Chapter 4
Safety and Efficacy of Nano/Micro Materials

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Abstract  Nano/micro materials have been used in various applications, and drug delivery is one of the areas where nano/micro particles have made differences. Nano/micro particulate delivery systems can be divided into different categories based on several parameters, such as nature of nanomaterials (inorganic and organic), biodegradability, hydrophilicity, structures, and processing method. Most of the nano/micro materials in drug delivery have been used without careful considerations in potential toxicity and safety issues. The size, surface area, chemistry, solubility, and shape of nano/micro materials all play significant roles in toxicity. It is time to consider potential problems that may result from unguided use of nano/micro materials. This chapter deals with potential sources of toxicity in development of various drug delivery systems.

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4.1 Introduction

Nanotechnology is considered to be one of the most important technologies in modern times. Its unique abilities are expected to revise conventional research and development (R&D) models. For example, cosmetics which have ultrafine clays and oil nanoparticles provide customers significantly improved feelings on their skin; plastics which are modified by carbon nanofibers are as strong as steel, yet as light as hair; and clothes composed of nanofibers are not dampened by the rain. All of these improvements in “incremental technology” [1] have so much potential that many countries are investing considerable resources in this area. Although many promising products, such as carbon nanotubes (CNTs), quantum dots (QDs), sculptured thin films, single-electron transistors, and nanofluidic sensors, have been developed, few of these are available in mass quantities for commercial applications. However, current research and future prospects can provide us with a picture that nanotechnology-based products will be commonly available for consumers within the next decade. It is time to consider potential dangers associated with the preparation, manufacturing and application of nanoparticles. Microparticles are also considered here, as there is no clear boundary separating nanoparticles from microparticles.

The safety protocols of using nanoparticles are urgently required, but have not been given much attention to date [2, 3, 4]. In 2006, the International Risk Governance Council (IRGC) surveyed the current situation of the nanotechnology governance [5]. According to this report, survey participants, consisting of governments of eleven countries, eleven industrial organizations, five research organizations, and nine non-government organizations (NGOs), recognized the importance of R&D activities as well as potential benefits resulting from nanotechnology. Nevertheless, most of respondents did not identify the need for any specified national or international regulations for nanotechnology.

With the ever-increasing R&D activities in nanotechnology, results and data indicating the risks of nanoparticles have been accumulating. Thus, appropriate regulatory action is urgently required to protect human health and the environment from
potential disasters [6, 7, 8, 9]. This chapter deals with the analysis of advantages and disadvantages of the preparation, manufacturing, and application of nanoparticles. In particular, the details of safety protocols for overcoming these disadvantages are discussed, along with guidance that are now in place.

4.2 Drugs

The selection of drugs for preparing and manufacturing of nano/micro particles is based on pharmacological activities and market needs. This section is focused on the strategy of maintaining the stability of drugs, especially biomolecular drugs, and on characteristics of non-organic nanoparticles.

4.2.1 Biomolecular Drugs

With advances in biotechnology, more and more biomolecular drugs have been developed for mass production. Clinical applications of these biomolecular drugs, however, have been limited due to their poor stability in formulations and short half-lives in blood. Biomolecular drugs, mainly protein drugs, are prone to denaturation by high temperatures, exposure to organic solvents, contact with solid surfaces, and chemical reactions with other molecules, leading to poor stability during manufacturing and storage of nanoparticulate formulations.

The purity of the protein plays an important role in protein stability/instability [10]. The stability of native proteins is more likely affected by the manufacturing process as compared with chemical modification of the proteins. Poly(ethylene glycol) (PEG) has been used widely for chemical modification (known as PEGylation) of proteins [11]. Methoxy-PEG (MPEG) conjugated proteins [12, 13] have been shown to be more stable than their native counterparts. There are several PEGylated proteins currently on the market, including PEG-adenosine deaminase (Adagen®, Enzon), pegfilgrastim (Neulasta®, Amgen), PEG-L-asparaginase (Oncaspar®, Enzon), pegvisomant (Somavert®, Pfizer), PEG-α-interferon-2b (PegIntron®, Schering-Plough), and PEG-α-interferon-2a (Pegasys®, Roche). As an alternative to the non-biodegradable monomethoxy-PEG, poly(sialic acid), a naturally occurring and biodegradable polymer, has been used [14, 15]. Poly(sialic acid) modified proteins were shown to have the same ability to increase the circulation half-life of catalase and asparaginase.

To decrease the degradation of proteins resulting from exposure to the interface during water/oil emulsion processes, anhydrous protein powders have been directly added to polymer-containing organic solvents or the solubility of proteins in organic solvents has been increased. A protein drug can be precipitated at its isoelectric point to make it neutral in charge for dissolution in organic solvents [16]. A protein drug can go through a freeze-drying or spray freeze-drying (SFD) process at a pH away from its isoelectric point [17, 18, 19, 20], resulting in an anhydrous form of
that protein. The protein solubility can also be increased based on an ion-pairing mechanism. An oppositely charged surfactant is used to bind the protein and give it a neutral hydrophobic surface. Negatively charged surfactants are usually used to neutralize the positively charged protein because of the toxic side effects of the cationic surfactants [21, 22, 23].

Crystallization is an alternative approach to improving protein stability during microencapsulation procedures, storage and delivery [24], because it only involves a one-step process and results in high purity proteins. Crystalline protein particles are even reported to be more active, stable, and acceptable than their spray-dried amorphous forms [25, 26]. However, few crystalline forms of proteins, especially glycoproteins, have been used as active pharmaceutical ingredients because most proteins are too large and flexible to be crystallized [27].

**4.2.2 Inorganic Drugs**

**4.2.2.1 Magnetic Nanomaterials**

Nanoparticles that possess magnetic properties have been extensively investigated as a useful tool for improving the quality of magnetic resonance imaging (MRI), hyperthermic treatment for malignant cells and targeted drug delivery [28]. Iron-containing nanomaterials are controlled by remote magnetic fields, and can be coated with various marker molecules or anti-cancer drugs for targeting within the body. Although neither iron oxide nanoparticle alone nor the coating material alone is known to be toxic, combining the two to create water-soluble nanomaterials produces a completely different effect. They can be toxic to nerve cells and encumber formation of their signal-transmitting extensions [29].

**4.2.2.2 Titanium Dioxide Nanomaterials**

Titanium dioxide (TiO₂), a noncombustible and odorless white powder, naturally exists in minerals like anatase, rutile, and brookite. It is widely used as a white pigment for paints, paper, plastics, ceramics, for example. TiO₂ is also used as a food additive, such as in toothpaste and capsules, and the Food and Drug Administration (FDA) established a regulation for TiO₂ as the color additive for food. Federal Regulations of the US government regulates the quantities of TiO₂ not to exceed 1% by weight of food. TiO₂ becomes transparent at the nanoscale (particle size < 100 nm), and is able to absorb and reflect UV light, making it useful in sunscreens. Nowadays, TiO₂ nanoparticles are used widely because of their high stability, anticorrosive character, and photocatalysis.

TiO₂ nanoparticles can produce free radicals with a strong oxidizing ability which can catalyze DNA damage both in vitro and in human cells [30]. TiO₂ nanoparticles also have pulmonary toxicity after endotracheal inhalation and instillation into the organism. It was reported that the TiO₂ nanoparticles (20 nm) penetrated more easily into the pulmonary interstitial area than the fine particles (250 nm).
of the same mass [31]. The size-dependent toxicity of TiO₂ particles may not be sig-
ificant if different routes of administration or different genders are used [32]. In an
inhalation exposure study, mice exposed to 2–5 nm TiO₂ nanomaterials revealed a
moderate inflammatory response among animals [33]. Pulmonary toxicity research
in rats with three forms of TiO₂ particles showed that exposures to ultrafine TiO₂
particles can induce typical pulmonary effects, based on their composition and crys-
tal structure [34].

4.2.2.3 Silica Nanomaterials
Nanomaterials of silica, a non-metal oxide, have been used in chemical mechani-
cal polishing, and as an additive to drugs, cosmetics, printer toners, varnishes, and
food, because it is a “generally regarded as safe” (GRAS) material. In recent years,
applications of SiO₂ nanomaterials have been extended to biomedical and biotechno-
logical fields, such as biosensors [35], biomarkers [36], cancer therapy [34, 37],
DNA delivery [38], and enzyme immobilization [39].
Recent literature searches indicate that silica nanomaterials are nontoxic at low
dosages but cell viability decreases at high dosages, because high dosages of sil-
ica induce cell membrane damage. On the other hand, silica-chitosan composite
nanomaterials are known to induce less inhibition in cell proliferation and less
membrane damage. The cytotoxicity of silica to human cells depends strongly on
their metabolic activities, but it could be reduced by combining with chitosan [40].
In addition, dose-dependent exposure to SiO₂ nanoparticles induced cytotoxicity
in human bronchoalveolar carcinoma-derived cells that was closely correlated to
increased oxidative stress. It appears that SiO₂ nanomaterials reduce cell viability
resulting from penetration of the particles into the cell nucleus [41].

4.3 Polymeric Carriers
Polymeric carriers are often used as drug delivery systems. They must not only be
biocompatible and immunocompatible, but also be readily eliminated from the body,
preferably through biodegradation. There are so many polymers that it is very diffi-
cult to classify each by certain criteria. For convenience, however, they are divided
into biodegradable and non-biodegradable polymers in this chapter. The biodegrad-
able polymers can be hydrophilic, hydrophobic, or amphiphilic.

4.3.1 Non-Biodegradable Polymers
Non-biodegradable polymers were frequently used as implant drug delivery sys-
tems in the early 1970s because of their long-lasting release and reduced host
response. Examples are poly(vinyl alcohol) (PVA), poly(ethylene vinylacetate)
(PEVA) [42, 43], and polysulfone capillary fiber (PCF) [44]. They were proven to be safe in rabbit eyes for months [43, 44].

4.3.2 Biodegradable Polymers

4.3.2.1 Hydrophilic Polymers

Hydrophilic polymers generally have little immunogenicity in clinical applications. However, most hydrophilic macromolecules have to be crosslinked or copolymerized to form hydrogels; otherwise they will be dissolved and cleared from the body. Physical hydrogels may be better than the chemical ones, because most crosslinking agents are toxic and the chemical crosslinking process may chemically affect the entrapped molecules. It is very important to remove any residual crosslinking agent before in vivo application.

At the end of the last century, synthetic polymers became more and more important. Synthetic polymers could be tailored for various physicochemical properties to suit various applications. The synthetic polymers used in biomedical applications must be biocompatible, i.e., they must not provoke a defensive, potentially dangerous reaction in vivo. Application of nanomaterials may need to be considered as “polymer genomics.” The term “polymer genomics” is defined as “an effect of synthetic polymers on pharmacogenomic responses to chemotherapeutic agents and the expression of transgenes delivered into cells” [45, 46]. Understanding of polymer genomics is expected to lead to safe and efficient nanoparticles for clinical applications.

Poly(2-hydroxypropyl methacrylamide) (PHPMA) is a potential water-soluble carrier. Rihova [47] reported that the molecular weight and the properties of the oligopeptidic side chains could result in some immunogenicity. PHPMA with molecular weight around 30 kDa is not recognized as a foreign molecule and has no recorded defense reaction to it. The attachment of pendant oligopeptide sequences to the HPMA backbone bestows a certain degree of immunogenicity, which depends on the composition of the oligopeptidic side chains, dose and route of application, molecular weight, and the genotype of the immunized individual.

4.3.2.2 Hydrophobic Polymers

Hydrophobic polymers are often used for long-term drug delivery, such as intraocular implants [48]. For convenience of avoiding removal of the system after completing drug release, biodegradable polymers are often preferred. Pure polyanhydrides can be degraded in 3 years unless it is copolymerized with sebacic acid (SA) [49]. Increasing the percent of the SA leads to faster degradation, and the copolymer with 80% SA can degrade in just a few days. The copolymer with SA is less hydrophobic and is known to be a good biocompatible material [50].

Poly(ortho ester) (POE) is a hydrophobic, biodegradable polymer. Currently, there are four (I–IV) families of POE [51, 52, 53]. There are acidic and basic
portions in POE. The acidic portion determines the degradation rate. The basic portion maintains the polymer backbone’s stability [54, 55]; moreover, it neutralizes the acidic microenvironment when the POE is degraded [56]. Different molecular weights of POE polymers have different release profiles in vitro [57]. Traditional gamma irradiation sterilization results in degradation of the POE III. So when a POE polymer is used, aseptic preparation is preferred [58]. During the storage of the POE III and its drug delivery system, they have to be sealed in glass bottles under an argon atmosphere [59].

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA) are the most widely used biodegradable polymers. The biggest problem of these polymers is that their hydrolysis results in acidic products, which induce protein degradation. In order to solve the problem, Jiang and Schwendeman [60, 61] used a blend of hydrophobic PLA and hydrophilic PEG for delivery of bovine serum albumin (BSA). With the PEG content in the blend, the degradation speed of PLA decreased, and BSA remained structurally intact without aggregation. PLGA/PEG blends showed the same effect [62]. There are other strategies, such as preparation through an o/o emulsion and co-encapsulation of additives [60, 61, 63], to alleviate the acidic microenvironment. The carboxyl end groups of the degradation products of the polymers can interact with positive charges of proteins to adversely affect the protein stability [64, 65].

During degradation of biodegradable polymers in the rabbit’s eyes, triphasic release patterns were observed: initial drug-burst, diffusive phase, and a final drug burst. The latest procedure is generally uncontrollable and poorly predictable. Yasukawa [66, 67] reviewed the attempts to improve the release procedures. The larger the molecular weight or the lower the glycolide content, the slower the biodegradation [66]. Using PLA polymers with two different molecular weights in different ratios resulted in decrease in the final drug burst with a pseudo-zero order kinetic of drug release [68].

4.3.2.3 Amphiphilic Polymers

Amphiphilic polymers are promising polymeric carriers as they can load the drug under a mild condition [69] as a polymer micelle or as a sol-gel phase reversible system. Amphiphilic polymers are divided into two parts: hydrophilic and hydrophobic segments. The properties of the two parts and the ratio of the two parts determine the in vivo fate of the micelles [70].

Poly(ethylene glycol) (PEG) [71, 72, 73], or poly(ethylene oxide) (PEO) [74], is the most commonly used polymer as the hydrophilic part of the polymer micelle. They endow the micelles a stealth surface by repelling the foreign substance, and thus increase the blood circulation half-life of the polymer micelles in vivo. It was reported that MPEG-PLA improved the efficacy of the direct nose–brain transport for drugs, which is especially important for peptides and proteins that are unable to penetrate through the blood-brain barrier [75].

A small difference between ethylene oxide (EO) and propylene oxide (PO) monomer units is the additional methyl group in the PO unit, which makes it more
hydrophobic. The hydrophobic segment of the polymer containing PO units can be used to adsorb and anchor the block copolymer molecule to the nanoparticle surface, while the hydrophilic EO-containing segment, or PEG sections, can extend into solution and shield the surface of the particle. It was found, however, that the physically adsorbed polymers can be desorbed [76], and thus covalent grafting to the surface may be necessary for improved stability [77, 78]. Properties of PEG chains, such as molecular weight [72, 79, 80, 81], surface chain density [81, 82, 83], and conformation [72], affect biodistribution and pharmacokinetics of nanoparticles. The optimal molecular weight of the PEG chain for surface coating is above 2000; otherwise the length of the PEG chain is too short to be flexible [72, 81, 84].

An increased surface coverage by PEO, e.g., using Poloxamer 407, resulted in not only a reduction of the amount of adsorbed serum proteins, but also affected the type of proteins adsorbed [74]. When the surface coverage was above 25%, high-molecular-weight proteins did not adsorb onto the nanoparticles. Even at the 5% surface coverage, the in vivo circulation time was longer than the uncoated nanoparticle.

The nature and state of the hydrophobic segment in the micelles have a significant impact on the in vivo stability as well as the pharmacokinetics and biodistribution of the micelles. The longer length of the hydrophobic segment and the higher proportion of the hydrophobic polymer endow a greater thermodynamic stability [85, 86]. The physical state of the core-forming polymer, such as amorphous, crystalline or semicrystalline, has an effect on the micelle stability [87, 88]. A micelle containing a hydrophobic block with a glass transition temperature \( T_g \) exceeding 37°C has a “frozen” core. The crystalline or semi-crystalline [88] core makes the micelle more stable and the duration of drug release becomes longer.

The ratio of the hydrophilic/hydrophobic will also affect the shape of the micelle. In general, when the hydrophobic part of the micelle outweighs the hydrophilic part [87] or the length of the hydrophilic segment is longer than that of the core block [89], the shape of the resulting micelles is spherical. On the other hand, non-spherical structures, including rods and lamellae, can be formed by increasing the length of the core segment beyond that of the corona-forming chains or the hydrophilic part of the micelle outweighs the hydrophobic part.

Amphiphilic β-cyclodextrin nanosphere suspensions [90] also reported to have unexpected good physical stability of the suspensions after 3 years of storage at room temperature after the secondary hydroxyl functions of the β-CDa glucopyranosyl units were grafted by hexanoyl carbon chains.

### 4.3.3 Others

The copolymer concentration is another factor affecting the effectiveness of the carrier. Three MPEG-b-poly(ε-caprolactone) (MPEG-b-PCL) copolymer concentrations, 0.2 mg/kg dose group (unimers), 2 mg/kg dose group (unstable micelle), and the 250 mg/kg dose group (stable micelle), were used to investigate the in vivo
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fate of mice following intravenous administration [88]. It was found that when the polymer was given in unimers, more copolymer penetrated into tissues. The thermodynamically unstable micelles (i.e., 2 mg/kg dose group) had a much longer circulation half-life and slower rate of elimination than the unimers. The thermodynamically stable micelles (i.e., 250 mg/kg dose group) had the slowest elimination rate from the plasma during the elimination phase. The 250 mg/kg dose group also had the lowest tissue to plasma concentration ratio during the elimination phase.

Copolymers with a low polydispersity index (PDI) (e.g., PDI < 1.1) usually lead to more stable micelle systems in vivo [88]. The polymer purity and molecular weight distribution are also known to affect microsphere morphology and in vitro cytotoxicity [91]. Microspheres showed decreased in vivo degradation rate and lower initial protein burst after ultrafiltration. Ultrafiltration appears to be a useful method to control the properties of microspheres.

4.4 Additives

Pharmaceutical additives are widely used to preserve the pharmacological activity of drugs and to prolong the shelf life of the dosage forms, especially of protein drugs. Stabilization of protein drugs by additives is based on the surface-active properties of some additives and/or electrostatic interactions. However, there is no general rule how to choose an optimal additive for a specific protein.

Sugars have been widely used as excipients for stabilizing protein drugs. No general rules have been established explaining how sugars stabilize proteins. Different sugars have different effects on the same protein, whereas the same sugar also has different effects on different proteins. For example, trehalose and mannitol have significant protective effects on the soluble non-aggregated interferon-γ (INF-γ) and growth hormone after emulsification and ultrasonication [16], whereas no or little protecting effect on insulin-like growth factor-I [92]. Trehalose, mannitol, and sucrose have no protecting effect against the degradation of lysozyme, whereas lactose and lactulose have significant protective effects [93, 94]. Cyclodextrins (CD) [95, 96] is also used as a special stabilizer. Hydroxypropyl-β-cyclodextrin is known to increase the stability of recombinant human INF-α-2a protein [97]. Generally, sugars are added in the inner aqueous phase in the w/o/w emulsion process.

A surfactant is another additive which is widely used in protein formulations. Non-ionic surfactants usually have better effects than ionic surfactants because binding of ionic surfactants to proteins can cause protein denaturation. Poloxamer 188 successfully prolonged the release of active INF-α when it was mixed with PLGA [98], whereas it had no effect on BSA secondary structure [17].

Albumins and gelatins are frequently added to the inner aqueous phase during emulsion process to protect the bioactivity of proteins. The protective function of albumins (i.e., bovine, human or rat serum albumins) results from their surface-active properties. Albumins occupy the interfaces to shield the pharmacological
protein from exposure to solvent [92, 99, 100]. Albumins are also known to remove
the protons formed during degradation of PLGA, avoiding protein aggregation
resulting from the acidic environment [101]. Albumins, however, may not work
in many other cases [102]. Gelatin is another protein which is used as an additive
to protect protein drugs from ultrasonication [92]. The gelatin protective effect is
dependent on the gelatin molecular weight and concentration; the higher the molec-
ular weight/concentration, the better the stabilization effect [103].

Some synthetic polymers are also used as an additive too. PEG has been used to
protect protein against degradation [102, 103, 104], although sometimes it resulted
in adverse affects [105]. PEG can be added to either aqueous or organic phases
[106, 107]. Two or more additives can be combined to enhance the protective func-
tion over individual additives [92, 108].

4.5 Structure

4.5.1 Quantum Dots

Quantum dots (QDs) are semiconductor nanocrystals with unique optical and
electrical properties. QDs have a longer durability and higher fluorescence than
conventional organic fluorophores, thus it can act as information and visual tech-
nologies to transfer in vivo imaging and diagnostics of living organisms [109].
Moreover, fluorescent QDs can be joined together with bioactive moieties (e.g.,
antibodies, receptor ligands) to target specific biological event and cellular struc-
tures and receptors.

To understand the potential toxicity of QDs, it is required to understand the
physicochemical properties of QDs. QDs consist of a metalloid crystalline core and
a shell that shields the core and makes the QD bioactive. Many QD core metals (e.g.,
Cd, Pb, Se) are known to be toxic to vertebrate systems even at low concentrations
(parts per million). For example, Cd, a bioaccumulative carcinogen, has a biologic
half-life of 15–20 years in humans, can cross the blood-brain barrier and placenta,
and is distributed throughout the body. Degradation of the QD coating may also
result in unexpected reaction of QD in vivo. Furthermore, some QD coating mate-
rials, such as mercaptoacetic acid, have been found to be cytotoxic. Till now, there
are no standardized protocols on QD synthesis and coating. The safety of QDs is
known to depend on QD size, charge, concentration, and bioactivity of outer coat-
ing (capping material and functional groups) [110, 111]. Protonation [112] or photo-
oxidation [113] is known to deteriorate the stability of QDs. In a relatively low pH
range, between 2 and 7, the deprotonated thiols or thiolates which are bound to cad-
mium chalcogenide QDs will become protonated and detached from the QD surface
causing the precipitation of the crystals [112]. Photooxidation of the surface ligands
also makes them detached from the surface, leading to precipitation. Increasing the
thickness and packing density of the ligand is useful to delay the initiation process
of photooxidation [113].
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4.5.2 Carbon Nanotubes

Carbon nanotubes (CNTs) consist exclusively of carbon atoms arranged in a series of condensed benzene rings rolled-up into a tubular structure. Various physicochemical properties of CNTs, such as ordered structure with high aspect ratio, ultralight weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semi-metallic behavior and high surface area, present unique opportunity for diverse applications [114, 115].

Non-functionalized CNTs are hydrophobic materials, requiring functionalization to be compatible in the biological media. Such function-groups can be obtained by adsorption, electrostatic interaction, or covalent bonding of different molecules to make CNTs more hydrophilic [116]. The physical state of the CNTs is also very important to the safety of the usage of the CNTs [117, 118]. The functionalized surface can prevent aggregation of the individual tubes that occurs through van der Waals forces.

4.5.3 Dendrimers

Dendrimers consist of a central core molecule acting as the root from which a number of highly branched, tree-like arms originate in an ordered and symmetric fashion [119]. Because of their unique molecular architecture, they have many distinctive properties which are different from other polymers, such as the gradual stepwise synthesis method through a divergent or a convergent one, a well-defined size and structure with a low PDI. The relatively empty intermolecular cavity and the highly dense terminal groups can be used to entrap host molecules.

Most dendrimers are known to have poor solubility in aqueous solutions, and a structure that would predict likelihood of cellular accumulation. They may present unacceptable toxicity and/or immunogenicity if administered parenterally. It is well known that the large surface area/volume of all nanosized materials can potentially lead to unfavorable biological responses if they are inhaled and absorbed into the body [7, 120]. It is widely known that dendrimers with –NH₂ termini display concentration- and generation-dependent cytotoxicity [121]. The exposure time is also an important factor which affects the morphology of the cell. When polyamidoamine (PAMAM) with generation 4 was incubated in B16F10 cells for 5 hrs, cell membranes were damaged [122], but it was reversible by removing the dendrimers [123].

Dendrimer cytotoxicity is not only dependent on the chemistry of the core, but is also influenced by the properties of the surface. PAMAM dendrimer and polypropyleneimine dendrimer have a cationic net surface charge. Cationic surface charges are in general more toxic than anionic or PEGylated dendrimers [124]. In order to decrease the toxicity, quaternization is always used as a strategy [125, 126]. Increased branching (or generation) and a greater surface coverage with biocompatible terminal groups, such as C12 lauroyl groups or PEG 2000, reduce the
dendrimer toxicity significantly [122]. However, when the surface of generation 4 PAMAM dendrimers was modified with lysine or arginine, the toxicity increased which was confirmed by incubation with HepG2 or 293T human embryonic kidney cells for 48 hrs, probably due to the increased density and molecular weight [127]. To improve the transfection efficiency, some compounds are added as additives into the dendrimer–DNA complexes, such as DEAE-dextran [128] and substituted cyclodextrin [129].

4.6 Processes

The particle size plays a key role in the final biodistribution and pharmacokinetics. It was reported [84] that a particle with hydrodynamic radius of over 200 nm will be cleared more quickly than particles with radius under 200 nm. Controlling the particle size may be one way to prepare safe nano/micro materials.

4.6.1 Emulsion

Emulsion methods have been used widely in preparing nano/micro particles. Water-in-oil-in-water (w/o/w) double emulsion method has been most widely used. Many hydrophilic drugs/proteins can be encapsulated by this method. The particles obtained in this method are very stable [130]. In emulsion methods, protein drugs can become denatured by exposure to the interface between water and solvent [99, 131]. The effect of the primary w/o emulsion has stronger denaturizing effect than that of the secondary w/o/w emulsion [132]. To avoid the exposure of proteins to organic solvent, protein particles in the solid state can be directly suspended in the organic phase to form s/o/w emulsion [23, 133]. For this s/o/w method to work, the hydrophilic drug/protein power has to be under an anhydrous condition before encapsulation [17, 18]. The anhydrous proteins can be obtained by freeze-drying or SFD before being encapsulated (See Section 2). The nature of the organic solvent also has an impact on protein stability. Use of blend solvents (such as acetone/ethylene chloride blend) could reduce surface tension between the organic and the water phases [20, 23, 102].

Although all ultrasound, sonication, vortexing, and homogenization operations can result in protein degradation, a good choice of the apparatus can minimize the instability of proteins [134]. Milling is a traditional method for micronization of drug powders. The standard micronization processes comprise crushing/milling, air micronization, sublimation, and recrystallization from solvents. There are various mills, such as ball mills, colloid mills, and jet or fluid energy mills. Most of them have advantages and shortcomings. The mechanical treatments can damage degrade particles due to high stresses (thermal and mechanical) generated by attrition. They result in particle adhesion, agglomeration, and loss of drug activity. Jet milling is a process to reduce the size of crystals or coarse particles by high velocity air [135].
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The majority of inhalation powders are prepared by jet mill [135, 136]. It can produce particles between 1 and 20 μm [136]. In addition to milling, crystallization, and lyophilization have been also widely used. For crystallization processes, coprecipitating solvent is often used [137]. Some solvent will result in the instability of the biopharmaceuticals, but use of optimal lyoprotectant can prevent aggregation and increase shelf life [138, 139].

4.6.2 Spray Drying

Spray drying transforms protein-containing solution to powder in a single step [140]. The major advantage of the process is that this technology can avoid thermal degradation. Though high temperature drying air is used, the drying time usually lasts less than 100 milliseconds to seconds [135, 136]. During the drying process, the material temperature remains significantly less than the drying air due to evaporative cooling.

There are some problems to consider with spray drying. Atomization may result in degradation and denaturation of proteins. This problem has been alleviated by adding suitable excipients, such as sucrose, trehalose, lactose, PVA, dipamitoylphosphatidylcholine, and even albumin [141, 142, 143, 144, 145, 146, 147]. The yield of spray drying is rather low in the range of 20–50% [136, 146]. This can be improved to 70% by introduction of high-performance cyclone for collecting the dried particles [135, 148]. It is difficult to control the mean droplet size during spray drying [149]. Though the use of ultrasonic nozzles can lighten the problem, it can also cause protein denaturation [150]. The spray drying technique is difficult with poorly water-soluble drugs. In that case, spray freezing into liquid (SFL) can be used. Careful selecting the operating parameters can play a significant role in obtaining high quality product in spray drying.

4.6.3 Spray Freeze-Drying

Spray freeze-drying is a process which takes advantage of the very low boiling point of nitrogen, oxygen, or argon to freeze a solution containing proteins, then to lyophilize the frozen droplets to obtain porous spherical particles. In contrast to the dense particles (~3 μm) produced by spray drying, SFD results in porous, fragile particles (~8-10 μm) with low aerodynamic size [151].

There are a few limitations of SFD. The process is time-consuming (taking 3 days) and expensive because of the safety issues resulting from the extremely low boiling point (below –195.8°C) of liquid nitrogen. The stress associated with freezing and drying, especially the adsorption of a protein at the air–liquid interface during atomization, results in irreversible damage to the protein. This problem can be reduced by limiting the time of exposure to the air–liquid interface during atomization [152]. SFL is an improved SFD technique.
Spray freezing into liquid [153, 154, 155, 156] has been developed for poorly water soluble or insoluble drugs. The advantage of the technique is that the aqueous protein solution is sprayed directly into the liquid nitrogen through an insulated nozzle instead of into the cold vapor in SFD. Another improved technique is spray-freezing with compressed CO₂ [135]. In this technique, biopharmaceutical and excipients mixture is atomized, then mixed with compressed CO₂, developing a CO₂-saturated aqueous solution. The droplets become frozen particles when they are sprayed through a nozzle of a sprayer. The goal of the method is to obtain stable, porous or hollow protein particles with a narrow size distribution [157].

4.6.4 Supercritical Fluid (SF) Technology

Supercritical fluid technique [135, 158] combines advantages of both liquid and gas. The density values and solvation power of a solute can be adjusted by the SF’s critical temperature and pressure. The viscosity of the solutes in SF is lower than liquid, while the diffusivity is higher. Most important is the SFs are highly compressible. The most widely used SF is CO₂ because of its low critical temperature (31.2°C) and pressure (7.4 MPa), non-flammable, non-toxic, and inexpensive. The only limitation of SF CO₂ is its limited solvation power though it can be changed into an advantage when the SF CO₂ is used as an anti-solvent. The solvation power of a SF CO₂ can be adjusted by incorporating a small amount of volatile cosolvent, such as acetone or ethanol, which acts as organic modifier.

In general, SFs can be divided into 3 groups.

- Precipitation from supercritical solutions composed of SF and solutions (rapid expansion of supercritical solution, RESS).
- Precipitation from gas saturated solutions (precipitation from gas-saturated solution, PGSS).
- Precipitation from saturated solutions using SF as anti-solvent (including gaseous anti-solvent, GAS; aerosol solvent extraction system, ASES; solution enhanced dispersion by SF, SEDS and precipitation by compressed anti-solvent, PCA) [159].

Recently innovative techniques have been developed where the SF CO₂ is used to assist spray drying. The SF and the solution are intimately mixed and sprayed in a drying atmosphere. This process allows the minimal decomposition of thermally labile drugs, no need of high pressure vessel and as small as 3 μm particles.

4.7 Workers’Safety

Although more than 35 countries have developed various R&D programs on nanotechnology since 2000, the importance of safety issues regarding nanoparticles
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was recognized only recently. In 2006, the National Institute of Occupational Safety and Health (NIOSH) published a document, “Approaches to safe nanotechnology.” It reviews potential risks of nanotechnology at workplace in order for workers, employers, researchers, and general public to be aware of the hazard of nanomaterials and to minimize exposure to nanostructures [160].

The way workers are exposed to engineered nanoparticles is directly related to safety and health. The exposure routes can be inhalation, dermal, and ingestion, which were reviewed already [8, 161, 162, 163]. Pathophysiology and toxicity of nanoparticles in the body include reactive oxygen species generation, oxidative stress, mitochondrial perturbation, inflammation, uptake by reticulo-endothelial system, protein degradation/denaturation, brain/peripheral nervous system injury, DNA damage, endothelial dysfunction/blood clotting, and alternation of cell cycle [8, 162, 164, 165]. Several possible mechanisms on nanoparticle–biological tissue interactions have been suggested. They include UV activation leading to radical production, impurities and defects to induce active electronic configuration, redox cycling and catalytic activity of surface metals and polymers, and particle dissolution in media [165].

Although exact mechanism of nanoparticle toxicity is not understood very well, several key factors have been suggested, which are size, surface area/chemistry, solubility, and shape [164]. It was reported that smaller particles were more penetrative into lung tissue than larger ones [166]. In addition, ultra-fine particles (UFP, < 100 nm) rather than fine (< 2.5 μm) or coarse particles (2.5–10 μm) could penetrate into even cells and be localized at mitochondria leading to oxidative stress [167]. Surface area, which is related to the dose of nanoparticles, is exponentially related to lung deposition of nanoparticle, tissue damage, and inflammation [164]. Insoluble nanoparticles were known to be retained in lung tissues and induce inflammation depending on the surface area, which was initiated by oxidative stress [168]. Special interest on the shape was initiated from single-walled nanotube (SWNT), which has 0.7–1.5 nm in diameter and several micrometers in length. In vitro incubation of SWNT with keratinocyte induced oxidative stress [169] and inflammation [170]. Toxicological aspects of nanoparticles should be considered before developing, preparing, and applying nanoparticles to avoid unexpected hazardous conditions of workers and patients.

4.8 The Related Guidance

Because of extremely fast advances in nanotechnology, making appropriate guideline on the biocompatibility of nanoparticles in clinical applications seems relatively very slow. A brief review of existing guidance would be beneficial for development of better and safer nanoparticles.

(1) The International Standards Organization 10993 (ISO 10993/FDA #G95-1/Japanese Guideline
ISO 10993 consists of 20 parts of harmonized standards for biocompatibility since 1986. However, the ISO 10993 has too many details and it is not free to access so that it may be considered only as a suggestion rather than a standard on biocompatibility of nanoparticles. The FDA published blue book memorandum #G95-1 entitled “Required biocompatibility training and toxicology profiles for evaluation of medical devices” in 1995. It lists a brief and broad guideline for biocompatibility tests properly based on ISO 10993, which includes cytotoxicity, sensitization, irritation or intracutaneous reactivity, acute system toxicity, sub-chronic toxicity, genotoxicity, implantation, and hemocompatibility. The guideline also includes detailed categories, such as contact time of engineered materials to host tissue and device types to be applied. Differences among the international Standards Organization 10993 (ISO 10993), FDA #G95-1 and Japanese Guideline were compared [171].

(2) A guideline about the critical path to medical device development [172]

The summary of this guideline published by FDA in 2004 is shown in Fig. 4.1. It shows the pathway to modify the nanoparticles for clinical applications. Three important points to be considered are safety, medical utility, and industrialization. Engineered nanoparticles should be safe enough to be applied to humans. The medical utility means efficacy of the developed nanoparticles for the benefit of human health. Moreover, if nanoparticles cannot be produced in large scale, it will be less useful for the public health. At each stage of development, obtained results and data
should be examined in terms of these three factors. The guideline did not present any detailed protocols, but general test methods were described in FDA memorandum No. G95-1.

(3) **FDA guideline for industry and FDA staff [173]**

Although appropriate guidelines and protocols for clinical applications of nanoparticles are urgently needed, the difficulty is in that nanoparticles can not only be used by themselves, but also be combined with different devices. Thus, a combination product defined by FDA in this guideline could be “a product comprised any combination of a drug and a device, a biological product and a device, a drug and a biological product, or a drug, device, and a biological product.” The report describes that combination products may require more careful consideration during development than conventional products due to their complexity. Such complexity may not only be simply due to combination of devices, drugs, and biological products, but also due to interactions between a combination product (as well as its constituents) and biological tissues.

(4) **FDA Guidance for industry, investigators, and reviewers [174] and (5) FDA Final guidance for industry and CDRH staff [175]**

For clinical applications, additional guidelines were suggested in the report, which described general approaches for investigating new drugs and for modification of existing devices or protocols.

**References**

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### Chapter 4

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