Review

Methods for Assessing Basic Particle Properties and Cytotoxicity of Engineered Nanoparticles

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Abstract: The increasing penetration of materials and products containing engineered nanoparticles (ENPs) to the market is posing many concerns regarding their environmental impacts. To assess these impacts, there is an urgent need of techniques for determining the health-related properties of ENPs and standards for assessing their toxicity. Although a wide number of systems for characterizing nanoparticles in different media (*i.e.*, gases and liquids) is already commercially available, the development of protocols for determining the cytotoxicity of ENPs is still at an infant stage, drawing upon existing knowledge from general toxicology. In this regard, differences in the preparation of ENP-containing solutions for cytotoxicity testing, as well as in the steps involved in the tests can result in significant deviations and inconsistencies between studies. In an attempt to highlight the urgent need for assessing the environmental impacts of nanotechnology, this article provides a brief overview of the existing methods for determining health-related properties of ENPs and their cytotoxicity.

Keywords: nanotechnology; engineered nanoparticles; cytotoxicity; human exposure

1. Introduction

Advances in nanotechnology over the past few decades have offered novel materials that would not have been possible using conventional methods. These materials have applications in a number of sectors including structural engineering, electronics, optics, consumer products, energy production and storage, soil and water remediation, and medicine [1]. The enhanced performance of these nanotech products is the result of the unique properties of their building blocks, namely the engineered nanoparticles (ENPs). These are man-made nanoparticles (*i.e.*, particles with at least one of their dimensions smaller than 100 nm), having properties that can differ markedly from those of their large-particle counterparts and of bulk materials [2].

Penetration to the market currently counts more than 1300 consumer products that employ ENPs in one way or another [3], whereas global investment in nanotechnology research and development by public and private sectors has reached few tens of millions of euros annually. Albeit the rapid growth of nanotechnology, assessment of the potential environmental and health risks is lagging behind [4]. In fact, predicting the risks of nanotechnology is difficult as ENPs can undergo diverse physicochemical transformations that change their properties, their fate, and their impacts once released into the environment. In addition, there is increasing concern that human exposure to some types of ENPs, inadvertently or intentionally, can lead to significant adverse health effects [5].

Human exposure to ENPs can occur in different stages during the life cycle of the nanoparticle-based products, *i.e.*, from synthesizing nanoparticle building blocks and fabricating nanomaterials, to using and disposing them. The stage with the highest risk for human exposure is during the synthesis of ENPs and the fabrication of ENP-containing products. Occupational exposure during production of ENPs can occur via inhalation (particularly important for powder preparations of ENPs), dermal exposure or ingestion by swallowing [6].

Details of the industrial processes for synthesizing ENPs, the quantities in which they are produced, as well as how they are handled and used remains largely uncontrolled. In addition, public awareness and governmental involvement in overseeing the developments of nanotechnology industry is not keeping up with the rapid pace of development and commercialization of nanoparticle-based products [5]. As a result, leading insurance companies have characterised nanotechnology as one of the highest emerging risks, indicating that a continued lack of data could increase the costs of nanotech companies [7].

To assess the risks involved in producing, using, and disposing nanomaterials and nanoparticle-containing products we need to fully understand their hazards (*i.e.*, their toxic effects) and quantify the levels of human exposure [8]. Although existing protocols and state-of-the-art techniques in toxicology can be adopted for ENPs, the complex behavior of colloidal systems poses great challenges [9]. As a result, to assess the hazards associated with ENP exposure it is very important to establish standard testing procedures where the conditions are well controlled.

Assessing human exposure to ENPs requires measuring their size and concentration in different media. To measure ENPs suspended in the air one can employ standard techniques for determining particulate matter pollution [10]. Advancements in this field over the last few decades have provided a wide number of instruments of high precision and accuracy. Techniques for measuring nanoparticles in liquid media have been developed mainly to determine the properties of ENPs in view of their

technological applications. However, recent advances in this field aim to expand the applicability of these methods for environmental studies [11].

In this paper we provide a brief overview of the available cytotoxicity assessment methods that have been applied for different types of nanoparticles and discuss state-of-the-art techniques for determining the health-related properties of ENPs in the environment, whilst examining the different human exposure scenarios.

2. Toxicity Assessment Methods for ENPs

2.1. Nanoparticle Synthesis and Dispersion Methods

Published results on nanoparticle cytotoxicity are quite often inconsistent and conflicting [12]. The most important reason of these inconsistencies is differences of the nanoparticle samples (which can be attributed to the different methods used to prepare them) employed in each study. These differences can be traced back to the way that the nanoparticles are synthesized, the way those are dispersed in the solute or the cell suspension, as well as the agents used in the suspension for stabilization.

Nanoparticles of well-defined size and chemical composition can be synthesized by a variety of methods [13]. These can be classified as *wet-chemistry* or *aerosol-based* methods depending on whether the synthesis occurs in a liquid or in a gas medium, respectively. Wet-chemistry methods involve the mixing of precursor compounds to form ionic solutions, which under certain conditions initiate nucleation of the ions [14]. A number of physical and chemical parameters of these solutions can be adjusted to control the size of the resulting particles, which can then be collected by evaporating the solute. Aerosol-based methods on the other hand involve evaporating bulk materials and subsequently cooling the vapors to form clusters and nanoparticles by nucleation and condensational growth [15]. ENPs generated in this way can be collected by filtration, impaction or electrostatic precipitation. In either case, the resulting nanoparticles are collected in the form of powders, which can then be employed in a number of applications including the preparation of colloidal solutions for toxicity tests.

Once the nanoparticles are in the powder form, they need to be dispersed in the culture media for the toxicity tests [16]. Despite that the process is seemingly simple, the extent to which ENPs are dispersed in the biological suspensions can significantly influence the cytotoxicity result [17]. Sonication is often used to disperse nanoparticles and reduce their agglomeration in liquid solutions [18]. Stabilizing agents can also be used to keep nanoparticles apart from one another if their concentration is high [19]. Concerns about the toxicity of these agents have required use of non-cytotoxic dispersing compounds, such as Darvan 7N, for the tests [20].

An alternative to dispersing nanopowders is to transfer them directly to the cell cultures. This can be achieved in a simpler manner when the ENPs are synthesized by aerosol-based techniques because the carrier gas can be easily replaced by the solute (*i.e.*, the cell-culture). Direct transfer of the aerosol nanoparticles into liquid solutions can be achieved by liquid impingement and bubbling [21]. In principle, these techniques can yield ENP suspensions of very high purity, which are highly desirable for toxicity tests.

2.2. Cytoxicity Assays

Cell culture assays are widely used to assess the toxicity of various compounds on cellular systems. *In vitro* methods have shown a significant potential for assessing the toxicity of both environmentally and occupationally occurring compounds [22]. These include methods for testing the integrity of the cell membrane, as measured by enzymatic activity released by damaged cells (e.g., LDH activity) and the metabolic activity of viable cells (used for example in colorimetric assays, such as the MTT and MTS assays). Bioluminescent methods (*i.e.*, methods using luciferase, which catalyzes the formation of light from adenosine triphosphate (ATP) are also very commonly used, as are cell viability assays on which the number of surviving cells is determined by measuring the uptake and accumulation of neutral red dye and Trypan blue after exposure to the toxicant.

The most widely used assays to assess nanoparticle cytotoxicity are the MTT, MTS, and the LDH. MTT (*3*-(4,5-Dimethylthiazol-2-yl*)*-2,5-Diphenyltetrazolium Bromide) is a yellow substance, which is reduced to purple formazan within the cell mitochondria, and measured by absorption spectrophotometry (between 500 and 600 nm wavelength). This reduction takes place when mitochondrial enzymes are active, and is directly related to the number of viable cells. Like most classical toxicity tests, a dose-response curve can be produced when comparing the amount of formazan causing cell death in cells treated with a toxicant, and that produced by untreated (control) cells. Although having certain limitations based on the state of cells and the mitochondrial dehydrogenase activity, MTT is a very useful method for measuring cell growth in cytotoxicity studies.

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay is a modification of the MTT assay, based on the conversion of a tetrazolium salt into a colored soluble formazan product by mitochondrial activity of viable cells. The formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in the culture. In addition, LDH (lactate dehydrogenase) leakage assay can be used to measure cell membrane integrity. This is a colorimetric assay, which quantitatively measures LDH released from damaged cells into the media, resulting in a fast and reliable method for determining cellular cytotoxicity and cytolysis.

In principle, all of the above classic cytotoxicity assays can be used to assess ENP cytotoxicity. However, not all assays may be suitable, as ENPs can interfere with assay reagents or detection systems to give false positive or negative results (e.g., in the case of the LDH assay, if ENPs only influence intracellular activities) [23,24]. When choosing a cytotoxicity method for ENP testing it is also important to consider the fact that different cell types exhibit different sensitivity to ENPs [25].

2.2.1. MTT Assay

Sayes *et al*. [26] used the MTT assay to show that nano-C_{60} is cytotoxic to HDF and HepG2 cells at 20 ppb. Exposure to carbon nanotubes (CNTs) in the human lung epithelial cell line A549 using the MTT assay over one-week *in vitro* tests showed significant cytotoxic effects, particularly at a low concentration of 0.1 mg/mL [27]. Single-walled carbon nanotubes (SWCNTs) induced toxic effects on HUVEC cells in a concentration- and time-dependent way, while COOH-SWCNTs (carboxylic acid functionalization) contributed to a higher toxicity [28]. MTT has also been used to test the cytotoxicity

of SiO_2 nanoparticles in A549 human lung cancer cells: 15-nm and 46-nm SiO_2 nanoparticles were shown to significantly reduce cell viability in a dose-dependent and time-dependent manner at 10–100 µg/mL dosage [29]. Using the MTT assay and an additional methodological step in the usual protocol on different cell lines (liver, kidney, and lung) showed that TiO_2, Al_2O_3, SiC ENPs induced a significant cell death [30]. Among ENPs, the smallest, anatase, and spherical nanoparticles induced the highest cytotoxic effects. Nano-C_{60} was found to be cytotoxic to human dermal fibroblasts, human liver carcinoma cells (HepG2), and neuronal human astrocytes at 50 ppb (LC_{50} = 2–50 ppb, depending on cell type) after 48 h exposure [31]. Nanodiamonds were not found to be cytotoxic using the MTT assay to neuroblastoma, macrophage, keratinocyte and PC-12 cells, and did not produce significant reactive oxygen species [32]. The MTT assay was also used in one of the earliest reports of fullerene toxicity [26], showing that nano-C_{60} was cytotoxic to HDF and HepG2 cells at the 20 ppb level.

2.2.2. LDH Assay

The LDH assay has most commonly used in combination with the MTT assay for a variety of ENPs, and has been used with many types of cell lines. Elevated LDH levels showed cell membrane damage after A549 human lung cancer cells were exposed to 15-nm SiO_2 nanoparticles for 48 h. LDH levels were increased by 8.7%, 12.5%, and 17.7% following exposure to 10, 50, and 100 µg/mL of SiO_2 nanoparticles, respectively [29]. Nano-C_{60} has also been shown to disrupt cellular function in the LDH assay after 30 h of exposure [26]. In these tests, HDF and HepG2 cells both exhibited signs of leaky membranes and lipid oxidation.

2.2.3. Other Assays

The A549 human lung epitheilal cell line exposed to CuO ENPs, using trypan blue staining, showed potential of cytotoxicity [33]. These particles also caused oxidative lesions. Using crystal violet assay for cell viability, Isakovic *et al.* [34] showed that nano-C_{60} is toxic to U251 human glioma cell lines. Nano-C_{60} caused rapid reactive oxygen species (ROS)-associated necrosis characterized by cell membrane damage without DNA fragmentation. The water-soluble, hydroxylated fullerene (fullerol, nano-$C_{60}(OH)_{22–26}$) has also been found to be cytotoxic to human lens epithelial cells [35].

ENPs have a large number of variables that may determine biological impact, which makes toxicity assessment time-consuming and challenging. Intrinsic particle properties such as size and morphology can have an effect on the outcome of the toxicity test, even if their chemical composition is the same. In addition, several secondary interactions of ENPs with the media (e.g., cell vision) can also affect the outcome of the tests. Cell vision for instance is the first contact point of the nanoparticle surface with the cells, and influences the amount of ENPs they can uptake, as well as their fate in the intracellular environment [36]. Since there is no standard protocol for ENP testing, comparisons between studies is often difficult and data is inconsistent. Effects on different cell lines are observed with higher ENP concentrations than those in animal models, highlighting the need for standardized ENP cytotoxicity assessment, with validated and evaluated *in vitro* methodologies. The development of reference material is necessary and will improve the comparability between *in vitro* studies.

3. Measuring Health-Related Properties of ENPs

Nanoparticles in the environment can enter the human body through the respiratory tract, the gastrointestinal tracts, or the skin [37]. To assess human exposure to ENPs during the different stages of the life cycle of nanomaterials, we therefore need to estimate the quantities the health-related properties of ENPs released in different media in the environment. Inhalation of ENPs suspended in the air is regarded as the most important route for their entrance into the human body [38]. Nanoparticles in the air can remain suspended over long periods of time, thereby spreading over long distances from the point of their release. As a result, humans can be exposed to airborne ENPs in an uncontrollable manner compared to the other routes. Nanoparticles can also be disposed to the environment in liquid solutions, making human exposure to ENPs possible through swallowing or direct contact with the skin. ENPs release to the terrestrial environment is also possible, but, in this case, human exposure is significantly less probable. The following paragraphs provide a brief overview of the techniques for measuring nanoparticles in the breathing air and in liquid solutions.

3.1. Microscopy Analysis

Microscopy analysis of ENPs is perhaps the most straightforward way for measuring their size and concentration [39]. For the analysis, the material to be investigated is applied on microgrids (typically a metallic mesh with a polymer membrane). If the particles are in a liquid medium, a droplet of the sample is placed on the substrate and allowed to evaporate. For nanoparticles in the air, sampling on the microgrids can be achieved by diffusional, thermophoretic, or electrostatic precipitation [40]. Once the sampled nanoparticles are laid on the microgrids, they can be observed by electron (transmission or scanning) or atomic force microscopy.

In Transmission Electron Microscopy (TEM), the image of the sample is constructed by determining its absorbance of a high-energy electron beam [41]. Alternatively, in high-resolution TEM (HRTEM) the image of the sample is constructed by measuring the interference of the sample with the beam. Scanning Electron Microscopy (SEM) also employs a high-energy electron beam, which is scanned over the surface of the sample. In this case, the image of the sample is constructed by measuring the back scattering of the electrons. An important aspect of SEM is that the surface of the samples has to be conductive in order to scatter electrons efficiently [42]. If the samples are not conductive, a conductive coating (typically gold) is applied by spattering. Both TEM and SEM are performed under high vacuum. Environmental TEM and SEM allows the measurements to be carried out under a relevant atmosphere [43]. This technique has proven very useful over the last years in probing physicochemical properties of nanoparticles.

Atomic Force Microscopy (AFM) is another way of measuring ENPs. AFM uses a cantilever with a very sharp tip that moves over the surface of the samples [44]. The forces between the tip and the sample surfaces induce deflections to the cantilever, which are measured by a laser detection system. Combining the measured deflections with the positioning of the cantilever over the sample offers 3D visualization of the systems. Apart from sample-gas interfaces, AFM can be used in liquid media, thereby allowing observation of the particles in environments that are more relevant to most of the

toxicity tests. However, care must be taken so that the nanoparticles adhere on the substrate (which has to be flat) and are not moved by the motion of the tip.

In all microscopy methods, a small number of particles can be viewed in every image, which may not be representative of the whole sample. This, however, can be tackled with online techniques that can be used both in gaseous and liquid media as described in the sections that follow.

3.2. Measuring ENPs in the Air

Monitoring and measuring the concentration of airborne nanoparticles can be achieved by either optical or electrical techniques. Optical techniques rely on the growth of nanoparticles by means of heterogeneous nucleation and subsequent counting of the particles by light-scattering techniques. These instruments, referred to as condensation particle counters (CPCs) [45] have found wide application in measuring airborne nanoparticles for monitoring and research purposes. Although CPCs can measure extremely low concentrations, their detection efficiency decreases markedly for particles having diameters below *ca.* 3 nm [10]. Measuring the concentration of airborne nanoparticles by electrical means, *i.e.*, by measuring the current produced by particles that have a known charge distribution, is another alternative for determining the concentration of airborne nanoparticles [46]. The advantage of this technique is that in principle it can detect particles of any size. The drawback, however, is that there is a threshold concentration of a few tens of particles per cm^3, depending on the operating conditions, below which the corresponding currents are too small to be measured.

Another important parameter for assessing the toxicity of ENPs is their size. Sizing particles in the gas phase can be achieved by a number of techniques depending on the desired range [15]. The most efficient instrument for determining the size of particles in the nanosize regime is the Differential Mobility Analyzer (DMA) [47]. DMAs classify airbone particles based on their mobility diameter, which under certain assumption (mainly the number of charges they carry and that they have spherical shapes) can be use to estimate their size. Although a wide range of DMA designs have been described in the literature [48], the most widely used is the cylindrical.

Toxicology studies indicate that the pulmonary effects induced by inhalation of ENPs are best correlated with the surface area rather than the concentration of the particles [8]. Instruments delivering signals that are more closely related to the particle surface than the mass are already available in the market. One of these, the Nanoparticle Surface Area Monitor (TSI Model 3550) estimates the surface area of sampled particles by ion attachment and measuring the electric current of charged particles in a continuous flow. Other more advanced instruments, such as the Grimm Nanocheck and the Philips Aerasense Nanotracer (also based on ion attachment and current measurement), determine the mean particle diameter and the number concentration in accordance with recent guidelines defining benchmarks or reference threshold values. To derive the surface concentration of the airborne nanoparticles, all these instruments rely on the assumption that the particles are spherical. This assumption, however, raises criticism since most airborne nanoparticles, and especially ENPs, are not spheres but rather flaky agglomerates of smaller primary particles.

The greatest challenge in monitoring ENPs in the air is to distinguish them from background/environmental nanoparticles. This task is challenging because the concentration of background nanoparticles can vary substantially from one time to another. Online distinction between

engineered and background particles can be made by probing nanoparticle properties such as size, morphology or even solubility and volatility, provided that these are detectably different among the two species. The Scanning Mobility Particle Sizer (SMPS) [49] can be employed to measure the particle size distribution. Tandem Differential Mobility Analysis (TDMA) techniques [50] can also be used to measure the intrinsic properties of particles, such as morphology, vapor uptake, and volatility [51,52]. Information on the chemical composition or the structure of the airborne nanoparticles can also be obtained by offline techniques such as electron microscopy [53]. These techniques, however, are time-consuming, expensive, and of high complexity, thereby making them inappropriate for monitoring purposes.

3.3. Measuring ENPs in the Aquatic Environment

Dynamic light scattering (DLS) is the most widely used set of techniques for measuring in situ the size and the shape of nanoparticles suspended in liquids [54]. In DLS the solution sample is illuminated by a monochromatic light source, and the light scattered by the particles is continuously measured by a photodetector. The recorded scattering fluctuations are then processed to determining the translational and rotational diffusion coefficients of the particles, which in turn are used to determine their size and shape [55]. A drawback of DLS is that the larger particles in the samples can mask the signal of the nanoparticles when performing dynamic light scattering [56]. This is because Rayleigh scattering depends on the 6th power of the particle diameter.

An alternative method for determining the size distribution of the particles in liquid suspensions is Nanoparticle Tracking Analysis (NTA). NTA also measures the scattering generated from particles undergoing Brownian motion [57]. In this case, however, the diffusion coefficients of the particles are estimated by tracking the motion of the particles for a certain amount of time. Similarly to DLS, the signal of the larger particles in NTA can mask that of the smaller ones due to the strong variation of scattering with particles size. However, the effect is less important than DLS [58].

A technique that has been developed rapidly over the past decade to allow measurement of particles with diameters down to the nanometer range is Flow Field-Flow Fractionation (Flow FFF) [59]. In Flow FFF the particles are separated in a cross-flow channel based on their diffusivity, where larger particles are immobilized in an expanding channel faster than the smaller ones [60]. A variation of this methods is the Asymmetric Flow FFF (AF4) which is capable of fractionating particles and macromolecules having diameters down to 2 nm at concentrations that range from 10^3 to 10^{10} g/mol [61]. AF4 can separate particles within a very wide size range thereby giving unique characterization possibilities for colloidal dispersions.

The wide number of instruments and analytical procedures that have been developed over the years typically provide different apparent sizes of particles thereby making it complicated to understand several size-dependent properties. This is particularly important when trying to understand the toxic effects of nanoparticles suspended in liquids, and highlighting the urgent need for standardizing the procedure.

4. Conclusions

In summary, assessing the environmental impacts of nanotechnology products involves the assessment of the toxicity and determining the health-related properties of ENPs. Cytotoxicity tests of ENPs thus far have drawn upon existing methods of general toxicology. Due to the high variability of ENPs with respect to their size, morphology and composition, it is important to strive for standardized protocols for *in vitro* tests. The high variability of the ENP-containing colloidal samples resulting from their preparation methods also highlights the importance for developing standards in order to improve the comparability among these tests. Regarding the techniques for assessing human exposure to ENPs, already existing methods for assessing the impacts of particulate pollution and the properties of nanoparticles for technological application can be easily adopted for measuring health-related properties such as size and concentration of nanoparticles in air and in liquid media.

Conflicts of Interest

The authors declare no conflict of interest.

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