

Review

Mechanisms Underlying Cytotoxicity Induced by Engineered Nanomaterials: A Review of *In Vitro* Studies

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Abstract: Engineered nanomaterials are emerging functional materials with technologically interesting properties and a wide range of promising applications, such as drug delivery devices, medical imaging and diagnostics, and various other industrial products. However, concerns have been expressed about the risks of such materials and whether they can cause adverse effects. Studies of the potential hazards of nanomaterials have been widely performed using cell models and a range of *in vitro* approaches. In the present review, we provide a comprehensive and critical literature overview on current *in vitro* toxicity test methods that have been applied to determine the mechanisms underlying the cytotoxic effects induced by the nanostructures. The small size, surface charge, hydrophobicity and high adsorption capacity of nanomaterial allow for specific interactions within cell membrane and subcellular organelles, which in turn could lead to cytotoxicity through a range of different mechanisms. Finally, aggregating the given information on the relationships of nanomaterial cytotoxic responses with an understanding of its structure and physicochemical properties may promote the design of biologically safe nanostructures.

Keywords: *in vitro* methods; cytotoxicity; nanomaterials; cell culture; nanosafety

1. Introduction

The most common definition of nanotechnologies includes materials with at least one dimension in the range of 1 to 100 nm [1,2], although some authors classify them as all submicronic particles up to 200 nm [3,4], or even up to 1000 nm [5,6]. At this size, nanomaterials (NMs) might interact in a unique fashion with biological systems and be easily taken up by cells [7], which opens up a wide range of interesting applications in medicine [8]. However, the same characteristics that make the NMs a promising approach for many new clinical therapies also make them very reactive structures that can generate undesirable cell interactions and adverse effects.

Similarly, nanotechnologies may change many sectors of industry for the better, though considerable concern has arisen in these different industrial fields about their side effects and possible risks to human life. It is clear that the potential toxicity of nanoparticles (NPs) *versus* biological systems has to be much more thoroughly investigated than has been done to date in order to define their future role in clinical, diagnostic, technological and environmental applications.

Concerning the unique properties of the NMs, a new research field of toxicology, namely nanotoxicology, was defined to address gaps in knowledge and also to assess the problems likely to be caused by nano-sized materials [9]. More specifically, nanotoxicology aims to understand the principles and mechanisms of interactions at the nano–bio interface and also to determine the relationship between NM physicochemical properties and the associate toxicological profiles [10]. It is a fact, with open discussions in the scientific community, that NM composition, size, geometry, and transport or durability in the body, can cause adverse effects on human health. Furthermore, due to their specific intrinsic properties, the NMs have a big tendency to suffer cellular uptake, which may also result in bioaccumulation and, thus, in increasing the adverse effects and toxic potential [11]. Moreover, it is widely described that NMs presented great chemical reactivity, which may lead to many kinds of toxic reactions, including mutagenicity by formation of DNA adducts or sensitization by hapten binding [1].

For most engineered NMs, toxicity data are unavailable or the data reported show very controversial and inconclusive results [1]. In such a context, an understanding of the toxicity mechanisms is crucial for both the design of more efficient NMs, and at the same time for the design of nanotechnologies that are biologically and/or environmentally benign throughout their life-cycle.

Although numerous *in vitro* nanotoxicity studies have already been published, most of the experiments carried out thus 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. Aspects related to the importance of a detailed characterization of the NMs prior to any *in vitro* studies are also reviewed and commented in this article in Section 3.

A wide range of *in vitro* approaches has been used to assess the toxicological behavior of different types of NMs. However, many traditional approaches might have even greater limitations for NP than for other conventional chemicals and also may not offer the throughput and velocity to handle the dynamic developments in nanotechnologies [1]. Therefore, it is worth mentioning that novel approaches need to be developed in order to cover the needs of nanotoxicology.

The issue of safety and risk assessment of NMs becomes complicated because they are not a uniform group of substances [12,13]. Therefore, a huge problem concerning the toxicological aspects of NMs is the wide range of nanotechnologies with different chemical compositions, overall physicochemical characteristics and sizes. Along these lines, emerging information on quantitative structure–activity relationship (QSAR) modeling of NMs' toxic effects is gaining force toward the construction of a database platform including the toxicological information of different NMs, interrelated with the physicochemical characteristics and chemical composition of each NM. The application of a structure–activity paradigm to NMs represents a promising approach to anticipate their toxicological properties in a fast and inexpensive way. Likewise, following this paradigm, it is possible to predict the toxic effects induced by NMs on the basis of some structural similarities with chemicals for which toxicological behavior have been previously determined [14].

Up until now, no NM-specific risk assessment paradigms have been produced, and therefore, researchers took liberal approaches to studying its toxicity [8,15]. For example, within the European Union, REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) is for the time being the basis of NM risk assessment, even though no specific legislation is assigned specifically to NM analysis [16]. The regulatory agencies continuously reported that efforts to address the human toxicity of NMs should be directed towards developing models that predict associations between changes to NM physicochemical characteristics and hazardous properties [17]. Following this line, high throughput *in vitro* cellular models have been receiving growing attention toward establishment of rapid and reliable approaches for nanotoxicology assessments.

Considering the special properties of NMs that can lead to toxic effects, many strategies have been applied to improve performance and reduce toxicity of NMs in medical design, *i.e.*, the use of coating materials and/or the development of biocompatible/biodegradable NPs [18]. Independently of the approach used to design a new drug delivery system, its toxicological properties must be assessed by using, for example, *in vitro* cellular models for a primary screening and also for the elucidation of some inherent mechanism underlying toxicity of each type of nanodevice. There is growing concern regarding the interrelationship between particle size, shape, chemical composition and toxicological effects of NMs, which are demonstrated by the increasing number of studies considering this subject [19,20].

As already highlighted, the number of research articles concerning nanotoxicology has been growing significantly in the last decade. However, most of the data published is related to toxicological phenomena and the comprehension of the underlying mechanisms for NM-induced toxicity are less explored and understood. Therefore, the increasing usage of engineered NMs, especially in health concerns, has emphasized the need for further mechanistic insight to predict the consequences of exposure to this new class of materials.

The characterization of the risks of the NMs is highly complex because of the special and specific physicochemical properties of such materials. In searching for a testing strategy that can rapidly and efficiently provide a screening approach for evaluating the potential hazard of NMs, researchers have been using a wide range of *in vitro* cellular models in an attempt to determine the toxicological behavior of NMs and to elucidate the mechanisms underlying this toxicity.

2. *In Vitro* Toxicity Assessment

The wide variety of *in vitro* and *in vivo* assays that are employed to assess NP toxicity has been used because these are the tools that were already available for molecular toxicology when nanoparticle toxicity questions first arose. The first major problem with using these assays is that the modes of NP toxicity might not be the same as those incurred by molecular toxicants. Because of the expense of animal testing in toxicology and pressure from both the general public and government to develop alternatives to *in vivo* testing, *in vitro* cell-based approaches might be more attractive and necessary for NM toxicity assessment [21]. *In vitro* testing is a common first step in assessing the health risks related with engineered NMs. Despite the frequent lack of consistency or predictability between *in vitro* models and *in vivo* observations, there is little rational or ethical justification to proceed directly from material synthesis to animal models [22]. Moreover, some effects can be seen only *in vitro* and thus hazards that are masked by current *in vivo* tests due to animal defenses should not be underestimated [1]. Therefore, it is desirable to develop and validate simple non-*in vivo* assays for the purpose of predicting *in vivo* responses in order to reduce and avoid extensive testing using laboratory animals [23].

The primary focus of the *in vitro* assays is to assess the cytotoxic effects of chemicals and, more recently, of nano-based forms of such chemicals. However, the *in vitro* assays have many features and advantages to be used in more specific studies, *i.e.*, to study the mechanisms underlying cytotoxicity induced by chemicals or NMs. *In vitro* approaches allowed researchers to obtain faster and more reliable mechanistic information on nanotoxicology. It is worth mentioning that the application of the *in vitro* methods to NMs do not follow a validated process. Hartung [1] described a number of alternative methods validated for chemicals and drugs that might be useful for NMs. Moreover, the author stated that efforts directed at the validation of the existing methods for NPs would not only expand the applicability domains of these validated methods, but might also allow a possible fast-track to obtaining regulatory acceptance for NP evaluation.

On the other hand, there are some concerns about the growing use of *in vitro* methods for nanotoxicity assessments. Discussions are constantly raised about the fact that such alternative methods are less applicable to particles than to soluble chemical substances, mainly due to their particularly physicochemical properties. Indeed, some of these concerns apply to NM structure and characteristics, *e.g.*, the *in vitro* kinetics of particles (their behavior in cell culture) might differ, including in terms of phenomena such as particle aggregation, binding to plastic and/or floating on the cell culture media surface [15,24]. Similarly, air exposure and specific artifacts used in cytotoxicity assays (*i.e.*, MTT or NR dyes) might interfere in the *in vitro* experiments [1,25,26]. In this case, intrinsic photometric absorbance or fluorescence of NMs may alter colorimetric or fluorometric assay reporting. Moreover, the high surface energy and surface area of NMs may also contribute to the binding of unanticipated amounts of assay reagent or analyte [22]. However, the nanotoxicology is a new subject that is likely a driving force and not a declining influence regarding the use of modern approaches in toxicology [1,27,28]. *In vitro* methods have many advantages over the *in vivo* experiments, especially concerning the comprehension of the mechanisms underlying the toxic effects of NMs.

There are some authors that believe the first approach to proceed with a toxicological assessment of NMs is to use acellular systems to explore the reactivity of the materials in such acellular environments [23,29,30]. NMs that do not produce reactive species are seen as having a lower capacity for inducing significant toxic responses in biological systems. After this initial characterization, it would be possible to proceed to *in vitro* cellular models by using testing methods with relevant endpoints such as cytotoxicity, apoptosis, cell-cycle alterations, skin and ocular toxicity, genotoxicity, potential carcinogenicity and effects on the immunological system [13]. The underlying mechanisms of toxicity for NMs are remarkably complicated, hence the need for dedicated and specific analytical methodology and tools.

Interestingly, many of the research articles that consider the mechanisms underlying cytotoxicity, using specifically *in vitro* assays, have focused on more than one mechanism of cell interaction [20,31]. This is important in order to obtain some correlation between each cell disturbance effect and also to determine a sequence of mechanisms and specific NM–cell interactions that would ultimately to toxic effect.

Despite the great importance and validity of the *in vitro* model to screen compounds and identify the type of effect induced on cells, it is necessary to recognize that they may not be sufficient for defining safe exposure limits [32]. Simple *in vitro* methods are totally relevant for initial toxicity screening studies of NMs, as well as determining some mechanisms underlying cell interaction that may be responsible for their cytotoxicity. However, more physiologically relevant *in vitro* models or even *in vivo* assays might be useful and necessary to better understand how NMs can impact human health at all.

2.1. Cytotoxicity Activity

2.1.1. Cytotoxicity Assays

The cytotoxicity data of most of the tested NMs have been generated by using tetrazolium salt-based endpoints. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), and the water-soluble tetrazolium salts (WST-8 and WST-1) assays are widely reported in the literature with this purpose.

The MTT approach is the most common assay, and has been extensively applied to assess the effect of a variety of NMs on cell viability [7,31,33–38]. This method measures the reduction of MTT salt to a colored insoluble formazan in active mitochondria in viable cells [39]. Moreover, this assay can be applied either for cytotoxicity and cell proliferation studies.

The cytotoxicity endpoint MTS was applied for monitoring the cytotoxic responses of gold NPs [40] and of cationic polystyrene nanosphere [41]. Moreover, MTS was also applied to determine the cell damage induced by bioadhesive poly(anhydride) NPs destined for oral antigen/drug delivery. HepG2 and Caco-2 were used as model cell lines, the last one also being used in studies to discriminate between cytoadhesion and cytoinvasion mechanisms of cell interaction [42].

Although to a lesser extent, the XTT assay has also been applied, e.g., to assess the cytotoxicity of citrate stabilized gold NPs in three cell lines [43] and of bio-active silica NPs in 19 different cell lines

representing all major organ types [44]. Specifically, in the later study, the results revealed little toxicity in any cell type analyzed and, therefore, the cell line characteristics did not influence the final toxic response. However, many authors have demonstrated that the organ and species of cell used in the cytotoxicity study have strong effects on the outcoming results [31,33,45]. The single characteristics of each cell line can make it less or more sensitive to the cytotoxic effects of a NM. As a general rule, the selection of a relevant cell type depends on the expected *in vivo* target organ and application of the NPs.

The WST assays have appeared to be advantageous over MTT in that they are reduced outside cells, and yield a yellow-colored water-soluble formazan, which is soluble in the tissue culture medium. WST-8 assay was conducted to determine the cytotoxic potential of two-dimensional carbon-based NMs, including graphene sheets (GS) and graphene oxide (GO) [38], as well as to measure metabolic activity of six cell lines from different organs and species after exposure to silica NPs [45], and carbon black (CB), single-wall carbon nanotube, silicon dioxide (SiO₂) and zinc dioxide (ZnO) NPs [19]. In another study, WST-8 endpoint was used to evaluate the cell proliferation of hepatic stellate cells (HSCs) treated with silver NPs [46]. Likewise, WST-1 derived assay is also a colorimetric technique that allows the quantitative determination of cell viability. It was used by Ghosh *et al.* [37] to determine the cytotoxicity of TiO₂.

The neutral red uptake assay (NRU) is also a simple endpoint commonly used to evaluate the cytotoxicity of different NMs [7,20,36,37]. It measures the uptake of the NR dye by viable cells with intact plasma membrane, and its concentration in lysosomes.

Trypan blue staining is a dye exclusion assay, used to measure cell membrane integrity, and has been applied to determine the effects of gold [47] and TiO₂ [37] NPs on cell membrane damage. Likewise, Nymark *et al.* [48] applied this simple cytotoxicity assay to determine the cytotoxicity of polyvinylpyrrolidone (PVP)-coated silver NPs by counting the number of living (unstained) cells using a phase-contrast microscopy. Moreover, Karlsson *et al.* [49] determined the potential of copper-containing NPs to alter the cell membrane integrity by using this approach. Alternatively, these authors used the hemolytic assay and hemoglobin interactions to assess the particle–cell membrane interactions and, thus its cytotoxicity. Finally, Chueh *et al.* [40] counted trypan blue-stained cells (dead) on a hemocytometer under a microscope for the determination of cell growth curve after gold NPs treatment.

Alternatively, cell membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside, *i.e.*, lactate dehydrogenase (LDH), which is commonly measured by the LDH leakage assay. This assay has been used in many kinds of studies on NM toxicity. Sun *et al.* [46] performed this assay to assess the acute toxicity of silver NPs on HSCs cells. Likewise, LDH release was measured to determine the cytotoxicity of cationic surfactant-based nanoparticles and micelles on neutrophils [50], and of multi-walled carbon nanotubes (MWCNTs) in four different cell lines [51].

Luminescent cell viability assay is another cytotoxicity assay employed in nanotoxicological studies, *e.g.*, on PVP-coated silver NPs [48] and citrate stabilized gold NPs [43]. This assay determines the number of viable cells based on the quantification of ATP, which signals the presence of metabolically active cells. Darolles *et al.* [52] also performed intracellular ATP measurements to determine the cytotoxicity of cobalt oxide particles against cell metabolism. The authors used a commercial kit that measures the luminescence luciferase conversion to oxyluciferin.

Additionally, the resazurin (Alamar Blue) reduction assay, with fluorometric measurement, has been used to monitor toxic effects of NMs, *i.e.*, of silver NPs [53], TiO₂ NPs [37], ZnO NPs [54], and of multi-walled carbon nanotubes (MWCNTs) [51]. The number of viable cells correlates with the magnitude of dye reduction and, thus, also gives an overview of the cell's metabolic condition.

CBQCA total protein cell viability test is a specific assay that assesses the survival/viability and is based on the capacity of the cells to incorporate and bind a fluorescent dye (ATTO-TAG CBQCA) to the protein amines. Robbins *et al.* [8] used this approach to assess the effects of bio-active NPs and polyplexes on hepatocytes cell growth. A toxic chemical will result in a reduction of the growth rate as reflected by cell number, and this response is highlighted by the decrease of fluorescence of the amine bound CBQCA.

Clonogenic assay were applied to count the colony numbers for determine cytotoxicity of gold NPs in two different studies [40,47]. Moreover, Darolles *et al.* [52] applied this assay to assess the cytotoxicity of cobalt oxide particles. In this assay, a colony is defined as at least 50 clones of one initial cell, and its count is performed through staining with crystal violet (0.5%). Besides, the cell growth assay, performed by using a Z1 counter, was applied to determine the effect of gold NPs on cytokinesis of three different cell lines [33]. Moreover, these authors used the colony-forming assays to determine the long-term cytotoxic of the same NPs.

Another popular method is to stain the entire cell or specific cellular components with fluorescent dyes. This approach allows rapid detection via flow cytometry, and can be combined with microscopy-based analysis to evaluate, *i.e.*, morphological features like cell spreading. In this line, viability measurements after zinc oxide NPs exposure have been performed using a mix of fluorescein diacetate (FDA) and ethidium bromide (EtBr) fluorescent dyes [55]. Cells were stained with a 1:1 solution of 80 µg/mL FDA plus 50 µg/mL EtBr and observed under a fluorescence microscope. In this double-staining method, living cells were stained in green, while dead cells exhibit their nucleus stained in orange. Furthermore, membrane integrity of human lung epithelial A549 cell line treated with engineered cadmium-coated silica NPs was assessed using calcein-AM/propidium iodide (PI) double-staining assay [56]. The cytotoxicity result was given after cell count using fluorescence microscopy. Likewise, quantification of cell viability was performed using PI and fluorescein diacetate (FdA) double staining after HeLa cells treatment with iron oxide magnetic NPs [57]. PI is normally excluded from the inside of healthy cells, but freely cross the membrane and stain intracellular components of cells with compromised membrane. The cells were analyzed by fluorescence microscopy and the cytotoxicity was established by the ratio between viable (green) and dead cells (red) counted on several microscopic fields. Some authors are also using commercial kits to determine the rate of cell proliferation after treatment with NPs. A cell proliferation assay based on the detection of fluorescence/red fluorescent dye that stains nucleic acids was used to determine the cytotoxicity of cobalt oxide particles [52].

A different approach based on a cell-culturing platform (Cell-IQ) has been used as a real-time cell-monitoring system to measure cell biological behaviors, including the total cell number, number of dead cells and cell movement. Sun *et al.* [46] applied this technology to study the behavior of HSCs cells after treatment with silver NPs. In this same line, an electrical measurement known as Electric Cell-Substrate Impedance Sensing (ECIS) was applied to real-time monitoring of *in vitro* cellular cytotoxicity of silica nanotubes [58] and gold NPs [47]. This technique has been used to study cellular viability and proliferation, and also has the advantages to be label-free and non-invasive in comparison

with other conventional viability assays. Likewise, real-time cell analysis (RTCA) system was used for continuous monitoring of changes in cell growth after treatment with gold NPs [40]. This system is also based on the measurement of cell impedance for continuous monitoring of cell growth.

Recently, a new approach, called single-cell mechanics, which is derived from atomic force microscopy-based single-cell compression, has been proposed to investigate NM-induced cytotoxicity [59]. The authors proved the validity of this approach by reading force–deformation profiles following known NP treatments utilized previously. Moreover, it was highlighted that this single-cell-based approach is advantageous in terms of being able to directly correlate to *in vivo* investigations.

Noteworthy is that the utilization of a simple *in vitro* endpoint can also give some valid information concerning the mechanisms underlying NM-induced cytotoxicity. For example, the widely used MTT assay measures the cell metabolic activity in the mitochondria of viable cells and can thus be used as a parameter to determine both cell metabolism and any damage to the mitochondrial compartments [20]. Moreover, the NRU assay reflects the functionality of the lysosomal membrane [60]. Finally, the LDH assay measures severe cell damage and is an indicator of plasma membrane integrity [19]. Therefore, due to their specific mechanisms, these different endpoints can display varied sensitivity to detect the cytotoxic effects of chemicals. The utilization of complementary assays in the same study, each one based on a different mechanism for toxicity detection, is therefore strongly recommended [61,62].

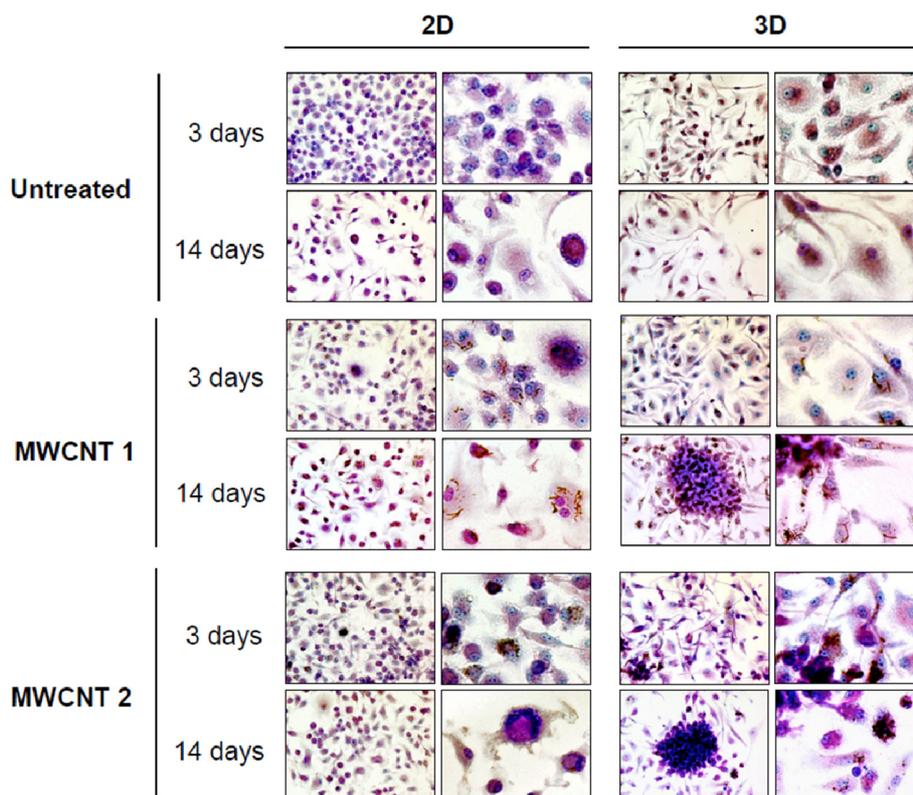
Finally, it is known that NMs interfere with several assay systems, leading some researchers to dedicate efforts to understanding the specific behavior of different NMs with cell viability endpoints. Monteiro-Riviere *et al.* [26] reported that aluminum NPs interact with the MTT assay, and to a lesser extent, with CellTiter 96[®] AQueous One (96 AQ; Promega Corp, Madison, WI, USA) and Alamar blue[®] (aB; Invitrogen, Carlsbad, CA, USA) viability assays. Likewise, it was demonstrated that carbon-based NPs interacted with some widely used viability assays, including Alamar blue, NRU and MTT [25]. These interactions led to an apparent increase in viability and thus to a misinterpretation of the cytotoxicity data, which highlighted the importance of the combination of more than one assay when determining NP toxicity for risk assessment. Recently, Guadagnini *et al.* [63] published a specific study on the interferences of NMs with assay processes and components of classic *in vitro* tests. The authors have provided a great overview of the main challenges of the conventional viability assays when applied to nanostructures.

2.1.2. Three-Dimensional (3D) Cell Culture Systems

Clinically relevant *in vitro* models, such as the 3D cell culture systems, have been manufactured to mimic the properties of tissue *in vivo*. Hashimoto *et al.* [64] developed a novel 3D system using murine macrophages (RAW 264.7 cell line) to assess the biological effects of polyvinylpyrrolidone-coated silver NPs. The authors observed that the cytotoxic results differ between 2D and 3D cultures, the 3D culture system being less sensitive to the NP cytotoxic effects.

In another study, a 3D *in vitro* model was proposed to evaluate the potential of carbon nanotubes to form epithelioid granulomas in nonadherent primary murine bone marrow-derived macrophages (BMDM), cultures in a 3D system [65]. The results of this study showed the distinctive morphological and phenotypic responses of the 3D culture, which suggest this *in vitro* model as a potential alternative to both traditional 2D monolayer cultures and to rodent bioassays for granuloma formation (Figure 1).

Figure 1. Light microscopic morphology and kinetics of macrophage aggregation in 2D and 3D cultures. BMDM were exposed to 0.5 $\mu\text{g/mL}$ (0.38 $\mu\text{g/cm}^2$) of particulates. Formation of stable cellular aggregates was evaluated at 3 and 14 days post-exposure. Macrophages were stained with May-Grünwald-Giemsa. Reprinted from [65]. Open Access article, under the terms of Creative Commons Attribution License. Copyright 2011, Licensee Biomed Central Ltd.



The 3D cell culture systems have also been applied as a useful tumor model to study the antitumor activity of a wide range of NMs. 3D-tissue-engineered tumor models have the potential to bridge the gap between monolayer cultures and patient-derived xenografts and, in this context, were applied to test different NP-based chemotherapeutics [66–68]. Xu *et al.* [69] developed a hydrogel-derived prostate cancer model, in which the cancer cells were entrapped in hyaluronic acid matrices to form distinct tumor-like multicellular aggregates. Finally, the application of 3D *in vitro* models to study the advantages of nanotechnology for cancer therapy has been recently reviewed [70].

2.2. Alterations of Enzymatic Activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are important enzymes in the area of neurobiology, toxicology and pharmacology. Inhibition of AChE causes the accumulation of acetylcholine, interfering with the function of the nervous system. On the other hand, inhibition of BChE could result in the accumulation of acetylcholine in neural synapses, which can disrupt the nervous system normal function. In a recent study, it was demonstrated that silver NPs inhibited both the activity of AChE and BChE *in vitro*, by using an enzyme assay with *o*-nitrophenyl acetate or *o*-nitrophenyl butyrate as substrates, respectively [71]. The extent of ChEs inhibition was shown to be

dependent on the surface coating of silver NPs, and these preliminary findings suggest that metallic NPs had an inhibitory potential on the metabolism of xenobiotics governed by the cholinesterases. Finally, the authors stated that the tested enzyme assays offer practical implementation in the toxicokinetic studies of metallic NPs.

The adsorption and inhibition of AChE [72] and BChE [73] by different NMs, including three metal NPs (Cu-C, Cu and Al), three oxides NPs (SiO₂, TiO₂ and Al₂O₃) and two carbon nanotubes (MWCNT and SWCNT) have been assessed. In these experiments, the Ellman assay [74] was used to measure the activity of both enzymes and calculate the inhibition rate, because NPs could adsorb the yellowish product, 50-mercapto-20-nitrobenzoic acid (5-MNBA) during the color development, which does not allow a simple colorimetric enzyme activity assay. The samples were finally read at 410 nm by spectrophotometry.

The effects of different classes of NPs on the enzymatic activity of the cytosolic protein human arylamine N-acetyltransferase 1 (NAT1) was recently investigated [75]. The importance of NAT1 lies in the fact that it is a drug-metabolizing enzyme responsible for the activation and detoxification of known carcinogens. NMs including metal oxides, synthetic clay NPs and a self-assembling thermo-responsive polymeric NP that differ in size and surface characteristics were used to perform the study. The results suggested that enzyme activity may be compromised in organs exposed to NPs and, thereby highlighting the importance of the evaluation of NP–enzyme interactions.

The binding of luciferase with citrated-coated silver NPs was studied by Kakinen *et al.* [76]. It was demonstrated that luciferase readily bound to NPs through electrostatic interactions and, thus, the authors analyzed whether the enzyme activity was hindered as a result of the interaction by using different analytical techniques such as UV-vis spectrophotometry, transmission electron microscopy, circular dichroism (CD) spectroscopy and inductively coupled plasma mass spectrometry. The combination of these mechanistic studies may give the basis for facilitating the understanding of NM implications at the molecular level.

Polyamidoamine dendrimers with different surface charges (positive, negative and neutral) were studied in respect to their ability to interact with porcine pepsin, a negatively charged protein [77]. The enzymatic activity of pepsin was measured by UV spectrophotometry, followed by CD spectroscopy analysis. The dendrimers with positive and neutral surface charges were able to inhibit enzymatic activity, whereas the negatively charged dendrimer had no influence on the activity of pepsin, probably due to the electrostatic repulsion.

On the other hand, novel polymeric NPs with mucoadhesion and enzymatic inhibition activity were developed for transnasal insulin delivery [78]. The inhibitory activity of these NPs toward α -chymotrypsin, elastase, trypsin and leucine aminopeptidase was tested. The NPs were incubated with substrate solutions and the progress of reaction was followed by monitoring the appearance of the absorption band of *p*-nitroaniline at 410 nm by UV-Vis spectrophotometry. The tested NPs showed strong inhibitory activity especially toward leucine aminopeptidase present on the nasal mucosa, which demonstrated their potential application as novel drug delivery systems for transnasal delivery of protein and peptide drugs.

2.3. Alterations on the Normal Cell Cycle

Assessment of alterations in the cell cycle has been commonly performed using a fluorescent solution of propidium iodide (PI) and flow cytometry analysis. Apart from the utilization of a PI solution, the cells were incubated with the enzyme RNaseA before flow cytometry analysis. Chuang *et al.* [47] and Paul *et al.* [34] evaluated the effects of gold NPs and PLGA NPs on normal cell cycle, respectively, by using this approach. Moreover, Darolles *et al.* [52] assessed the effects of cobalt oxide particles on the normal cell cycle of BEAS-2B human bronchial epithelial cell line.

Staining the DNA with PI, followed by flow cytometry analysis, was also used to perform the cell cycle analysis of A549 cells after treatment with silver NPs [79]. Ten thousand cells were analyzed, and the percentage of the cells in the sub-G1 phase of the cell cycle was calculated from the total 10,000 cells (100%) in the assay, while the percentages for cells in the G0/G1, S and G2/M phases were calculated from the total cells excluding the sub-G1 cells.

2.4. Induction of Apoptosis and Necrosis

The fluorescein isothiocyanate (FITC)-annexin V and PI-annexin V double-staining methods have been widely used to detect apoptosis induced by a variety of NMs. These approaches are based on the determination of phosphatidylserine externalization during apoptosis and leakage from necrotic cells. The ratio of apoptotic and necrotic cells after exposure to silica NPs was measured with the annexin V/PI assay by Foldbjerg *et al.* [45]. This approach analyzed the cells at different stages, *i.e.*, early apoptosis (annexin V+, PI-), late apoptosis/necrosis (annexin V+, PI+) and live (annexin V-, PI-) cells. Alternatively, apoptotic cell death prompted by gold NPs [40,47], photocopyers emitting NP [80] and food-relevant inorganic NPs [81] were determined using annexin V-FITC binding assays.

McCracken *et al.* [81] also performed a specific assay to evaluate necrotic cell death (cellular membrane damage) in NP-treated cells. The authors performed a flow cytometric analysis using Sytox Red dead cell stain. As a positive control for cell death, some cells were treated with 10 or 20 mM hydrogen peroxide.

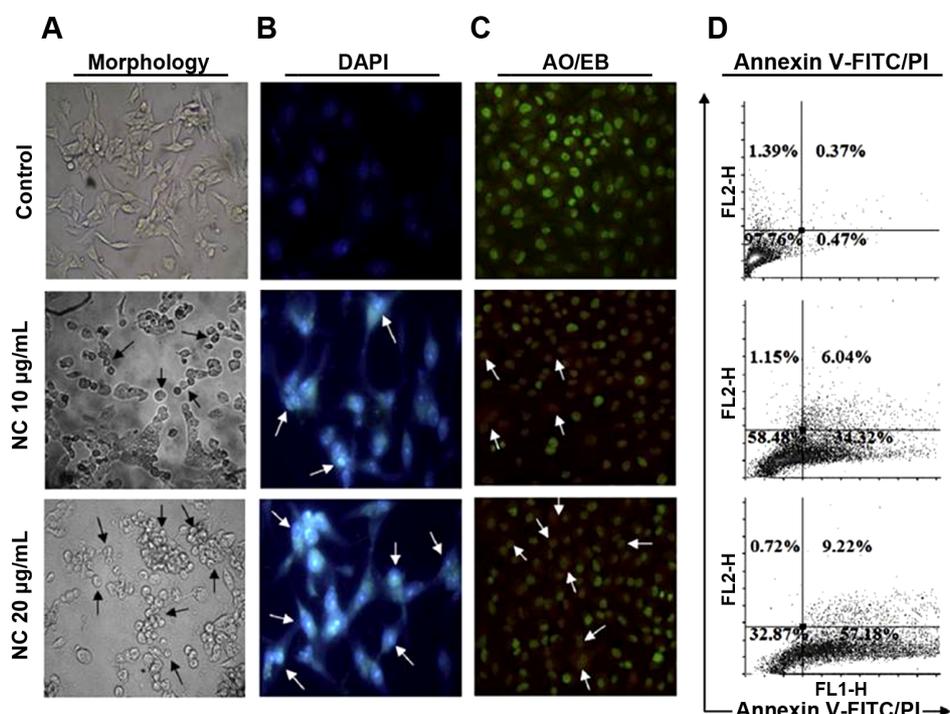
Another double-staining method for apoptosis study is based on the use of the fluorescent dyes acridine orange (AO) and ethidium bromide (EB). By this approach, the treated cells were analyzed by fluorescence microscopy. Nogueira *et al.* [7,20] used this assay to assess the apoptotic and necrotic potential of pH-sensitive polymeric NPs and nanovesicles, respectively, in both tumor and non-tumor cell lines. Moreover, A549 cells were double stained with AO/EB after treatment with plant latex-capped silver NPs, and their apoptotic potential examined under a fluorescence microscope [82].

Western blot analysis was used to determine if exposure to gold NPs induced expression of various proapoptotic-related proteins [33]. By this assay, procaspase-9, one of the late apoptosis-initiating proteins of the intrinsic pathway, was analyzed, along with the effector poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme commonly cleaved as a result of caspase-3 and caspase-7 activation. Alternatively, immunofluorescence detection of activated caspase-3 was performed in order to detect the apoptotic pathway of A549 cells after incubation with engineered cadmium-coated silica NPs [56]. The cell samples were incubated with specific antibodies and were counterstained for DNA with PI, and finally examined under a fluorescence microscope.

Propidium iodide, a fluorescent DNA intercalating agent, has been used to stain and quantify the proportion of cells in the sub-G1 phase of the cell cycle, which is indicative of apoptotic cells. This assay was used by Coulter *et al.* [33] in order to determine the apoptotic potential of gold NPs and thus to confirm the expression of various proapoptotic proteins previously detected by Western blot analysis.

Paul *et al.* [34] used various approaches to determine the apoptotic potential of biodegradable poly(lactide-co-glycolide) (PLGA) NPs. Different methods based on cellular morphology analysis under phase contrast microscopy, nucleosomal fragmentation study with DAPI staining and analysis under fluorescence microscopy, and more specifically on AO/EB and annexin V-FITC/PI double staining, were considered for a detailed characterization of apoptosis (Figure 2). Moreover, change in expression of some key apoptotic genes in both mRNA and protein levels, *i.e.*, p53, Bax, Apaf-1, cytochrome c, caspase-9, caspase-3 and Bcl-2, was determined by immunoblot analysis.

Figure 2. (A) Morphological changes of nano-chelidonines (NCs) (10 and 20 $\mu\text{g/mL}$)-treated HepG2 cells observed by phase contrast microscope; (B) nuclear condensation assessment of control and treated cells by DAPI staining were analyzed through fluorescence microscopy; (C) the increased apoptotic cells were determined by AO/EB staining through fluorescence microscopy. The nuclear condensation and transformation of color green to reddish orange with fragmented nuclear membrane represents the induction of apoptosis in the treated cells with respect to control ones; (D) assessment of cellular apoptosis by externalizing phosphatidyl serine through Annexin V/PI assay by flow-cytometric analysis. Reprinted with permission from [34]. Copyright 2013, Elsevier.



Finally, a cell death detection enzyme-linked immunosorbent assay (ELISA) kit was used to differentiate late apoptosis from necrosis after incubation of J774A.1 mouse macrophages with chromium oxide NPs [83]. The specific enrichment of mono- and oligo-nucleosomes in cell lysates

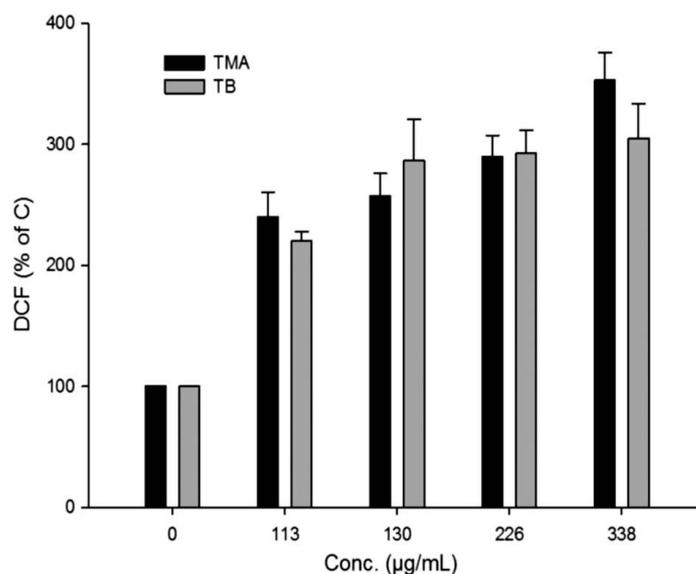
(reflecting the level of late apoptosis), and in culture supernatants (reflecting the level of necrosis), was presented as an enrichment factor for each experimental condition.

2.5. Induction of Oxidative Stress

Nanomaterial-induced reactive oxygen species (ROS) play a key role in cellular and tissue toxicity. The intracellular ROS production by NM can follow two different pathways: direct ROS generation through NM-catalyzed free-radical reactions in cells, or indirect ROS generation through disturbing the inherent biochemical equilibrium in cells [84]. Because of these different mechanisms, some cell-based assays seek to quantify the ROS species production by cells directly, while others seek to quantify its effects on cell behavior or further production of other cell biochemical reactions.

The intracellular generation of ROS after NM exposure was commonly measured by using the fluorescent marker 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). This DCF assay was used to assess the generation of ROS after treatment with different kinds of bovine serum albumin (BSA)-stabilized silica NPs [45] (Figure 3) and of multi-walled carbon nanotubes (MWCNTs) [51], being the cells analyzed by flow cytometry. On the other hand, cell fluorescence was analyzed by fluorimetry to detect ROS production after treatment with silver NPs [53] and CB, single-wall carbon nanotube, SiO₂ and ZnO NPs [19]. Likewise, the derivative fluorescent probe 5-6-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate acetyl ester was applied to measure ROS level after cell incubation with gold NPs [33].

Figure 3. Measurement of ROS production in A549 cells after 24 h NP exposure. The DCF fluorescence of treated cells was normalized to that of untreated controls and reported as mean \pm SD. Reprinted with permission from [45]. Copyright 2013, Elsevier.



Alternatively, ROS production after cell exposure to NMs has been assessed with the fluorescent probe dihydroethidium (DHE). DHE assay is able to detect superoxide radicals. In cells, DHE reacts with superoxide anion to form ethidium, which exhibits red fluorescence. This assay was applied to determine the dependence of oxidative stress on the size of silica NPs [85] and to assess whether local

ROS contribute to impair endothelium-dependent vasodilatation in coronary arterioles after NP inhalation [86].

As a more specific assay to detect intracellular peroxide formation, Shen *et al.* [87] used the MitoSOX Red-based assay to evaluate the mitochondrial superoxide levels as a measure of oxidative stress and intracellular ROS generation. Mitochondria are a major intracellular source of ROS and, thus this assay appears to be more specific and sensitive.

Yang *et al.* [19] also used the intracellular glutathione (GSH), superoxide dismutase (SOD) activity and malondialdehyde (MDA) measurement to indicate the oxidative damage caused by CB, single-wall carbon nanotube, SiO₂ and ZnO NPs. The concentration of intracellular GSH, determined by colorimetric assay, was also used by De Simone *et al.* [56] to evaluate the oxidative stress induced by cadmium-coated silica NPs. Alternatively, GSH depletion induced by silver NPs in HepG2 cells was evaluated through luminescence measurement using a plate reader [88].

In another study, despite the measurement of intracellular GSH, it was performed specific assays to determine the activities of glutathione reductase (GR) and glutathione peroxidase (GPx), two crucial enzymes involved in GSH metabolism [89]. The results of each assay were obtained through spectrophotometric readings.

The measurement of reactive nitrogen species (RNS) have also been used to determine oxidative stress induced by NMs. Roy *et al.* [90] applied this assay to measure the RNS generation after macrophages incubation with ZnO NPs. After a defined incubation time, RNS was measured by using Griess's reagent and absorbance was measured at 540 nm in a plate reader.

Superoxide anion production induced by plant latex-capped silver NPs was evaluated by nitro blue tetrazolium (NBT) reduction method [82]. The results were obtained after absorbance readings at 570 nm.

The reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) has been applied to determine the lipid peroxidation elicited by ROS. It is known that cell surface and organelle membrane lipids may undergo peroxidation in response to oxidative stress [91]. In this line, the oxidative damage induced by nanovesicles containing pH-sensitive surfactants was studied by using this simple approach, being the responses measured using a microplate reader at 532 nm [20]. The measurement of MDA was also used by Tang *et al.* [92] to determine the oxidative stress induced by nanosized titanium dioxide.

2.6. Induction of Injury in Specific Cell Organelles: Mitochondria and Lysosome

2.6.1. Mitochondrial Injury

The mitochondrial injury has been commonly evaluated by the rhodamine 123 (R123) assay, which is based on fluorometric readings of cell responses after incubation with the NMs under test. Sahu *et al.* [53] used this approach to assess the induction of any damage to the mitochondria after cell treatment with silver NPs. The uptake and retention of the fluorescent dye R123 by viable living cells is directly proportional to their mitochondria membrane potential [93] and thus any injury in the mitochondrial compartment will be detected by alteration in this potential.

This same approach was used to assess the effect of TiO₂ NPs on the mitochondrial integrity, the cells being re-suspended in PBS containing 25 mM rhodamine 123 after each NP treatment [37].

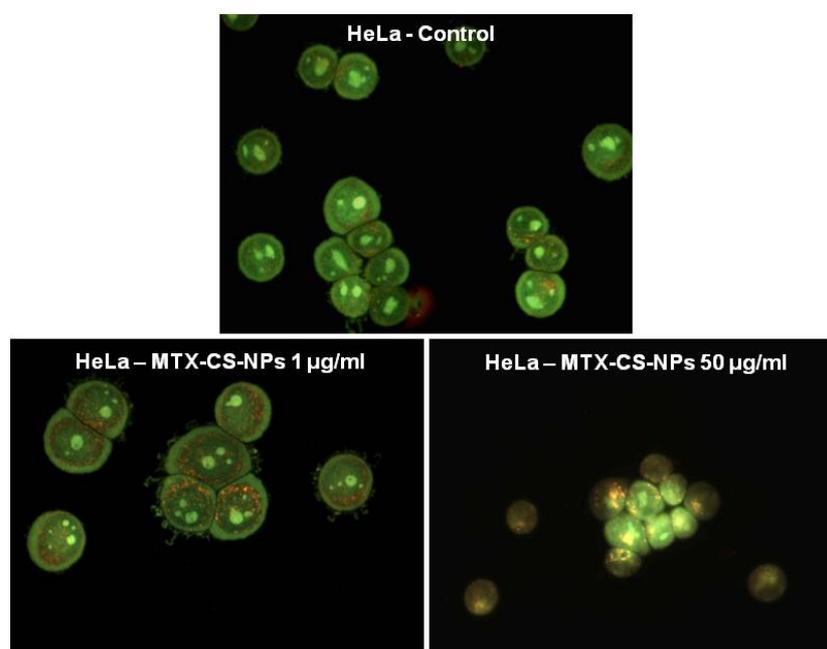
Whereas many authors use the ATP luminescence assay to detect NM cytotoxicity, Tang *et al.* [92] applied this assay as a measure of mitochondrial integrity. Intracellular ATP level is a sensitive readout of the mitochondrial state and was determined using a firefly luciferase-based ATP assay.

Tetramethyl rhodamine ethyl ester (TMRE) assay has been applied to measure the mitochondrial membrane potential (MMP) after NM treatment. Chairuangkitti *et al.* [79] evaluated the changes in the MMP after silver NPs exposure by using the TMRE assay. TMRE is a red–orange fluorescent permeable cationic and lipophilic dye, which can be readily taken up by active mitochondria into the negatively charged mitochondrial matrix. Thus, the intensity of the fluorescent signal obtained is indicative of the MMP. In another study, changes in the MNP, after cell incubation with titanium dioxide NPs, were measured using the lipophilic cationic dye, 3,3'-dihexyloxycarbocyanine (DiOC₆), with cell analysis performed by flow cytometry [94].

2.6.2. Lysosomal Injury

The integrity of the lysosomal membrane after cell treatment with pH-sensitive chitosan NPs encapsulating methotrexate (MTX) has been assessed using the acridine orange (AO) relocation technique [7]. AO is a lysosomotropic base that produces a red fluorescent emission when accumulated in acidic lysosomes, and the disruption of the lysosomal membrane can be assessed by measuring the change in intracellular AO fluorescence (*i.e.*, the loss of the lysosomal red signal and gain of cytoplasmic green) (Figure 4). Following the treatment with NPs, the cells were stained with the dye and analyzed under fluorescence microscopy.

Figure 4. Assessment of the effects of chitosan NPs encapsulating MTX (MTX-CS-NPs) on lysosomal membrane permeabilization in HeLa cells as visualized via AO staining. In untreated control cells, lysosomes can be seen as red–orange granules and cytoplasm has a diffuse green fluorescence. In cells with lysosomal membrane damage (HeLa cells treated with 50 mg/mL MTX-CS-NPs), lysosomes exhibit a shift from red–orange to a yellow–green fluorescent color. Reprinted with permission from [7]. Copyright 2013, Elsevier.



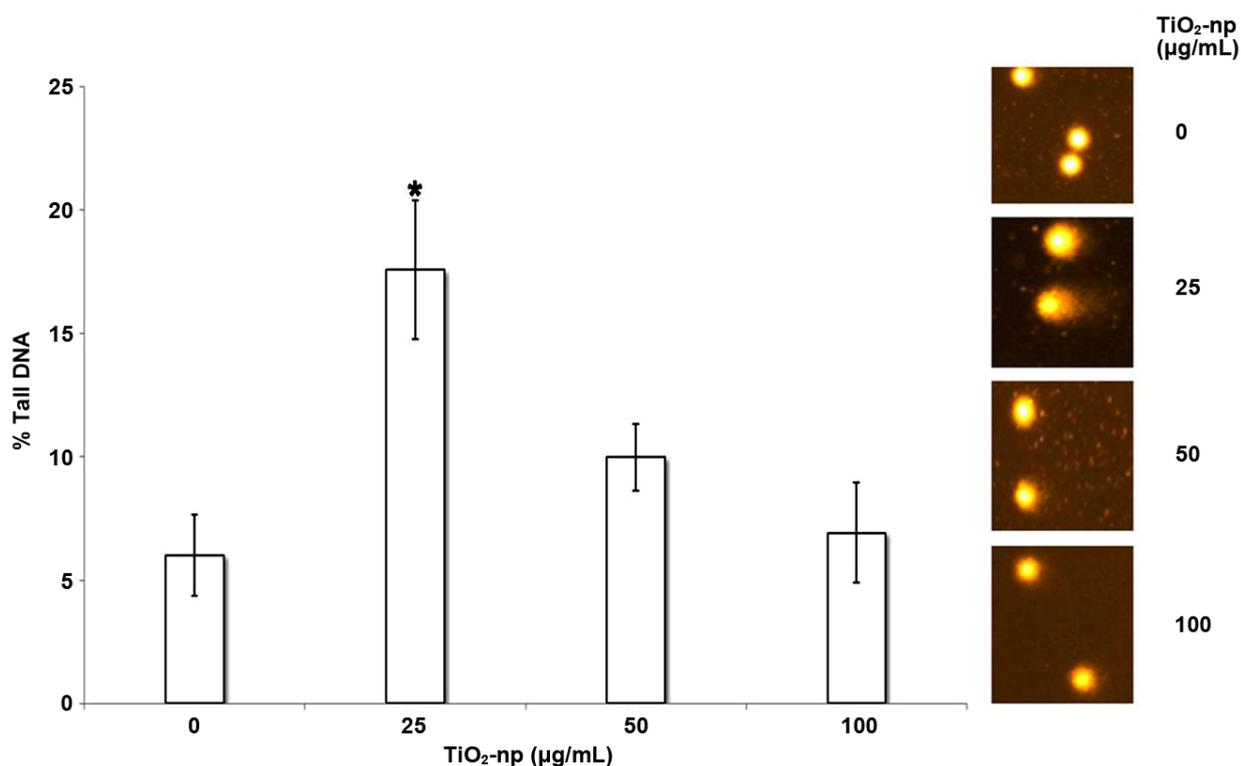
Sohaebuddin *et al.* [95] also applied this same assay to determine the effects of TiO₂ and SiO₂ NPs, and multi-wall carbon nanotubes (MWCNTs) with differing sizes, on lysosomal membrane integrity of three established cell lines. In another study, the effect of the polystyrene nanosphere on lysosomal stability was determined by using AO staining, but with cell analysis performed by flow cytometry [41].

Alternatively, Fröhlich *et al.* [96] used lucifer yellow to assess the lysosomal integrity after cell exposure to polystyrene NPs of different sizes, the results being obtained after analysis by confocal laser scanning microscopy. Moreover, the authors used an acidotropic probe that accumulates in acidic compartments of cells as a result of protonization to assess the lysosome function/pH. Accumulation in the acidic environment of lysosomes results in a pH-dependent increase in fluorescence, which was quantified by confocal laser scanning microscopy. Finally, the lysosome function was also assessed by measuring the activity of lysosomal enzymes cathepsin B and sulfatases through fluorometry.

2.7. Induction of DNA Damage and Genotoxicity

The alkaline single-cell gel electrophoresis (comet) assay has been used to study DNA strand breaks and alkaline labile sites in different cell lines after NM treatment. This assay was performed to assess the genotoxicity potential of different types of NMs, including CB, single-wall carbon nanotube, SiO₂ and ZnO NPs [19]. Moreover, Khatri *et al.* [80] used this assay to assess the DNA damage prompted by photocopiers emitting NPs, while Ghosh *et al.* [37] monitored the DNA damage in human lymphocytes incubated with TiO₂ NPs (Figure 5), and Nogueira *et al.* [20] assessed the genotoxic potential of pH-sensitive lipid-based nanovesicles.

Figure 5. Comet data (% tail DNA) of human lymphocytes treated with different concentrations of titanium dioxide (TiO₂) nanoparticle; * $P < 0.05$. Reprinted with permission from [37]. Copyright 2013, John Wiley and Sons.



Cellular DNA damage induced by silver NPs was determined fluorometrically by the cellular double-stranded DNA (ds-DNA) content. In this assay, the nonfluorescent dye Hoechst 33258 becomes highly fluorescent when it binds to the ds-DNA, the ds-DNA content being determined from a standard plot [53].

Genotoxicity of PVP-coated silver NPs in BEAS 2B cells was assessed by three different approaches: the alkaline comet assay, the chromosomal aberration (CA) assay, and the cytokinesis-block micronucleus (CBMN) assay [48], which can be used to study chromosomal damage that occurs due to exposure to toxic agents.

The *in vitro* DNA damage induction by short multi- and single-wall carbon nanotubes was assessed by comet assay, by the CBMN assay and also by using the immunoslot blot assay for the detection of malondialdehyde (M1dG) DNA adducts [97].

Finally, detection of genotoxicity induced by mesoporous silicon NPs was made using the BrdU (5-bromo-20-deoxyuridine) ELISA-based assay [98]. The results were obtained after absorbance measuring using a microplate reader at a wavelength of 450 nm.

2.8. Inflammatory Responses

Enzyme-linked immunosorbent assay (ELISA) is the main method used to measure the inflammatory responses of different kinds of NMs. The conditioned medium of HSCs cells treated with silver NPs were analyzed using ELISA for the presence of different mediators, such as hepatocyte growth factor (HGF), interleukin (IL)-6, transforming growth factor (TGF)- β 1, tumor necrosis factor (TNF)- α , matrix metalloproteinase (MMP)-2 and MMP-9 [46]. An ELISA kit was also used to quantify the levels of the proinflammatory IL-8 after cell treatment with ZnO NPs [99].

The measurement of enhancement in secretions of cytokines, in particular proinflammatory cytokines, following the incubation of cells with NPs coated with either non-ionic poly(ethylene glycol) or zwitterionic poly(carboxybetaine), was performed by using a multiplex assay that monitor the expression of several cytokines, including interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (P40), IL-12 (P70), IL-13, IL-17, granulocyte-colony-stimulating factor(G-CSF), granulocyte macrophage-colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) [100]. Likewise, a multiplex assay was used to assess the release of 13 cytokines (IL-8, G-CSF, IL-1 α , IL-1 β , IL-6, IFN γ , and others) after THP-1 cells treatment with photocopiers emitting NPs [80].

In another study, cytokines such as IL-12, TNF- α , IL-10, IL-6, IL-1 β and IL-8 were measured by a cytometric beads array set system, the samples being incubated with antibodies for fluorescence detection and analyzed by flow cytometry [51]. This assay was applied to assess the inflammatory response induced by multi-walled carbon nanotubes. Likewise, Roy *et al.* [90] used this approach to measure IL-6, IL-10, IL-17, TNF- α and IFN- γ release after cell treatment with ZnO NPs. Moreover, these same authors also examined the role of proteins opsonization of ZnO NPs with IgG in phagocytosis. With this intend, fetal bovine serum (FBS) opsonized particles (ZnO NPs disperse in 10% FBS-supplemented RPMI (Roswell Park Memorial Institute) media) were incubated with IgG and its binding was measured by ELISA.

Lastly, elastase release assay was also used to measure inflammation because activated neutrophils degranulate to release inflammatory mediators such as elastase. This assay was applied to evaluate the response of human neutrophil after exposure to cationic surfactants in the form of NPs and micelles [50].

2.9. Interactions with Blood Components

Hemolysis and agglutination assays using EDTA-stabilized rat blood were performed to determine the hemocompatibility of lipid nanovesicles modified with cationic lysine-based pH-sensitive surfactants [20]. For the typical hemolysis assay, the samples were incubated with erythrocyte suspension, the measure of cell damage and hemoglobin release having been performed by spectrophotometry. On the other hand, the agglutination was determined by phase contrast microscopy. In this same study, the authors also analyzed the adsorption of human plasma proteins to the surface of the nanocarriers. For this purpose, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed. These same hemolysis and agglutination assays were also used by the authors to determine the blood compatibility of chitosan NPs modified with a pH-sensitive lysine-based anionic surfactant [7].

Hemolysis assay was also employed to evaluate the *in vitro* blood compatibility of GO and GS intended for biomedical applications, using fresh ethylenediaminetetraacetic acid (EDTA)-stabilized human whole blood samples [38]. Recently, Shahbazi *et al.* [98] performed the hemolysis assay using human erythrocytes to study the hemocompatibility of mesoporous silicon NPs. Moreover, to evaluate the morphological changes and also the NP–erythrocyte interactions, the diluted erythrocyte suspension were evaluated by scanning electron microscopy (SEM) after incubation with NPs.

An extended study on the effects of TiO₂ NPs on peripheral blood has been performed by Ghosh *et al.* [37]. Human peripheral blood cells were incubated with different concentrations of NPs and blood count tests were performed using a hematology analyzer to determine, among other parameters, the white and red blood cell count, hemoglobin, hematocrit value, platelet count, neutrophils, lymphocyte, monocyte, eosinophil, basophil and reticulocytes.

It is known that NPs are subject to the inspection of the immune system and, the complement system is a rapid-acting host defense mechanism that protects the intravascular space and other biological compartments from foreign attackers [101]. In this context, Pham *et al.* [102] employed a modified *in vitro* hemolysis-based assay to examine the variations in NP-mediated complement activity between individuals. The assay was applied to perfluorocarbon NPs of varying size, charge and surface chemistry, and might provide the tools for an in-depth structure–activity relationship study.

Hyperbranched polyglycerol hybrid nanostructures were tested for their probable effects on blood coagulation time by using Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) tests, the blood biocompatibility of NMs being measured by means of a coagulation analyzer with mechanical end point determination [103]. Moreover, the complement activation was evaluated using a commercial single radial immuno-diffusion (SRID) immunoassay kit, which monitors the cleavage of complement components C3 and C4.

3. Nanomaterials Characterization Needed for Reliable Nanotoxicological Assessment

In vitro nanotoxicology has many advantages (as already described in Section 2), but also presents special problems that are directly related to the specific characteristics and physicochemical properties of NMs. Not everything called *nano* is actually nano, because secondary structures with significantly higher sizes are formed due to NP agglomeration or aggregation in aqueous media [104]. Nanoparticle size is a significant parameter to evaluate, not only in determining the release profile and degradation manners, but also in determining the efficacy of the therapeutic agent in terms of tissue penetration and cellular uptake [105]. This is only one example that highlights the importance of a detailed physicochemical characterization before the performance of any *in vitro* study. Structural alterations of NPs in aqueous solutions, *i.e.*, in cell-culture medium, might also affect and change the final results of the *in vitro* toxicological studies. Likewise, the surface charge of NPs plays an especially important role in cell–NP interactions because cell membranes themselves are charged [38]. Understanding the behavior of NMs at the time of toxicological assay may play a crucial role in the interpretation of its results [106]. Finally, the results obtained from studies in which the NM characterization was not properly performed might be very controversial, making difficult the gain of conclusive data and the elucidation of the main mechanisms underlying toxicity.

Nanoparticles with similar chemical compositions may have totally different sizes, shapes, crystal structures, surface coatings, and surface reactivity characteristics, thereby creating a quite difficult situation for analysis by chemists, medical scientists and toxicologists [10]. Therefore, a detailed and comprehensive physicochemical characterization of the test NMs is stated as the first step before any toxicological screening [2,15].

A testing system to assess NM toxicity has been suggested [23,29]. Different stages are described, including an emphasis on detailed physicochemical characterization prior to and during subsequent testing in cell-free, *in vitro* cell-based and *in vivo* assays. The careful evaluation of NM solubility, chem-reactivity, size, hydrodynamic diameter, agglomeration/aggregation, zeta potential and polydispersity is recommended.

The unique physicochemical properties of NMs might represent major problems during *in vitro* assays. Even the utilization of well-established *in vitro* models can lead to false-positive or false-negative results, as well confounding or conflicting data. Furthermore, the properties of NMs, such as nanosize, high absorption capacity, catalytic activity, magnetic properties, dissolution and alkalinity/acidity, might introduce substances into cytotoxicity studies, which could interfere with assay components or detection systems [107]. Taken together, these unique characteristics of NMs must be considered for their *in vitro* toxicological evaluation. Because of the predicted variability and inconclusive data, a combination of different techniques and endpoint assays might be required in order to achieve more reliable data about NM risk assessment.

A suitable characterization of NMs, either in the original dispersion or when suspended in cell culture media, is needed prior to any *in vitro* assay in order to achieve detailed knowledge concerning general and specific bio–nano interactions. Size characterization can be performed by scanning electron microscopy (SEM) [108,109] or transmission electron microscopy (TEM) [31,48,110]. Dynamic light scattering (DLS) is widely used to determine the hydrodynamic diameter and the polydispersity of NM suspensions [20,111]. Other less conventional methods have also been applied to

size characterization of NMs. Helfrich *et al.* [112] developed a reliable method for the size characterization of gold NPs by using liquid chromatography coupled on-line to inductively coupled plasma–mass spectrometry (ICP-MS). Surface morphology and particle size of the NPs can be also determined by atomic force microscopy (AFM), as described by Paul *et al.* [34] to characterize PLGA NPs. Liao *et al.* [38] also used AFM to measure the particle size of graphene sheets (GS) and graphene oxide (GO).

Izak-Nau *et al.* [106] performed a detailed characterization of silica NPs by assessing their colloidal stability in water, standard biological buffers, and cell culture media containing either bovine or human sera. Techniques such as DLS, zeta potential measurements and TEM have also been used. Moreover, interactions of the particles with biological media were investigated by SDS-PAGE in bovine and human sera, and extracted proteins were assessed using matrix-assisted laser desorption/ionization-time of flight technique (MALDI-TOF). The authors demonstrated that the NPs tended to agglomerate, and that this phenomenon depended on NP functionalization as well as on their concentration and the incubation time. Altogether, the results of this study showed that the surface charge, ionic strength and biological molecules alter the properties of NPs and potentially affect their resulting biological effects.

For the polymeric NPs, Fourier transform infrared (FTIR) spectroscopic studies to ascertain whether the encapsulation procedure altered the characteristics of polymer or drug, and whether interactions occur between the drug and the polymer after encapsulation is very commonly applied. Furthermore, FTIR data also speak about whether appropriate polymerization had occurred or whether monomers were present in the physical mixture. Among many authors, Paul *et al.* [34] characterized chelidone-loaded PLGA NPs by using this analytical approach. Likewise, attenuated total reflectance-FTIR spectroscopy has been applied to evaluate the attachment of a reducing and stabilizing agent to silver NPs [98]. Moreover, in this same study, the kinetics of thermal decomposition of the nanocomposites was investigated using different heating rate thermo-gravimetric analysis.

More specifically, information about the molecular structure and chemical speciation can be obtained using techniques such as synchrotron radiation circular dichroism spectroscopy (SRCD) and X-ray absorption fine structure (XAFS), respectively [10]. X-ray photoelectron spectroscopy (XPS) was also used to measure the chemical composition of NMs such as GO and GS [38]. Moreover, Raman spectroscopy is a noninvasive technique that can be applied for the characterization of structural and electronic properties of NMs [113,114].

Information concerning the specific surface area per mass unit ($\text{m}^2 \cdot \text{g}^{-1}$) has been obtained by means of Brunauer, Emmet and Teller (BET) analysis (adsorption of nitrogen in cryogenic condition). The surface area is an important type of metric to be considered during toxicological studies and, thus, the BET analysis has been applied to characterize NMs such as silica NPs [115], nanoclusters of poorly soluble drugs [116], and calcium carbonate NPs [117].

4. Relationships between Cell Internalization of Nanomaterials and Their Toxicological Responses

The varied NM–cellular localization and interaction might lead to varied modes of toxicity. Even NPs of the same material can show completely different intracellular behavior due to, for example,

slight differences in surface coating, charge and size. It has been reported that especially the NM size determines the efficiency of cellular uptake and subsequent intracellular processing [118].

Cellular uptake and localization of NPs will almost certainly be different than internalization of molecular species, and this fact will likely lead to different modes of toxicity. While there are some examples in the literature exploring NP uptake and localization, this is currently far from being well understood and, evidently varies significantly based on the NP being used. In order to obtain more reliable data concerning cellular uptake and localization, a number of imaging methods are available to greatly advance the understanding of NM behavior in the cellular compartments.

Not only the cell internalization, but also the disruption of the NM inside the cell greatly influences the resulting toxic effects. The cytotoxicity could be attributed to the chemical composition of the nanostructure, but it should be considered that the NM destabilization results in release of its content into intracellular compartments, which can present synergic cytotoxic effects. This cytotoxicity can be desired, *i.e.*, in antitumor treatments [7,119,120], or undesired, *i.e.*, during NM application for diagnostics purposes [121,122].

The internalization efficiency of NMs is commonly assessed by using flow cytometry and/or fluorescent microscopy. For this purpose, a range of different dyes have been using in order to control and/or visualize the intracellular trafficking and final localization of NPs. Rhodamine [123], FITC [41] and acridine orange [124] are molecules usually used as fluorescent markers in this kind of study. Considering the drugs with autofluorescent behavior, doxorubicin is the most commonly used as model molecule [125,126].

When the NM is designed for an intended release of its content inside the cell in order to achieve therapeutic effects, one common strategy is to exploit the intracellular pH gradients [127]. Fluorescent dyes and drugs are frequently used as markers for cell uptake and intracellular behavior studies. Among fluorescent dyes, calcein has been widely applied as a tracer molecule, which is internalized by the cell through endocytosis and is thus used to monitor the stability of endosomes following NM uptake [7,20].

Cellular visualization of NPs can be assessed through analytical approaches such as fluorescence microscopy, flow cytometry, confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), soft X-ray scanning transmission microscopy (STXM), X-ray fluorescence (μ -XRF) and X-ray absorption near edge structure (μ -XANES) [10]. Additionally, Ng *et al.* [128] demonstrated the applicability of Energy-Dispersive X-ray (EDX) spectroscopy to reveal cellular uptake of gold NPs and their aggregate forms. Likewise, inductively coupled optical emission spectrometry (ICP-OES) was used to determine the cellular uptake of cadmium NPs in renal cells [36]. Moreover, inductively coupled plasma–mass spectrometer (ICP-MS) has been applied to measure the intracellular content of silver NPs [88].

5. Conclusions

Nanotechnology is considered a technology of the future, with especially important potential applications in the biomedical sciences. Nanotechnology, nanomedicine and nanotoxicology are complementary fields that in conjunction aim to improve human life. The discipline of nanotoxicology aims to study how properties of NMs define their interactions with cells, tissues and organs in exposed

humans. In this context, understanding NM behavior in live biological systems is of increasing importance for determining their potential toxicity.

The need for more toxicological information concerning the risks of exposure to nano-based structures is reflected in the growing number of studies leaning toward the evaluation of NM toxicity and elucidation of the mechanisms underlying these hazardous effects to environmental and human health. The *in vitro* cell-based approaches are more attractive for NM testing, especially due to ethical aspects and the expense of animal testing. Therefore, relevant *in vitro* toxicological models based on established cell lines have become the basis of a highly needed screening approach and high-throughput toxicity testing protocols essential for the preliminary risk assessment of NMs. In line with the *in vitro* toxicology, it is worth mentioning that all toxicity data must be interpreted in the context of the physicochemical characteristics of the nano-sized materials, in order to assure the reliability of the obtained data, and also to possibly establish a structure–toxicity relationship.

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Author contributions

The authors have made equal contributions in the preparation of this review.

Conflicts of Interest

The authors declare no conflict of interest.

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