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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60 ## 4.1 Introduction
61

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

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

88 With the ever-increasing R&D activities in nanotechnology, results and data indi-
89 cating the risks of nanoparticles have been accumulating. Thus, appropriate regula-
90 tory action is urgently required to protect human health and the environment from

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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 those 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 deamidase (Adagen®, Enzon), pefilgrastim (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

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136 that protein. The protein solubility can also be increased based on an ion-pairing
137 mechanism. An oppositely charged surfactant is used to bind the protein and give
138 it a neutral hydrophobic surface. Negatively charged surfactants are usually used
139 to neutralize the positively charged protein because of the toxic side effects of the
140 cationic surfactants [21, 22, 23].

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

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4.2.2 *Inorganic Drugs*

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4.2.2.1 Magnetic Nanomaterials

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

163

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4.2.2.2 Titanium Dioxide Nanomaterials

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166 Titanium dioxide (TiO_2), a noncombustible and odorless white powder, naturally
167 exists in minerals like anatase, rutile, and brookite. It is widely used as a white pig-
168 ment for paints, paper, plastics, ceramics, for example. TiO_2 is also used as a food
169 additive, such as in toothpaste and capsules, and the Food and Drug Administration
170 (FDA) established a regulation for TiO_2 as the color additive for food. Federal Reg-
171 ulations of the US government regulates the quantities of TiO_2 not to exceed 1% by
172 weight of food. TiO_2 becomes transparent at the nanoscale (particle size < 100 nm),
173 and is able to absorb and reflect UV light, making it useful in sunscreens. Nowa-
174 days, TiO_2 nanoparticles are used widely because of their high stability, anticorro-
175 sive character, and photocatalysis.

176 TiO_2 nanoparticles can produce free radicals with a strong oxidizing ability
177 which can catalyze DNA damage both in vitro and in human cells [30]. TiO_2
178 nanoparticles also have pulmonary toxicity after endotracheal inhalation and instil-
179 lation into the organism. It was reported that the TiO_2 nanoparticles (20 nm) pene-
180 trated more easily into the pulmonary interstitial area than the fine particles (250 nm)

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181 of the same mass [31]. The size-dependent toxicity of TiO_2 particles may not be sig-
182 nificant if different routes of administration or different genders are used [32]. In an
183 inhalation exposure study, mice exposed to 2–5 nm TiO_2 nanomaterials revealed a
184 moderate inflammatory response among animals [33]. Pulmonary toxicity research
185 in rats with three forms of TiO_2 particles showed that exposures to ultrafine TiO_2
186 particles can induce typical pulmonary effects, based on their composition and crys-
187 tal structure [34].
188
189
190 #### 4.2.2.3 Silica Nanomaterials
191
192 Nanomaterials of silica, a non-metal oxide, have been used in chemical mechani-
193 cal polishing, and as an additive to drugs, cosmetics, printer toners, varnishes, and
194 food, because it is a "generally regarded as safe" (GRAS) material. In recent years,
195 applications of SiO_2 nanomaterials have been extended to biomedical and biotech-
196 nological fields, such as biosensors [35], biomarkers [36], cancer therapy [34, 37],
197 DNA delivery [38], and enzyme immobilization [39].
198 Recent literature searches indicate that silica nanomaterials are nontoxic at low
199 dosages but cell viability decreases at high dosages, because high dosages of sil-
200 ica induce cell membrane damage. On the other hand, silica-chitosan composite
201 nanomaterials are known to induce less inhibition in cell proliferation and less
202 membrane damage. The cytotoxicity of silica to human cells depends strongly on
203 their metabolic activities, but it could be reduced by combining with chitosan [40].
204 In addition, dose-dependent exposure to SiO_2 nanoparticles induced cytotoxicity
205 in human bronchoalveolar carcinoma-derived cells that was closely correlated to
206 increased oxidative stress. It appears that SiO_2 nanomaterials reduce cell viability
207 resulting from penetration of the particles into the cell nucleus [41].
208
209
210 ## 4.3 Polymeric Carriers
211
212 Polymeric carriers are often used as drug delivery systems. They must not only be
213 biocompatible and immunocompatible, but also be readily eliminated from the body,
214 preferably through biodegradation. There are so many polymers that it is very diffi-
215 cult to classify each by certain criteria. For convenience, however, they are divided
216 into biodegradable and non-biodegradable polymers in this chapter. The biodegrad-
217 able polymers can be hydrophilic, hydrophobic, or amphiphilic.
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219
220
221 ### *4.3.1 Non-Biodegradable Polymers*
222
223 Non-biodegradable polymers were frequently used as implant drug delivery sys-
224 tems in the early 1970s because of their long-lasting release and reduced host
225 response. Examples are poly(vinyl alcohol) (PVA), poly(ethylene vinylacetate)

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226 (PEVA) [42, 43], and polysulfone capillary fiber (PCF) [44]. They were proven to
227 be safe in rabbit eyes for months [43, 44].
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230 ## *4.3.2 Biodegradable Polymers*
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232 ### 4.3.2.1 Hydrophilic Polymers
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234 Hydrophilic polymers generally have little immunogenicity in clinical applications.
235 However, most hydrophilic macromolecules have to be crosslinked or copolymer-
236 ized to form hydrogels; otherwise they will be dissolved and cleared from the
237 body. Physical hydrogels may be better than the chemical ones, because most cross-
238 linking agents are toxic and the chemical crosslinking process may chemically affect
239 the entrapped molecules. It is very important to remove any residual crosslinking
240 agent before in vivo application.
241 At the end of the last century, synthetic polymers became more and more impor-
242 tant. Synthetic polymers could be tailored for various physicochemical properties
243 to suit various applications. The synthetic polymers used in biomedical applications
244 must be biocompatible, i.e., they must not provoke a defensive, potentially danger-
245 ous reaction in vivo. Application of nanomaterials may need to be considered as
246 "polymer genomics." The term "polymer genomics" is defined as "an effect of syn-
247 thetic polymers on pharmacogenomic responses to chemotherapeutic agents and the
248 expression of transgenes delivered into cells" [45, 46]. Understanding of polymer
249 genomics is expected to lead to safe and efficient nanoparticles for clinical applica-
250 tions.
251 Poly(2-hydroxypropyl methacrylamide) (PHPMA) is a potential water-soluble
252 carrier. Rihova [47] reported that the molecular weight and the properties of the
253 oligopeptidic side chains could result in some immunogenicity. PHPMA with
254 molecular weight around 30 kDa is not recognized as a foreign molecule and has no
255 recorded defense reaction to it. The attachment of pendant oligopeptide sequences
256 to the HPMA backbone bestows a certain degree of immunogenicity, which depends
257 on the composition of the oligopeptidic side chains, dose and route of application,
258 molecular weight, and the genotype of the immunized individual.
259
260 ### 4.3.2.2 Hydrophobic Polymers
261
262 Hydrophobic polymers are often used for long-term drug delivery, such as intraocu-
263 lar implants [48]. For convenience of avoiding removal of the system after complet-
264 ing drug release, biodegradable polymers are often preferred. Pure polyanhydrides
265 can be degraded in 3 years unless it is copolymerized with sebacic acid (SA) [49].
266 Increasing the percent of the SA leads to faster degradation, and the copolymer with
267 80% SA can degrade in just a few days. The copolymer with SA is less hydrophobic
268 and is known to be a good biocompatible material [50].
269 Poly(ortho ester) (POE) is a hydrophobic, biodegradable polymer. Currently,
270 there are four (I–IV) families of POE [51, 52, 53]. There are acidic and basic

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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

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316 hydrophobic. The hydrophobic segment of the polymer containing PO units can be
317 used to adsorb and anchor the block copolymer molecule to the nanoparticle sur-
318 face, while the hydrophilic EO-containing segment, or PEG sections, can extend
319 into solution and shield the surface of the particle. It was found, however, that the
320 physically adsorbed polymers can be desorbed [76], and thus covalent grafting to
321 the surface may be necessary for improved stability [77, 78]. Properties of PEG
322 chains, such as molecular weight [72, 79, 80, 81], surface chain density [81, 82, 83],
323 and conformation [72], affect biodistribution and pharmacokinetics of nanoparti-
324 cles. The optimal molecular weight of the PEG chain for surface coating is above
325 2000; otherwise the length of the PEG chain is too short to be flexible [72, 81, 84].
326 An increased surface coverage by PEO, e.g., using Poloxamer 407, resulted in
327 not only a reduction of the amount of adsorbed serum proteins, but also effected
328 the type of proteins adsorbed [74]. When the surface coverage was above 25%,
329 high-molecular-weight proteins did not adsorb onto the nanoparticles. Even at the
330 5% surface coverage, the in vivo circulation time was longer than the uncoated
331 nanoparticle.
332 The nature and state of the hydrophobic segment in the micelles have a significant
333 impact on the in vivo stability as well as the pharmacokinetics and biodistribution of
334 the micelles. The longer length of the hydrophobic segment and the higher propor-
335 tion of the hydrophobic polymer endow a greater thermodynamic stability [85, 86].
336 The physical state of the core-forming polymer, such as amorphous, crystalline or
337 semicrystalline, has an effect on the micelle stability [87, 88]. A micelle containing
338 a hydrophobic block with a glass transition temperature (T_g) exceeding 37°C has a
339 "frozen" core. The crystalline or semi-crystalline [88] core makes the micelle more
340 stable and the duration of drug release becomes longer.
341 The ratio of the hydrophilic/hydrophobic will also affect the shape of the micelle.
342 In general, when the hydrophobic part of the micelle overweighs the hydrophilic
343 part [87] or the length of the hydrophilic segment is longer than that of the core
344 block [89], the shape of the resulting micelles is spherical. On the other hand,
345 non-spherical structures, including rods and lamellae, can be formed by increas-
346 ing the length of the core segment beyond that of the corona-forming chains or the
347 hydrophilic part of the micelle overweighs the hydrophobic part.
348 Amphiphilic β-cyclodextrin nanosphere suspensions [90] also reported to have
349 unexpected good physical stability of the suspensions after 3 years of storage at
350 room temperature after the secondary hydroxyl functions of the β-CDa glucopyra-
351 nosyl units were grafted by hexanoyl carbon chains.
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355 ### *4.3.3 Others*
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357 The copolymer concentration is another factor affecting the effectiveness of the
358 carrier. Three MPEG-b-poly(ε-caprolactone) (MPEG-b-PCL) copolymer concen-
359 trations, 0.2 mg/kg dose group (unimers), 2 mg/kg dose group (unstable micelle),
360 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 signification positive 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 molecular 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 function 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 technologies 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 structures 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 materials, 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 coating (capping material and functional groups) [110, 111]. Protonation [112] or photooxidation [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 cadmium 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 $-NH_2$ 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 cytotoxity is not only dependent on the chemistry of the core, but is also influenced by the properties of the surface. PAMAM dendrimer and polypropylenimine 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

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496 dendrimer toxicity significantly [122]. However, when the surface of generation 4
497 PAMAM dendrimers was modified with lysine or arginine, the toxicity increased
498 which was confirmed by incubation with HepG2 or 293T human embryonic kid-
499 ney cells for 48 hrs, probably due to the increased density and molecular weight
500 [127]. To improve the transfection efficiency, some compounds are added as addi-
501 tives into the dendrimer–DNA complexes, such as DEAE-dextran [128] and substi-
502 tuted cyclodextrin [129].
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4.6 Processes
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The particle size plays a key role in the final biodistribution and pharmacokinetics.
508 It was reported [84] that a particle with hydrodynamic radius of over 200 nm will
509 be cleared more quickly than particles with radius under 200 nm. Controlling the
510 particle size may be one way to prepare safe nano/micro materials.
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4.6.1 Emulsion
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516 Emulsion methods have been used widely in preparing nano/micro particles. Water-
517 in-oil-in-water (w/o/w) double emulsion method has been most widely used. Many
518 hydrophilic drugs/proteins can be encapsulated by this method. The particles
519 obtained in this method are very stable [130]. In emulsion methods, protein drugs
520 can become denatured by exposure to the interface between water and solvent
521 [99, 131]. The effect of the primary w/o emulsion has stronger denaturizing effect
522 than that of the secondary w/o/w emulsion [132]. To avoid the exposure of pro-
523 teins to organic solvent, protein particles in the solid state can be directly suspended
524 in the organic phase to form s/o/w emulsion [23, 133]. For this s/o/w method to
525 work, the hydrophilic drug/protein power has to be under an anhydrous condition
526 before encapsulation [17, 18]. The anhydrous proteins can be obtained by freeze-
527 drying or SFD before being encapsulated (See Section 2). The nature of the organic
528 solvent also has an impact on protein stability. Use of blend solvents (such as ace-
529 tone/ethylene chloride blend) could reduce surface tension between the organic and
530 the water phases [20, 23, 102].
531 Although all ultrasound, sonication, vortexing, and homogenization operations
532 can result in protein degradation, a good choice of the apparatus can minimize the
533 instability of proteins [134]. Milling is a traditional method for micronization of
534 drug powders. The standard micronization processes comprise crushing/milling, air
535 micronization, sublimation, and recrystallization from solvents. There are various
536 mills, such as ball mills, colloid mills, and jet or fluid energy mills. Most of them
537 have advantages and shortcomings. The mechanical treatments can damage degrade
538 particles due to high stresses (thermal and mechanical) generated by attrition. They
539 result in particle adhesion, agglomeration, and loss of drug activity. Jet milling is a
540 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.

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586 Spray freezing into liquid [153, 154, 155, 156] has been developed for poorly
587 water soluble or insoluble drugs. The advantage of the technique is that the aque-
588 ous protein solution is sprayed directly into the liquid nitrogen through an insu-
589 lated nozzle instead of into the cold vapor in SFD. Another improved technique
590 is spray-freezing with compressed CO_2 [135]. In this technique, biopharmaceutical
591 and excipients mixture is atomized, then mixed with compressed CO_2, developing
592 a CO_2-saturated aqueous solution. The droplets become frozen particles when they
593 are sprayed through a nozzle of a sprayer. The goal of the method is to obtain stable,
594 porous or hollow protein particles with a narrow size distribution [157].
595
596
597 ### *4.6.4 Supercritical Fluid (SF) Technology*
598
599 Supercritical fluid technique [135, 158] combines advantages of both liquid and gas.
600 The density values and solvation power of a solute can be adjusted by the SF's criti-
601 cal temperature and pressure. The viscosity of the solutes in SF is lower than liquid,
602 while the diffusivity is higher. Most important is the SFs are highly compressible.
603 The most widely used SF is CO_2 because of its low critical temperature (31.2°C)
604 and pressure (7.4 MPa), non-flammable, non-toxic, and inexpensive. The only lim-
605 itation of SF CO_2 is its limited solvation power though it can be changed into an
606 advantage when the SF CO_2 is used as an anti-solvent. The solvation power of a SF
607 CO_2 can be adjusted by incorporating a small amount of volatile cosolvent, such as
608 acetone or ethanol, which acts as organic modifier.
609 In general, SFs can be divided into 3 groups.
610
611
- Precipitation from supercritical solutions composed of SF and solutions (rapid
612 expansion of supercritical solution, RESS).
613
- Precipitation from gas saturated solutions (precipitation from gas-saturated solu-
614 tion, PGSS).
615
- Precipitation from saturated solutions using SF as anti-solvent (including
616 gaseous anti-solvent, GAS; aerosol solvent extraction system, ASES; solution
617 enhanced dispersion by SF, SEDS and precipitation by compressed anti-solvent,
618 PCA) [159].
619
620
621 Recently innovative techniques have been developed where the SF CO_2 is used
622 to assist spray drying. The SF and the solution are intimately mixed and sprayed in
623 a drying atmosphere. This process allows the minimal decomposition of thermally
624 labile drugs, no need of high pressure vessel and as small as 3 μm particles.
625
626
627 ## 4.7 Workers'Safety
628
629 Although more than 35 countries have developed various R&D programs on nan-
630 otechnology since 2000, the importance of safety issues regarding nanoparticles

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 ($< 2.5\ \mu$m) 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

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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

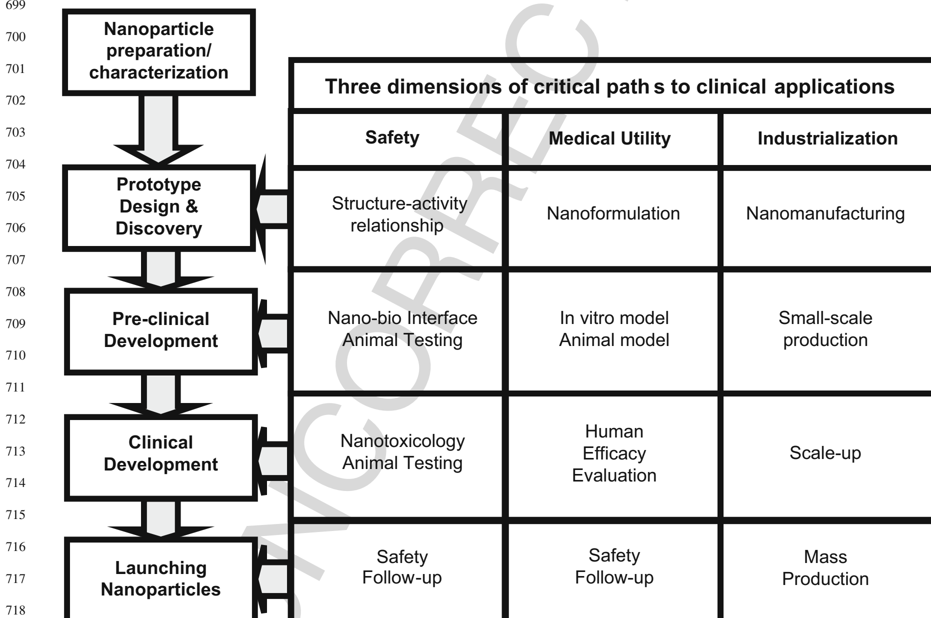

Fig. 4.1 Three dimensions of critical paths to clinical applications of nanoparticles

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721 should be examined in terms of these three factors. The guideline did not present any
722 detailed protocols, but general test methods were described in FDA memorandum
723 No. G95-1.

724
(3) FDA guideline for industry and FDA staff [173]
725

726 Although appropriate guidelines and protocols for clinical applications of nanopar-
727 ticles are urgently needed, the difficulty is in that nanoparticles can not only be
728 used by themselves, but also be combined with different devices. Thus, a combina-
729 tion product defined by FDA in this guideline could be "a product comprised any
730 combination of a drug and a device, a biological product and a device, a drug and a
731 biological product, or a drug, device, and a biological product." The report describes
732 that combination products may require more careful consideration during develop-
733 ment than conventional products due to their complexity. Such complexity may not
734 only be simply due to combination of devices, drugs, and biological products, but
735 also due to interactions between a combination product (as well as its constituents)
736 and biological tissues.

737
738 *(4) FDA Guidance for industry, investigators, and reviewers [174] and (5) FDA Final guidance for industry and CDRH staff [175]*
739

740 For clinical applications, additional guidelines were suggested in the report,
741 which described general approaches for investigating new drugs and for modifi-
742 cation of existing devices or protocols.

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744
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Chapter 4

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