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Review article

Current *in vitro* methods in nanoparticle risk assessment: Limitations and challenges

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ABSTRACT

Nanoparticles are an emerging class of functional materials defined by size-dependent properties. Application fields range from medical imaging, new drug delivery technologies to various industrial products. Due to the expanding use of nanoparticles, the risk of human exposure rapidly increases and reliable toxicity test systems are urgently needed. Currently, nanoparticle cytotoxicity testing is based on *in vitro* methods established for hazard characterization of chemicals. However, evidence is accumulating that nanoparticles differ largely from these materials and may interfere with commonly used test systems. Here, we present an overview of current *in vitro* toxicity test methods for nanoparticle risk assessment and focus on their limitations resulting from specific nanoparticle properties. Nanoparticle features such as high adsorption capacity, hydrophobicity, surface charge, optical and magnetic properties, or catalytic activity may interfere with assay components or detection systems, which has to be considered in nanoparticle toxicity studies by characterization of specific particle properties and a careful test system validation. Future studies require well-characterized materials, the use of available reference materials and an extensive characterization of the applicability of the test methods employed. The resulting challenge for nanoparticle toxicity testing is the development of new standardized *in vitro* methods that cannot be affected by nanoparticle properties.

1. Introduction

Nanoparticles are generally defined as engineered structures with at least one dimension less than 100 nm. They are manufactured as multifunctional particles in a variety comparable to that of conventional chemicals. In recent years, an increasing number of products composed of these tiny structures have entered the market. Metal oxide nanoparticles such as TiO_2, for instance, are frequently used in products which range from cosmetics to food packaging [1].

Nanoparticles also offer an extraordinary opportunity for applications in pharmacology and medicine. Carbon nanoparticles, such as fullerenes (e.g., C_{60}), or carbon nanotubes are currently tested for site-specific targeted drug delivery [2,3]. Nanoshells, composed of a dielectric core such as silica surrounded by a metallic shell (e.g., Au, Cu), can act as photoabsorbers and have been successfully used for photothermal ablation of tumours in mice [4]. Additionally, they have been applied in bioimaging with optical and photoacoustic tomography (for review see [5]). Even more attractive for the use in medical diagnostics are quantum dots (QDs), semiconductor nanocrystals with unique fluorescent properties. QDs of different sizes are excitable with one single light source but – depending on their size – emit light at different wavelengths, which enables an optical "bar-coding" of targets [6,7]. Polymer nanoparticles, like chitosans, have been used in gene therapy [8], drug delivery [9], and water treatment [10].

With the ongoing commercialization of nanotechnology products, human exposure to nanoparticles will dramatically increase and an evaluation of their potential toxicity is essential. A number of manufactured nanoparticles have recently been shown to cause adverse effects *in vitro* and *in vivo* (reviewed by [11–13]). Their large surface area per unit mass may lead to an increased biological reactivity that enhances any intrinsic toxicity. Nanoparticles of TiO_2, for instance, have been shown to induce a much greater pulmonary–inflammatory response than larger particles of the same chemical content at equivalent mass doses [14]. Consequently, screening techniques commonly used for toxicity testing of macro-scale substances may not be appropriate for nanoparticle hazard characterization, but may have to be adapted or modified with regard to their nanospecific properties [15].

Although numerous *in vitro* nanotoxicity studies have already been published, most of the experiments carried out so far have used particles not well characterized regarding their composition and physicochemical properties. However, such a characterization is mandatory since nanoparticles might interact with assay components or interfere with detection systems resulting in unreliable data [16]. In this review, we shortly reflect the general use and

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limitations of *in vitro* assays in toxicology, provide an overview of currently used *in vitro* cytotoxicity methods, and focus on nanoparticle properties that likely interfere with these *in vitro* test systems. Furthermore, we discuss novel technologies enabling marker-free toxicity testing that may provide new approaches to nanoparticle risk assessment.

2. General use and limitations of *in vitro* assays in toxicology

In vitro toxicity assays are primarily utilized to investigate the generic cytotoxicity or genotoxicity of chemicals. While the traditional application of *in vitro* tests has been screening of chemicals, recent developments in molecular biology have helped to provide mechanistic information on toxicity [17]. The use of *in vitro* toxicity methods for the assessment of nanoparticles was a subsequent development and the applicability of a prevalent *in vitro* test to nanoparticles is reviewed below. Sutter et al. identified six general applications of *in vitro* toxicity assays, among which are the selection of the most appropriate animal model of humans and the rapid screening of series of toxicants [18]. In comparison to animal models, *in vitro* assays allow for a simpler, faster and more cost-efficient assessment of defined toxicity endpoints [19]. However, *in vitro* test systems lack the complexity of animal models or the human body [17], and the metabolic activity of standardized cell lines has often not been comprehensively characterized [20]. According to Snodin et al., *in vitro* systems have no value for the prediction of biodistribution and target organ toxicity for the applied chemical and its metabolites [21]. Furthermore, a dose–response relationship will probably only be reliably determined *in vivo* [22]. Besides these considerations, scientists have been striving to determine the correlation between the results obtained from *in vitro* and *in vivo* toxicity assessments. In the domain of particle toxicology, for example, Sayes et al. found little correlation between *in vitro* and *in vivo* pulmonary toxicity of several fine and nanoparticles [23]. On the other hand, Donaldson et al. reported that the threshold for inflammation onset was identical *in vitro* and *in vivo* when the "particle surface area burden per unit of proximal alveolar region surface area" of different low-toxicity, low-solubility particles was used as reference [24]. Contradictory *in vitro* results on the same test substance accumulating in the literature as well as the continuous public concern for animal welfare and safe handling of substances have driven diverse international efforts to standardize and validate *in vitro* tests (e.g., [25]). The OECD validation harmonization report [26], programs as the MEIC (Multicenter Evaluation of *In vitro* Toxicity, e.g., [27]), the German Center for the Documentation and Validation of Alternative Methods (ZEBET), and standard protocols made available via the INVITTOX database run by the European Centre for the Validation of Alternative Methods (ECVAM), for example, are important aspects of an international standardization and validation process (reviewed e.g., by [28]).

In vitro assays have general limitations for reliable risk assessment in the aspects of validation by *in vivo* experiments and a lack in standardized testing procedures, which are verified by reference materials or interlaboratory validation.

3. Current *in vitro* cytotoxicity assays

3.1. Cell viability

Cell viability is the most commonly investigated parameter in cytotoxicity testing. As cell viability is determined by various cellular processes, different endpoints are currently utilized to assess the actual state of cultured cells *in vitro*.

Detection of mitochondrial activity. The colorimetric MTT assay is a widely used cell viability assay based on the reduction of the yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple water insoluble formazan in cells bearing intact mitochondria [29,30]. It has been improved and applied in numerous cytotoxicity studies [31], and employed to validate other methods [32] and to determine nanoparticle toxicity. Results from MTT assays have been reported, e.g., for nanoparticles composed of titanium dioxide [33], iron oxide [34], zinc oxide [23], chitosan [35], silica [36], for fullerenes [37], and for naked or chitosan-coated QD [38]. Assay protocols described in the literature differ strongly [39], which has to be considered when comparing results reported by different laboratories. The absorption spectrum of reduced MTT is pH-dependent [40], and metal ions such as Zn^{2+} interfere with the MTT reduction reaction [41]. Nanoparticles may interact with the substrate, thereby depleting free MTT and causing false negative results as described for single-walled carbon nanotubes (SWCNTs) [42–44] (Table 1). Therefore, the suitability of MTT for *in vitro* toxicity assessment of SWCNTs has come into question [45]. Due to their optical properties, nanoparticles present in the reaction mixture, in or on cell culture cells may directly influence the readout by increasing the light absorption, which has already been demonstrated for sodium titanate nanoparticles [46].

Table 1
Nanoparticle interference with cytotoxicity assays

Cytotoxicity assay	Detection principle	NP interference	Altered readout	Particle/Reference
Cell viability				
MTT	Colorimetric detection of mitochondrial activity	Adsorption of substrate	Reduced indication of cell viability	Carbon nanoparticles [42,44,79]
LDH	Colorimetric detection of LDH release	Inhibition of LDH	Reduced indication of necrosis	Trace metal-containing nanoparticles [59,98]
Annexin V/ propidium iodide	Fluorimetric detection of Phosphatidylserine exposure (apoptosis marker)	Ca^{2+}-depletion	Reduced indication of apoptosis	Chitosan nanoparticles [61,114]
	Propidium iodide-staining of DNA (necrosis marker)	Dye adsorption	Reduced indication of necrosis	Carbon nanoparticles [71]
Neutral red	Colorimetric detection of intact lysosomes	Dye adsorption	Reduced indication of cell viability	Carbon nanoparticles [45]
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of apoptosis	Trace metal-containing nanoparticles, especially Zn^{2+} [92,93,98,115]
Stress response				
DCF	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles [104]
Inflammatory response				
ELISA	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles [79] Metal oxide nanoparticles [112]

Detection of LDH release upon necrosis. The colorimetric lactate dehydrogenase (LDH) assay which is based on the oxidation of the yellow tetrazolium salt INT to a red formazan has a long tradition in the clinic to evaluate tissue or cell damage [47,48]. As significant amounts of LDH are released from the cytosol upon cellular necrosis [49], LDH activity is measured in the cell culture supernatant. In the cellular metabolism, LDH catalyses the balance reaction pyruvate – lactate generating $NADH+H^+$ [50]. Instead of pyruvate, INT (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride) is used as an alternative substrate for dehydrogenases such as LDH [51], lactyl-CoA dehydrogenase [52], and succinate dehydrogenase [53].

LDH assays using INT as substrate have been applied to assess the cytotoxic potential of various nanoparticles, for instance, silica [54], iron oxide [34], TiO_2 [55], ZnO [23], SWCNTs [56], and fullerenes [57].

LDH is significantly deactivated under low pH conditions [51], whereas a high basic pH destabilizes the substrate [58]. Furthermore, metal ions (e.g., copper) have been shown to interfere with the LDH assay [59]. Single-walled carbon nanotubes, however, do not seem to interact with the substrate INT [42].

Annexin V/propidium iodide staining for apoptotic and necrotic cells. Annexin V (VAC alpha), which is regularly used to detect apoptotic cells [60], binds strongly to phosphatidylserine in a calcium-dependent manner [61]. Phosphatidylserine is normally excluded from the extracellular side of the plasma membrane [62], but flips between the inner and the outer side upon the onset of apoptosis [63]. Fluorescently labelled Annexin V can therefore be used to detect apoptotic cells [64]. Necrotic cells will allow Annexin V to bind PS on the inner part of the plasma membrane resulting in false negative results due to cell disintegration. Hence, cells have to be co-stained with propidium iodide (3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide) which will exclusively stain necrotic cells [65,66]. Investigation of apoptosis via Annexin V staining and flow cytometry has been reported, e.g., for TiO_2 nanoparticles [67], for pure and polyhydroxylated fullerenes [68], for SWCNTs [42], and for quantum dots [69].

As apoptotic cells may easily detach from their substrate, it is essential to collect adherent and floating cells for Annexin V staining [70]. Consequently, nanoparticles in the cell culture supernatant will be present in the cell suspension despite several washing steps. Gold nanoparticles have been shown to bind propidium iodide and to be taken up by intact cell culture cells. This process leads to false positive results in the detection of necrotic cells [71] (Table 1).

Detection of intact lysosomes via neutral red uptake. Since 1894, neutral red has been used as a viability stain [72] and has since been implemented in numerous cytotoxicity, cell proliferation and adhesion assays [73,74]. Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is weakly cationic, and is thought to be taken up into the cytosol by non-ionic diffusion through the cell membrane to then accumulate in the lysosomes of viable cells, whereas it is excluded from dead cells [75]. The uptake of neutral red may be detected via fluorescence or absorption measurement. So far, the neutral red uptake (NRU) in NIH3T3 mouse fibroblasts is the only validated *in vitro* method for toxicity testing [76] and has been incorporated into the REACH (Registration, Evaluation, Authorisation of Chemicals) directive by the European Commission for the *in vitro* toxicity assessment of chemicals [77]. A number of different nanoparticles such as TiO_2 [78], SWCNTs [43], multi-walled carbon nanotubes (MWCNTs) [79], and chitosan nanoparticles [35] have been tested in NRU assays. The colour and intensity of light emission of neutral red is pH-dependent [80]. It is known that neutral red has a high affinity to lipophilic structures (such as suberin and lipids [81] or phenolic substances [82]), the protonated dye interacts with negative charges [82], and covalently binds to cellular structures [81]. These properties were utilized to study the adsorption of neutral red to mercaptoethane sulfonate-protected gold nanoparticles. The optical properties of neutral red were significantly influenced, the nanoparticles exerted static and dynamic energy transfer quenching of neutral red fluorescence [83]. Furthermore, SWCNTs have been shown to interact with neutral red and deplete the dye from the cell supernatant leading to false positive results [45] (Table 1). Hence, properties that are useful for technical applications may be deleterious in cytotoxicity testing.

Detection of the apoptosis marker Caspase-3. The detection of active Caspase-3 is one of the most commonly used apoptosis assays. Apoptosis may be triggered by different elicitors activating two main signalling cascades that converge in the activation of Caspase-3 [84]. The cystein protease Caspase-3 is produced as a zymogen in the cytosol and is activated in the terminal apoptotic cascade by cleavage [85]. As soon as Caspase-3 is activated, cell death is inevitable. Activated Caspase-3 can be detected by measuring the cleavage of a Caspase-3 substrate (preferably the amino-acids DEVD) linked to a chromophore (pNA) or fluorophore (AFC, AMC) that absorbs or emits light when separated from the substrate [86]. As yet, the Caspase-3 assay has been utilized to examine apoptosis in cell culture cells upon exposure, e.g., to fullerenes [87], SWCNTs [88], silica nanoparticles [89], quantum dots [90], and to TiO_2 nanoparticles [91].

Caspase-3 is inhibited by trace metal ions, especially by Zn^{2+} ions [92,93] (Table 1). On the other hand, Caspase-3 is relatively unsusceptible to changes in pH [92]. As described above, apoptotic cells may easily detach from their substrate, so that adherent and floating cells have to be used for Caspase-3 activity assays [70]. Nanoparticles in the cell culture supernatant will therefore remain in the cell suspension during measurement.

3.2. Stress response

Detection of reactive oxygen species. Cellular stress response is often investigated with H_2DCF-DA (2′,7′-dichlorodihydrofluoresc(e)in diacetate), which is a widely used probe for the *in vitro* detection of intracellular reactive oxygen species (ROS) [94,95]. The acetylated non-fluorescent molecule is taken up by cell culture cells, is presumably trapped in the cytosol by deacetylation and becomes fluorescent upon intracellular oxidation [96,97]. Several studies on the exact mechanism responsible for H_2DCF oxidation offer varying conclusions, consequently it has been suggested that DCF should be applied as a qualitative marker for cellular oxidative stress in general [97]. A possible increase in DCF fluorescence has been investigated after cell culture exposure, e.g., to TiO_2 [96], SWCNTs [98], fullerenes [99], and to silica particles [100].

Internalized, deacetylated H_2DCF does not exclusively remain in the cytosol but may accumulate in the extracellular space and react with catalytically active substances outside the cells [101]. DCF fluorescence is strongly pH-dependent [102,103]. Additionally, nanoparticles such as carbon-based materials may absorb light so that DCF fluorescence may be quenched (Table 1). Controls including nanoparticles and oxidized DCF have been suggested [104], but need to be verified for a linear relationship between nanoparticle and dye concentration versus the decrease in fluorescence.

3.3. Inflammatory response

In vitro study of inflammatory marker production via enzyme-linked immunosorbent assay (ELISA). The ELISA method was first described in 1971 [105], and enables simple and accurate quantification of inflammatory markers in cell culture supernatants through antibodies and enzymatic detection reactions. ELISA results have been reported for nanoparticles of different composition and origin,

e.g., for titanium dioxide [106], iron oxide [107], zinc oxide [23], carbon black [79,108], carbon nanotubes [43], fullerenes [109], silica [110], and for quantum dots [111]. The most commonly tested human and murine inflammatory markers are the chemokine Interleukin-8 (IL-8), followed by TNF-α and IL-6. In some cases, IL-1β as well as a few other cytokine and stimulating factor concentrations are measured. The chemokine MIP-2 is usually quantified in rat model systems together with TNF-α and/or IL-6.

Recently, nanoparticles have been reported to interfere with enzymatic immunoassays. Adsorption of cytokines by nanoparticles *in vitro* was discussed for carbon nanoparticles (IL-8, [79]) and for metal oxides (IL-6, [112], Table 1). Even depletion of trace nutrients or growth factors from cell culture media due to the high adhesive surface area of nanoparticles was considered [79,113].

Since many nanoparticles are manufactured under non-sterile conditions, they might be contaminated with bacteria or endotoxins that may also induce inflammatory responses. Particles should therefore be characterized and treated accordingly to remove any interfering contaminants [14,16].

4. Nanoparticle properties influencing *in vitro* toxicity assays

Nanoparticles exhibit several unique physicochemical properties that are exploited in a variety of high-performance products or novel applications. These properties, however, may represent major obstacles for cytotoxicity studies and have to be carefully characterized in advance. An increasing number of nanoparticle toxicity studies conducted with well-established *in vitro* models have generated confounding or even conflicting data, and evidence is accumulating that nanoparticles may interfere with assay components or detection systems. Depending on their size and the dispersion agent, nanoparticles will either diffuse within the liquid or sediment onto exposed cell culture cells [116]. Their presence during the assay, together with their unique properties associated with their nanosize might introduce artefacts into cytotoxicity studies. In this section, we focus on nanoparticle properties that are likely to influence *in vitro* toxicity testing.

High adsorption capacity. Due to their large surface per unit mass, nanoparticles display an increased adsorption capacity and biological reactivity as compared to the bulk material. Therefore, many nanoparticles become coated with a set of different proteins when entering a biological fluid (for review see [117]). The composition of the resulting protein corona is not only determined by protein identity, but strongly depends on particle surface, size, aggregation state and even on particle concentration [118]. In turn, the coating process determines the effective size, charge and therefore the behaviour of nanoparticles under physiological conditions [16,119]. Fullerenes, for instance, are capable of specific interactions with proteins as shown by the production of fullerene-specific antibodies [120], and even seem to affect peptide configurations [121]. Hence, nanoparticles could potentially confound cytotoxicity data by inducing indirect effects through the adsorption of nutrients and growth factors from culture media [113]. In addition, nanoparticles may directly influence the assay outcome when protein concentration or activity is utilized to evaluate particle toxicity [112]. Furthermore, nanoparticles have been shown to interact with other assay components (e.g., substrates, dyes) thereby introducing artefacts into a number of different *in vitro* studies [42,45,79]. Hydrophobicity determines how nanoparticles become dispersed in aqueous solutions, and this in turn modulates their ability to adsorb proteins or other assay components. Similarly, adsorption by nanoparticles will be influenced by surface charge. Negatively charged chitosan nanoparticles, for instance, have been shown to bind Ca^{2+} [114], which may lead to interference with Ca^{2+}-dependent assays depending on the dispersion agent.

Finally, high adsorptive particles could potentially bind contaminating compounds during the production process. Carbon nanotubes as hollow cylinders contain metal contaminants (predominantly Fe, Ni, Co) used as catalysts during manufacturing. These impurities are likely to induce an increased immune response and oxidative stress, and have to be eliminated before conducting *in vitro* experiments [98,122].

Optical properties. If light absorption or fluorescence detection is used to evaluate particle toxicity, it has to be considered that many nanoparticles display optical properties potentially interfering with the detection system. Due to their light-absorptive features, metallic nanoparticles like sodium titanate directly influence the readout in cell viability assays [46]. Moreover, the proximity of gold nanoparticles and fluorescent dyes, like Cy5, has been shown to result in reduced fluorescence signal intensity [123]. Finally, nanoparticles used for medical imaging, such as QD or nanoshells, can absorb and emit light of different wavelengths, and might distort the signal intensity in assays with an optical readout, which is the case for most of the commonly used cytotoxicity methods.

Catalytic activity. The high surface/mass relationship of nanosized materials results in an excess surface energy enhancing any catalytic activity. A number of different nanoparticles such as metal oxide nanoparticles, fullerenes and silica particles have been shown to produce ROS in cell-free systems [68,124–126]. ROS production was 100–1000 times faster on 2–4 nm-sized nanoparticles than on 100 nm-sized particles [127], demonstrating that the increased catalytic activity of nanoparticles is size-dependent. Photoactivated TiO_2 and ZnO nanoparticles have been shown to degrade anionic dyes like erythrosine [128], while SWCNTs may interfere with MTT viability tests by oxidizing the substrate MTT [44]. Hence, redox-active nanoparticles may cause false signals in assays based on substrate oxidation.

Acidity/alkalinity. Another issue that has to be considered is the pH of nanoparticles in solutions since the most widely used *in vitro* assays are pH-dependent (see chapter 2), and may thus be influenced by acidic or basic nanoparticles if these remain in considerable amounts in the assay mixture.

Magnetic properties. Some metal oxide nanoparticles like Fe_2O_3 are superparamagnetic and generate strong, local magnetic fields which lead to the production of free radicals that in turn may interfere with cytotoxicity methods based on redox reactions [129,130].

Dissolution. Nanoparticles that are designed to dissolve in aqueous solutions, like water-soluble QD [131], or particles that show an intrinsic, size-dependent dissolution in aqueous media, like ZnO [115], will release metal ions or trace metals when introduced into biological media. Cytotoxicity assays that are sensitive to metal ions may therefore be perturbed in the presence of dissolving nanoparticles.

Taken together, nanoparticles display several unique properties that may lead to interferences in cytotoxicity assays. Therefore, current *in vitro* methods should not be used for nanoparticle toxicity testing without a detailed characterization of the particle properties in advance [16]. Since particle interference with assay components cannot be determined in detail *a priori*, different screening techniques and adequate controls must be employed to generate reliable data for nanoparticle risk assessment.

5. Challenges for nanoparticle *in vitro* test methods

Physicochemical properties of nanoparticles often limit the use of established *in vitro* toxicity assays for risk assessment. Studies designed to determine nanoparticle toxicity should ideally be performed using test systems that cannot be influenced by nanospecific properties [132]. Currently, however, nanoparticle risk analysis

is impaired by the lack of standardized test systems which fulfil these criteria.

New test systems for toxicity screening address new endpoints or new toxicity biomarkers. For instance, cell culture systems better reflecting *in vivo* toxicity parameters have recently been developed [133]. New subdisciplines of toxicology, such as toxicogenomics, focus on studies of cellular products controlled by the genome (RNA, proteins, metabolites) and provide new approaches to assess adverse biological effects of exogenous agents [134–136]. These technologies will enable a deeper understanding of biochemical pathways and cellular responses.

However, many of these strategies depend on conventional detection systems and may still be influenced by nanoparticle-specific properties. Novel technologies allowing marker-free cytotoxicity testing might overcome these obstacles. For example, cellular analysis can be performed using physical cell properties such as electrical resistance or refractive index. Digital holographic microscopy, e.g., detects the integral refractive index of living single cells in cell culture medium resulting in a phase shift of visible light [137]. Cell morphology reconstruction of the digitally captured holograms is performed by application of a spatial phase shifting, non-diffractive reconstruction method [138]. This method can be applied to a marker-free online analysis of processes induced by drugs or toxic agents that lead to altered cell morphology including apoptosis and cell swelling [139,140]. Electrochemical impedance spectroscopy measures cellular dielectric parameters such as membrane capacitance and conductivity that can change rapidly after exposure to toxic substances [141]. Tight cell layers grown on top of an electrode form an electrical resistance, which is altered after drug induced cell morphology alteration such as cell rounding or cell–cell contact dissociation [142,143]. The online monitoring of cellular resistance can be integrated in conventional high-throughput plate formats [144]. Compared with a conventional MTT assay for cytotoxicity screening, electrochemical impedance spectroscopy was shown to be more sensitive [145].

After evaluation and validation, these new marker-free and live measurement technologies may define a new standard for a general *in vitro* toxicity testing of nanoparticles unaffected by specific nanoparticle properties.

In conclusion, nanoparticles exhibit size-specific properties limiting the application of established *in vitro* assays. Nanoparticle *in vitro* toxicity testing therefore requires a careful characterization of particle properties and an extensive validation of assay systems when applying established methods for risk assessment. Future studies should only present data obtained with well-characterized particles and include reference materials when available. A major deficiency considering the wide range of nanoparticles to be assessed is the current lack of nationally or internationally agreed reference nanoparticles or nanomaterials and standardized test protocols. Finally, the definition of dose is not yet standardized with respect to mass, number, surface area and other metrics, and this hinders the objective analysis of the data and comparisons between materials [22].

Since current *in vitro* test methods are likely to be influenced by nanoparticle-specific properties, nanoparticle risk assessment needs an adaptation of existing cytotoxicity methods or the development of new test systems. Technologies based on marker-free detection of cellular endpoints have the potential to overcome nanomaterial-dependent assay limitations and will provide new standards for nanoparticle risk assessment.

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