Nanoparticles and toxicity in therapeutic delivery: the ongoing debate

"In order to move the debate on nanoparticles and toxicity ahead, it is necessary for the exchange of concepts, methods and know-how and, therefore, a close collaboration is required between those working in drug delivery and particle toxicology."

Keywords: carbonate apatite ■ drug-delivery system ■ nanoparticle ■ therapeutics ■ toxicity

Nanoparticles & toxicity

In nanotechnology, a particle is defined as a small object that behaves as a whole unit in terms of its transport and properties. According to the American Society for Testing and Materials standard definition, nanoparticles are particles with lengths that range from 1 to 100 nanometers in two or three dimensions [1]. Nanoparticle therapeutics are typically particles comprised of therapeutic entities, such as small-molecule drugs, peptides, proteins and nucleic acids, and components that assemble with the therapeutic entities, such as lipids and polymers, to form nanoparticles. Nanoparticles possess tremendous potential for therapeutics delivery. Early clinical results suggest that nanoparticle therapeutics can show enhanced efficacy, while simultaneously reducing side effects, owing to properties such as more targeted localization in tumors and active cellular uptake. Therefore, human exposure to nanoparticles is inevitable as nanoparticles become more widely used. Since nanoparticles have tremendous potential for being extensively used, especially in biomedical engineering for therapeutics delivery, tissue remodeling and diagnosis, evaluation of their biocompatibility is an indispensable task considering their safety issue in human beings [2]. For biomedical purposes, especially *in vivo* applications, toxicity is a critical factor to be considered while evaluating their potential. Especially as nanoparticles for therapeutics delivery are often coated with bioconjugates such as DNA, proteins and monoclonal antibodies depending on the target cells. Since these nanoparticles are intentionally engineered to interact with cells, it is important to ensure that these enhancements do not cause any adverse effects. The important issue is that whether naked or coated, nanoparticles will undergo biodegradation in the cellular environment, and thus cellular responses will be induced owing to the degradation of nanoparticles. For example, biodegraded nanoparticles may accumulate within cells and lead to intracellular changes such as disruption of organelle integrity or gene alterations causing severe toxicity. A proper understanding about how an agent will react in the body often involves cell-culture studies. Cytotoxicity is a rapid, sensitive and inexpensive test to detect the potential ability of nanoparticles to induce sublethal or lethal effects at the cellular level. Cytotoxicity may not be the only adverse effect of nanoparticles; nanoparticles may also affect the immunological response of cells. One simple cytotoxicity test involves visual inspection of the cells with bright-field microscopy for changes in cellular or nuclear morphology [3]. However, the majority of cytotoxicity assays used throughout published nanoparticle studies measure cell death via colorimetric methods. These colorimetric methods can be further categorized into tests that measure plasma membrane integrity and mitochondrial activity.

Scope of nanoparticles in therapeutics delivery

Nanoparticulate delivery systems have been developed for proper delivery of therapeutics with limited toxic effect. Several researches have shown that conventional drug formulations are obviously more toxic and less efficient than nanoparticle drug-delivery systems. Therefore their scope in therapeutics delivery in terms of design and efficacy in treating several diseases has been the focus of several researchers.

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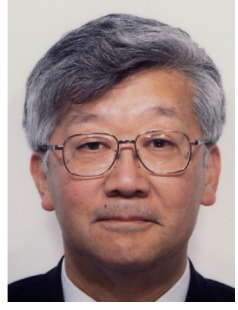

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Nanoparticles in drug & gene delivery

Drug delivery is one of the most advanced application areas for nanoparticles. The primary research goals of nanoparticles in therapeutics delivery include:

- Highly specific drug targeting and delivery;
- Reduced toxicity while maintaining therapeutic effects;
- Greater safety and biocompatibility;
- Faster development of new safe medicines.

The basic prerequisites for the design of new materials for drug delivery includes drug incorporation, cellular interaction and intracellular release, formulation stability and shelf-life, biocompatibility, biodistribution and targeting, and functionality. Moreover, the possible adverse effects of residual material after drug delivery should be considered as well when used solely as a carrier. Therefore, biodegradable nanoparticles with a lifespan as long as therapeutically needed would be optimal. The basic objective of drug incorporation with the nanoparticles is to retain the enhanced delivery to, or uptake by, target cells and/or to reduce the toxicity of the free drug to nontarget organs. Nanoparticulate delivery will moderate the margin between the doses resulting in therapeutic efficacy; for example, tumor cell death and toxicity to other organ systems which will result in an increase of the therapeutic index. Therefore, design of target-specific and long-circulation nanoparticles is needed. Most of the compounds recently used for drug delivery are biodegradable polymers resulting in drug release after degradation. One of the problems associated with the use of particulate drug carriers, including nanomaterials, is the entrapment in the mononuclear phagocytic system as present in the liver and spleen [4–7]. However when treating liver diseases such as tumor metastasis or hepatitis, targeting strategies of nanoparticles may be favorable. It was found that surface modification of nanoparticles with polyethylene glycol (PEG) resulted in long circulation in the bloodstream by inhibiting recognition and phagocytosis by the mononuclear phagocytic system [8–10]. In addition to altering the distribution in the body, the PEG modification also reduced *in vitro* toxicity when gold nanorods were modified using PEG. Coating of nanoparticles may also prevent agglomeration, which was reviewed recently [11]. Sometimes, nanoparticle size can influence the distribution of nanoparticles as was demonstrated for lipid vesicles for which a lower liver uptake was found for the smaller vesicles (200–300 vs 25–50 nm). Even the actual distribution may be influenced by the small size differences and thus affect the overall bioavailability of nanoparticles [12].

Potential uses of nanoparticles in various diseases

Since selective delivery of drugs still remains the major hurdle, especially for cancers and other diseases thereby avoiding the adverse side effects to healthy organs, the use of nanoparticles has become popular to combat such challenges. Moreover, some therapeutics such as DNA, RNA and proteins that cannot enter the cells by normal diffusion require incorporation into nanoparticles for effective delivery. The success of polymer- and liposome-based drug-delivery systems focuses their potential, many of which are in clinical use today. Polymer-based drug-delivery systems can be categorized as polymeric drugs, polymer–protein conjugates, polymer–drug conjugates and polymeric micelles [13]. Polymers can also be emulsified into nanometer-size particles within which drugs can be trapped. Polymeric drugs consist of natural polymers having antiviral or antitumor characteristics. The basic aim of polymer–drug conjugation includes the improvement of solubility and specificity of low molecular weight drugs. Polymer–protein conjugates most commonly use PEG because of its high water solubility and excellent biocompatibility. Therefore, its attachment to drugs results in increased solubility. Lastly, polymeric micelles are typically created with amphiphilic polymers that form micelles in solution with a drug entrapped inside the micelles. A dendrimer is a synthetic polymeric macromolecule of nanometer dimensions, composed of multiple highly branched monomers that emerge radially from the central core. Fascinating properties associated with these dendrimers, such as their monodisperse size, modifiable surface functionality, multivalency, water solubility and available internal cavity make them attractive for drug delivery [14]. Polyamidoamine dendrimer, the dendrimer most widely used as a scaffold, was conjugated with cisplatin [15]. The easily modifiable surface characteristic of dendrimers enables them to be simultaneously conjugated with several molecules such as imaging contrast agents, targeting ligands, or therapeutic drugs, yielding a dendrimer-based multifunctional drug-delivery system [14].

Liposomes are self-assembling closed-colloidal structures composed of lipid bilayers having a spherical shape in which an outer lipid bilayer surrounds a central aqueous space. Currently, several kinds of cancer drugs have been applied to this lipid-based system using a variety of preparation methods. Among them, liposomal formulations of the anthracyclines doxorubicin (Doxil, Myocet®) and daunorubicin (DaunoXome®) are approved for the treatment of metastatic breast cancer and AIDS-related Kaposis sarcoma [16–18]. Besides these approved agents, many liposomal chemotherapeutics are currently being evaluated in clinical trials. The next generation of liposomal drugs may be immunoliposomes, which selectively deliver the drug to the desired sites of action [19]. Carbon nanotubes are carbon cylinders composed of benzene rings that have been applied in biology. These are used as sensors for detecting DNA, protein, diagnostic devices for the discrimination of different proteins from serum samples and carriers to deliver vaccines or proteins [20]. These nanotubes can be linked to a wide variety of active molecules such as peptides, proteins, nucleic acids and therapeutic agents through the introduction of chemical modification making them more water-soluble and functionalized [21]. Carbonate apatite, cationic liposomes, cationic polymers such as polyethylenimine, poly-L-lysine, polyallyamine, chitosan and dendrimers are used for gene delivery. From 1989 to March 2009, over 1540 preclinical and clinical trials using nonviral vectors in gene therapy have been approved or are in progress worldwide. The majority of these trials represent Phase I or II (~79%), with Phase II trials accounting for approximately 16.5% and Phase II/III trials for 4.2% [101]. Moreover, nanoparticles have made a tremendous impact in the treatment of various types of cancer, neurodegenerative diseases, HIV/AIDS, ocular diseases and respiratory diseases, as evidenced by the numerous nanoparticle-based drugs and delivery systems that are in clinical use.

Nanoparticles & the blood–brain barrier

From several perspectives, the brain is a challenging organ for drug delivery. Extensive efforts have been made to develop strategies for delivering drugs to the CNS by enhancing their ability to cross the blood–brain barrier (BBB), well-known as the best gatekeeper in the body toward exogenous substances [22]. The delivery of drugs, peptides, proteins and genes to the brain depends on brain-specific vectors. The development of such vectors requires the identification of new receptor–ligand and antigen–antibody interactions that are selective for the BBB. The use of vectors employing a transporter or acting in a nonspecific manner may find an increased application, especially when combined with a nanoparticle or liposome containing the drug(s) for delivery. This type of approach using particulate systems potentially allows large payloads of a drug to be delivered, which may have particular application in the delivery of cytotoxic agents, neurotrophic peptides/proteins, enzymes, gene vectors and other large molecules to the brain. Some current approaches for drug delivery to the CNS have been reviewed by Hossain *et al.* [23]. Knowledge of the pharmacogenomics of the BBB will undoubtedly lead to the discovery of new ways of promoting the delivery of pharmaceuticals to the CNS.

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Intracellular targeting

Apart from the cellular targeting, the fate of the nanoparticles along with the incorporated therapeutics at the cellular level is of great importance. Efficient delivery of small interfering RNA (siRNA), for example, to the cytosol of target cells depends on both the translocation of the nonviral vectors through the plasma membrane and their subsequent escape from endosomal/lysosomal compartments. Endocytosis, the vesicular uptake of extracellular macromolecules, has been established as the main mechanism for the internalization of nonviral vectors into the cells. However, after endocytosis, the internalized molecules tend to be trapped in intracellular vesicles and eventually fuse with lysosomes where they are degraded. It is, therefore, important for a delivery system to avoid the fate of lysosomal degradation by facilitating release of the internalized siRNA into the cytoplasm. The release of therapeutics from the carrier is essential for binding with specific mRNA (in the case of siRNA) in the cytoplasm for efficacy. The approach taken for carbonate apatite nanoparticles for siRNA delivery is to rapidly dissolve the particles in the endosomal acidic pH conditions so that the associated siRNA can be released into the cytoplasm. The

siRNA–apatite complexes, following endocytosis, are very quickly almost completely dissolved below pH 7.0 [24]. This might have contributed to the destabilization of endosomes, resulting in the quick escape of therapeutics from the endo/lysosome in a short timeframe. Such a pH-sensitivity approach to nanoparticle delivery increases both the biodegradability and escape of therapeutics from the endosome for maximum drug efficacy. Therefore, carbonate apatite shows higher biocompatibility and efficacy than lipofectamine. Most of the universal therapeutics such as DNA, siRNA, mRNA, protein and drugs were successfully delivered by these pH-sensitive nanoparticles [24–28]. Mechanism elucidation of intracellular targeting and efficient transfection mediated by pH-sensitive carbonate apatite-facilitated drug/siRNA delivery is illustrated in **Figure 1**.

Carbonate apatite was characterized to investigate its comparatively much lower cytotoxicity. It was found that calcium, one of the important components of carbonate apatite, is pumped out from the cells immediately after entering, thereby shielding the adverse side effect on the cell. Sometimes surface modification such as surface charge of nanoparticles favors intracellular targeting. Surface functionalization of gold nanoparticles with PEG resulted in the efficient internalization of nanoparticles in endosomes and cytosol, and localization of nanoparticles in the nuclear region [29]. Poly(D,L-lactide-co-glycolide) nanoparticles were found to be ingested by cells via endocytosis [30]. The escape from these endosomes into the cellular cytoplasm was suggested to be caused by a change in surface charge from negative to positive of the poly(lactic-co-glycolic acid) nanoparticles resulting in cytoplasmic delivery of the incorporated drugs. Moreover, targeted cellular delivery can be achieved by coating the nanoparticles with various ligands for specific cell receptors thereby avoiding the adverse side effects using the concept of active targeting. PK2 FCE28069, a *N*-(2-hydroxypropyl)methacrylamide–polymer–Gly-Phe-leu-Gly–doxorubicin conjugate that also contains the sugar galactosamine, was the first ligand-targeted nanoparticle to reach the clinic. The galactose-based ligand was used to target the asialoglycoprotein receptor, which is expressed on hepatocytes, in the hope that its high expression is retained on primary liver cancer cells. However, as asialoglycoprotein receptors are also expressed on healthy hepatocytes, the targeted nanoparticles accumulated in normal liver cells as well as in the tumor [31].

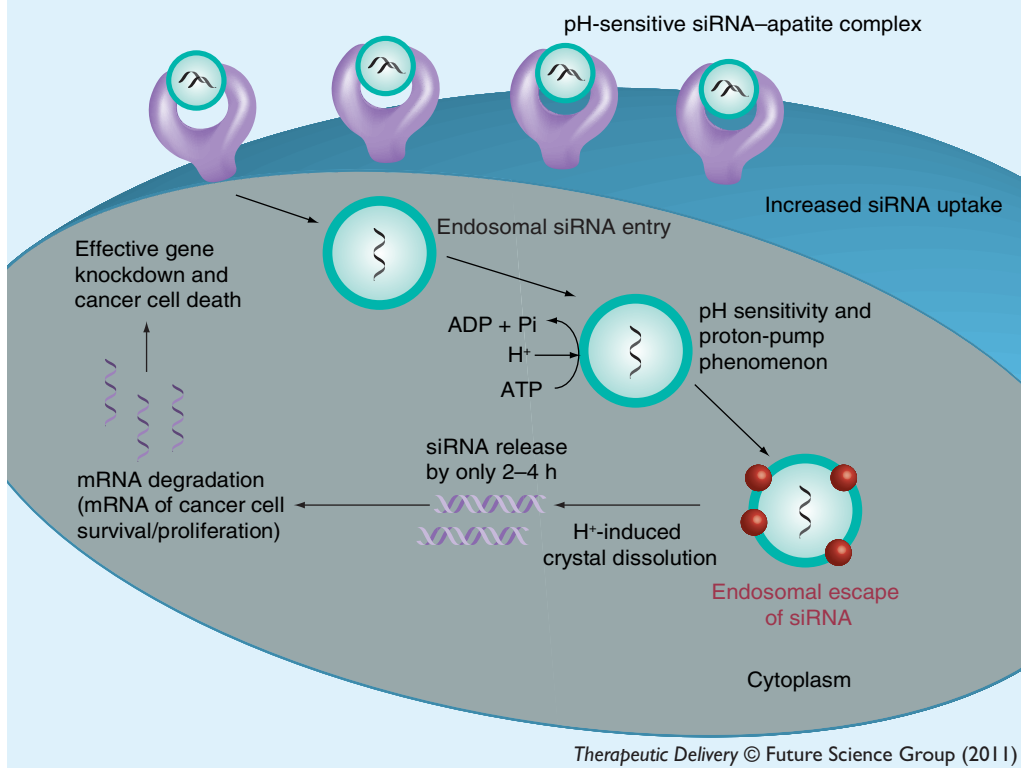

Figure 1. Biocompatible pH-sensitive carbonate apatite-facilitated therapeutics delivery: mechanism elucidation of efficient transfection and intracellular targeting [24].

Toxicological hazards of nanoparticles

With the increase in potential usefullness of nanoparticles in therapeutics delivery, manufacture of nanoparticle-containing merchandise, and the constant discovery of new applications of nanoparticles, it is surprising that knowledge on the health effects of nanoparticle exposure is still limited. Thus, several researchers have tried to investigate the toxicological effect of these nanoparticles in their research.

Biological adverse effects of nanoparticles

After reaching the blood circulation by any favorable administration route, nanoparticles can be internalized in the cells throughout the body, and therefore should be able to interact with signaling processes. Nanoparticles below 40 nm have a size similar to a large protein and may form complexes with endogenous proteins and interfere with their physiological functions depending on nanoparticle surface properties [32]. Moreover, if the particle is not degradable and its size is above 5 nm, the particle cannot be cleared through renal elimination and may exert more adverse effects in the body. Among the indirect effects, inflammation in the lung due to particle deposition may affect target organs by mediators that become systematically available. The inflammatory mediators may trigger systematic hypercoagulability of the blood, thereby increasing the risk of cardiovascular events [33]. Progression of atherosclerosis and increased vulnerability to plaque rupture has also been suggested for particle inhalation-mediated cardiovascular death [34]. Nanoparticles may also affect the CNS, since they can be transported along axial nerve endings to the brain. When nanoparticles with different surface characteristics were evaluated, neutral nanoparticles and low concentrations of anionic nanoparticles were found to have no effect on the BBB integrity, whereas high concentrations of anionic nanoparticles and cationic nanoparticles were toxic for the BBB. Nanoparticles have been shown to induce the production of reactive oxygen species and cause oxidative stress [35] and this has been confirmed in the brain after inhalation of manganese dioxide nanoparticles [36]. Oxidative stress has been implicated in the pathogenesis of neurodegenerative diseases such as Parkinson's and Alzheimer's. Apart from the systemic toxicity, the lung is a major target organ for particle-induced toxic effects, such as pulmonary inflammation and carcinogenicity. Among the mechanisms by which nanoparticles could cause an enhanced inflammatory response, direct effects have been reported on alveolar macrophages such as inward leaching of calcium, impairment of phagocytosis and cytoskeletal changes [37]. Epithelial and nerve cells may also contribute to airway inflammation by producing pharmacologically active compounds such as capsacein. Low-toxicity, poorly-soluble particulates such as carbon black and titanium dioxide (TiO_2) induce chronic inflammation, fibrosis, neoplastic lesions and lung tumors in rats [38].

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Toxicity of therapeutic nanoparticles

A number of papers have described the toxicology of newly engineered therapeutic nanomaterials, including fullerenes, carbon nanotubes and quantum dots and have illustrated that apart from size and surface area, many more parameters describing the material (surface) properties have to be included. In particular, the ability of the particle surface to generate free radicals. When metallic cobalt (20 nm), metallic nickel (20 nm) and TiO_2 (28 nm) nanoparticles of similar specific surface areas were instilled in equal mass doses in a rat, nickel demonstrated significantly greater inflammatory responses than either cobalt or TiO_2 and cobalt was more inflammogenic than TiO_2. Nickel also produced a marked increase in lymphocytes in bronchoalveolar lavage fluid. The chemical surface modification of gold nanoparticles can, however, impact toxicity. Attachment of a cationic polymer monolayer (alkyl thiol with a quaternary ammonium group) onto gold nanoparticles can render them cytotoxic [39]. The effect of gold nanoparticles was tested on the proliferation, nitric oxide and reactive oxygen species production of RAW264.7 macrophage cells [40]. After 48 h of up to 100 μm gold-nanoparticle treatment, RAW264.7 macrophage cells showed greater than 90% viability with no increase in pro-inflammatory cytokines TNF-α and IL-1β and the overall cell viability decreased to 85% after 72 h [40]. For gold nanorods, the cytotoxicity could be attributed to the presence of the

stabilizer cetyl trimethylammonium bromide of which even residual presence after washing resulted in considerable cytotoxicity [41]. Carbon nanotubes are completely insoluble in all solvents, generating some health concerns and toxicity problems. Platelet aggregation was induced by both single and multiwall carbon nanotubes, but not by the C_{60}-fullerenes that are used as building blocks for these carbon nanotubes as shown by a recent study on carbon-derived nanomaterials [42]. In a study exposing human embryo kidney cells to single-wall nanotubes for 1–5 days, a dose- and time-dependent decrease in cell-adhesion ability, cell proliferation and increases in induction of apoptosis were observed [43]. Multiwall carbon nanotubes also elicit proinflammatory effects in keratinocytes. Several studies using intratracheal instillation of high doses of nanotubes in rodents demonstrated chronic lung inflammation, including foreign-body granuloma formation and interstitial fibrosis [44]. The induction of lung granulomas after intratracheal administration was demonstrated by two *in vivo* studies on single-wall carbon nanotubes. The nanotubes were more toxic than quartz particles on a dose-per-mass basis and are well known for their lung toxicity [44,45]. Cationic liposomes showed severe *in vitro* cytotoxicity for siRNA delivery compared with carbonate apatite [24].

> "*Nanoparticles have made major contributions to clinical medicine in the areas of medical imaging and drug/gene delivery. However, the risk of nanoparticulate carrier systems to the patient has not been well realized.*"

In the case of quantam dot nanoparticles, the main toxicological risk associated with the use of quantam dots *in vivo* is the exposure of the inorganic core by deterioration of the organic layer. However, *in vitro* studies have indicated that quantum dots may be toxic, of which some toxicity could be attributed to the surface coating [46]. Quantum dot toxicity was reduced after surface modification with *N*-acetylcysteine, while the nonmodified cadmium telluride quantum dots induced lipid peroxidation in the cells [47]. It was demonstrated that nonmodified or naked quantum dots are supposed to be cytotoxic by induction of reactive oxygen species resulting in damage to plasma membranes, mitochondria and the nucleus [48]. Silica nanoparticles showed both *in vitro* toxic and nontoxic responses; however, *in vivo* cytotoxicity has not been studied. Silica nanoparticles showed dose-dependent cytotoxicity *in vitro* in a time-dependent manner. SiO_2 exposure resulted in increased reactive oxygen species levels and reduced glutathione levels, indicating an increase in oxidative stress [49]. Silica nanoparticles were shown as toxic at high doses by a reduction in cell viability or cell proliferation and by lactate dehydrogenase release from the cells, indicating membrane damage [50].

Conclusion

Nanoparticles have made major contributions to clinical medicine in the areas of medical imaging and drug/gene delivery. However, the risk of nanoparticulate carrier systems to the patient has not been well realized. The number of efforts aimed at determining the health risks associated with nanoparticle exposure continue to grow. For pharmaceutical-specific drug delivery, formulations may be used to increase the so-called therapeutic ratio or index, this being the margin between the dose needed for clinical efficacy and the dose inducing adverse side effects (toxicity). This is particularly true for the application of nanoparticles for drug delivery. The types of hazards that are introduced by using nanoparticles for drug delivery are beyond those posed by conventional hazards using chemical delivery matrices. However, little is known about the adverse effect of nanoparticles when interacting with living cells, organs and organisms, and the scientific paradigm for the possible (adverse) reactivity of nanoparticles is lacking. In most cases, proper *in vitro* and *in vivo* cytotoxic data of nanoparticles are inadequate. Nanoparticles useful for cellular therapies using genes may undergo an *in vitro* cytotoxicity assay, but those useful for drug delivery must be evaluated for *in vivo* toxicity. Therefore, it is very much necessary to properly characterize nanoparticles in terms of toxicity before their clinical use, and for that reason a conceptual understanding of biological responses to nanomaterials is needed. In order to move the debate on nanoparticles and toxicity ahead, it is necessary for the exchange of concepts, methods and know-how and, therefore, a close collaboration is required between those working in drug delivery and particle toxicology.

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