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Experimental considerations on the cytotoxicity of nanoparticles

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Abstract

Engineered nanoparticles are one of the leading nanomaterials currently under investigation due to their applicability in various fields, including drug and gene delivery, biosensors, cancer treatment and diagnostic tools. Moreover, the number of commercial products containing nanoparticles released on the market is rapidly increasing. Nanoparticles are already widely distributed in air, cosmetics, medicines and even in food. Therefore, the unintended adverse effect of nanoparticle exposure is a growing concern both academically and socially. In this context, the toxicity of nanoparticles has been extensively studied; however, several challenges are encountered due to the lack of standardized protocols. In order to improve the experimental conditions of nanoparticle toxicity studies, serious consideration is critical to obtain reliable and realistic data. The cell type must be selected considering the introduction route and target organ of the nanoparticle. In addition, the nanoparticle dose must reflect the realistic concentration of nanoparticles and must be loaded as a well-dispersed form to observe the accurate size- and shape-dependent effect. In deciding the cytotoxicity assay method, it is important to choose the appropriate method that could measure the toxicity of interest without the false-negative or -positive misinterpretation of the toxicity result.

Keywords

agglomeration; cell type; cytotoxicity assay; dose; nanoparticle; toxicity

With the rapidly growing interest in nanoparticle research, the toxicity of nanoparticles is becoming an increasingly important issue in nanotechnology. Based on an ISI Web of Science search, it can be seen that the number of publications concerning nanoparticle toxicity has grown exponentially over the years, with 2735 papers being published on the subject since 2000 (Figure 1). The reason for the growing concern for nanoparticle toxicity is the rise in both the number and types of nanoparticles being encountered today. Engineered nanoparticles are one of the leading materials under investigation in the various fields of nanotechnology. The use of nanoparticles for biomedical applications, such as drug and gene delivery, biosensors, cancer treatment and diagnostic tools, has been extensively

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studied throughout the past decade [1–11]. The commercialization of nanoparticles for nanomedicine is also in progress with a significant growth rate. The National Science Foundation has proposed that market size for pharmaceutical nanoproducts will reach approximately US\$180 billion per year between 2010 and 2015 [12]. In addition, nanoparticles are already broadly distributed around us in ambient air and in hundreds of nanoparticle-containing products on the market, including cosmetic products, printer toners, varnishes, drug and even food products. However, little is known about the health risks and toxicity of these nanomaterials.

Due to the increased production and use of nanoparticles in various fields, the unintended adverse effect of nanoparticles is an emerging and growing concern both academically [13–15] and socially [16]. Several studies have investigated the toxicity of nanoparticles based on various characteristics, such as shape, size, surface chemistry, chemical composition, surface activity and solubility [17–21]. Recommendations for the use of nanoparticles in various fields has emerged as a result of these initial toxicity studies. For example, the Royal Society recommended the conditional use of nanoparticles in cosmetics upon a favorable assessment by the European Commission safety advisory committee after identifying that nanoparticles are capable of penetrating the skin [22]. However, a more rigorous evaluation of toxicity is needed in order to confidently regulate the safe use of all forms of nanoparticles.

Despite the growing interest in nanoparticles and their effect on the body, standardized procedures have not yet been outlined for the evaluation of nanoparticle toxicity [23–25]. *In vivo* toxicity studies of nanoparticles are more desirable since direct verification of the effect of nanoparticles toward the human body is achieved. To date, however, nanoparticle toxicity studies have mainly focused on *in vitro* examinations due to the ease in execution, control and interpretation of the experiments compared with *in vivo* tests. By carefully considering the experimental conditions, *in vitro* toxicity studies with well-defined nanoparticles can mimic the *in vivo* system and function as a simple method to investigate the toxic effect of such materials.

Therefore, more consideration must be placed on improving the experimental procedures of nanoparticle toxicity assays in order to gain reliable data. Several critical issues should be addressed when designing an experiment to study the cytotoxicity of nanoparticles. What cell type should be chosen? What would be the most suitable cytotoxicity assay method to identify the toxic effect of the nanoparticle? What would be the proper dose to test the practical toxic effect of nanoparticles? How would the properties of the nanoparticle be influenced when placed in a biological environment? Several papers have investigated these issues in an attempt to optimize the experimental conditions of toxicity studies [26–36]. This article will review the most significant factors that affect cytotoxicity assays and the methods used to address them, especially for the nanoparticles that people are extensively exposed to through various routes, such as intravenous administration, respiratory tract, dermal exposure and ingestion. In general, cell types should be selected based on the expected introduction route and target organ of nanoparticles in the body. In deciding the nanoparticle dose for a toxicity test, it is important to estimate the anticipated concentration of nanoparticles to which someone would be exposed. Choosing the appropriate cytotoxicity assay method is also an important consideration, since some may interfere with the actual toxic effect produced by the nanoparticle [26–29]. The alteration of nanoparticle properties is also a notable issue since nonspecific protein adsorption and aggregation of particles can change the surface charge, surface composition and the size compared with the initially prepared particle when placed in the biological media [30–36]. In order to perform more reliable experiments and obtain undistorted results, a thorough understanding and ability to

track the transport of the nanoparticle in a biological system is essential. We will also present the future outlook for the study of nanoparticle toxicity.

Choice of cell types

The toxic effect of nanoparticles is highly dependent on the organs, and more specifically the type of cell, encountered. This is due to the variation in cell physiology (e.g., epithelial or lymphoid), proliferation state (tumoral or resting cells), membrane characteristics and phagocyte characteristics among different cell types [37]. Cancer cells, for instance, are more resilient towards nanoparticle toxicity than normal cells due to an increased rate of proliferation and metabolic activity [21,38]. The difference in toxic effects is even observed for nanoparticles of the same material. Therefore, selection of the appropriate cell type based on target introduction methods of nanomaterials is an important factor in cytotoxicity assays.

In order to identify the exact effect of nanoparticles on the organs or cells of interest, the cytotoxicity assay must include cells that represent the exposure route and organs targeted by nanoparticles. Given that nanoparticles will encounter different cell types depending on the exposure route and cell types vary in their response to nanoparticles, different levels of toxicity can be observed with different exposure routes. For example, fullerene, a carbon-based nanostructure, showed minimal dermal and oral toxicity in animal studies, but displayed notable toxicity upon intravenous administration [39–41].

Nanoparticles can be introduced into the human body through various routes. The respiratory tract is the most probable entry portal of nanoparticles for the wide variety of airborne nanoparticles found in work places and air pollutants, and cardiovascular or extrapulmonary organs are greatly affected by the inhalation/instillation of nanoparticles [15,42–45]. In the case of biomedical applications, nanoparticles are often introduced into the human body through the intravenous, subcutaneous, intramuscular or intraocular pathway. Dermal exposure and ingestion are also significant exposure routes since the occurrence of nanoparticles in various commercial products, such as cosmetics and foods, has become increasingly more common [46]. Based on the variety of affected organs, numerous cell types ranging from endothelium, blood, spleen, liver, nervous system, heart and kidney are all of interest in nanoparticle cytotoxicity studies [47–51].

In addition to understanding the effect of nanoparticles on specific cell types, it is essential to identify the systemic responses they elicit. Although nanoparticles accumulate in certain areas of the body based on their composition and exposure route, they can also be distributed throughout the body. The delocalization of nanoparticles can lead to adverse effects, such as oxidative stress, inflammation and immune response [52–55]. For example, macrophages play a key role in the cellular response to foreign material, such as nanoparticles. Macrophages are a type of white blood cell that act as scavengers designed to identify and remove foreign material from the body. They are also one of the main cells responsible for regulating the development of inflammation. When a macrophage identifies a foreign body it secretes signaling molecules to attract more cells to defend against the foreign material. Thus, it is necessary to study the response of macrophages and other defense cells toward nanoparticles in order to understand the systemic effect that may result.

An understanding of the immune system response to nanoparticles is also significant for the advancement of nanomedicine developments. Nanoparticles are used as drug-delivery vehicles to improve the solubility and transport of drugs in the body. They are also used to limit the immunotoxicity of drugs by storing them in an enclosed vesicle until the target site is reached. By understanding the factors that activate an immune response, a nanoparticle capable of evading the immune system can be developed, which will result in a more effective nanoparticle drug-delivery system.

Due to the wide variety of affected cells and elicited responses by nanoparticle exposure, cytotoxicity assays should extend beyond single cell study to the coculture of multiple cell types. The combination of various cells, such as macrophages, epithelial and endothelial cells, in a single assay will more closely mimic the *in vivo* situation, since numerous types of cells of *in vivo* systems react systematically and simultaneously toward the nanoparticle [56,57].

Selecting cell types that reflect the introduction route of nanoparticles and the affected site of *in vivo* system is important. For airborne particles, they are often introduced through the respiratory tract, and the pneumocyte or alveolar macrophages must be used for the toxicity study. Nanoparticles used in the cosmetics are introduced by the dermal contact and, therefore, various dermal cells should be selected to observe the toxicity of these particles. In addition to choosing the appropriate cell type for a cytotoxicity study, it is also necessary to emphasize the importance of optimizing the numbers and conditions of cells. Cells with the different culture status show different rates of proliferation, cellular concentration and proportion of death. All of these factors may greatly alter the susceptibility toward the nanoparticle and ultimately obscure the assay results.

Characterization of nanoparticle & sample preparation

In addition to the type of cell exposed to the nanoparticles, the nanoparticle properties, such as size and shape, also play a major role in cytotoxicity. Numerous studies on the size- and shape-dependent toxicity of nanoparticles have demonstrated the biokinetic and biological impacts of these properties [13,17,18,21,58–61]. These parameters have been shown to alter cellular uptake, protein adsorption, accumulation in organelles and distribution throughout the body. One reason for these effects is the correlation between particle size and surface area. The surface area of a particle increases as the size of the particle decreases and the ratio of surface to total atoms or molecules increases exponentially as the particle size decreases. The number of molecules expressed on the particle surface also increases as the surface area increases. For example, when the particle size decreases from 30 to 3 nm, the number of surface molecules expressed increases from 10 to 50% [15]. Since it is the surface atoms and molecules that play a significant role in determining the bulk properties of the nanoparticle, most of the previous toxicity studies have demonstrated an inverse relationship between particle size and toxicity. For various nanoparticles ranging from quantum dots to silica nanotubes that our group has studied extensively, the toxicity increases as the particle size decreases [13,21,59].

However, the agglomeration of nanoparticles can randomly and dramatically change the original size and shape of the particles. Agglomeration is the assemblage of particles that are attached by contact at their corners and edges. Nanoparticles tend to easily agglomerate due to their high surface energy but can easily be redispersed by an ultra sonication or vortexing method when loosely bound. However, strongly bound particle agglomerates are difficult to restore to their original, well-separated form. This type of uncontrolled agglomeration alters the size and shape of nanoparticles, which greatly influences the cell–particle interactions. This presents an obstacle when preparing the nanoparticle suspension for a cytotoxicity study since it significantly hinders the study of the actual effect of particle size and shape on toxicity [18,37,62–64]. The problem is further challenged by the culture medium for *in vitro* studies, which induces the agglomeration of nanoparticles due to its fixed pH, ionic strength and presence of proteins. Iron/graphite, magnetite/silica, bare silica and magnetite/faujasite zeolite particles were all highly agglomerated in the cell culture medium of 10 wt% fetal calf serum [37]. In addition to agglomeration, the uncontrolled adsorption of proteins can widely alter the cell–particle interaction and eventually prevent the nanoparticles from reaching the target site [65–68]. Therefore, maintaining the original size, shape and surface composition

of the particle with a well-suspended state is crucial for obtaining reliable data on size- and shape-dependent toxicity of the nanoparticles.

Recently, surface modification of nanoparticles has been used to limit agglomeration of nanoparticles and nonspecific adsorption of proteins. Surface coatings with polyethylene glycol (PEG) or polyelectrolytes prevents the nonspecific adsorption of protein and helps maintain a well-separated nanoparticle dispersion [65]. Moreover, the PEGylated particles showed improved nanoparticle–blood compatibility and suppressed the disruption of red blood cells and platelet aggregation and coagulation [37]. The formation of electrostatically induced repulsive capping through surface modification is also thought to be an efficient method of attaining a well-dispersed nanoparticle suspension. In the case of fullerene, specific surface treatments are required to disperse the fullerenes in suspensions for *in vitro* and *in vivo* toxicity studies [69].

Several efforts have focused on achieving a well-dispersed nanoparticle suspension through the addition of surfactants or additives [70–74]. For example, rapid cellular uptake by C3A hepatocyte cell and primary rat hepatocytes was observed with polystyrene nanoparticles dispersed in cell media containing 10% fetal calf serum, while cellular uptake did not occur with particles dispersed in serum-free medium [75]. Purification through dialysis was also used as a convenient method to avoid aggregation. This resulted in improved ability to form well-dispersed suspensions with the various synthesized and commercial silica nanoparticles [76].

Although these methods have proven to successfully limit agglomeration of nanoparticles and adsorption of biomolecules, it is important to note that their use may impede the identification of the toxic effect of the nanoparticle itself. Surface-bound molecules and additives that adsorb to the particle alter the surface properties of it and can greatly influence the cell–nanoparticle interaction. In addition, the additive may interact with the cell and give rise to unintended toxic effects, which are unrelated to the nanoparticle. Thorough preliminary studies on the conditions that cause the agglomeration and the adsorption of molecules, as well as the effect of preventative measures, are essential in order to improve future nanoparticle toxicity studies.

The actual size and shape of any agglomerations formed should also be extensively investigated to clearly identify the true size and shape effect on nanoparticle toxicity. Two common methods of investigating these properties are dynamic light scattering (DLS) and electron microscopy. However, DLS is incompatible with biological media due to the presence of various light scattering components. Electron microscopy methods also suffer from some limits, since the electron microscopy analysis, such as scanning electron microscope (SEM) and transmission electron microscopy (TEM), are operated under the vacuum condition, which alters the particle size from its hydrodynamic size. In order to overcome this limitation, the cryogenic-temperature electron microscopy (cryo-EM) is often used to image the samples in aqueous system. The cryo-EM uses the cryo-fixation to rapidly freeze the sample so as not to destroy its aqueous environment. Therefore, the cryo-EM could be a useful tool to acquire high-resolution direct images of nanoparticles and cells in their undisturbed aqueous state. In this context, there is an effort to measure the true size and aggregation of particles using fluorescence single particle tracking techniques [77].

Another significant factor to consider during sample preparation is the presence of trace impurities within the nanomaterial formulation that may lead to additional toxic effects. A recent cytotoxicity study with gold nanorods revealed that free cetyltrimethylammonium bromide (CTAB) in solution caused toxic effects in human colon carcinoma cells [78]. Similarly, unpurified carbon nanotubes, iron oxide and gold nanoparticles induced an

inflammatory response resulting in cytokine secretion [79], while purified nanoparticles did not induce inflammatory responses [80–82]. Therefore, thorough purification of impurities from the nanoparticle suspension is required prior to any cytotoxicity assay.

Recently, various kinds of surface coatings have been invented for biomedical nanoparticles, which improve their biocompatibility and allow for successful transport to the target site. In addition to these abilities, surface coatings that are capable of maintaining the original, well-dispersed form of nanoparticles are also necessary. Toxicity tests of these surface-modified nanoparticles must be performed as well, since the surface coatings could greatly affect the biological interaction and toxicity of nanoparticles. For example, we have synthesized silica nanotubes and investigated their toxicity. The cytotoxicity of silica nanotubes was tested using nanotubes with bare and aminofunctionalized outer surfaces. At the same concentration, the aminofunctionalized nanotubes demonstrated increased toxicity due to the increased affinity of the positively charged nanotube surface for the negatively charged cell surface [21]. Modifications to the inner surface of nanotubes and other hollow nanoparticles may affect the toxic effects observed. Iron oxide is often incorporated into the pores of hollow nanoparticles to generate a magnetic nanoparticle for biomedical applications [6–8]. Moreover, toxic impurities that may be present after the formation of the nanoparticles and the coating of the nanoparticle surface must be finely purified before the toxicity test is performed in order to exclude the effect of impurities. Similarly, the toxicity of the surfactants added to improve the dispersion of nanoparticles must also be carefully tested in advance.

Right standard for dose

Nanoparticle toxicity has been shown to function in a concentration-dependent manner [13,21]. As a result, determination of an appropriate nanoparticle dose to utilize in a cytotoxicity assay is key to understanding the toxic effects of the nanoparticles under real world conditions. The nanoparticle dosage employed should be based on how and in what quantity people are exposed to the nanoparticles. Examining the effects of a realistic dose of nanoparticles, limits the probability of observing artificial toxicity induced by an unrealistically high dose and allows for the observation of the actual toxic effects of the nanoparticles. The concentration of particles introduced into the cell cultures of cytotoxicity assays widely varies depending on the parameter used to determine the dose of nanoparticles. In most studies, the dose has been expressed as mass per unit volume (e.g., $\mu\text{g}/\text{ml}$). However, in contrast to soluble chemicals, the nanoparticles have a tendency to diffuse, settle and agglomerate in dispersion media. The extent of these processes depends on the nanoparticle size, shape, charge and density, as well as the viscosity and density of solution [25]. These properties influence the transportation rate of nanoparticles to adherent cells on the culture plate and therefore affect the effective dose within cells (Figure 2) [25]. As a result, defining the dose for *in vitro* study is rather complicated.

Considering the gravitational settling and expressing the dose in terms of mass per unit surface area of the culture dish ($\mu\text{g}/\text{cm}^2$) may alleviate some of the discrepancies among doses. Moreover, the impact of cell density on cell behavior should be considered as it affects the actual dose of particles that reach each cell. The dose could be expressed in terms of mass per cell numbers ($\mu\text{g}/10^6$ cells) to account for this effect. In addition to stating the mass of nanoparticles administered to the cell media, the number of nanoparticles administered is also important to note during the cytotoxicity assay. Thus, in our previous study on the cytotoxicity of silica nanotubes, the mass per unit volume concentration was converted to the number of particles and reported in the article. At equivalent concentrations the number of 200 nm nanotubes was approximately 2.5-times higher than that of 500 nm nanotubes. We hypothesized that the increased number of 200 nm nanotubes, along with the

smaller size, contributed to the increased toxicity observed [21]. Indicating the dose in numerous forms concerning the property of the nanoparticle may be helpful for the quantitative estimation of particle–cell interaction and may improve the comparability among cytotoxicity assays.

Although the metrics mentioned above allow for a better description of the administered nanoparticle concentration, they can only represent the nominal media concentration. None of these methods can accurately reflect the concentration of nanoparticles directly delivered to the cells. For the precise identification of cellular dose rather than exposure, a systematic consideration of dose–response assessment is required. The transport of nanoparticles to cells is influenced by factors such as the diffusion rate, gravitational settling and agglomeration of the particle. These factors are determined by the size, shape and surface charge of the particle and the density and viscosity of the media. Numerous analytical tools, including microscopy [83], mass spectrometry (MS), inductively coupled plasma MS (ICP-MS) [17], liquid chromatography MS (LC–MS) and radioactive isotope [84], are used to estimate the cellular dose of nanoparticles. In addition to estimating the *in vivo* dose of particles, these computational dosimetry methods have also been proposed as a promising tool to describe the kinetics of nanoparticles in the biological systems [85,86].

In order to estimate the cellular dose of nanoparticles more accurately, the concerted tools of the above mentioned methods are thought to be helpful. For example, the MS could quantitatively measure the dose and the microscopic method would qualitatively visualize the translocation of nanoparticles throughout the cell organelles. The absence of agreed definition of dose with respect to mass, volume, number and surface area also hinders the ability to achieve the objective analysis and comparison of toxicity between various nanoparticles. To overcome this obstacle, stating the dose of nanoparticle with at least two metrics would be beneficial in generating a more standardized concept of dose. Especially, indicating the parameter of dose both in mass per unit volume ($\mu\text{g/ml}$) and number of particle per unit volume (number of particle/ml) would be helpful to have a sense on the density of particle and quantity of cell–particle interactions.

Choice of cytotoxicity assay

Selection of the appropriate cytotoxicity assay is vital to the accurate assessment of nanoparticle toxicity. Various assays can be used to study the toxic effects of nanoparticles on cell cultures, including lactate dehydrogenase (LDH) leakage, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and identification of cytokine/chemokine production. In choosing the correct assay, all potential interferences must be considered to avoid obtaining false-positive and false-negative results. Interactions between the nanoparticles and the chosen dye have been cited as a major potential interference leading to inaccurate results. In a cytotoxicity evaluation of single-walled carbon nanotubes (SWCNTs), the SWCNTs attached to the MTT-formazan crystals that were formed after the reduction of MTT and stabilized their chemical structure so that the crystals were not soluble in the solvents used to dissolve the MTT-formazan, such as 2-propanol, hydrochloric acid, sodium dodecylsulfate or acetone. As a result, reduced cell viability was observed in the MTT test [26]. In other studies, the WST-1 did not form crystals with SWCNTs as in the case of MTT, but the absorbance was increased when the concentration of SWCNTs was increased demonstrating the interaction between SWCNTs and dye used for WST-1 [87]. The disturbance of toxicity assays with metallic nanoparticles has also been studied by several groups. For gold nanoparticle-treated cells, the dead cells were imaged with the commonly used fluorescent propidium iodide (PI). Normally, the fluorescent PI molecules cannot penetrate the cell membrane. However, in the experiment, the PI entered the cell during the endocytosis of the nanospheres and resulted in a false-positive toxicity result

[88]. Aluminum nanoparticles showed a strong interaction with the MTT dye causing significant misreading of the cell viability data; however, no interference was observed in the CellTiter 96 Aqueous One and almar blue assays [89]. In the case of silver nanoparticles, the MTT, CellTiter 96 Aqueous One and almar blue assays were simultaneously tested. It was confirmed that the almar blue method was the most suitable method because it was the only method that showed no interaction between the nanoparticles and assay medium [90]. Moreover, several copper-containing compounds, including CuCl_2 , CuSO_4 and Cu powder, have been shown to interact with LDH [91]. When the copper compounds were incubated with LDH, the inhibition of LDH calibrator detection was observed depending on the dose of Cu salts.

In addition to the potential interferences mentioned above, light absorption and scattering by the nanoparticles can lead to inaccuracies in the data since many nanoparticles absorb in the UV-Vis range. For example, absorbance at 525 nm, the same wavelength used in MTT assays, has been observed for some nanoparticles (iron/graphite magnetic particles, super-paramagnetic magnetite/silica nanoparticles, bare and PEGylated silica nanoparticles and magnetic composites magnetite/FAU zeolite) in culture medium in the absence of cells. This absorbance increases with the nanoparticle concentration and can greatly interfere with MTT assay results [37]. Therefore, extra care for complete removal of nanoparticles is needed for similar types of toxicity assays. In another case, fluorescence quenching was observed with carbon black, resulting in a decrease in fluorescence as the concentration of carbon black increased [92].

As can be seen from the above examples, determining potential sources of interference is an important issue in the preliminary study of nanotoxicology. Variability in the data among different assays was found to be the result of interferences such as nanoparticle–dye interactions and absorption by the nanoparticle. An in-depth evaluation of all potential interferences, including the absorbance range of the nanoparticle and expected interactions with assay components, should be completed before selecting a cytotoxicity assay. The occurrence of false-positive and false-negative results highlights the importance of cross-checking the data with alternative assays to ensure reliability of the results. For instance, the toxicity of the same SWCNTs measured with WST-1 and MTT showed different results [26], and by cross-checking the data, the interference of nanoparticles in specific assay methods was revealed. Therefore, to avoid misinterpreting the results, cytotoxicity data should be verified with at least two or more independent tests.

There have been several attempts to eliminate the interference of the nanoparticles during the cytotoxicity assays. In some cases, the absorbance of the cell culture media and the nanoparticle-dispersed cell culture media was previously measured and the effect of both were subtracted from the absorbance data attained from particle-treated cells [93]. Moreover, to remove the effect of cells and particles during the MTT assay, the mixture of particle, cell and detecting dye dispersed in cell culture media was centrifuged and the absorbance was measured with the separated supernatant of the mixture [91]. These additional treatments are clearly helpful but may not be perfect in some cases due to residual particles.

In addition to interferences, the type of toxic effects being studied is also an important factor to consider when selecting an appropriate cytotoxicity assay. *In vitro* toxicity assays often measure the extent of cell death caused by the nanoparticles. However, in some cases, sub-lethal cellular changes may occur, which alter certain functions of the cell but do not result in cell death. These less obvious toxic effects must be carefully characterized by genomic and proteomic array tests. Other forms of cell damage, such as membrane damage, metabolic abnormality and inflammatory responses, can be characterized with the LDH

leakage, MTT assay and cytokine/chemokine measurements. There are several papers finely describing the various assay methods classified by the type of nanoparticles and by the kind of toxic effect that we are interested in. However, it is difficult to comment about the false-positive or -negative effect that occurs due to the unintended interferences of nanoparticles and how we could handle this problem [13,23,94–96]. As suggested previously, the cytotoxicity assessment with at least two or more independent tests would help to avoid errors. In addition, the false-positive or -negative effect occurring due to the interference of nanoparticles while measuring the luminescence must be subtracted from the resulting data. The selection of cytotoxicity assays that can identify the diverse range of potential particle-induced responses is critical in fully understanding the toxic effect of nanoparticles. Moreover, the careful and thorough characterization of particle properties and the intensive validation of assay methods are required prior to the toxicity assessment. Internationally agreed upon protocols for the toxicity tests are necessary to attain more standardized data on the wide variety of nanoparticles.

Conclusion

Based on the analysis of the literature, it is clear that the experimental conditions of a cytotoxicity assay play a significant role in the outcome of the results. Therefore, a thorough investigation into all of the potential factors and interferences involved in the assay is mandatory. The determination of parameters, such as cell type and nanoparticle concentration, requires an extensive understanding of the anticipated exposure to the nanoparticle, as well as its distribution throughout the body. Selection of the appropriate method to study nanoparticle toxicity involves a preliminary investigation into the interactions between the nanoparticles and assay components. More than one method may be required to fully evaluate the toxic effects of the nanoparticle due to many sublethal cellular changes that may occur. In addition, all toxicity studies require the verification of alternative assays to confirm the accuracy of the data. These issues mentioned above are important while planning the toxicity test of nanoparticles and the experimental facts that the researchers should consider in each step of the experiment are suggested as a flow chart in Figure 3.

Future perspective

In order to identify the toxic effect of nanoparticles more clearly, more advanced characterization methods that can identify the nanoparticle properties in the biological system are required. In the case of microscopy methods, TEM and SEM only show the static condition of nanoparticles within the cell and the resolution limit of confocal microscopy prevents the observation of particles smaller than the diffraction limit. However, recent advances in optical microscopy have improved the imaging resolution to approximately 20 m. It is anticipated that further progress in imaging technology will allow for a more clear and direct study of endocytosis of nanoparticles in real time. Moreover, a greater variety of techniques, such as ICP-MS or computational methods, are expected to be employed to attain more precise information on the nanoparticle and its toxicity. In addition to these advances in the characterization methods, standardized protocols for the toxicity assessment are required to achieve more objective data on the toxicity of nanoparticles. Recently, in response to this requirement, there are several attempts on defining the standard protocols for characterization and toxicity assessment of nanomaterials. The technical committee 229 of the International Organization for Standardization (ISO) is devoted to developing the harmonized standards related with the nanotechnologies, and the Committee E56 of the American Society for Testing and Materials (ASTM) is also putting efforts on forming the standard for nanotechnology and nanomaterial. There are tremendous nanoparticles with various shapes and materials and the requirements on the standard protocols for

characterization and toxicity assessment for these particles are still demanding. More reliable and in-depth toxicity data will enable the development of regulations on the safe use of nanoparticles in extensive applications throughout a person's everyday life.

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▪ of interest

▪▪ of considerable interest

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Executive summary

- Due to the immensity of nanoparticle type, assay method and cell lines used for cytotoxicity test, careful considerations are necessary to attain more consistent and reliable data on toxicity of nanoparticles.
- Cell types used for the toxicity test must reflect the introduction path of nanoparticles and comprehensive reactions of body, including immune response and inflammation induced by the nanoparticles.
- Careful sample preparation methods that could prevent nanoparticle aggregation and the adsorption of proteins in the biological media are required to study the accurate effect of interested nanoparticles.
- The toxicity assay method must be selected carefully concerning the false-negative or -positive effect that is induced by the interference of nanoparticles, and efforts to subtract these effects need to be preceded.

Figure 1.
Articles published on the toxicity of nanoparticles.

Figure 2. Defining dose for nanoparticles *in vitro*

(A) Dose for nanoparticles *in vitro* can be defined differently since the particle–cell interaction changes as the particle moves from the cell culture media to cells. (B) Fluorescence images of an alveolar epithelial cell grown in culture and exposed to fluorescence-tagged 500 nm amorphous silica particles, demonstrate the principles shown in (A). (B1) A single green particle on the cell surface represents the delivered dose (arrow). (B2) As the focal plane moves to the cell interior, the particle is no longer visible but (B3) internalized particles, representing the cellular dose, are observed.

SA: Surface area.

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LDH: Lactate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.