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Nanoparticles in Biomedical Applications and Their Safety Concerns

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1. Introduction

In this chapter, we will discuss the fate of nanoparticles when they are introduced into a system. Recent advances in synthesis and functionalization of nanoparticles have brought a significant increase in their biomedical applications, including imaging of cell and tissues, drug delivery, sensing of target molecules, etc. For example, iron oxide nanoparticles (Feridex) have been clinically administered as a contrast agent in magnetic resonance imaging (MRI).

Their superb magnetic properties provide a significant contrast of tissues and cells where particles were administered. The use of Feridex as a MRI contrast agent enables a facile diagnosis of cancers in diverse organs in their early stages of development. As the range of different nanoparticles and their biomedical applications continue to expand, safety concerns over their use have been growing as well, leading to an increasing number of research on their *in vivo* toxicity, hazards, and biodistributions.

While the number of studies assessing *in vivo* safety of nanoparticles has been increasing, a lack of understanding persists on the mechanisms of adverse effects and the distribution pathways. It is a challenge to correlate reports on one type of particles to reports on other types due to their intrinsic differences in the physical properties (particle size, shape, etc.) and chemical properties (surface chemistry, hydrophobicity, etc.), methods of preparation, and their biological targets (cells, tissues, organs, animals).

Discrepancies in experimental conditions among different studies is currently bewildering the field, and there exists a critical need to arrive at a consensus on a gold standard of toxicity measure for probing *in vivo* fate of nanoparticles. This chapter summarizes recent studies on *in vivo* nanoparticle safety and biodistribution of nanoparticles in different organs. An emphasis is placed on a systematic categorization of reported findings from *in vivo* studies over particle types, sizes, shapes, surface functionalization, animal models, types of organs, toxicity assays, and distribution of particles in different organs.

Based on our analysis of data and summary, we outline agreements and disagreements between studies on the fate of nanoparticles *in vivo* and we arrive at general conclusions on the current state and future direction of *in vivo* research on nanoparticle safety.

2. Nanoparticles in biomedical applications

Particles in nanosize have significantly different characteristics from particles not in nanoscale. Since these nanoparticle properties are often in many applications, they have been applied in a wide variety of medical research. (Bystrzejewski, Cudzilo et al. 2007; Yu 2008; Nune, Gunda et al. 2009; Yaghini, Seifalian et al. 2009)

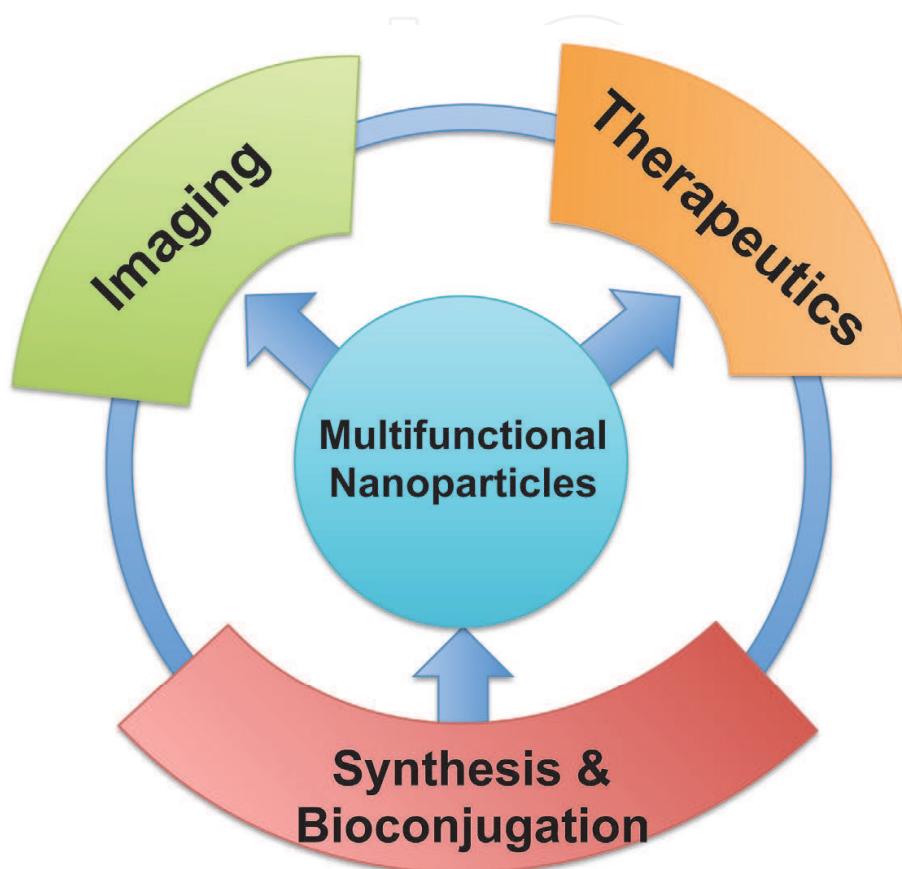


Fig. 1. Multifunctional nanoparticles in bioimaging and medicine. Developed synthesis and bioconjugation strategies for multifunctional nanoparticles helps enabling applications of multifunctional nanoparticles in *in vivo* imaging and therapy.

In this chapter, nanoparticles of different kinds will be reviewed for their applications in biomedical imaging and therapeutics. Popular nanoparticles in biomolecular and biomedical imaging include fluorescent particles for optical imaging, such as quantum dots, gold nanoparticles and magnetic particles for MRI. Nanoparticle derives therapeutics includes heat ablation of target tumours, or delivery of drugs. Figure 1 summarizes the attributes of multifunctional nanoparticles that have attracted the field of bioimaging and medicine. Multiple modalities of these particles enable the accurate, less-invasive diagnosis and therapeutic approaches.

2.1 Imaging

Nanoparticles in imaging applications have been increasingly developed in last 20 years. Because of the superior photo stability, narrow range of emission, broad excitation wavelength, multiple possibilities of modification, quantum dots have gathered much

attention from engineering and scientists who are interested in bio markers, sensors or drug targeting. (Willard and Van Orden 2003; Qi and Gao 2008; Ghaderi, Ramesh et al. 2010; Han, Cui et al. 2010; Li, Wang et al. 2010) Commercially available binary quantum dots from Qdot have been successfully applied for above purposes during the last 10 years and reported in a vast number of literatures. Small size comparable to biomolecules (antibody, RNA, virus, etc.), high quantum yields and high magnetism are few representative advantages of nanoparticles that makes them to be a next generation imaging tools for *in vivo* imaging applications.

2.1.1 Nanoparticles for optical imaging

The most widely used nanoparticles in optical imaging are semiconductor nanocrystals, known as quantum dots. Their size dependent optical properties are unique in their applications to the efficient labelling of biomolecules and tissues where the traditional fluorescent labels have been hardly accessible to because of the size restrictions. In contrast, the size and shape of fluorescent nanoparticles can be rather easily controllable during their synthesis. Semiconductor quantum dots are about 100 times brighter, have narrow emission spectra and broader excitation than traditional organic dye molecules. Since the quantum dots share the similar excitation wavelength and the emission is size tunable, multiple color imaging with single excitation.

Recent developments of conjugating particle surface with biomolecules allowed cell targeting using quantum dots. (Hoshino, Hanaki et al. 2004; Jaiswal, Goldman et al. 2004) Targeting of cells with quantum dots, however, often faces the issues in their accessibility of internalization. Larger size particles will affect protein trafficking and the viability of the cells.

Whether fluorescent nanoparticles are uptaken into the cell or not is critical decision maker in application of them for *in vivo* imaging. The number of nanoparticles in the cell cytoplasm should be to enough to enlighten the cell in the deep tissue. Although there have been efforts to enhance the fluorescent signal in the deep tissue by using a two-photon microscope or upconversion nanoparticles, it is still important to have enough number of nanoparticles per cell to be able to clearly visualize the target. A difficulty here is, the increased number of nanoparticles will increase toxicity of them to the cells. Therefore, the development of fluorescent nanoparticles for *in vivo* imaging is still an open challenge.

In vivo imaging of the target cells by fluorescent nanoparticles are often achieved by first labeling cells with particles then injecting them in the target. Loading of nanoparticles into human cancer cells *in vitro* has been shown successfully (Sage 2004; Li, Wang et al. 2006; Xing, Smith et al. 2006) and their *in vivo* application in mice model (Kim, Jin et al. 2006; DeNardo, DeNardo et al. 2007; Goldberg, Xing et al. 2011) was evaluated as well. It showed the division of human cancer cells and their reforming of tumour tracked by fluorescence. In imaging of lymphatic or cardiovascular systems, fluorescent nanoparticles have shown their potentials. Sentinel lymph systems in small animals were imaged by using a near infrared emitting quantum dots. (Parungo, Colson et al. 2005; Soltesz, Kim et al. 2006; Frangioni, Kim et al. 2007) Trafficking of quantum dots in those lymphatic systems was rather investigated by other groups as well. Lymph node imaging is beneficial to the surgeons for them to locate the exact position of the target.

Another example of *in vivo* imaging application using fluorescent nanoparticles is imaging of cardiovascular systems. Sensitivity and stability of fluorophore is always been a challenge in cardiovascular imaging. Coronary vasculature of a rat heart has been imaged with near IR emitting nanoparticles with high sensitivity. (Morgan, English et al. 2005)

Early detection of cancerous cells is the topic of interest for applications of quantum dots. Multiplexing of quantum dots for the better targeting and sensitivity has been a candidate for this purpose. Surface receptors are available on cancer cells that can be targeted by the multiplexed nanoparticles. Antibody coated quantum dots that are specific to the surface markers on cancer cells were demonstrated to label them in mice. Currently, targeting tumours are based on such an approach that functionalizing quantum dots with molecules specific to the target.

Since *in vivo* imaging requires high quantum efficiency of quantum dots to penetrate deep tissue and organs, its bioconjugation strategy should also be compatible to keep the initial brightness. In that regards, near IR emitting quantum dots are believed to be the optimal candidates for *in vivo* optical imaging. Infrared has the long wavelength that it can penetrate the deep tissues relatively better than other visible lights. It will also minimize the possible false positive signal by autofluorescence from the background since near IR is not relatively absorbed well by water or hemoglobin in the system.

Gold nanoparticles have been the popular choice for near IR emitting nano fluorophores since it is relative biocompatible and easy to synthesize. (Lee, Cha et al. 2008; Shang, Yin et al. 2009) The surface plasmon resonance is dependent on the size of the nanoparticles that it moves towards red with increasing particle size. Other types of gold nanomaterials such as gold nanorods and gold nanoshells were also popularly used in bioimaging because of its tunable surface plasmon bands and controllable position of the resonance by varying the synthesis conditions.

Several imaging methodologies were developed to be able to use gold nanoparticles and their derivatives in bioimaging. Optical Coherence Tomography (OCT) uses the scattering function of gold nanoshells for *in vivo* imaging. (Agrawal, Huang et al. 2006; Adler, Huang et al. 2008; Skrabalak, Chen et al. 2008) The accumulation of gold nanoshells at the tumour increases scattering at that location that provides the contrast. Another imaging tool for gold nanomaterials is using photoacoustic imaging. The photoacoustic imaging adapts a pulse of near IR that causes thermal expansion nearby and sound wave detectable at the surface. Distinctive sound wave generated by gold nanoparticles can be separated from background signal by surrounding tissues and organs.

Another approach of adapting gold nanomaterials for *in vivo* imaging is using a two-photon fluorescence spectroscopy. Since gold nanomaterials possess the strong surface plasmon resonance, it can increase occurrence rate of two-photon excitation and relaxation of energy through fluorescence.

Lastly, Raman spectroscopy can be used for enhanced Raman effect at the surface of gold nanomaterials. Location of gold nanoparticles in animal model was demonstrated by using a Raman effect of reporter dye on the gold surface of particles. (Christiansen, Becker et al. 2007; Lu, Singh et al. 2010)

Although quantum dots are useful as a tagging material, they also have several disadvantages. First and the most serious demerits of binary quantum dot is that it is toxic to cells. Most popular components of binary quantum dots are cadmium / selenide which are deleterious to cells. Because of the intrinsic toxicity of binary quantum dot, very thick surface coating is required. The final size of quantum dot is almost twice as thick as the initial core size and hinders the applications of quantum dots in a cell. Another drawback of binary quantum dot is its blinking behavior when a single binary quantum dot is observed with confocal fluorescent microscope. (Durisic, Bachir et al. 2007; Lee and Osborne 2009; Peterson and Nesbitt 2009) Its blinking behavior hinders the tracking of quantum dot targeted bio molecule in a bio system.

Because of drawbacks of binary quantum dots, silicon nanocrystal has been studied to overcome the demerits of commercially available quantum dots and be used as a substituting fluorophore with traditional organic dyes. Silicon nanocrystals’ superiorities as a fluorophore are summarized in Table 1. Silicon is basically non-toxic to cells so that it does not require a thick surface coating to prevent exposure of core to the environment. Therefore, its average size remains close to its core size.

	Silicon Nanocrystal	Binary Quantum dot	Organic dye
Average Size	1~4 nm (in diameter)	10~20 nm (in diameter)	0.5~10 nm
Quantum Yield	< 60 %	>50 %	>90 %
Photostability	> 6 month	No data	1 day
Blinking	No data	Micro second	No data

Table 1. Comparison of characteristic properties of Silicon nanocrystal with binary quantum dots and traditional organic dyes.

2.1.2 Magnetic nanoparticles

Recently, various non-invasive imaging methods have been developed by labeling stem cells using nanoparticles such as magnetic nanocrystals, quantum dots, and carbon nanotubes. Among these, magnetic nanocrystals provide the excellent probe for the magnetic resonance imaging (MRI), which is widely used imaging modality to present a high spatial resolution and great anatomical detail.

In the last decade, superparamagnetic iron oxide (SPIO) nanoparticle has become the gold standard for MRI cell tracking, and has even entered clinical use. However, in many cases, SPIO-labeled cells producing hypointensities on T_2/T_2^* -weighted MR images, cannot be distinguished from other hypointense regions such as blood clots or scar tissues in some experimental disease models. Moreover, the susceptibility artifact or “blooming effect” resulting from the high susceptibility of the SPIO may distort the background images.

Gd complex based contrast agents can be good alternative MRI contrasts to generate the unambiguous positive contrast (hyper-intensity) and developed. Even if they produce positive contrast and increase the visibility of cells in low signal tissue, they have short residence time and can’t pass through the cell membrane easily. Therefore, there have been developed some of Gd ion based nanoparticulate contrast agents to overcome these disadvantages of the complex agents. (Ananta, Godin et al. 2010)

MnO nanoparticles have also been recently explored as a new T_1 MRI contrast agent and fine anatomical features of the mouse brain were successfully obtained. These MnO nanoparticles were also used to demonstrate feasibility of cell labeling and *in vivo* MRI tracking. (Baek, Park et al. 2010) However, under existing MnO based nanoparticle systems, the contrast is weak and the duration of signal is short for the long time *in vivo* MRI tracking.

Therefore, it is required the further development of the MnO based contrast agent with high relaxivities and improved cellular uptake to stem cells which is more difficult to label due to the lack of substantial phagocytic capacity. (Kim, Momin et al. 2011)

2.2 Multifunctional nanoparticles in therapy

Multifunctional nanoparticles are in the process of being evaluated as new tools for therapy in biomedical research. In the United States 15 out of every 100,000 persons are diagnosed with brain cancer every year.

The most common type of adult brain tumor is malignant glioma with median survival rate of 10-12 month. Due to the complexity of the brain, the most practical treatment remained surgical removal of the tumor that frequently results in reoccurrence of the disease.

A new type of nanoparticles is suggested that it cannot only be used for imaging but also can be used as a therapeutic agent. These new nanoparticles can be activated either by using radiofrequency (RF) pulses or infrared light to release the drug.

2.2.1 Hyperthermia

In order to implement hyperthermia treatment, magnetic nanoparticles can be introduced in the body through magnetic delivery systems (high gradient magnetic fields) or local injection to the affected area. (Corchero and Villaverde 2009)

MRI utilizes RF pulses to generate coherent magnetization from the magnetic moments of water molecules in the sample that can then be detected. Since RF energy can also be converted into heat (e.g. similar to using a microwave to boil water) if the MRI agents can absorb RF energy efficiently, then a localized heating is possible during MR image collection. This idea of RF induced hyperthermia, or in other words, RF ablation has been studied in cancer research since the 1950's.

Certain parameters should be evaluated before deciding the contrast agent for the best hyperthermia applications. The best candidate nanoparticles are selected following these categories; specific absorption rate, size, biocompatibility.

Tumor treatment by hyperthermia has limitations, however, that the most of nanoparticles do not have high specific absorption rate. At least 10% of tumor weight should be absorbed in order to be effective to heat-ablate tumors through hyperthermia.

Treatment of malignant tumors at any site in the body is expected to be possible if agents that convert RF energy into heat can be delivered to the malignant cells. However, RF ablation suffers from the disadvantage that it is an invasive method that often requires insertion of electrodes into the body to deliver RF to the tumor sites.

Superparamagnetic iron oxide nanoparticles of a correctly determined size are appropriate for *in vivo* hyperthermia applications, as they have no net magnetization without the external magnetic field. No net magnetization without the external magnetic field would eliminate the possible particle aggregation in the system. Aggregated particles often experience non-specific engulfment by reticular endothelial system that will significantly reduce the contrast.

Plasmonic photothermal therapy is another new technology to treat tumor by using nanoparticles. (Chen, Frey et al. 2010) Plasmonic photothermal therapy is based on the surface plasmon resonance effect in nanoparticles when the light activates them. Most common example of this therapy is using gold nanoshells that we discussed before to achieve localised, irreversible thermal ablation of the tumor.

In future direction of the research, the MRI will be used passively to visualize the tumor and actively to eradicate it. Multifunctional nanoparticles have a tremendous potential for RF activated heating and triggering since they possess magnetic properties to generate MRI contrast, have the ability to absorb remote RF energy, and can deliver/release anti-cancer drugs in a controlled manner.

2.2.2 Photodynamic therapy

Singlet oxygen ($^1\text{O}_2$), as a part of reactive oxygen species (ROS) is useful tool to destruct cancer cells at the local site when singlet oxygen is concentrated. Photodynamic therapy is a new technology to treat tumor based on nanoparticle generated ROS at the tumor site. (Takahashi, Nagao et al. 2002; Oberdanner, Plaetzer et al. 2005) Photosensitizers, such as nanoparticles, can produce ROS when they are activated with the appropriate wavelength of excitation light. Nanoparticles as photosensitizers must be in close proximity to the tumor cells that they are usually administered at the tumor site directly. Photodynamic therapy is desirable in that it is relatively non-invasive and low toxicity. The major technical barrier, however, of this therapy is its difficulty in systemic introduction of photosensitizer to the tumor site and local irradiation to activate them. Tumors that have disseminated throughout the whole body may not be adequate for this therapy since the current technology is not available to irradiate the whole body. In addition, UV light is the wavelength of choice for the most of traditional photosensitizers that cannot efficiently penetrate into deep tissue.

Therefore, the new class of nanoparticles called up-converting nanocrystals was introduced to alleviate these issues. (Vetrone, Naccache et al. 2010) Up-converting nanoparticles are excited by near infrared light that can efficiently penetrate tissues deeper than UV-VIS light, which allows for the non-invasive application of the method. Functionalized surface on up-converting nanoparticles can direct particles to the specific tumor site that will concentrate ROS production.

There are still few disadvantages of up-converting nanoparticles that their size is intrinsically large. The size of them is usually around 100 nm that may not be appropriate for *in vivo* imaging. Furthermore, ROS are produced at the surface shell of up-converting nanoparticles that its efficiency may be degraded while diffusing out to the surrounding environment.

3. Toxicity

Production and exposure of nanoparticles less than 100 nm in diameter may pose unknown risks since the responses of biological systems to novel materials of this size have not been adequately studied.

The high surface area to volume ratio makes nanoparticles particularly good catalysts and such particles readily adhere to biological molecules. The size and surface charge of nanoparticles enable them to access places where larger particles may be blocked, including passage through cellular membranes. However, the wider application of semiconductor quantum dots as biological probes has been held back by their inherent chemical toxicity, which necessitates encapsulating them in a robust inert shell that increases the diameter of the probe.

Although there are studies (Zhu, Oberdorster et al. 2006; Rogers, Franklin et al. 2007; Teeguarden, Hinderliter et al. 2007; Warheit, Hoke et al. 2007; Clift, Rothen-Rutishauser et al. 2008; Prow, Bhutto et al. 2008; Simon-Deckers, Gouget et al. 2008; Zhu, Zhu et al. 2008; Crosera, Bovenzi et al. 2009; Kramer, Bell et al. 2009; Marquis, Love et al. 2009; Monteiro-Riviere, Inman et al. 2009; Simeonova and Erdely 2009; Warheit, Reed et al. 2009; DeVoll 2010; Li, Muralikrishnan et al. 2010; Maurer-Jones, Lin et al. 2010; Samberg, Oldenburg et al. 2010; Yang, Liu et al. 2010; Zhu, Chang et al. 2010) on both known and unknown hazards of several kinds of nanoparticles, many questions remain unanswered. Furthermore, there are

few systematic studies dealing with both cytotoxicity and inflammatory responses of cells treated with nanoparticles.

How will a biological system react when exposed to nanoparticles? What is the fate of the nanoparticles once they are presented to a population of cells? If the nanoparticles enter into the cell, what effects do they exert internally? These questions must be answered in order to ensure safety to the patient if nanoparticles are incorporated in biomedical applications.

In this chapter, we will discuss nanoparticles as for any diagnostic or medicinal tool and point out that nanoparticles can only be applicable to *in vivo* applications on humans when they pass the assessment for their toxicity. To the fact that the number of different nanomaterials synthesized and potentially targeted for *in vivo* applications is much more than the number of toxicity assessment for them, these investigations are only at the very early stage.

Noticeable conclusions from those studies have been already distress the field and public to strengthen the extended investigation requirement before pursuing any further research. Recent reviews on the toxicity assessment of nanoparticles keep pointing out that the experimental conditions, preparations of nanoparticles and protocols the investigators use all affect the results. These discrepancies between studies even for the same kind of nanoparticle result from the complexity of the investigated systems and potential interference of nanomaterials to the assay techniques.

3.1 Nanoparticle toxicity assessment in *in vitro* assays

Growing public concern regarding the unknown toxicological effects of nanoparticles has spawned cooperative efforts by government agencies and academia to closely investigate these issues.

<i>In vitro</i> assay	Assay mechanism	Detection	Tested nanoparticles	Pros	Cons
MTT (or MTS)	Dead cells cannot reduce MTT (MTS).	Absorption	Quantum dots, gold nanoparticles	Widely used, relatively simple, low cost	Metabolic activity can be affected by multiplexed effects
Calcein AM	Dead cells cannot cleave the acetomethoxy group of calcein AM	Fluorescence	Gold nanoshells	Widely used fluorescence assay, relatively simple, low cost	Fluorescent nanoparticles interfere with calcein dyes.
Protease activity assays (e.g., CytoTox-Glo)	Substrates bind to dead cells' proteases in the media to produce a fluorescence signal.	ELISA/fluorescence colorimeter	Fullerene, carbon black, quantum dots	Cytotoxicity can be probed based on the activity of various proteases	Expensive; fluorescent nanoparticles in the cell interfere with the signal.
Blood contact properties (e.g., hemolysis)	Hemoglobin released from cells is oxidized and quantified by its absorbance	ELISA/absorption colorimeter	PAMAM dendrimers, TiO ₂ nanoparticles	Widely used, relatively simple, low cost	No established positive control for nanomaterials; possible interference
Macrophage functions (cytokine induction)	Nitric oxides, various cytokines (e.g., interleukins, TNF- α) are induced	ELISA/absorption/fluorescence colorimeter	Si nanoparticles	Widely used fluorescence assay, relatively simple, low cost	Fluorescent nanoparticles interfere with detection dyes

Table 2. Summary of popular cytotoxicity and inflammatory response assays used for nanoparticles

Nanoparticles may not be filtered by the body's defense mechanisms because of their small size, which suggests that they may cause inflammatory and/or toxic responses. The reported cytotoxicity and immune response studies on nanoparticles have been based mainly on *in vitro* assays such as cell viability tests, cytokine release analyses and cell

function degradation analyses upon the exposure of a bulk culture of cells to nanoparticles (see available assays: http://ncl.cancer.gov/working_assay-cascade.asp).

However, no validated standard or protocol has yet been established to test biological responses to nanoparticles. Table 2 lists a representative selection of cytotoxicity and inflammatory response assays used to test biological responses to nanoparticles.

3.1.1 Nanotoxicity: Complex system to investigate

The number of reports on assessment of the nanoparticle toxicity has been growing with the number of biomedical research associated with them. It is noticeable that the reports are not consistent in terms of particles' toxicity results. Some reports on popular nanoparticles such as cadmium selenide, iron oxide, gold and silica nanoparticles all have non-consistent conclusions about their toxicity to the biological system. These inconsistent conclusions result from the fact that there is currently no standard protocol for the assessment of the toxicity of nanomaterials. Variation of experiment parameters as well as interference of nanoparticles to the measuring instruments is prime reasons that make it impossible to compare the results between different studies.

3.1.2 Cytotoxicity

The cytotoxicity of a nanomaterial is influenced by the following parameters: cell line, culture conditions for *in vitro* studies, how to introduce particles in *in vivo* studies, nanoparticle concentration, size and duration of exposure. No standard protocol is available at the current stage in terms of setting those parameters relatively be consistent. It is challenging, furthermore, to decide whether the reported range of particle concentration is physiologically relevant to the *in vivo* system.

The cell line to test *in vitro* is a critical factor determining the degree of cytotoxicity of nanomaterials. In one study, nanoparticle uptake rate and resulted cytotoxicity was compared in the same cell line but prepared by following different protocols. It was found that the cytotoxicity could be varied among the cell lines depending on how they were prepared.

Another factor for observed discrepancies between the results of toxicity assays is different testing methods applied on the same nanomaterial.

In most of the *in vitro* cytotoxicity studies, cell death is investigated using colorimetric assays such as shift of absorption or emission of markers. For example, Trypan blue dye exclusion assay provides information of cell death by showing dye staining on cells that were ruptured. Potential issue here is that nanoparticles applied are usually strongly emit or absorb light. Nanoparticles absorb or emit light may give false positive signal.

Cytotoxicity assays commonly used are to measure the effect of test compound that can rather quickly diffuse into the target cell that they can be assayed within the time frame when the dye still can be effective. Therefore, cytotoxicity assays are rarely run for over few days. Another potential issue here is that nanoparticles are less mobile than the most chemical compounds resulting that they will require longer duration of assays. This would require the modification of the cytotoxicity protocols that should be used for nanoparticles and nanomaterials.

Physical and chemical characterizations of nanoparticles are critically important for cytotoxicity assays. For size analysis, either dynamic light scattering (DLS) or transmission electron microscopy (TEM) is the method of choice for the most of nanoparticles. The nanoparticles that are well dispersed in water would not show a significant aggregation or morphological variations in TEM images.

However, it is often pointed out in many studies that there are some discrepancies in mean particle size between that measured by TEM or by DLS. (Teeguarden, Hinderliter et al. 2007; Warheit, Hoke et al. 2007; Marquis, Love et al. 2009; Monteiro-Riviere, Inman et al. 2009; Vippola, Falck et al. 2009) These discrepancies may be due to differences in both preparation and the inherent limitations of nanoparticle sizing methods, and emphasize the necessity to apply multiple techniques for determining particle sizes in polydisperse batches. While TEM can serve as a tool to capture the size of each individual particle, it is limited in that it can only measure particles after they have been suspended and then dried, it requires measurements of many different particle regions to appropriately represent the entire particle batch, and complex geometries of particles or agglomerates may make characterization difficult. The solvent used to disperse the particles prior to drying for TEM analysis may also affect the measurements.

While dynamic light scattering is performed in solutions, the suspending media and how the particle sample was mixed (i.e. sonication intensity and exposure time) and pre-filtered can significantly affect the particle hydrodynamic size analysis. Moreover, particle agglomeration and time-dependent sedimentation of large (i.e. > 100 nm) and dense silver particles may affect the DLS measurement reliability even during the short measurement time period (2-5 min).

DLS measurements of highly polydispersed particle solutions are also dependent on the main analysis parameter. In an intensity-based DLS analysis of a polydisperse particle sample, smaller particles are under-represented due to weaker light scattering and particle shape effects. For this reason, a number-based DLS analysis would be more appropriate to highlight the most abundant particle size population so that one could make limited comparisons between the different particles, especially when the particles are not pre-filtered and the effect of media on nanoparticle size, agglomeration, and polydispersity are significant.

In general, all of the aqueous particles demonstrate an increase in particle size and/or agglomeration, either by DLS measurement or visually, when mixed with DPBS media due to reaction with chloride ions and the presumable formation of poorly soluble silver chloride.

Surface chemistry of nanoparticles is also another important factor that will affect cytotoxicity of nanoparticles. Citrate is the conjugate base of citric acid, which is a popular reducing agent used in silver and gold production, and provides a negatively charged surface moiety that stabilizes nanoparticle colloids through Columbic repulsion. The citrate-stabilized nanoparticles suspended in water acquired a significant negative charge and acidified the aqueous solution.

However, in comparing the nano-sized particles, it was found that particles share the similar pH and zeta potentials when they are diluted in PBS solution regardless of the degree of citrate coating on each particle. Furthermore, no significant differences in cytotoxicity levels between the nano-sized particles argues in favor of particle size as a stronger determinant of toxicity rather than initial surface chemical properties. This also emphasizes the potential importance of plasma proteins in altering the surface properties of nanoparticles by coating them and affecting their biocompatibility.

3.2 Nanoparticle toxicity analysis toward its *in vivo* applications

In general, the smaller the nanoparticle is the greater the toxicity. This is due in part to the fact that small nanoparticles are more readily uptaken into the cell or even near the nucleus. Larger nanoparticles may therefore be less cytotoxic simply because their cellular uptake is limited at that same concentration.

In order to consider and predict possible nanoparticles toxicity in *in vivo* applications, few things should be carefully examined.

First of all, *in vitro* studies for cytotoxicity should carefully be used to extrapolate expected results in *in vivo* studies. Nanoparticles in *in vivo* system would experience much more complicated perturbations because of a wide variety of proteins and small biomolecules present around them. Because of these neighboring biomolecules, nanoparticles can be degraded, engulfed by phagocytic cells, or traveled away from the target site by lymphatic system. Assay responses obtained from well-controlled environment such as in culturing plate may not always present the same results obtained in *in vivo* environment. Therefore, it will be inadequate to draw any conclusions from the *in vitro* assay for nanoparticle responses in *in vivo* system until following experiments at least in animal model is performed.

Second of all, limitations of current assays performed for cytotoxicity or inflammatory responses of cells to the nanomaterials should be carefully recognized and further endeavors to advance technologies for better assaying nanoparticles should be invested. Studies of *in vitro* cytotoxicity and the inflammatory response to nanoparticles have adopted conventional assays developed for chemical toxins or microparticles. These reports provide little insight into how individual cells react when exposed to nanoparticles. Also, the analysis of these assay results is prone to error because cells can behave differently depending on the assays employed.

The limitations of current cytotoxicity and immune response assays for the assessment of nanoparticles can be summarized as follows. First, cells cannot be recovered after the single assay readout; thus the possibilities for time-dependent monitoring of changes in a cell's activity are limited. Second, the assays' readings are averaged over all the cells present. Therefore, a single cell's responses to the nanoparticles cannot be individually recorded from the assay. Third, nanoparticles inside a cell may interfere with the fluorescence signal produced by the dye used in the assay. Additionally, nanoparticles may interact with and/or bind to dyes, altering their absorption and/or fluorescence. Nanoparticles can also adsorb to proteins and other biomolecules in the cell culture medium, which can interfere with the particles' normal interactions with cells. Furthermore, nanoparticles can bind to cytokines released from the cells; this may artificially reduce an assay's positive signal. Flow cytometry is a commonly used method in biological response assays, but the technique requires that cells be detached from the cell culture plate, which may alter the cells' mortality. Finally, multiplexed analyses of nanoparticles in the same well with single cells have not been performed. Because of these limitations, there is an emergent need to develop a solid assay that overcomes the above-mentioned problems with conventional assays and is able to evaluate biological responses to nanoparticles in a multiplexed, high-throughput manner.

Cutting-edge single-cell assay techniques have been developed for assessing cytotoxic and inflammatory responses to nanoparticles in a multiplexed manner. The multiplexed analysis strategy will be used in safety studies of various nanoparticles. Time-dependent analysis of a single cell's responses to nanoparticles may elucidate the mechanism of toxicity for nano-sized particles. Such single-cell analyses will be used in concert with conventional bulk assays. The approaches discussed will benefit nanotoxicological studies and help the broader nanotechnology community by providing proof of concept for an efficient analytical tool with which to investigate the safety of nanoparticles at the single-cell level in a high-throughput and multiplexed fashion.

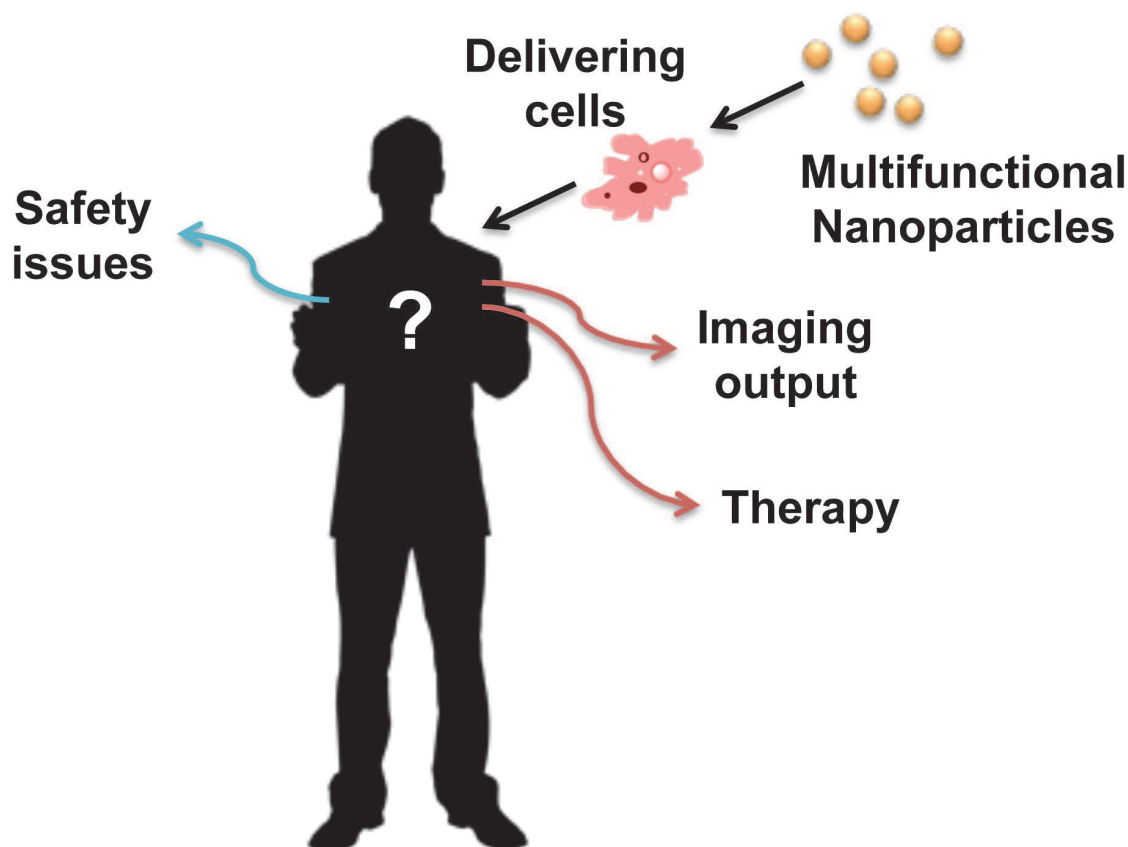


Fig. 2. Safety concerns about nanoparticles in *in vivo* applications grows and it is still a “black box” that has not been clearly shown its potential hazards.

4. Conclusion

The toxicity of nanoparticle is critically important topic for researchers both in material science and biomedical fields. Toxicity assessment so far has been informative but it could not catch up the development of technology especially in biological application of nanoparticles as covered in earlier sections in this chapter. Even in *in vitro* assays, assay results were often challenged by their inconsistencies. For *in vivo* application it is even more important to have well defined, consistent assay protocol and techniques so that one can try to discover the key to the unknown, “black box” of particle toxicity *in vivo* (Figure 2). The immediate need in this regard will be the standardization of assessment protocols for nanoparticle toxicity. Government, academics and worldwide cooperation are desirable to facilitate this process for standardization of assays. *In vitro* findings should be carefully integrated to the *in vivo* behavior of nanoparticles since it is fairly different environment that nanoparticles will experience. For *in vivo* applications, therefore, extra care should be taken in prediction of potential toxicity of nanoparticles before their actual implementation.

5. Acknowledgment

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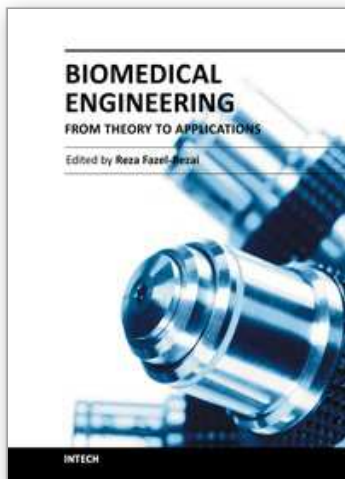
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In all different areas in biomedical engineering, the ultimate objectives in research and education are to improve the quality life, reduce the impact of disease on the everyday life of individuals, and provide an appropriate infrastructure to promote and enhance the interaction of biomedical engineering researchers. This book is prepared in two volumes to introduce a recent advances in different areas of biomedical engineering such as biomaterials, cellular engineering, biomedical devices, nanotechnology, and biomechanics. It is hoped that both of the volumes will bring more awareness about the biomedical engineering field and help in completing or establishing new research areas in biomedical engineering.

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