De Vos et al., 1997). Alginate microspheres have also been prepared with other polymers such as chitosan. The addition of chitosan alters the microparticle characteristics and the release profile for potential gastrointestinal tract delivery via an oral route (Hari et al., 1996).

Gelatin. Gelatin is the denatured form of animal-derived collagen, and has excellent biocompatibility and biodegradability. Gelatin microparticles have been studied for sustained release applications (Ratcliffe et al., 1984; Tanaka et al., 1963). Micropellets of gelatin were first prepared in the early 1960s by Tanaka et al. (Tanaka et al., 1963). Since then, gelatin microparticles have been explored for the delivery of mitomycin C, 5-fluorouracil or adriamycin (Oner and Groves, 1993), and used commercially in agglutination tests for diagnostic detection of HIV-1, syphilis, and measles (Hesketh et al., 2006; Barbara et al., 1989; Sato et al., 1997). Mikos and co-workers encapsulated different growth factors inside gelatin microparticles for applications in cartilage tissue engineering. Growth factors such as transforming growth factor-beta 1 (TGF- β 1) (Holland et al., 2005, 2007; Park et al., 2007a), insulin-like growth factor-1 (IGF-1) (Holland et al., 2007; Pham et al., 2008), bone morphogenetic protein-2 (BMP-2) (Patel et al., 2008b,c), and vascular endothelial growth factor (VEGF) (Patel et al., 2008a,c) have been successfully encapsulated. Dual growth factor delivery systems devised to deliver two growth factors simultaneously result in improved cell growth and bone matrix regeneration, in comparison to single growth factor delivery systems (Holland et al.,

TABLE 1.2.19.1 Examples of Materials Used in Microsphere Drug Delivery Applications

Polymer	Product Name	Active Ingredient	Application	Synthesis Method	Reference
PLGA	Sandostatin LAR® Depot	Octreotide acetate	Acromegaly	Emulsion solvent evaporation/extraction	Mundargi et al., 2008
	Nutropin Depot®	Growth hormone	Growth hormone deficiency	Spray freeze-drying	Mundargi et al., 2008
	Lupron® Depot	Leuprolide acetate	Prostate cancer	Emulsion solvent evaporation/extraction	Mundargi et al., 2008
	–	DNA, protein antigen, siRNA, CpG oligos, monophosphoryl lipid (MPL)	Delivery of vaccines and immunomodulatory agents	Emulsion solvent evaporation/extraction, spray drying	Bates et al., 2006 Singh et al., 2008 Malyala et al., 2009
PHEMA	–	Vasopressin	Oral delivery	–	Lee et al., 2007
Alginate		Protein	Drug delivery	Ionic gelation	Wheatley et al., 1991
		Pancreatic islets	Diabetes	Ionic gelation	De Vos et al., 1997
		Recombinant fibroblasts	Non-autologous somatic gene therapy	Ionic gelation	Chang et al., 1994
SPCL	–	Dexamethasone (DEX)	Drug delivery and tissue engineering	Emulsion solvent evaporation/extraction	Balmayor et al., 2009
Poly sebacic anhydrides	–	Rhodamine B	Controlled drug release	Spraying with acoustic excitation	Berkland et al., 2004
Poly orthoesters	–	Plasmid antigens	DNA delivery	Emulsion solvent evaporation/extraction	Nguyen et al., 2008
Poly caprolactones	–	Antigen, anti-hypertensive drugs, Taxol, antibiotics, ribozymes, nerve growth factor (NGF), insulin, heparin	Drug delivery	Emulsion solvent evaporation/extraction, spray drying, solution enhanced dispersion method Hot-melt process	Heller et al., 2002
Collagen		Hydroxyapatite	Osteoblast-based grafting material	Water-in-oil emulsion	Wu et al., 2004
Chitosan		Anti-cancer drugs, anti-inflammatory drugs, cardiac agents, calcium channel blockers, antibiotics, antithrombotic agent, steroids, proteins, antigens, antidiabetic agents, growth factors, DNA encapsulation, cytokines	Drug delivery, gene therapy	Ionic gelation, emulsification and ionotropic gelation, coacervation and complex-coacervation, precipitation–chemical cross-linking, thermal cross-linking, solvent evaporation method, spray drying	Sinha et al., 2004b
Hyaluronic acid		Hydrocortisone	Drug delivery	Solvent evaporation	Benedetti et al., 1990
Hyaluronic acid/chitosan		Gentamicin sulphate	Mucoadhesive nasal drug delivery	Emulsion solvent evaporation/extraction	Lim et al., 2000

PHEMA: poly(2-hydroxyethyl methacrylate)

PLGA: poly(lactic-co-glycolic acid)

SPCL: starch-poly-ε-caprolactone

2005, 2007; Patel et al., 2008c; Park et al., 2008). Glutaraldehyde is a common cross-linker used to form microparticles, and its concentration can control the release rate of biomolecules. To slow degradation, enhance sustained release of drug, and enhance *in vivo* biocompatibility, some researchers have utilized the naturally-occurring

cross-linker genipin to cross-link gelatin microparticles. The resulting particles had a reduced degradation rate and inflammation *in vivo*, in comparison to glutaraldehyde-cross-linked microparticles (Liang et al., 2003).

Dextran. Dextran is a branched homopolysaccharide of glucose, and is synthesized naturally from sucrose

by some bacterial strains. Numerous biomedical studies have employed dextran as a biomaterial since it is biocompatible, biodegradable, available in different molecular weights, and can be easily derivatized. Microparticles of dextran have been studied for biomedical applications ranging from drug delivery (Chen et al., 2006; Cheung et al., 2005; Diwan et al., 2001) and cell therapy (Demetriou et al., 1986) to diagnostics and biosensors (Zhang et al., 2008). In a number of studies, dextran has been employed in the preparation of beads that were later functionalized with antibodies or biomolecules for diagnostic applications (Zhang et al., 2008; Tansey and Cammer, 1998). For example, hydroxyl ethyl methacrylated dextran microspheres have been studied for the delivery of human growth factor (Vlugt-Wensink et al., 2006, 2007). Adding different excipients changed protein loading efficiency and release. Collagen-coated dextran microparticles have also been used for liver tissue engineering. Hepatocytes were attached to their surfaces and injected intraperitoneally into rats, in an attempt to devise therapies for hepatic failures (Demetriou et al., 1986).

Other Natural Materials. In addition to the above mentioned materials, other natural materials have also been employed for microparticle synthesis. For example, collagen microparticles have been used for delivery of glucocorticosteroids (Berthold et al., 1998) and lipophilic drugs (Rossler et al., 1995; Swatschek et al., 2002). Similarly, hyaluronic acid microparticles have been utilized to deliver biomolecules (i.e., recombinant human growth hormone) (Kim et al., 2005), and antibiotics (Lee et al., 2002). Natural materials are biodegradable and well-tolerated in the body, which renders them suitable for delivery applications. See Chapter I.2.7 for details on natural polymers used in medicine.

Synthetic Polymers

Poly(lactic acid) (PLA), Poly(glycolic acid) (PGA), and Copolymers (PLGA). Bioresorbable aliphatic polyesters of lactic acid and glycolic acid are used clinically for controlled drug delivery (Lewis, 1990). Structural details of homopolymers (PLA, PGA) and copolymers (PLGA) have been discussed (Chapter I.2.6). Optically inactive poly(D,L)lactic acid is amorphous in nature, and thus is mechanically weaker than L-PLA or D-PLA, making it a suitable candidate for drug delivery applications. PLGA microparticles were first developed as drug delivery carriers in the late 1970s (Tice and Lewis, 1980). Since then, these polymers have been synthesized in various morphologies, including microparticles, microneedles, pellets, nanocapsules, films, and implants (Park et al., 2007b; Siegel et al., 2006; Teixeira et al., 2005; Marchais et al., 1998; Gumusderelioglu and Deniz, 2000; Webber et al., 1998). As discussed (Chapter I.2.6), there exists no linear relationship between homopolymer ratios of PLGA and degradation behavior of the copolymer. The degradation rate depends on several factors that facilitate water accessibility to the ester linkages in the

microspheres (Park, 1995; Shive and Anderson, 1997). The crystallinity of the homopolymer or copolymer plays a critical role; hydrolytically-cleavable ester linkages in PLGA are more accessible to penetrating water molecules compared to linkages in PLA, thus resulting in faster degradation kinetics. In a classic study, microspheres formed using PLGA copolymers with homopolymer ratios PLGA 50:50 and 70:30 degraded much faster than PLGA 80:20 and 90:10 (Figure I.2.19.1) (Park, 1995). This study illustrated that PLGA microparticles degrade in a heterogeneous fashion (Figure I.2.19.1A). During the degradation process the microspheres retain their shape until they completely disintegrate (Figure I.2.19.1A). Because the end products of PLA or PLGA microparticles are primarily lactic or/and glycolic acids, L-lactic acid content in the degradation medium was quantified. Figure I.2.19.1C shows an increase in L-lactic acid content with an increase in PGA homopolymer content, because the PGA linkage is the primary hydrolytically cleavable site in these polyesters (Park, 1995). Nonetheless, degradation is highly tunable. For example, in separate *in vivo* studies, PLA microspheres injected in rat gastrocnemius muscles degraded between 360–480 days, whereas PLGA (50:50) microspheres degraded in 63 days (Visscher et al., 1985, 1986, 1987, 1988). Because of their excellent biocompatibility and the ability to fine-tune monomer ratio to obtain varying degradation, this polymer has been studied extensively for delivery of a range of biomolecules, including nucleic acids, proteins and peptides, and hydrophilic and hydrophobic drugs (Singh et al., 2008).

Poly(ϵ -caprolactone) (PCL). PCL is a biodegradable, semicrystalline polymer with a glass transition temperature of $\sim 60^\circ\text{C}$. The ring-opening polymerization of PCL was first studied in 1934 (Van Natta et al., 1934), but the biodegradable nature of the polymer was not recognized until the 1970s. Since then, various groups have explored microparticles of PCL for drug delivery applications because of its degradation characteristics, biocompatibility, high permeability with drugs, and lack of toxicity (Murthy, 1997). Further, their slower degradation rates and non-acidic degradation environment, compared to PLA/PLGA, make PCL microparticles suitable for prolonged drug release. PCL blends well with other polymers, which results in altered permeability and degradation rates, and therefore, altered drug release profiles (Pitt et al., 1979a,b). For example, release kinetics of fertility-regulating agents from PCL microparticles mixed with cholesterol, which serves as a viscosity thickener and enhances the drug solubility or by PCL microparticles made from polymer pretreated with formic acid, have been compared to PLA/PGA/PLGA microparticles (Benagiano and Gabelnick, 1979). PCL microparticles have also been studied for encapsulation and release of antibacterial agents (Dubernet et al., 1987), and for combined delivery of antigens and adjuvants for enhanced oral immunization (Youan et al., 1999; Murillo et al., 2002). Lipophilic and hydrophilic drugs have been co-encapsulated in PCL microparticles using a double emulsion technique (Hombreiro Pérez

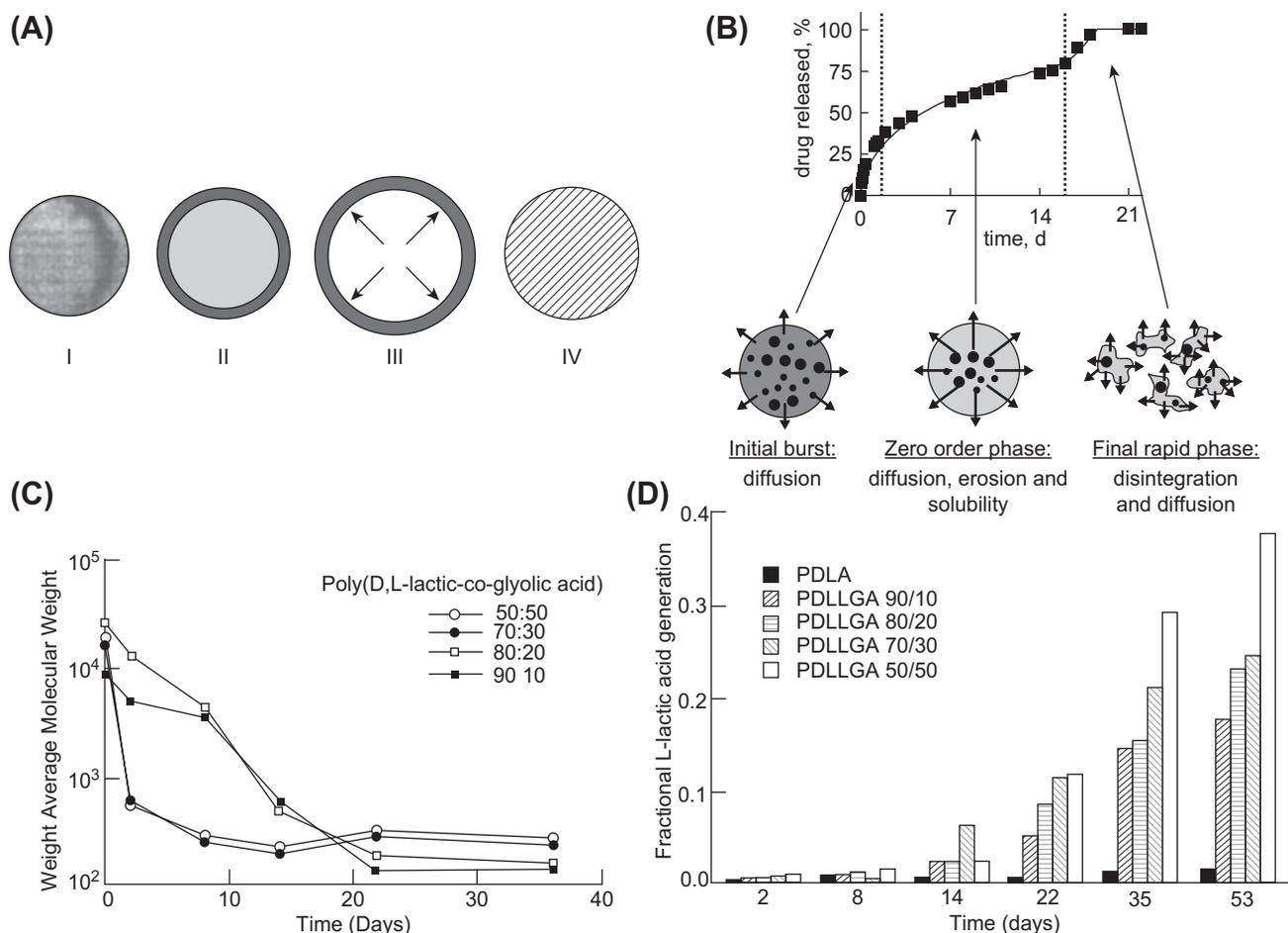


FIGURE 1.2.19.1 (A) Schematic diagram of microsphere degradation mechanism (Park, 1995); (B) drug release from and drug release mechanisms in 5-fluorouracil (5-FU)-loaded, poly(lactic-co-glycolic acid) (PLGA)-based microparticles (Siepmann and Siepmann, 2006); (C) decrease in molecular weight of microspheres with varied PLA/PGA ratio, as a function of time (Park, 1995); (D) fractional L-lactic acid concentration in the medium as a function of time, with varied PLA/PGA ratio (Park, 1995).

et al., 2000). For a detailed review on PCL microparticles, refer to the review by Sinha et al. (Sinha et al., 2004a).

Poly(ortho ester) (POE). Another class of biodegradable materials developed specifically for controlled drug delivery is the poly(ortho esters) (POEs). The biocompatibility and biodegradability of POEs have been studied extensively. There are four different families of POEs, which have been reviewed in detail by Heller et al. (Heller et al., 1983, 2002). POEs degrade by surface erosion, as opposed to the bulk erosion of PLGA polymers, resulting in slower degradation and rendering devices made from this polymer highly stable. Degradation time can be shortened by modifying the polymer backbone with short segments of lactic acid or glycolic acid. Varying the number of glycolic acid or lactic acid segments produces a range of degradation rates suitable for a wide range of delivery applications (Heller et al., 2002). POE microparticles have been examined for protein and nucleic acid delivery (Yang et al., 2001; Wan et al., 2001). For example, the release of plasmid DNA vaccines yielded a potent immune response. Slower surface erosion produced few degradation products, which may have otherwise caused DNA degradation, diminishing the immune response. Microparticles

have also been synthesized from the triblock copolymer POE-PEG-POE employing the double emulsion process. The incorporation of PEG in the POE chain resulted in increased hydrophilicity, leading to higher bovine serum albumin (BSA) encapsulation and faster protein release compared to unmodified POE particles.

Other Synthetic Materials. A number of synthetic materials have been examined as microparticles. For example, polyacrylamide microparticles were prepared almost three decades ago. Their distribution and elimination have been extensively studied in rodent models (Sjoholm and Edman, 1979), and they have been used in drug delivery applications (Kriwet et al., 1998). Similarly, poly(vinyl alcohol) microparticles (Ficek and Peppas, 1993) and poly(2-hydroxyethyl methacrylate) (PHEMA) microparticles (Lehr et al., 1992) have been used for controlled protein delivery and for the release of peptide drugs, respectively. Polyketal microparticles have been examined for the release of therapeutic agents after microparticle phagocytosis, in which release is mediated by microparticle degradation in the acidic phagosome environment (Lee et al., 2007). These materials have also been mixed with other natural or synthetic materials to

achieve desired characteristics, such as varying degradation rate, release profile, and selectivity for target tissue.

MICROPARTICLE PREPARATION

Polymer microparticles are prepared from synthetic monomers, natural polymers, and sometimes semi-synthetic polymers, using techniques that create polymer matrices of desirable sizes and structures. State-of-the-art techniques include single and double emulsion solvent evaporation/extraction methods, coacervation, spray drying, ionic gelation, and suspension cross-linking.

Single and Double Emulsion Solvent Evaporation

The emulsification solvent evaporation technique is one of the most commonly used in microparticle preparation, particularly for drug encapsulation. The two-step technique calls for initial emulsification of polymer solution in a volatile organic solvent, followed by internal phase solvent evaporation or extraction that results in hardening and precipitation of microparticles. Solvent evaporation is generally performed at atmospheric pressure (or sometimes under reduced pressure) to promote evaporation of the volatile solvent. The solvent extraction process, however, involves quenching in excess water or relevant quench medium, allowing for solvent diffusion through oil droplets. Commonly used solvents include methylene chloride (also called dichloromethane or DCM) (Singh et al., 2008), ethyl acetate (Cho and Sah, 2005), and acetone/methanol mixtures. The single emulsion technique is generally employed for synthesis of microparticles encapsulating hydrophobic drugs. The polymer is dissolved in one phase (e.g., PLGA in oil phase or chitosan in aqueous phase), followed by either addition of water to oil (W/O emulsion) or oil to water (O/W emulsion). To prevent coalescence of emulsion droplets, surfactants such as polyvinyl alcohol (PVA) are used. The two phase system is then homogenized at speeds of 8,000–20,000 rpm for 2–5 minutes. Emulsifying agents (i.e., surfactants) play an important role in emulsion selection for microparticle synthesis. If the emulsifier is oil soluble then it favors W/O type emulsion, and *vice versa*. Since the stability of the emulsion also depends on the difference in specific gravities between oil and water phases, W/O emulsions are sometimes difficult to create. However, stable systems can be created (Okochi and Nakano, 2000). For an O/W system, final microparticles are obtained as the volatile organic solvent diffuses outward through the solvent-saturated aqueous phase from the internal organic phase, eventually evaporating and producing hardened microparticles. Microparticle porosity is controlled by rate of evaporation, ratio of internal aqueous phase, and viscosity of the polymer. Rapid solvent evaporation, as in the solvent extrac-

tion process, often results in porous microparticles as compared to the slow solvent evaporation process.

Unlike the O/W single emulsion, which is suitable for encapsulating lipophilic molecules like steroids, the double emulsion process or water-in-oil-in-water (W/O/W) method is commonly used to synthesize microparticles encapsulating hydrophilic biomolecules and drugs such as peptides, proteins, and antigens (vaccines). The W/O/W double emulsion is a three-phase system in which polymer is dissolved in the oil phase with an internal aqueous phase, and immersed in an external aqueous phase containing surfactant or emulsifying agent. Although various techniques to create W/O/W double emulsions exist (e.g., one-stage emulsification method, two-stage emulsification method, phase inversion method), microparticle synthesis for biomedical applications has primarily focused on the two-stage method, because of its superior drug loading capability (Okochi and Nakano, 2000). In a typical W/O/W double emulsion process, polymer (e.g., PLGA, PCL, PEG-PLA copolymer) is dissolved in an organic (oil phase) solvent followed by addition of a small volume (100–500 μ l) of aqueous phase. This solution is subjected to vigorous homogenization or sonication to yield the primary emulsion (Dorati et al., 2008). This primary emulsion is then poured into an excess of aqueous solution containing emulsifier (e.g., PVA), and further homogenized. The volatile organic solvent is eliminated from the resultant W/O/W double emulsion through solvent evaporation or extraction. Similarly, chitosan microparticles are often prepared by an extended W/O emulsification process in which chitosan in the aqueous phase is emulsified in oil with surfactant, and then cross-linked using glutaraldehyde or ethyleneglycol diglycidyl ether to form hard droplets (Agnihotri et al., 2004).

The formation mechanism of initial emulsion droplets and resultant microparticles in an oil-in-water (O/W) single emulsion and W/O/W double emulsions has been examined (Figure I.2.19.2) (Rosca et al., 2004). During the initial stage of the O/W emulsion, emulsion droplets are large because the aqueous phase is initially saturated with solvent (Figure I.2.19.2B). As solvent evaporates, solvent concentration in the droplet decreases, causing a rapid shrinkage of droplet size and the formation of hardened microparticles (Figure I.2.19.2C). In contrast, in the W/O/W emulsion, different types of droplets are formed (Figure I.2.19.2D): (1) microcapsule with no inner aqueous phase entrapped; (2) microcapsule with only one aqueous phase; and (3) a single droplet containing multiple microcapsules. The inner and outer structures of these microcapsules are further clarified by the scanning electron micrograph (SEM) image (Figure I.2.19.2E). Droplets without any inner aqueous phase form plain polymer microparticles, indicated as “p,” thin-walled open microcapsules “c1” are formed when the emulsion droplet is similar in size to the inner droplet, and microcapsules “c2” are formed when a small difference exists between the microdroplet and emulsion droplet size.

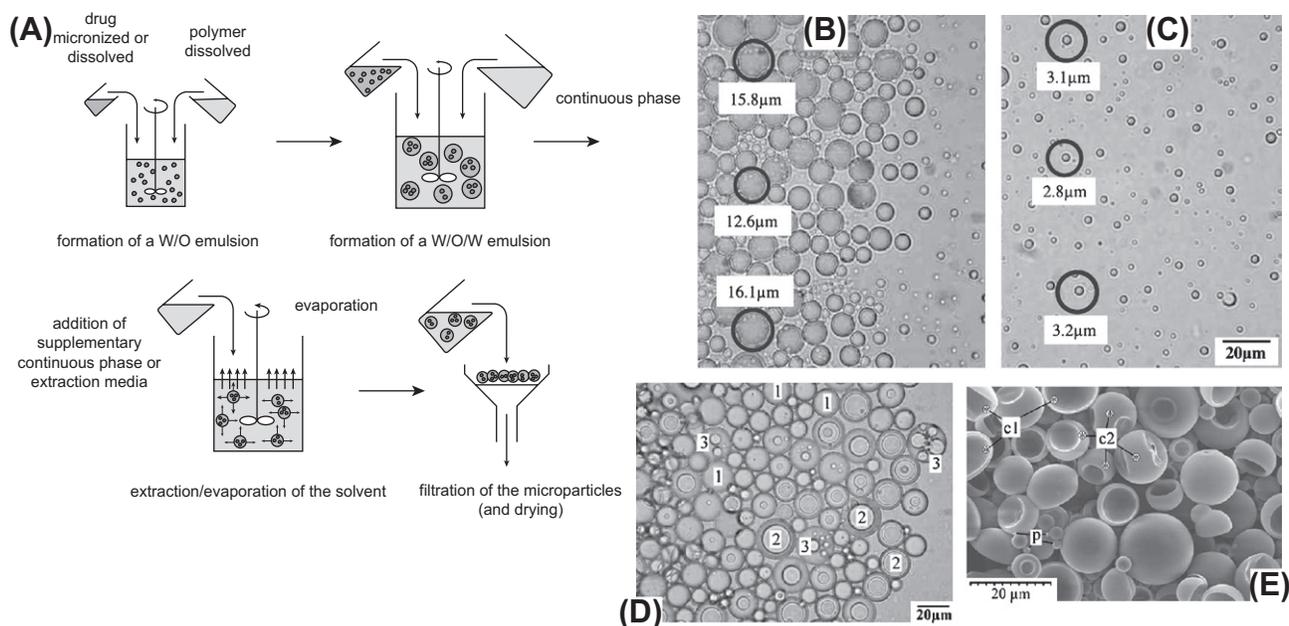


FIGURE 1.2.19.2 (A) Preparation of microparticles using a water-in-oil-in-water (W/O/W) technique (Siepmann and Siepmann, 2006); (B,C) the first and last images, respectively, of the emulsion microdroplets transformation into the final microparticles; (D) initial W/O/W microdroplets; and (E) final microparticle SEM (scanning electron micrograph) image (Rosca et al., 2004). See text.

Precipitation and Coacervation

Coacervation uses the physiochemical characteristics of polymers to form microparticles. Coacervation is a three-step process in which a W/O emulsion is formed with polymer dissolved in the organic phase and drug dispersed in the aqueous phase. The solubility of the polymer is altered (e.g., by mixing with another polymer or organic non-solvent or by changing pH or ionic strength). The non-solvent used in the initial steps should be miscible with the organic solvent used to dissolve the polymer; however, the polymer and/or drug should not be soluble in the non-solvent (e.g., vegetable oil, paraffin oils, silicone oils). This results in phase separation to form a polymer-rich coacervate phase surrounded by a dilute supernatant phase, essentially producing a coating of coacervate around the dispersed drug molecules. Stabilization of the coating occurs with solidification of microparticles in another organic non-solvent followed by extensive washing, centrifugation, and freeze drying (Jain, 2000).

Spray Drying

Conventional microparticle fabrication methods like double emulsion and coacervation often have limitations, including particle aggregation as a result of exceptionally high surface energies and mutual adhesion between particles, inefficient encapsulation, multi-step processing, and residual organic solvents (Jain, 2000; Jalil and Nixon, 1990). Spray drying is a faster, more efficient process for encapsulating drugs in polymeric microparticles (Jain, 2000). This technique involves dispersion (emulsification)

of aqueous drugs or direct addition of hydrophobic drugs in a polymer solution, which is then sprayed through a fine nozzle into a chamber (generally heated) where the solvent evaporates and particles are collected (Figure 1.2.19.3A). This technique generally yields microparticles from 1–100 μm in diameter and drug encapsulation of up to 99%, depending on drug–polymer interaction, solution viscosity, and nozzle characteristics (Jain, 2000; Wagenaar and Muller, 1994). Spray drying has been used for synthetic polymers such as PLGA, PLA, and PCL, and also for natural polymers including gelatin and polysaccharides (Lorenzo-Lamosa et al., 1998). For polysaccharides (e.g., chitosan), the aqueous acetic acid-based solution containing a mixture of polysaccharide, drug, and cross-linker is atomized similarly in a heated chamber. Cross-linkers such as glutaraldehyde form an aldehyde–amine bond with the polysaccharide that results in hardened microparticles; however, cross-linker addition is not essential for microparticle formation. Various parameters influence particle size, including nozzle radius, chamber temperature, flow rate and pressure, and cross-linker concentration (Agnihotri et al., 2004). Microparticles may be lost because of agglomeration and adhesion to the chamber walls, and some research groups have developed a novel dual-nozzle system that simultaneously delivers both the polymer–drug mixture and an anti-adherent solution (e.g., mannitol) to prevent adhesion and aggregation (Takada et al., 1995).

The low temperature spray drying method reported by Alkermes, Inc. (ProLease® technology) was specifically developed to maintain integrity and activity of encapsulated biomolecules during the synthesis process. In this technique, powdered biomolecules and stabilizing

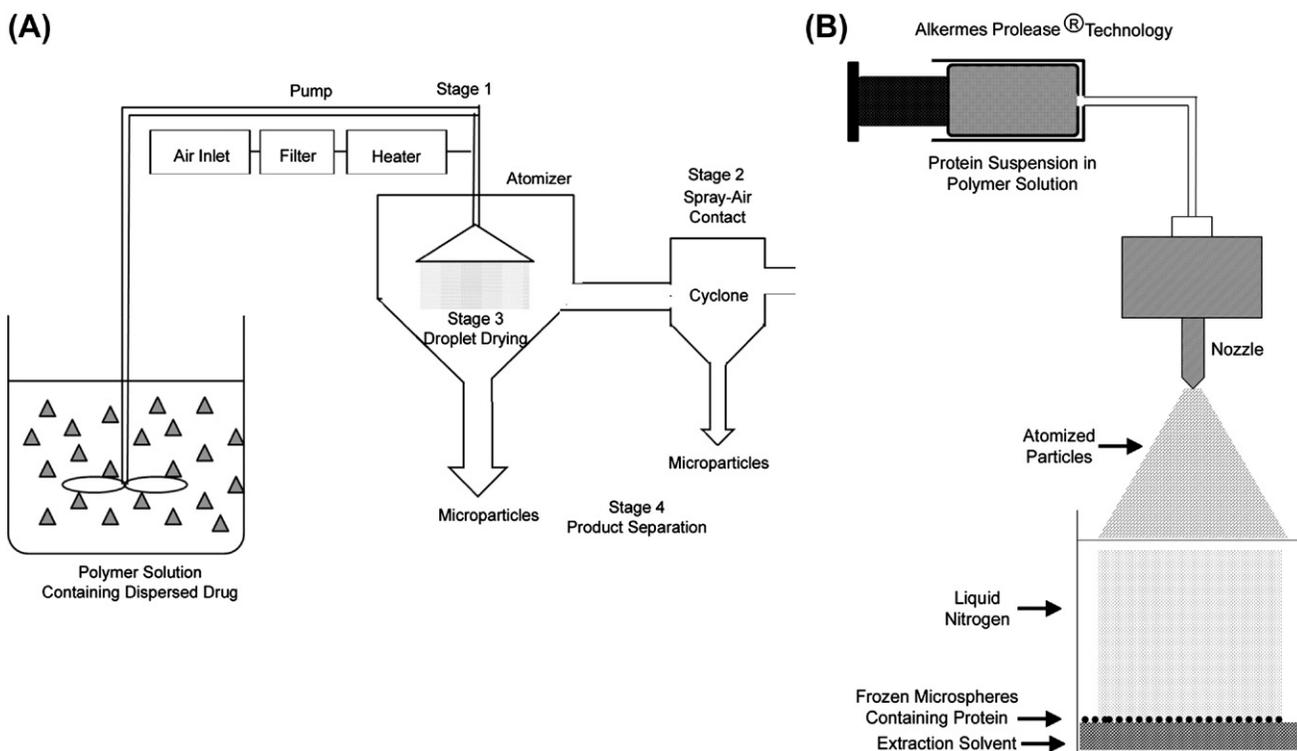


FIGURE I.2.19.3 Preparation of microparticles using spray drying techniques: (A) conventional spray drying method (adapted from Re, 2006); (B) low temperature spray drying method (adapted from Johnson et al., 1997).

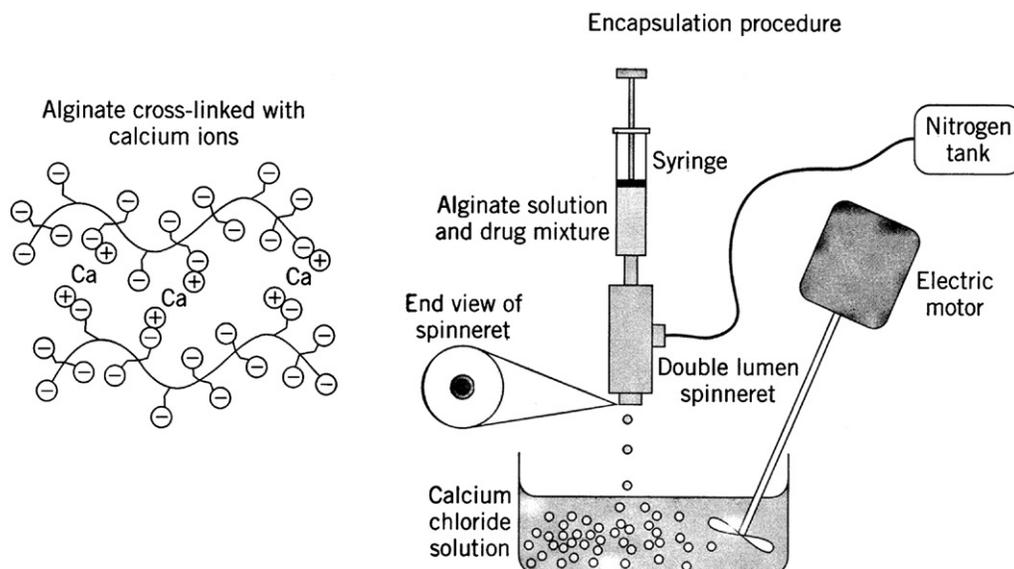


FIGURE I.2.19.4 Schematic showing the synthesis of microparticles by the ionic gelation method.

excipients are suspended in a polymer solution in an organic solvent (e.g., DCM, acetone, ethyl acetate) and sprayed into a vessel containing liquid nitrogen followed by evaporation of the liquid nitrogen (Figure I.2.19.3B). The vessel also contains a frozen solvent such as ethanol for extraction of the polymer solvent from liquid nitrogen frozen droplets. With this technique, microparticles with high encapsulation efficiency (~95%) and sizes ranging from 50–60 μm can be readily obtained (Johnson et al., 1997).

Ionic Gelation

Ionic gelation is a physiochemical technique in which ionic polyelectrolytes (or charged polymers) are chelated with multivalent counterions, producing cross-links (Figure I.2.19.4). Ionic gelation is widely used with polysaccharides such as chitosan and the sodium salt of alginic acid, because of its simplicity and mild conditions (Bodmeier et al., 1989; Li et al., 2008; Park et al., 2004; Shu and Zhu, 2001). Chitosan is a positively charged polysaccharide

which forms cross-links in anionic sodium triphosphate or tripolyphosphate (TPP) or $[\text{Fe}(\text{CN})_6]^{4-}$ or $[\text{Fe}(\text{CN})_6]^{3-}$ (Li et al., 2008). The ionic gelation process is fairly simple and involves drop-wise addition of polymer solution to the counter ion solution with dispersed drug, under continuous stirring. The ionization degree of TPP is dependent on the solution pH. At slightly acidic pH, only $(\text{P}_3\text{O}_{10})^{5-}$ anions are available, favoring ionic gelation with the acetic acid-based chitosan solution (Ko et al., 2002). The ionic gelation process can result in both nano- and microparticles ranging from several nanometers to 700 μm . The process has been extensively applied for encapsulating drugs (e.g., insulin, cyclosporine A, felodipine) with encapsulation efficiencies ranging from 55% to 90%, depending on deacetylation of chitosan and reaction conditions (Agnihotri et al., 2004; Ko et al., 2002). Ionic gelation performed at high concentrations of TPP results in slower drug release and *vice versa*. Drug release is further influenced by the extent of cross-linking (Agnihotri et al., 2004).

SUBMICRON-SIZED PARTICLES

Liposomes and Micelles

Liposomes (Figure I.2.19.5) are submicron-sized spheres with an aqueous core and a bilayer membrane (e.g., lamella), whereas micelles consist of an aqueous core surrounded by a single layer of lipids. Liposomes are formed by one or several lipids, including sphingomyelin, egg phosphatidylcholine (PC), cholesterol, monosialoganglioside (GM1), distearoylphosphatidylethanolamine (DSPE) or dioleoylphosphatidylethanolamine (DOPE). Liposomes can be classified as multilamellar (0.5–5 μm diameter), small unilamellar (~0.1 μm), or large unilamellar (0.2–0.8 μm) vesicles. Liposomes have excellent biocompatibility, and can encapsulate hydrophobic drug between the lipophilic bilayers and hydrophilic drug in

the core. Liposomal delivery systems have been used for therapeutics, including rifampicin, budesonide, diclofenac, methotrexate, vaccine antigens, nucleic acids, peptides, and immunomodulatory agents (Torchilin, 2005).

Conventional liposomes have a short blood circulation time and are quickly removed by the reticuloendothelial system (RES), as was demonstrated in early studies with immunoliposomes (i.e., liposomes modified with antibodies) that resulted in mass accumulation in the liver. Extensive research has been performed to develop long-circulating liposomes. Surface immobilization of PEG imparts “stealth” properties to liposomes (Figure I.2.19.5C), which minimizes RES uptake. Liposomes modified with PEG have been used in delivering anticancer drugs such as doxorubicin and paclitaxel (Skubitz, 2003), and have demonstrated improved drug bioavailability and pharmacokinetics (Allen and Hansen, 1991). Long-circulating liposomes have also been used in infectious disease treatment and diagnostic applications (Bakker-Woudenberg, 2002). PEG can also be combined with other targeting methodologies (i.e., antibodies) through covalent modifications or hydrophobic interactions to produce long-circulating immunoliposomes (Figure I.2.19.5D,E) (Torchilin, 2005; Blume and Cevc, 1990; Abra et al., 2002).

NANOPARTICLES

Nanoparticles are generally acknowledged as particles with dimensions less than ~100 nm. Nanoparticles may be composed of virtually any material, but unlike those used in microparticles, some of the most common materials are semiconductors and metals. Nanoparticles have generated significant interest as biomaterials, primarily because of their unique size. From a purely physical perspective, nanoparticles are small enough to interface with objects that control some of the most basic cellular functions (e.g., ~2 nm diameter DNA). In comparison,

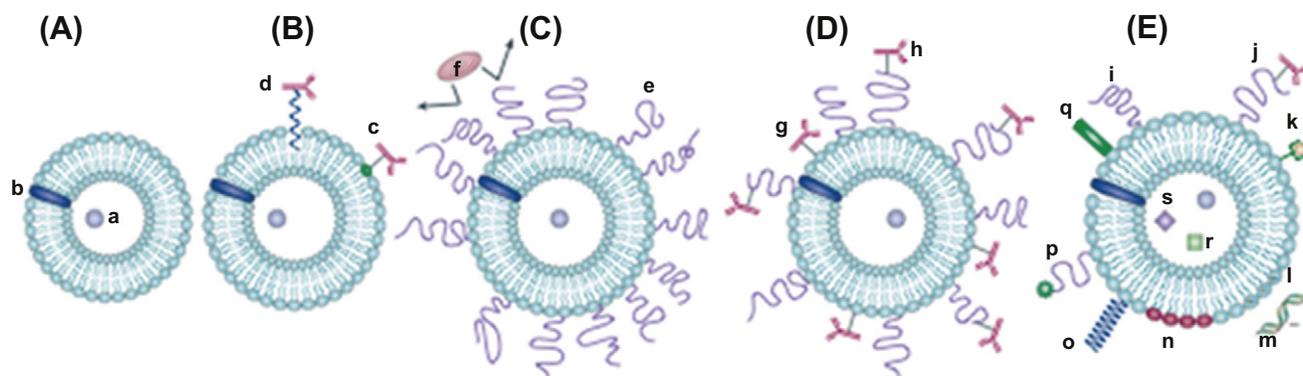


FIGURE I.2.19.5 Evolution of liposomes: (A) “Plain” phospholipid liposomes. Water soluble drug (a) is entrapped in the aqueous interior, and water-insoluble drug (b) is incorporated in the liposomal membrane (these designations are not repeated on other figures); (B) antibody-targeted immunoliposome. Antibody is covalently coupled (d) or hydrophobically anchored (c) to the liposomal membrane; (C) long-circulating liposome. Protective polymer (e.g., PEG) (e) shields the surface from opsonizing antibodies (f), which may otherwise lead to liposome phagocytosis; (D) long-circulating immunoliposome. Antibody is attached to the surface (g) or, preferably, to the grafted polymeric chain (h); (E) new-generation liposome. Multivalent liposomal systems can contain surface-bound protective polymer (i), targeting ligands (e.g., antibodies) (j), diagnostic labels (k), positively charged lipids (l) that permit complexation with DNA (m), stimuli-sensitive lipids (n) or polymers (o), cell-penetrating peptides (p) or viral components (q). The internal cavity can contain drug, magnetic particles (r) for magnetic targeting, and/or colloidal gold or silver particles (s) for electron microscopy (Torchilin, 2005).

cells are much larger (average diameter $\sim 10\ \mu\text{m}$). Also, their small size allows them to interface with existing microelectronic components, such as field effect transistors (FETs), permitting the development of diagnostic biosensors.

In addition to these direct benefits of size, nanoparticles are also interesting biomaterials because of their unique, size-dependent physical properties. For example, semiconductor quantum dots and gold nanoparticles produce optical responses that are not evident in bulk materials. Iron oxide particles display a different type of magnetism when presented as nanoparticles than when presented as microparticles or in bulk. These properties originate from different principles, but are all derived from the size of the material, which lies between bulk and atomic composition. Because of these interesting properties, nanoparticles have found application as contrast agents in biomedical imaging, signaling molecules that enhance diagnostic ability, carriers for drug delivery, and as therapeutic elements.

MATERIALS USED FOR NANOPARTICLE SYNTHESIS

Noble Metals

Noble metal nanoparticles have a long history. They were first discovered by the Romans (Wagner et al., 2000), who used them to create colored glass. More recently, they were extensively studied by Michael Faraday

(Faraday, 1857), whose samples are still on display at the Faraday Museum in London. In biomedical applications, the mostly commonly investigated materials have been gold and silver, which are created primarily through chemical reduction. For example, gold nanoparticles (Figure I.2.19.6A) can be created by the reduction of chloroauric acid (HAuCl_4) by sodium citrate (Turkevich, 1951) and silver nanoparticles by the reduction of silver nitrate (AgNO_3) by sodium borohydride (NaBH_4). In both cases, the reducing agent serves also as a passivating ligand, coating the surface of the nanoparticles.

Noble metals are widely regarded as non-toxic to humans; in fact silver nanoparticles are noted for their anti-bacterial properties (Sondi and Salopek-Sondi, 2004). As a result, noble metals have been applied as carriers for drug and gene delivery, and in anti-fouling coatings. In addition, they are attractive for imaging because they exhibit surface plasmon resonance (SPR) (Ritchie, 1957). Metals have many free electrons that typically reside in the conduction band. These electrons form a “cloud” surrounding the surface of the material that can deflect incoming light, producing a reflective surface. However, as we know from quantum mechanics, electrons can also behave as waves. If light of the same frequency as the oscillations of the electron cloud is absorbed, the cloud vibrates or resonates, producing an absorbance signal. This signal is altered by the thickness of the material and, in the case of nanomaterials, is enhanced by the large fraction of atoms at the material surface. This signal is also altered by adsorption of

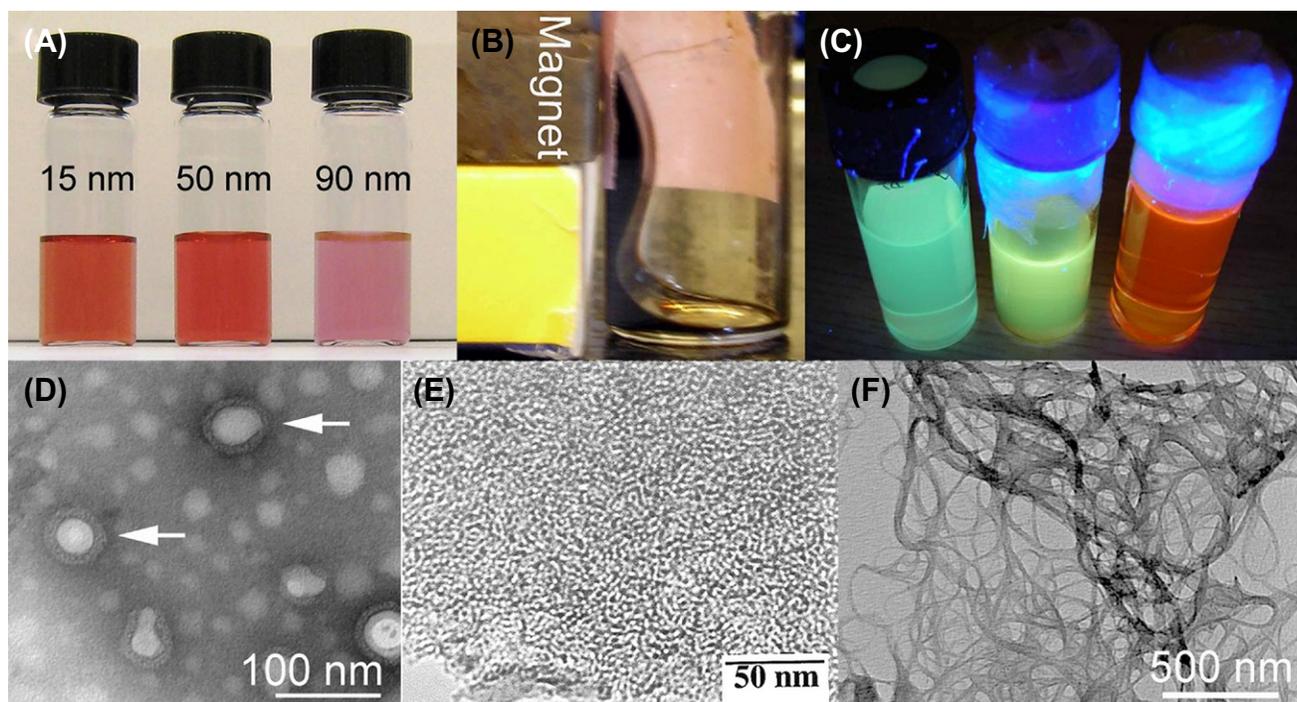


FIGURE I.2.19.6 Nanoparticles and their properties: (A) gold nanoparticles experience a color change with increasing size; (B) magnetic nanoparticles are attracted to magnets; (C) quantum dots fluoresce under UV excitation; (D) antibody-conjugated micelles (arrows) are shown in negative TEM (transmission electron microscopy) staining; (E) mesoporous silica contains multiple nanosized pores; (F) TEM image of single-walled carbon nanotubes. (Figures A–D courtesy of Dr. Jessica Winter, The Ohio State University; Figure E reproduced from Huo et al., 1997; Figure F courtesy of Dr. Maurizio Prato, University of Trieste, Trieste, Italy.)

other molecules to the material surface and nanoparticle aggregation; a property that has been exploited for the development of noble metal biosensors (He et al., 2000). Similarly, the strong absorbance produced by SPR has been used in photoacoustic imaging (Wang et al., 2003), which uses light to produce a signal detected via ultrasound.

Magnetic Materials

Although early research on magnetic nanoparticles dates back several decades (Crick and Hughes, 1949), interest in their use as a biomaterial is more recent (e.g., 1980s) (Sun et al., 2008). Superparamagnetic iron oxide nanoparticles (SPIONs), which are usually <15 nm in diameter, are the most commonly employed magnetic materials for these applications (Figure I.2.19.6B). SPION synthesis was originally performed through precipitation and microemulsion routes (Gobe et al., 1983), but more recently a high temperature precursor decomposition method (Hyeon et al., 2001) has been developed that yields small, uniform nanoparticles. One of the most important features of SPIONs is their unique magnetic response. Unlike larger nano- and micro-sized particles (e.g., >~15 nm), which are ferromagnetic, SPIONs display superparamagnetic behavior. This arises from their small size, which is below that of magnetic domain boundaries. Large particles consist of several magnetic domains that can be aligned in an external field, producing permanent magnetization. However, SPIONs have a single magnetic domain. They respond to a magnetic field, but do not display permanent magnetization, as evidenced by their lack of hysteresis on a magnetization (M) versus magnetic field strength (H) plot. This is because thermal fluctuations prevent permanent magnetic alignment of the single domain. Thus, SPIONs have the appearance of paramagnetic behavior (i.e., no permanent magnetization), while still responding to a magnetic field. This behavior is called superparamagnetism.

These magnetic properties have been used for a number of biomedical applications. The force generated on SPIONs in an external magnetic field has been harnessed for cell separation, drug delivery, and biomolecule manipulation (Pankhurst et al., 2003). Additionally, SPIONs have been used as contrast agents for magnetic resonance imaging (MRI) (Weissleder et al., 1990). MR imaging is accomplished by examining either the time that it takes for the spin magnetization of an individual proton to return to its original state (T1) or the time for the net magnetic moment for all protons to return to zero (T2). The magnetization produced by SPIONs interacts with water molecules in the body to shorten their T2 relaxation time. This produces an area of negative contrast in an image. In addition to MRI, SPIONs have been used for magnetic hyperthermia. When SPIONs are placed in an alternating magnetic field, they attempt to align their magnetic moments with that field. This produces internal friction that manifests itself as heat. If magnetic

nanoparticles are placed in a tumor and a temperature above 45°C is reached, the tumor cells can be killed in a process known as hyperthermia (Pankhurst et al., 2003). Alternatively, if the temperature is raised to ~42°C, the efficiency of chemotherapy can be improved.

Quantum Dots

Quantum dots are small, fluorescent nanocrystals made from semiconductor materials, usually of spherical shape and <10 nm in diameter (Figure I.2.19.6C). The most commonly studied quantum dots are composed of a cadmium selenide (CdSe) core surrounded by a zinc sulfide (ZnS) shell, and are commercially available. Quantum dots are synthesized primarily through high temperature precursor decomposition (Peng and Peng, 2001); however, some aqueous arrested precipitation routes are also available (Winter et al., 2005). Quantum dots were first identified by Louis Brus at Bell Labs (Rossetti et al., 1983), and first employed for biomedical applications in 1998 (Chan & Nie, 1998; Bruchez Jr. et al., 1998). Quantum dots are so named because they experience quantum confinement, which means that the electron wavefunction is limited by the size of the particle. Because of this, quantum dots have a band gap energy (i.e., energy difference between the ground and excited states of an electron) that is dependent on particle size. Optical properties of quantum dots can thus be tuned by adjusting their size (Alivisatos, 1996).

Quantum dots have been used in a broad range of biological applications, including single molecule biophysics (Courty et al., 2006), optical barcoding (Han et al., 2001), and molecular (Courty et al., 2006), cellular (Jaiswal et al., 2003), and *in vivo* imaging (Gao et al., 2004). Compared to organic dyes and fluorescent proteins, quantum dots are about 10–100 times brighter, mainly because of their large absorption cross-sections, 100–1000 times more stable against photobleaching, and show narrower and more symmetric emission spectra. In addition, a single light source can be used to excite quantum dots with different emission wavelengths. CdSe has become the prototypical quantum dot material for biological applications, because it can produce fluorescence at wavelengths from green (500 nm wavelength) to red (650 nm wavelength) simply by adjusting the nanocrystal diameter from 2 to 8 nm. Other materials may be used to span other spectral regions, such as the ultraviolet region (CdS, ZnS, ZnSe), the near-infrared (CdTe, CdSe_xTe_{1-x}, PbS), and the mid-infrared (PbSe). A major limitation of quantum dots is their relatively large size compared with organic dyes, which may lead to steric hindrance in some applications (Alivisatos et al., 2005).

Polymers and Lipids

Polymers (Marty et al., 1978) and lipids (Gregoriadis et al., 1971) were among the first nanomaterials employed

in biological applications. Polymer nanoparticles can consist of solid, porous polymer spheres or of nanocapsules consisting of an external polymer layer surrounding a liquid, aqueous core. Polymers can also form vesicles or micelles, sometimes called polymersomes. Lipid particles can consist of liposomes, lipid micelles or microemulsions (Figure I.2.19.6D). Polymeric nanoparticles can be fabricated via emulsion routes, in which case particle size is determined by the size of the suspended droplets, or via interfacial interactions with another solution, in which case size is determined by droplet size and mixing (Pinto Reis et al., 2006). Lipid-based microemulsions can be created by sonication of lipids in water, where size is determined by the extent of sonication. Vesicles/micelles of either lipids or polymers can be formed by self-assembly, in which case the size is determined by the thermodynamics of the system.

Polymer and lipid nanoparticles have been primarily used as carriers for therapeutic and imaging agents, and offer several advantages in this application. Polymer and lipid biomaterials can be used to encapsulate and protect therapeutic agents while they are transported to desired locations by passive or active targeting mechanisms. Furthermore, therapeutic agents (e.g., small molecule drugs, antibodies, small interfering RNAs) can be released from the nanocarriers in a controlled manner, for optimum therapeutic efficacy with minimal side effects. Additionally, these nanoparticles can incorporate a variety of factors, such as targeting, controlled release, imaging, and therapy into a single device.

Silica

The most common type of silica employed as a biomaterial is mesoporous silica (Figure I.2.19.6E). These particles, which have been created in both micro- and nanoparticle form, consist of silica (SiO_2) containing pores ranging from 2–10 nm in diameter. Mesoporous silica particles were first created in 1990 (Yanagisawa et al., 1990), and were improved by the development of controlled templating (Kresge et al., 1992) and synthetic advances to enhance particle monodispersity (Huo et al., 1997). In addition to mesoporous silica particles, silica is also widely-used as a nanoparticle coating. For example, silica has been employed to render quantum dots and iron oxide nanoparticles biocompatible. Most commonly, silica is deposited using the Stöber method (Stöber et al., 1968), which is a condensation reaction occurring in an alcohol–water system.

The primary advantages of silica as a biomaterial are its biocompatibility, easy surface modification, and porous structure. These properties have led to applications in imaging, drug delivery, and gene therapy. The surface of silica can be readily altered using siloxanes (R-SiOCH_3), a number of which are commercially available (e.g., terminal $-\text{SH}$ or $-\text{NH}_2$ modification). This property has permitted silica nanoparticles to be

conjugated to biomolecules that can specifically target certain cells or tissues. Additionally, the highly porous nature of silica materials has been used to encapsulate other materials, including imaging agents, drugs, DNA, and even other nanoparticles (Tallury et al., 2008). These materials can be slowly released at the target site to provide therapeutic benefit or a method of biodegradability.

Carbon

Several carbonaceous nanomaterials have been investigated as biomaterials including fullerenes, carbon nanotubes, and carbon nanodiamonds. The first of these to be discovered were carbon nanodiamonds, which were identified as an explosion by-product in 1961 (DeCarli and Jamieson, 1961); however, these materials were not investigated as a biomaterial until much later (Kossovsky et al., 1995). Carbon nanodiamonds are usually <10 nm diameter and display the diamond crystal structure. The next carbonaceous materials to be identified were fullerenes (Kroto et al., 1985), also known as buckyballs because of their structural similarity to geodesic domes designed by architect Richard Buckminster Fuller. Their discovery earned the Nobel Prize in Chemistry in 1996 for co-inventors Richard Smalley, Robert Curl, and Harold Kroto. Work on fullerenes led to the discovery of carbon nanotubes (CNTs) (Iijima, 1991), which can be viewed as rolled-up sheets of graphene (Figure I.2.19.6F). CNTs can consist of a single sheet (i.e., single-walled carbon nanotubes (SWCNTs)) or multiple sheets (i.e., multi-walled carbon nanotubes (MWCNTs)), and can be either semiconducting or metallic depending on the orientation of the rolled sheet. Carbonaceous nanomaterials can be produced through detonation (Danilenko, 2004), arc discharge (Kroto et al., 1985), laser ablation (Yang et al., 1998; Guo et al., 1995), and chemical vapor deposition (Feldman et al., 1995). Native carbonaceous materials are generally extremely hydrophobic; however, acid treatment can produce $-\text{COOH}$ functionalization that renders particles water soluble (Chen et al., 1998). Because of their extreme hydrophobicity there have been substantial concerns regarding the biocompatibility of carbonaceous materials (Cui et al., 2005; Schrand et al., 2007). However, toxicity appears to be related to a number of controllable factors including dimensions, surface functionalization, manufacturing method, and processing (Helland et al., 2007).

Carbonaceous nanomaterials exhibit a number of unique, size-dependent properties, including high strength (e.g., CNTs are 100× as strong as steel (Treacy et al., 1996)), and electrical and optical characteristics, which have been exploited in biomedical applications ranging from molecular separation, biomolecule delivery and therapeutic treatment to imaging. For example, CNTs have been used for gene (Pantarotto et al., 2004) and drug (Kam et al., 2005) delivery, because they can penetrate cells through a mechanism believed to be independent

of endocytosis (i.e., penetration of the lipid membrane) (Pantarotto et al., 2004). Because of their conductive and semiconductive properties, CNTs have been examined as scaffolds for nerve regeneration (Mattson et al., 2000), and as electrodes for neural prostheses (Lovat et al., 2005; Gheith et al., 2005). Conductivity has also been used to create biosensors (Kong et al., 2000) with detection down to the single molecule level (Besteman et al., 2003). However, two of the more interesting properties of carbonaceous materials are their ability to generate reactive oxygen species (ROS) and to produce multiple imaging signals.

ROS are oxygen or oxygen-containing molecules that have a highly reactive unpaired electron (e.g., O_2^1 , singlet oxygen). ROS can cause significant damage to cells by interacting with the mitochondrial respiration system or DNA, and can be used to kill targeted cells (e.g., photodynamic therapy). Fullerenes generate ROS by light-induced excitation of a free electron to the singlet excited state, which is quickly followed by decay to the triplet state (e.g., the spin of the electron is reversed) (Yamakoshi et al., 2003). Conversely, fullerenes not exposed to light can neutralize ROS. Despite numerous π -bonded rings, fullerenes are electron acceptors, rather than donors (Dugan et al., 1996), and easily react with the ROS-excited electron. This property can be used to reduce damage to cells, for example, neutralizing ROS generated by UV irradiation.

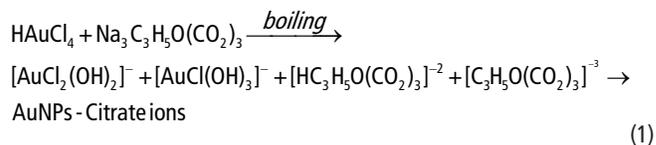
In addition to ROS generation, carbonaceous materials produce a number of optical signals, which, when coupled with a low toxicity, make them attractive candidates for *in vivo* imaging. Carbon nanodiamonds exhibit fluorescence that is believed to arise from excitation of nitrogen vacancies (known as N-V centers) (Holt, 2007), and can also produce electron spin resonance (ESR/EPR) signals (Balasubramanian et al., 2008). Similarly, semiconducting CNTs can exhibit bandgap fluorescence, as well as fluorescent emission resulting from surface states. CNTs also produce strong Raman scattering signals, which result when light striking an object loses or gains energy from the collision. In the case of CNTs, Raman scattering occurs from interactions of light with the electron cloud surrounding the CNT structure. Both of these properties have been used for cellular imaging (Heller et al., 2005).

NANOPARTICLE PREPARATION

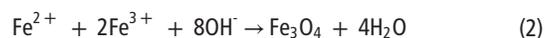
Arrested Precipitation

This method has been applied for the synthesis of semiconductor quantum dots (e.g., CdS), metals (e.g., Au), and iron oxide nanoparticles. In the absence of stabilizing agents, particles produced using these reactions exhibit very low solubility in water, hence they precipitate. However, when another agent is added to the reaction system that is water-soluble and can also bind to the nanoparticle surface, stable nanoparticles are produced. For example, in the presence of a Cd-binding ligand, mixing Cd^{2+} and S^{2-} salts produces CdS nanocrystals

with size-dependent fluorescence (Rossetti et al., 1984). Similarly, gold nanoparticles can be produced using the Turkevich procedure (Scheme 1) (Ji et al., 2007):



In this process, a sodium citrate solution is added to a $HAuCl_4$ solution with heating and vigorous stirring. Sodium citrate first acts as a reducing agent, and then the negatively-charged citrate ions bind to the gold nanoparticle surface, preventing aggregation (Turkevich, 1951). Similarly, iron oxide nanoparticles can be synthesized by reduction in base (Gupta and Gupta, 2005):



Iron ions are obtained from iron chloride salts (e.g., $FeCl_2$, $FeCl_3$), and sodium hydroxide (NaOH) serves as the base. The unifying feature of all these procedures is that particles are produced in the presence of a solubilizing agent, or ligand, that prevents aggregation and controls growth through steric hindrance and occupation of potential reaction sites on the particle surface.

High Temperature Precursor Decomposition

In 1993, the high temperature precursor decomposition method was developed to synthesize high quality quantum dots (Murray et al., 1993). This method relies on the use of a solid precursor, which decomposes at high temperature to yield reactive ions. As an example, to produce CdSe quantum dots, dimethylcadmium precursor and Se shot are mixed in solvents (e.g., trioctylphosphine, trioctylphosphine oxide, hexadecylamine) at high temperature. These solvents also serve as ligands whose basic functional groups (e.g., phosphines, phosphine oxides, amines) attach to the quantum dot surface, displaying their hydrophobic alkyl chains to the exterior. Slow growth and annealing results in uniform particle size and regularity in core structure. However, fluorescence quantum yield of quantum dots produced by this procedure decays over time, due to surface defects. In 1997, a higher band gap energy material (e.g., CdS) was used to coat CdSe quantum dots; the CdSe/CdS core shell structure resulted in a dramatic improvement in the fluorescence quantum yield (as much as 10 times) (Peng et al., 1997). Recently, the high temperature synthesis method has been adapted to produce other types of nanoparticles (e.g., iron oxide nanoparticles) (Hyeon et al., 2001).

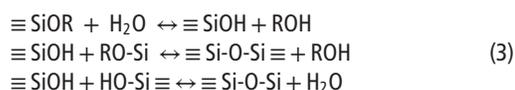
Self-Assembly

The self-assembly method is driven primarily by thermodynamic considerations. For example, amphiphilic

molecules, which possess both hydrophobic and hydrophilic segments, will attempt to minimize exposure of their hydrophobic segments in aqueous solution, spontaneously forming micelles, vesicles, rods, lamellae, tubules, and other nanoparticulates. The shape and size of these particles are mainly determined by the molecular structure of the amphiphiles employed (Zhang & Eisenberg, 1995). In practice, the self-assembly process can be conducted using different methods, such as direct dissolution, dialysis, and film hydration. The direct dissolution process simply involves adding amphiphile to water, but can only be used for water-soluble amphiphiles. Dialysis can be used when the amphiphile is not easily soluble in water, but can be readily dissolved in a water-miscible organic solvent such as dimethylformamide. In this case, a mixture of the amphiphile and water-miscible solvent is dialyzed against water, driving the organic solvent to the aqueous phase and inducing nanoparticle formation. If the amphiphile is only soluble in non-polar solvents, film hydration can be used. In this process, a thin film is formed by dissolving amphiphile in a low boiling point, non-polar solvent (e.g., chloroform), which is removed by evaporation. Water is then added, hydrating the film and driving spontaneous nanoparticle formation (Allen et al., 1999; Lasic, 1993).

Stöber Method

The Stöber method is a classic technique for producing silica nanoparticles, films, and coatings discovered in 1968 by Werner Stöber and colleagues (Stöber et al., 1968). The technique is a sol-gel method based on the hydrolysis and subsequent condensation of silicon-containing precursors (Scheme 3).



Typically, tetramethoxysilane (TMOS) or tetraethylorthoxysilane (TEOS) are used as the silicon source. Both of these compounds contain alkoxide groups (e.g., $-\text{OCH}_3$ for TMOS, $-\text{OCH}_2\text{CH}_3$ for TEOS) that react with water to yield Si-OH intermediates and R-OH by-products. Si-OH can then react with other Si-OH or Si-OR groups to yield silica and ROH and H_2O by-products. The reaction is usually performed in an alcohol-water mixture, with alcohols being methanol, ethanol, *n*-propanol or *n*-butanol. Ammonia is used as a catalyst for colloidal growth. This reaction produces nearly monodisperse particles in sizes ranging from the micron to nanometer scale. This method was further modified by Unger in 1997 (Grun et al., 1997) to create monodisperse, porous silica spheres. This was accomplished by adding cetyltrimethylammonium bromide (CTAB), a cationic surfactant, which forms micelles in the reaction solution. Micelles are encapsulated during sphere formation. This surfactant is subsequently removed during particle calcination (i.e., heating), producing pores

within the silica spheres. This method of particle formation is known as the modified Stöber method.

Methods to Produce Carbonaceous Nanoparticles

Carbonaceous materials can be produced via explosion, arc discharge, laser ablation, and chemical vapor deposition. A unifying feature of these methods is the heating of carbon precursors to high temperatures. Explosion is primarily used to create carbon nanodiamonds (DeCarli and Jamieson, 1961; Danilenko, 2004), and produces high temperatures and pressures that force carbon into the diamond region of its phase diagram. The carbon source may consist of a separate substrate or originate from the explosives themselves (Shenderova et al., 2002). Nanodiamond is manufactured in several commercial operations, primarily in Russia, its former republics, and Japan.

Arc discharge (Iijima, 1991; Krätschmer et al., 1990) and laser ablation (Kroto et al., 1985; Guo et al., 1995) are used to produce fullerenes and CNTs. Each method produces a carbon gas cloud, either by passing current through graphitic electrodes (i.e., arc discharge) or by using lasers to heat solid, carbon substrates (i.e., laser ablation) (Shenderova et al., 2002), reaching temperatures of 3000–4000°C. Gaseous carbon then condenses, producing carbonaceous nanostructures. This method is often performed in the presence of a catalyst, for example cobalt, yttrium or nickel, to control the shape of the resultant nanostructures. Critical parameters for arc discharge are the identity and pressure of the gas separating the electrodes, and the strength of the current. High gas pressure and low current tend to favor CNT production, whereas lower gas pressures favor fullerene formation. Laser ablation often produces a mixture of desired particles and undesired products. Separation processes, such as reflux in nitric acid, have been developed to isolate desired materials (Liu et al., 1998).

Chemical vapor deposition (CVD) has been used to produce carbon nanodiamonds (Eto et al., 1992) and CNTs (Kong et al., 1998). For nanodiamond, the CVD method primarily yields films, rather than freestanding particles (Shenderova et al., 2002). In this method, a hydrocarbon gas is deposited on a metal catalyst (e.g., iron, cobalt, nickel), which breaks the carbon-containing molecules into smaller components. These molecules serve as nucleation centers for additional particle growth. Reaction takes place at temperatures substantially below those of arc discharge or laser ablation (e.g., near ~1000°C). Acid treatment can be used to purify the materials produced.

SURFACE MODIFICATION OF MICRO/NANOPARTICLES

Surface properties of particles are governed by the charge and hydrophilic or hydrophobic behavior of the starting materials. For example, the surface charge of PLGA microparticles is slightly negative because of the presence of

carboxylic groups, and the surface of polystyrene-based microparticles is essentially hydrophobic because of hydrophobic monomers. Surface properties can modulate particle interactions with the body permitting passive targeting to specific biological elements (e.g., through charge or hydrophobic interactions) (Singh et al., 2000). Particles can also be modified with biomolecules (e.g., antibodies, peptides) for active targeting to specific organs, cells or cellular components. Passive adsorption is one of the oldest surface modification methods and has been extensively employed to attach proteins, antibodies, antigens, nucleic acids, and polymers to particles (Bates et al., 2006). Loading is initiated by the interaction of biomolecule functional groups with the micro/nanoparticle surface (e.g., interaction of nucleic acid amines with carboxylic groups on PLGA). To obtain high loading efficiency and retain bioactivity, adsorption should be performed at a pH near the isoelectric point of the biomolecule. In addition to passive loading, numerous bioconjugation strategies exist (see Chapter I.2.17). One class of molecules that have been particularly important in particle surface modification are poly(ethylene oxides) (PEOs) and their shorter chain polyethylene glycol (PEG) derivatives. PEO/PEG modification reduces protein adsorption to the particle surface, prolonging circulation time (Cui et al., 2001; Coombes et al., 1997), and reducing uptake by clearance systems (Akerman et al., 2002).

CHARACTERIZATION OF MICRO/NANOPARTICLES

Particle behavior depends on a number of factors. Surface charge and morphology, size, chemical composition, and aggregation tendency all play a crucial role, with minor alterations in any of these properties potentially resulting in significant changes. The most common methods for physiochemical characterization of micro/nanoparticles are electron microscopy (i.e., SEM, TEM), dynamic light scattering (DLS), zeta-potential measurement, X-ray photoelectron spectroscopy (XPS) analysis, atomic force microscopy (AFM), and optical light or fluorescence microscopy. Microscopy techniques, including optical, fluorescence, and transmission and scanning electron microscopy, are often utilized to study particle surface morphology, size, and shape (Champion et al., 2007). It should be noted, however, that EM techniques may involve sample preparation and pre-handling steps, including dehydration and coating, which might alter particle properties. Further, high vacuum renders imaging of aqueous samples problematic and may cause structural damage. To obtain size information on a distribution or large number of particles, light scattering is preferred. Light scattering measures particle size based on the scattering intensity, which changes with variation in scattering angle, size of particles, and refractive indices of particle and the medium. However, there are some limitations associated with this method, such as the difficulty

of evaluating particles with a large size distribution. Also, measurements are dependent on algorithms that require particles to be spherical, and to have a large difference between particle refractive index and dispersion medium. Therefore, light scattering is often combined with other techniques such as microscopy methods. AFM is used less frequently and can provide similar information on particle shape, size, and morphology. In addition, AFM can gather quantitative data on elastic modulus, and particle adhesion and deformation (Vakarelski et al., 2001; Tagit et al., 2008). XPS can provide information on the particle surface chemical composition (Xie et al., 2006). In particular, XPS has been exploited to detect the presence of biomolecules/drugs on the particle surface (Xie et al., 2006; Chesko et al., 2008). Zeta potential measures net surface charge, and is used to determine particle stability in suspension. A large absolute value of zeta potential indicates more stable particles that are less likely to form aggregates. Zeta potentials can also be used to determine whether a surface is positively or negatively charged, which is important for particle modification. For example, cationic microparticles can be prepared by modifying PLGA microparticles with surfactants, with the surface positive charge confirmed by zeta potential studies. Positively charged surfaces permit tethering of negatively-charged DNA for DNA delivery applications (Singh et al., 2000). Similarly, anionic microparticles of PLGA have been created with similar techniques (Singh et al., 2004). Apart from these commonly exercised characterization techniques, differential scanning calorimetry (DSC) has been used to assess changes in polymer thermal properties, infrared spectra (IR or FTIR) has been used to analyze interactions between multiple materials, and surface tension measurements have been used to evaluate particle aggregation. Chapters I.1.5 (surface characterization) and II.3.8 (microscopy) will be useful in understanding the characterization of microparticles and nanoparticles.

APPLICATIONS OF MICRO/NANOPARTICLES

Detection and Imaging

Microparticles have been used for detection and imaging applications, however, to a significantly lesser extent than nanoparticles. Fluorescent microparticles have been employed to detect diseased tissues. For example, fluorescent microparticles (1–10 μm diameter) were used for the detection of myocardium at risk of infarction in dogs (Fieno et al., 2000). Furthermore, because of the presence of multiple fluorescent dyes, microparticles with different fluorescent tags can be imaged in the same specimen. Fluorescent particles have also been used to image patterned scaffolds (Lu et al., 2006). Patterned scaffolds were fabricated with fluorescent microparticles as a prototype to demonstrate the feasibility of patterning scaffolds with multiple regions with varying properties (i.e., regions with

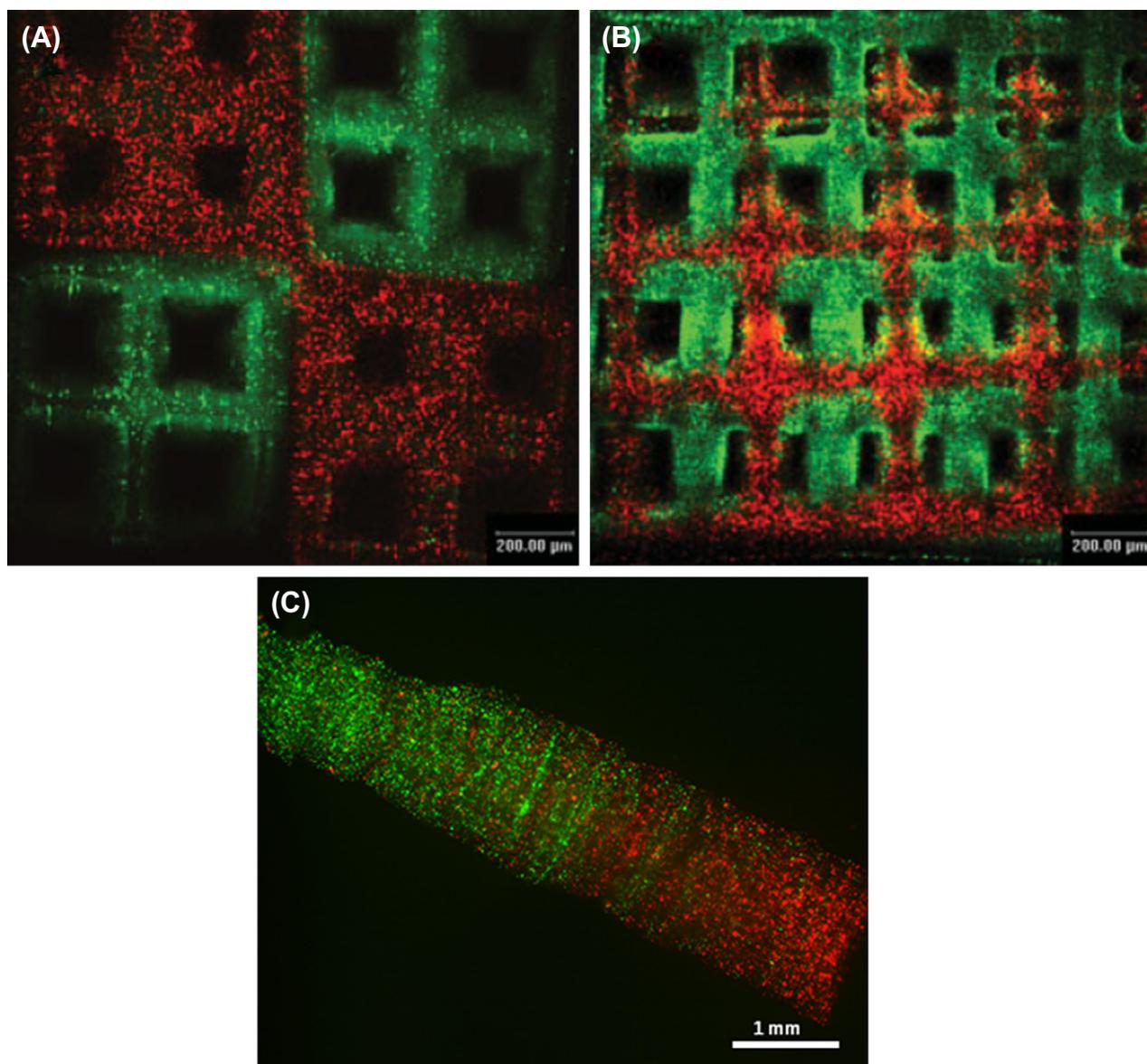


FIGURE I.2.19.7 Spatial patterning in PEG scaffolds using fluorescent microparticles (A and B) (Lu et al., 2006); (A) single-layered scaffold with spatially-patterned regions; (B) multi-layered scaffold with each layer fabricated with different fluorescent microparticles; (C) scaffold exhibiting the gradient of two different fluorescent microparticles running in opposite directions. (Figure C courtesy of Shalu Suri, Li Hsin Han, Shaochen Chen, and Christine Schmidt from The University of Texas at Austin, TX, USA.)

different biomolecules, growth factors, and cell types). For example, Cyt5- and FITC-labeled polystyrene microparticles were utilized to create spatially patterned multilayered PEG scaffolds (Figure I.2.19.7). Microparticles have also been used to create growth factor gradients as a proof of concept to create biomolecule gradients (Figure I.2.19.7C).

In addition to fluorescent microparticles, metal iron oxide microparticles (IOMPs) have also had an impact on *in vivo* cellular imaging and tracking (McAteer et al., 2007; Shapiro et al., 2004). Because of their larger size, in comparison to iron oxide nanoparticles, IOMPs are not suitable for molecular detection applications. However, IOMPs have successfully been used for imaging of single cells, mouse embryos, and tissue samples such as the brain. These microparticles are advantageous since the iron oxide content is orders of magnitude higher

than that in nanoparticles, which results in improved MRI contrast. Antibody-conjugated IOMPs are endocytosed by cells and provide quantifiable contrast even at very low concentration in cells, unlike nanoparticles. In addition, IOMPs are less susceptible to nonspecific cellular uptake than nanoparticles. Furthermore, fluorescent IOMPs offer the advantage of being able to perform MRI and fluorescence microscopy in the same specimen (Shapiro et al., 2004).

Nanoparticles in particular can play a critical role in molecular detection by serving as probes of biomolecular processes. Their size similarity to biomolecules and their unique size-dependent properties make them particularly attractive agents for imaging, detection, and treatment. For example, quantum dots (Chan & Nie, 1998; Bruchez Jr. et al., 1998) have achieved tremendous

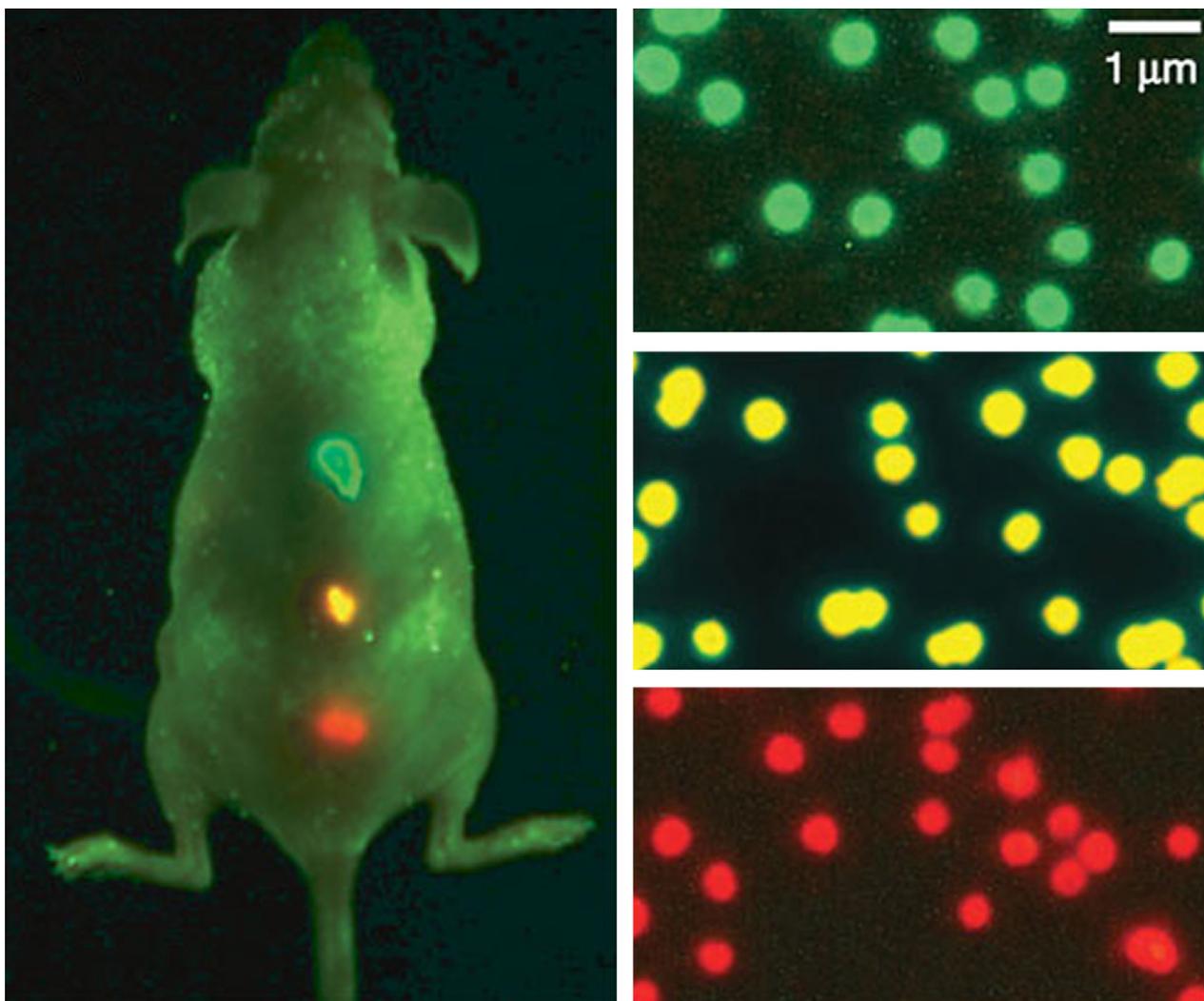


FIGURE I.2.19.8 Multiple color imaging. Three different colors of quantum dot microbeads (right) were imaged simultaneously (left). Approximately 1–2 million beads in each color were injected subcutaneously at three adjacent locations on a host animal. (Image from Gao et al., 2004.)

success in biomedical imaging because of their unique fluorescence properties. They exhibit a bright signal that has led to dramatically improved signal-to-noise ratios (compared with organic fluorescent dyes). Additionally, because of their narrow fluorescence emission peaks, they can be used to image multiple biomolecules simultaneously (i.e., multiplexed imaging). This is important because many diseases are often best identified by multiple, rather than single, biomarkers. The first clinically relevant biomolecular investigation using quantum dots was the detection and imaging of an important breast cancer marker, her-2 (Wu et al., 2003), the presence of which is used to direct treatment (e.g., eligible to receive herceptin) and can impact patient survival. Quantum dots of different emission wavelengths have been used to detect and quantify as many as four biomarkers simultaneously in tissue sections (Xing et al., 2007; Nie et al., 2007). *In vivo*, simultaneous targeting and imaging of three distinct tumors has been demonstrated (Gao et al., 2004) (Figure I.2.19.8). Further, quantum dots have

extraordinary stability against photobleaching, making them ideal for tracking dynamic biological processes. Quantum dots have been used to observe development of *Xenopus* frog embryos (Dubertret et al., 2002), trafficking of glycine receptors in the neural synapse (Dahan et al., 2003), motion of individual kinesin motor proteins (Courty et al., 2006), and intracellular dynamics of the Tat peptide, which is responsible for the cellular invasion of HIV virus (Ruan et al., 2007).

Whereas quantum dots have made a significant impact in imaging, gold nanoparticles have been instrumental in molecular detection. The SPR effect of gold nanoparticles has been extensively employed for detection of nucleic acids (e.g., DNA) (Elghanian et al., 1997). Gold nanoparticles are easily conjugated to oligonucleotides which, upon exposure to complementary target sequences, cross-link, reducing particle separation distance. This change in distance leads to an SPR effect, resulting in a solution color change detectable by the naked eye (Figure I.2.19.6A). Another property which has been exploited

for molecular detection is surface-enhanced Raman scattering (SERS), which is the enhancement of the Raman scattering signal upon binding of a reporter molecule to the gold nanoparticle surface. The enhancement factor can be as large as 10^{14} – 10^{15} , which has permitted *in vivo* tumor imaging (Qian et al., 2008). Compared with fluorescence detection, an important advantage of SERS-based detection is its potential for greater multiplexing. Each SERS spectrum is unique, and is composed of multiple bands that are much narrower than fluorescence spectra of dyes or quantum dots permitting many more signals to be simultaneously discriminated. It has been reported that up to three respiratory viruses in the same sample were detected by SERS-based gold nanoparticles (Boisselier and Astruc, 2009), and in theory multiplexing capacity could be greatly expanded.

In addition to these two nanoparticles, several others have made an impact in molecular medicine. SPIONs have been used as a contrast agent for MR imaging because of their ability to shorten T2 relaxation times (Pankhurst et al., 2003; Yang et al., 2009). SWCNTs have been used as tips in AFM imaging to selectively detect DNA from single-base mismatch sequences (Woolley et al., 2000). CNTs have also been used to create sensors. For example, the electrochemical signal of guanine bases was amplified for label-free electrochemical detection of DNA (Wang et al., 2004).

Drug and Gene Delivery

Microparticles are of immense interest in the field of drug and gene delivery, and have been researched predominantly for delivery applications. Microparticles offer versatile advantages including: multiple routes of administration; encapsulation of a range of drugs including hydrophobic, hydrophilic or neutral drugs; protection of the biomolecule from degradation in the stringent environment and from body clearance; and provision of site-specific controlled release. The drug or biomolecule can either be encapsulated in the bulk of the particle or conjugated outside using surface modification techniques. Multiple biomolecules can also be delivered from the same microparticles to target more than one application. When encapsulated, the drug release occurs either via diffusion through swelling or via bulk or surface degradation. Therefore, varying release profiles can be obtained simply by modulating the microparticle degradation rate. For example, PLA/PGA microparticles were used for 5-fluorouracil delivery; different PLA/PGA ratios resulted in altered release profiles (Park, 1995) (Figure I.2.19.1).

One of the most active and thoroughly investigated areas of microparticle drug delivery is for vaccine applications. Particularly, polymeric microparticles in the size range of 1–5 μm are easily recognized by immune cells as foreign and easily phagocytosed, thus making microparticles a popular system for vaccine delivery. Microparticles of natural and synthetic origin have been utilized

to deliver nucleic acids such as DNA, short interfering RNA (siRNA), proteins, and peptides. Immunogenic DNA antigens have been extensively encapsulated inside or conjugated outside PLGA microparticles for preclinical studies with Hepatitis B and various cancer models (Luo et al., 2003). Advancement in the synthesis/fabrication techniques has opened the field towards the delivery of multiple biomolecules. For example, modification of conventional PLGA microparticles has facilitated delivery of DNA with siRNA by encapsulating siRNA in the core of the particles and conjugating DNA to the surface (Singh et al., 2008). Further, hybrids of PLGA and poly β amino esters (PBAE, pH sensitive polyesters) have resulted in microparticles with superior intracellular delivery of DNA antigens for tumor rejection, as compared to conventional DNA encapsulated PLGA microparticles (Little et al., 2004).

Microparticles have also been extensively used for oral delivery of proteins and peptides. Mucoadhesive chitosan-based microparticles have been used for delivery of proteins to the gastrointestinal tract, where they can be taken up by the M cells of the Peyer's patch (van der Lubben et al., 2001c; Ahire et al., 2007). These particles exhibit prolonged retention in the mucous layer, thus increasing the availability of microparticles for cellular uptake. Further examples of microparticle applications based on material properties have been summarized in the section "Materials Used for Microparticle Synthesis" and in Table I.2.19.1.

Nanoparticles are attractive biomolecule carriers because of their small size, which provides several benefits. First, the dosing potential of nanoparticles is significant. Nearly 1 million, 5 nm particles could fit within the cytoplasm of a 10 μm cell versus ~1–10 micron-sized particles, with typical numbers in the range of tens of thousands. Second, nanoparticles consist primarily of surface (e.g., surface-to-volume ratio is very high), which is easily modified with biomolecules to be delivered. Thus, the loading capability of nanoparticle carriers is high on a volume basis. Additionally, easily modified surfaces permit conjugation to targeting molecules that can direct nanoparticles to the cell/organ of interest. Because nanoparticles are much smaller than cells, they can be directed not only to specific cell types, but even to specific regions of a cell (i.e., nucleus for gene delivery). Finally, the small size of nanoparticles has proven an advantage in evading the body's normal uptake and clearance system, the RES, which permits nanoparticle carriers to circulate for much longer than drug alone.

One of the most active areas of drug delivery has been in the field of cancer treatment, primarily because of the ability of nanoparticles to take advantage of the enhanced permeation and retention (EPR) effect (Matsumura and Maeda, 1986). Tumor vasculature is commonly poorly organized and "leaky" compared to native vasculature. As a result, particles with diameters $< \sim 200$ nm can pass through the blood vessels and accumulate at the tumor site. Polymer and lipid nanoparticles were

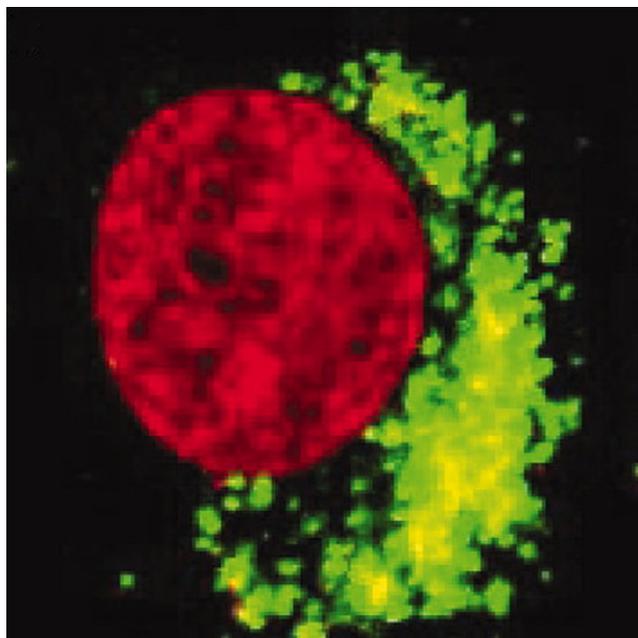


FIGURE I.2.19.9 Dual detection of fluorescent-DNA-CNT (green) internalized into a HeLa cell with the nucleus stained by DRAQ5 fluorescent dye (red). (Photo from Kam et al., 2005).

among the first studied as drug delivery carriers (Marty et al., 1978), primarily for delivery of doxorubicin, a classic chemotherapy drug. Silica (Roy et al., 2003), gold (Paciotti et al., 2004), quantum dots (Gao et al., 2004), and CNTs (Figure I.2.19.9) (Kam et al., 2005) have all been used as simple drug carriers, whereas magnetic nanoparticles and gold nanoshells have demonstrated enhanced delivery effects as a result of their unique properties. Accumulation of magnetic nanoparticles at the target site can be improved by the application of magnetic fields (Lubbe and Bergemann, 1998). Gold nanoshells, which are grown on an inert core, generate heat when exposed to near-infrared light that can be used to expand temperature-sensitive polymers and release drug at selected intervals (Sershen et al., 2000).

Nanoparticles have also been actively studied for gene delivery, and have several advantages as gene carriers. Despite the fact that their transfection efficiency can be considerably lower than their viral counterparts, they are often much less toxic. Additionally, it has been shown that the increased bulk of nanoparticles versus free DNA can enhance *ex vivo* transfection (Luo & Saltzman, 2000). Finally, many nanoparticles possess additional functionalities that can enhance delivery or permit simultaneous monitoring of transfection. As with drug delivery, the first use of nanoparticles included liposomes (Fraley et al., 1980) and polymers (Cohen et al., 2000), although silica (Kneuer et al., 2000), fullerenes (Nakamura et al., 2000), gold (McIntosh et al., 2001), magnetic nanoparticles (Scherer et al., 2002), CNTs (Pantarotto et al., 2004; Kam et al., 2005), and quantum dots (Srinivasan et al., 2006) have also been employed. One interesting application of gene carriers specific to

nanoparticles is magnetotransfection (Scherer et al., 2002), where magnetic nanoparticle gene carriers are exposed to a magnetic field that enhances their uptake at the target site.

Therapeutic Treatment

In addition to serving as carriers of drugs and other therapeutic compounds, occasionally the unique properties of nanoparticles themselves can be applied for treatment. Two excellent examples of this are the use of nanoparticles for hyperthermia and interactions with ROS for photodynamic therapy and antioxidant activity. Hyperthermia is the death of tissue caused by elevated temperature (e.g., $>41^{\circ}\text{C}$), and is primarily used for cancer treatment. It was originally induced by direct application of heat or by circulating heated fluids through the vasculature; however, both of these methods produced significant collateral damage. Nanoparticles, because of their small size and ability to be precisely targeted, offer the possibility of localized hyperthermia. Two types of nanoparticles have been targeted in this application. The first are gold nanoshells (O'Neal et al., 2004), which strongly absorb light in the near-IR and convert it to thermal vibrations (e.g., heat) (Figure I.2.19.10). Light absorption in the near-IR is advantageous because the near-IR and IR offer greatest tissue penetration. The second type of nanoparticles used for hyperthermia are iron oxide magnetic nanoparticles (Chan et al., 1993), which produce heat when placed in an alternating magnetic field. In both cases, heat is localized primarily to the tumor site, reducing collateral damage and producing near microscale targeting of cancer treatment.

The second application of nanoparticles as a direct therapy is their interaction with the ROS pathway. Usually nanoparticles are examined for photodynamic therapy, which is the light-induced generation of ROS for producing cell death, but they have also been applied as anti-oxidants that neutralize ROS. In photodynamic therapy, light excites an electron from a sensitizer molecule to an excited state. The energy in this excited electron can be transferred to oxygen or oxygen-containing species to produce ROS. Traditionally, photodynamic therapy has been accomplished using molecular sensitizers; however, nanoparticles offer a significant advantage in that their excitation wavelengths can be precisely tuned by adjusting nanoparticle size. Both quantum dots (Samia et al., 2003) and fullerenes (Tabata et al., 1997) have been used to produce ROS.

CHALLENGES FACING NANOPARTICLES

Despite their success as biomaterials, there are several challenges facing nanoparticles. Greatest among these are questions regarding their fate in the body. Although most of the materials used have been widely characterized in bulk form, there is evidence that nanoparticles

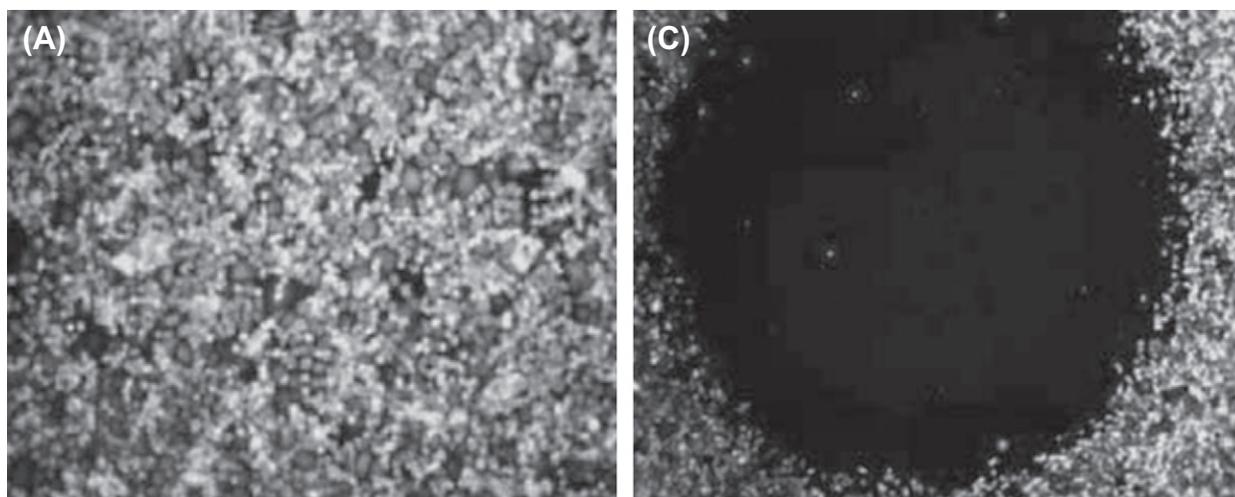


FIGURE I.2.19.10 Breast carcinoma cells were treated with nanoshells conjugated with either: (A) control; or (C) targeting biomolecule. Upon NIR (near infrared) laser treatment, cells exposed to targeted nanoshells were effectively destroyed (C), as demonstrated by the circular region corresponding to the laser spot that lacks staining with a fluorescent viability marker, while the nanoshells bound to the control antibody did not produce this effect (A). Silver staining was then performed to visualize nanoshell binding to cells. (Photo from Hirsch et al., 2006).

of the same material can exhibit substantially different behavior. For one, the surface-to-volume ratio is significantly enhanced in nanomaterials, altering the chemical interface with the body. Also, many nanoparticles exhibit unique properties not found in the bulk that can influence biological response. Finally, because of their small size they can be internalized by cells and organs, especially those of the RES, yet they are often too large to be cleared by the kidneys. Understanding the biological behavior of nanosized versions of well-known biomaterials is crucial to their continued application.

Toxicity

A major concern for nanoparticles is their potential toxicity. Although some materials (e.g., gold, silica, many polymer and lipid formulations, magnetic nanoparticles) have demonstrated exceptional biocompatibility, there are lingering questions regarding the toxicity of carbon nanostructures and quantum dots. Carbon nanostructures offer several potential modes of toxicity. They are often synthesized in the presence of metallic catalysts, such as nickel and iron, which can provoke cellular responses (Kagan et al., 2006). Careful isolation of the final product can minimize these effects. Also, they are usually extremely hydrophobic. Unmodified carbon nanostructures can penetrate the cell membrane (Monteiro-Riviere et al., 2005) and form granulomas in tissue (Warheit et al., 2004). This effect can be mitigated by acidic oxidation to yield hydrophilic surfaces. Finally, carbon nanostructures have been shown to cause direct toxicity, most likely through the generation of ROS (Oberdorster, 2004). Surface coatings are especially important in controlling these responses.

Quantum dots have evoked similar toxicity concerns (Kotov et al., 2009), most notably because a primary

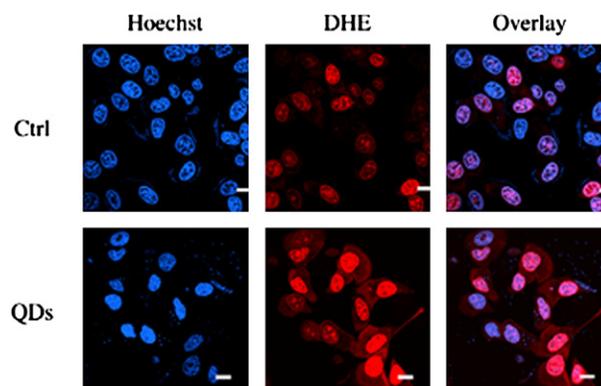


FIGURE I.2.19.11 Quantum dots induce generation of ROS. MCF-7 cells were stained with a marker for ROS (DHE, red) and for the cell nucleus (Hoechst, blue). Four hours after quantum dot exposure, quantum dot-treated cells (bottom) produce more ROS (red) than control cells. The scale bar represents 10 μm . (Figure from Lovric et al., 2005).

constituent material is often cadmium. Cadmium can cause acute toxicity, resulting in death in large doses, and in small doses is a carcinogen disrupting DNA mismatch repair. However, cadmium in nanoparticle form is presented as a complex with another material. Primary modes of quantum dot toxicity are believed to be their ability to generate ROS, and also given the structural similarity of Cd and Zn, to bind metallothionein, disrupting zinc metabolic pathways. Toxicity of quantum dot nanostructures can be minimized by judicious use of surface coatings (Derfus et al., 2004), but biocompatibility remains a significant concern hindering clinical application in patients (Figure I.2.19.11).

Targeting

Another significant challenge for nanoparticles is their controlled targeting both *in vitro* and *in vivo*. In theory,

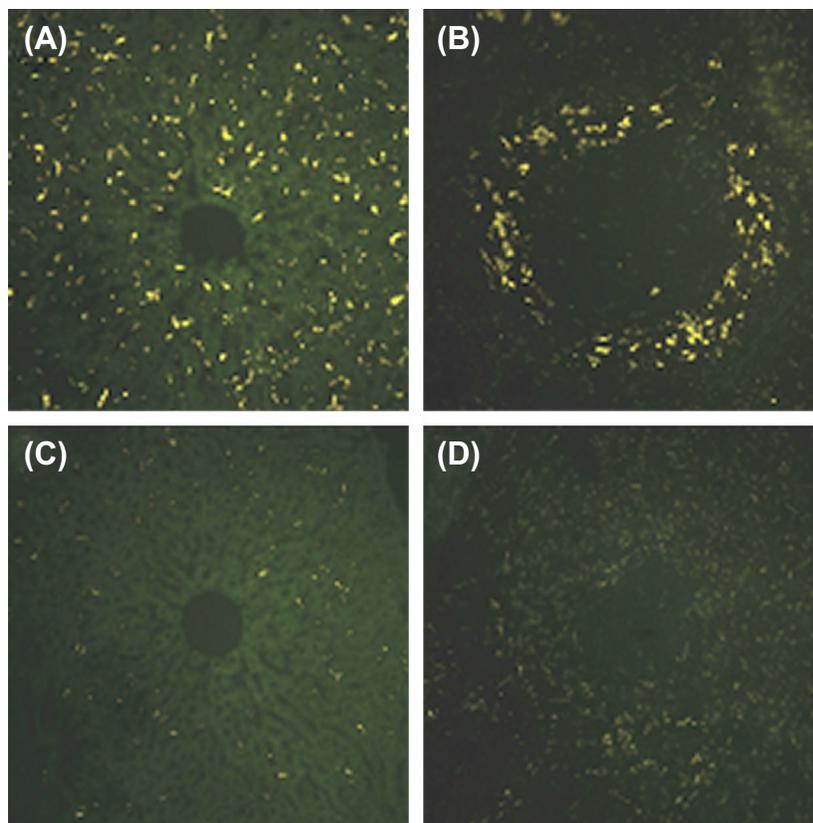


FIGURE I.2.19.12 Nanoparticle uptake by the RES is reduced by PEG coating. Green quantum dots without (A,B) or with (C,D) an added PEG coating were injected into the tail vein of mice. Quantum dot localization was observed by epifluorescent microscopy of the liver (A,C) and spleen (B,D). Original magnifications: 200 \times . (Figure from Akerman et al., 2002.)

nanoparticles can be precisely directed to a specific location using antibodies, peptides or other biorecognition molecules. However, in practice, nanoparticles are subject to non-specific binding and endocytosis *in vitro* (Gomez et al., 2005), and uptake by the RES *in vivo* (Akerman et al., 2002). Non-specific binding usually results from the nanoparticle charge, and can be reduced by using appropriate blocking agents or by encapsulating nanoparticles in another protective material. Endocytosis, however, is more problematic. Free nanoparticles in solution and nanoparticles targeted to cell surface receptors are readily internalized by cells through endocytotic pathways. It has been shown that nanoparticles accumulate in the perinuclear region (Shukla et al., 2005), where they presumably remain indefinitely or until metabolic dissolution. Endocytosis is related to nanoparticle size (Osaki et al., 2004), with optimal uptake for particles ~ 50 nm. *In vitro*, endocytosis can be prevented by performing experiments or analyses at low temperature (e.g., 4°C). The *in vivo* picture is more complicated. Since most of the accumulated data on cellular uptake is from *in vitro* systems, it is not clear that particles would be internalized *in vivo* at the same rate. Nonetheless, the possibility of nanoparticle internalization and permanent residency in the cell is troubling. Finally, many nanoparticles never make it to their cellular destinations *in vivo*, having been filtered from the blood by the RES. Evasion

of RES uptake is possible, usually using PEG as a coating (Figure I.2.19.12) (Akerman et al., 2002). PEG increases nanoparticle circulation time because it resists protein adsorption (Eugene, 2004), preventing recognition by immune cells of the RES.

Clearance

Nanoparticle biodistribution is a key issue for clinical application. Unlike bulk materials that remain in the body indefinitely (e.g., hip implant, pacemaker), nanoparticles have the potential to interact with the body's native clearance system, the RES. The RES consists of the lymph nodes, spleen, and liver, and is comprised of immune cells responsible for identifying and removing foreign matter from the blood. Alternatively, very small molecules may be filtered by the kidneys. Since many applications of nanoparticles utilize blood circulation to promote particle delivery, nanoparticles have ample opportunity to interact with the RES. Polymer, lipid, and magnetic nanostructures degrade *in vivo*, and their ultimate biological fate is less of a concern; however, there is little evidence that quantum dots, metallic nanoparticles, silica or carbon nanostructures are eliminated through natural degradation processes. The fate of particles is controlled not only by their composition, but also by their surface coating, further

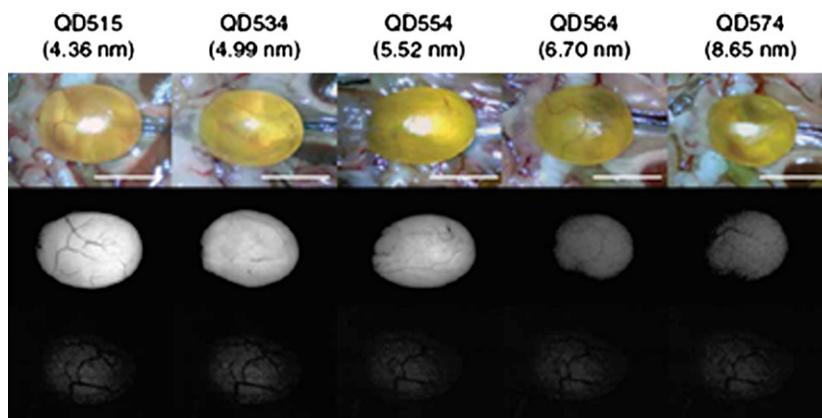


FIGURE I.2.19.13 Mouse bladders after intravenous injection of quantum dots of defined diameter. Shown are frames from a color video (top) and fluorescence images for bladder 4 hours after injection (middle) and for uninjected control bladder (bottom). Scale bar: 1 cm. (Figure from Choi et al., 2007.)

complicating the issue; and as this field is just emerging, current understanding of the fate of nanoparticles in the body is incomplete.

Gold nanoshells (James et al., 2007) and nanoparticles (Kim et al., 2007), quantum dots (Fischer et al., 2006), silica nanoparticles (He et al., 2008), nanodiamond (Yuan et al., 2009), and fullerenes (Yamago et al., 1995) have all been found to accumulate in RES organs. It has been shown that particles with diameters <6 nm (Choi et al., 2007; Burns et al., 2009), can be cleared through renal pathways (Figure I.2.19.13), but most nanoparticles intended for clinical use are >30 nm. There is some evidence that quantum dots (Chen et al., 2008) and silica (He et al., 2008) can be at least partially metabolized by the liver, and excreted through feces or urine. Carbon nanotube distribution has been more controversial, with several conflicting reports claiming elimination via kidney (Singh et al., 2006) versus uptake by the RES with little clearance (McDevitt et al., 2007). A recent report provides some clarity, demonstrating that individual CNTs can cross renal barriers for excretion, whereas CNTs that are aggregated are taken up by the RES (Lacerda et al., 2008).

CONCLUDING REMARKS

Despite their identical composition to bulk materials, microparticles and nanoparticles represent a new class of biomaterials with distinct properties, interactions with biological components, and distribution within the body. Microparticles and nanoparticles have been used for a range of applications in the biomedical field. Their properties can be easily modulated by varying their composition, shape, size, and surface chemistry. Microparticles were first used in the 1950s, and are one of most extensively studied systems in the biomedical field. Microparticles are most commonly employed in drug and vaccine delivery. Nanomaterials represent an emerging field; careful study of their biological activity

will be required before there is large-scale clinical application. Because of their small size scale and unique physical properties, nanoparticles offer great hope for the future, particularly in the areas of imaging and drug delivery. They have already been successfully applied in the clinic as MRI contrast agents and drug delivery vehicles. These applications will undoubtedly continue to expand as more materials are moved from the bench to the bedside.

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