Chapter

How to Assess Nanomaterial Toxicity? An Environmental and Human Health Approach

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Abstract

Nanomaterials had been used because of the properties they exert in such scale (<100 nm), and they have been used in a wide variety of products like paints, electronics, fabrics, and also personal care products. Recent manuscripts available in the literature demonstrate the potential benefits of nanotechnology with these products. However, when released in the environment or when in contact with the human body, it is hard to track their final destination and their influence over the living beings. So, since nanomaterials were considered an important technology, a concern about its risks also started. Due to the variety of sizes, physicochemical properties, and uses, many researchers are aiming to assess the possible toxicity of this class of particles. Because of that, the chapter objective is to gather which assay, performed in vivo and in vitro, is the most frequently used and recommended to measure nanomaterial toxicity. Also, it is important to know which is the most suitable test to evaluate the toxicity over the environment, through direct effect and after biodegradation, and also related to human health. This chapter presents a concise review about the accepted methods to assess nanomaterial toxicity and also discuss about the need for regulamentation.

Keywords: toxicity assays, test organisms, in vitro models, in vivo assays, nanoscale assessment

1. Introduction

Emerging and promising nanotechnology represents a field of multidisciplinary knowledge responsible for development and application of materials, which measure less than 100 nm [1, 2]. The Royal Society and Royal Engineering Academy proposed this concept in 2004, which was associated to nanoscience as the branch responsible for studying the phenomenon of materials with atomic, molecular, and macromolecular scales, whose properties differ significantly from those with major scales [3, 4].

Nanoparticles can be generally described as ultrafine small material with 1–100 nm; however, several types of systems not limited only by small particles of certain material are included in this definition, as nanotubes, nanospheres, and nanocapsules [4, 5]. The properties exhibited by nanomaterials are unique and are being applied in many fields, from industrial to medicine [6, 7]. According to Arora et al. [8], the use of nanomaterials is increasing for commercial purposes as fillers,
opacifiers, water filtration agents, cosmetic ingredients, semiconductors, electronic parts, and others. However, these same authors report that nanomaterials are being used in the medical area, mainly as agents for drug delivery, biosensors, and imaging contrast, i.e., human contact can happen both indirectly and directly, also being administered by ingestion or injection [8]. Once nanomaterials are used, environmental releasing turns dependent on the incorporation form of this product in each matrix, intrinsic material properties and also environmental conditions [9]. When there is human exposure or direct intake of nanomaterials, nanoparticles’ physicochemical properties and its possible modifications can influence absorption, distribution, and organism metabolism. Besides the potential to accumulate in some organs, relevant rates of nanomaterials are excreted, being released to the environment [10]. About the nanomaterials presence in the environment, a detailed description regarding its sources and fates can be found in the review of Part [11].

Due to the new scale of some materials, new physicochemical interactions may occur bringing unexpected and also adverse effects because these elements generally become highly reactive [12]. Physicochemical properties observed in engineered nanomaterials are attributed to small size, chemical composition (purity, crystallinity, electronics characteristics, etc.), structural surface (reactivity, organic or inorganic coating, etc.), solubility, form, and agglomeration potential [8].

In view of the properties that the nanomaterials present, studies that evaluate the toxicity, their behavior in different environments, and the interactions with the biological system are of extreme importance. According to Dusinska [6], the safety assessment of nanomaterials is based on principles of risk assessment of “bulk” chemical substances. However, it is known that the behavior of these materials, both in the environment and in the cells, is different from such crude samples, and therefore the monitoring needs to be more specific. Catalán et al. [13] emphasize that the damaging potential of biodurable nanomaterials is not well demonstrated, and thus the classical toxicity evaluation trials must undergo adaptations.

2. Brief history of nanotoxicology

According to Maynard et al. [14], until the 1990s, many studies that focused on environmental epidemiology indicated a relationship between exposure to aerosols and increased mortality and morbidity of organisms. The relationships between particle size, chemical nature, and toxic effects were demonstrated, with the most pronounced effects observed in the lungs and heart due to exposure to smaller particles. These same authors argue that only in this decade has there been evidence that environmental particles with a diameter of less than 2.5 μm could cause deleterious health effects due to their reduced size [14]. Now it is known that engineered nanoparticles can perform these same activities [12].

Since the inception of this science, the studies and applications of nanoparticles have grown exponentially and, to the same extent, heightened concerns about environmental and health implications. In this context, the term nanotoxicology was formalized by a proposal of Donaldson et al. in 2004 [15] in an editorial in the journal Occupational and Environmental Medicine [5] and, since then, has been used to describe specifically the harmful effects of nanomaterials on environmental, animal, and human health. In 2005, nanotoxicology was consolidated as an area of expertise, with the launch of the journal Nanotoxicology, with the first article published by Oberdörster et al. in 2007 [16]. This article discusses the history of nanotoxicology as a science and presents some challenges to be faced by researchers.

Considering that nanoparticles have a greater potential to travel through the body than conventional-sized materials, researchers warn of the possibility of
numerous interactions with biological fluids, cells, and tissues. Therefore, in vitro tests are recommended for an initial evaluation of the cytotoxicity and genotoxicity of nanomaterials, as well as for the identification and understanding of cellular mechanisms of toxicity [3]. In vivo methods are also used and, for both, some methods have already been developed by the Organization for Economic Co-operation and Development (OECD) and can be used for regulatory purposes.

3. Toxicological aspects of nanomaterials

According to Paschoalino et al. [3], the growing investment in nanoscience boosted the world market, as well as increased the use and consumption of products and processes aimed at this area. Despite this, it is true that research aimed at evaluating the toxicity of nanomaterials is still necessary, since the same properties that make nanomaterials so attractive may also be responsible for harmful effects on living organisms.

In this context, there is a recommendation for the analysis of physicochemical properties of nanomaterials in relation to human health and environmental safety (Table 1). In 2006, the OECD established a working party on manufactured nanomaterials to determine the appropriate methods for evaluating nanomaterials. According to the guidance manual developed, 26 physicochemical properties should be considered [6]. However, according to these same authors, only a few

| Property                                      | Relevance          |
|----------------------------------------------|--------------------|
| Particle size distribution                   | Essential          |
| Degree/state of agglomeration                | Important          |
| Particle shape                               | Important          |
| Chemical composition/purity                  | Essential          |
| Solubility                                   | Essential (if applicable) |
| Physical properties                          |                     |
| Density                                      | Matrix dependent    |
| Crystallinity                                | Matrix dependent    |
| Microstructure                               | Matrix dependent    |
| Optical and electronic properties            | Matrix dependent    |
| Bulk powder properties (important for dosimetry/exposure) | Matrix dependent    |
| Concentration (can be measured as mass, surface area, or number concentrations) | Essential |
| Surface properties                           |                     |
| Specific surface area/porosity               | Essential          |
| Surface chemistry/reactivity                 | Essential          |
| Surface adsorbed species                     | Important          |
| Surface charge/Zeta potential (especially in aqueous biological environment— may change according the environment) | Important |
| Surface hydrophobicity                       | Essential          |

*Adapted from Powers et al. [17]*

**Table 1.** Properties used for nanomaterial characterization regarding toxicity evaluation.
methods are available for the characterization of the toxicological properties of the nanomaterials, and the association of the effects with the physicochemical characterization is still a challenge.

Nanomaterials encompass a broad spectrum of materials with different physical, chemical, and biological properties. Thus, they do not constitute a homogeneous group and are usually defined by the type of core, which may be organic, such as fullerenes (carbon derivatives) and carbon nanotubes (single and/or multilayer), or inorganic, such as those of metal oxides (iron, zinc, titanium, etc.), metals (mainly gold and silver), and quantum dots [4].

According to Ju-Nam and Lead [4], some nanomaterials can have their surfaces manipulated in order to introduce specific functionalities for new applications. Thus, a vast field of possibilities opens up for materials with different properties and, therefore, also for infinite interactions with organisms and environment. However, the major challenge of nanotoxicology is to understand and prevent the risk of the use and/or exposure to nanomaterials that can cause toxicity by mechanisms not yet known or not yet explained by traditional toxicology [5].

Concern about the toxicity of nanomaterials lies primarily in production and commercialization on such a large scale as at present. Thus, the risk of these compounds reaching the different environmental compartments (atmosphere, water, and soil), becoming bioavailable, is very large [3, 17].

Since 2005, the European Commission Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has published reports on the impacts of nanoparticles on human health. The aforementioned committee focused its efforts on the evaluation of nanoparticles physically capable of entering the human body via inhalation, ingestion, and dermal absorption and reported that the size, shape, surface area, and chemical composition of the nanoparticle are closely associated with its toxicity. In addition, it has been explored how these characteristics affect bioavailability and interactions, as well as influence on exposure and dose. Therefore, the dose, the physicochemical properties, and the biokinetics are also important parameters to be evaluated when considering the toxicology of nanomaterials [14].

4. Deposition and interactions of nanomaterials with cells and the environment

In view of the numerous properties and characteristics of nanomaterials, products that are increasingly light, resistant, and often of lower cost are daily produced and marketed by the most different segments, such as electronic, medical, pharmaceutical, cosmetic, food, and agricultural [3]. In this context, when considering the ecotoxicology of particles whose components are nontoxic in the micro- or macro-metric scales, studies that elucidate the routes of exposure and effects of nanomaterials on environmental compartments and different organisms are fundamental.

According to Laux et al. [7], the entry of nanomaterials into the environment occurs by the release of their components during use and by final disposal, so it is important to track and understand the kinetics and transformation of these materials in organisms and the environment. Knowledge of the influence of biopersistence on biokinetics and environmental fate is of utmost importance when determining the toxic potential.

When a nanomaterial comes in contact with the human body or the environment, it is difficult to track it again. In the environment, some nanomaterials such as metallic (e.g., Ag and Cu) and metal oxides (e.g., ZnO and Fe₂O₃) can dissolve rapidly, while others are more persistent (e.g., TiO₂, SiO₂, carbon nanotubes, and
graphene) [18]. However, according to these authors, soluble nanomaterials present the best scenario of toxicity evaluation, since their behavior is generally similar to that presented by their ions. However, when cells internalize them, they can solubilize and release toxic metals through a mechanism known as “Trojan Horse” [18].

The aquatic ecosystem is the main route of exposure to a nanomaterial, since this type of environment is usually the final destination of nanocomposites introduced in natural systems [19]. After the aquatic environment, the atmosphere (troposphere), soil, and sediment follow an order of priority as routes of exposure. When present in aquatic environments, nanomaterials can be absorbed by cells, especially during filtration by aquatic organisms, directly interfering with their physiology and/or their ability to feed and breathe [3]. According to these same authors, other pathways of entry of nanomaterials into receptor organisms occur through cellular uptake, inhalation, or ingestion (Figure 1).

According to Bhaskar et al. [20] and Dusinska et al. [6], nanomaterials can enter the cells actively or passively, overcoming any protective barrier of the organism, including the blood-brain barrier. The capture mechanisms are related to the intrinsic physicochemical characteristics of the nanomaterial, as well as its route of exposure. Considering human health, generally the main route of exposure is inhalation, in which smaller particles reaching the alveoli and depending on their physicochemical properties cross the blood-air barrier of the lungs and reach the liver, heart, spleen, and kidneys [7]. There is a challenge when it comes to nanoparticles developed to cross human body barriers, such as those applied in medicine. There are materials that are developed to pass through barriers, not to enter cells, while there are others that are designed to act within them [10].

The first contact of the cell with any extracellular material occurs through the lipid (e.g., phospholipid) and protein components (e.g., membrane receptors) present in the cell membrane. For Paschoalino et al. [3], the nanomaterials present greater permeability to the skin, mucous membranes, and cell membranes due to their diminutive size.

Figure 1. Main sources, routes of exposure, and possible interactions between nanoparticles with the environment and organisms (adapted from Paschoalino et al. [3]).
Conner and Schmid [21] stated that most nanomaterials are actively incorporated by cells through endocytosis. This is one of the most important mechanisms of cellular communication with the external environment, since it involves the transmembrane and bidirectional flow of vesicles, through the movement of extracellular content internalization [1]. According to Radaic et al. [1], the shape, size, characteristics (such as porosity) of the surface, surface charge, and composition of nanoparticles directly influence endocytosis. According to Drasler et al. [22], cell size, proliferation rate, and surface receptor growth and expression characteristics are the major factors involved in the entry of nanomaterials into cells. Generally, endocytic pathways are essential in this process, where large particles or agglomerates of nanomaterials are obtained by phagocytosis (diameter greater than 250 nm), whereas smaller particles (diameter smaller than 150 nm) are obtained by pinocytosis, specific or not. Valsami-Jones and Lynch [18] have beautifully illustrated the possible mechanisms of nanomaterial uptake by cells.

Collins et al. [23] have described that besides penetrating cells, many nanomaterials are able to cross nuclear membranes and gain access to chromatin at any stage of the cell cycle. Thus, in addition to direct damage to DNA, nanoparticles can induce the formation of reactive oxygen species (ROS), such as hydroxyl radicals (·OH), causing oxidative stress (redox imbalance) and serious damage to the cell. Oxidative stress can be the result of the simple cellular response to the presence of the nanomaterial or a secondary effect of the inflammation generated by them [23]. It is also known that the dissolution of certain nanomaterials may be able to release toxic ions and/or other components, which may induce toxicity [22].

ROS are highly reactive molecules that disrupt intracellular medium homeostasis, since they interact with cellular macromolecules such as DNA, proteins, and lipids [24]. Singh et al. [24], Louro et al. [25], and Radaic et al. [1] stated that nanomaterials can induce genotoxic damage mediated by oxidative stress and through their interaction with cellular constituents, including mitochondria and NADPH oxidases bound to the cell membrane; by the depletion of antioxidants; or by the release of the metallic ions present in the constitution of many nanomaterials, which can promote the conversion of cellular oxygen metabolites into ROS. When considering the DNA molecule, the major damage induced by ROS is single-strand breaks, double-strand breaks, base modifications (such as the formation of 8-hydroxydeoxyguanosine adducts), and DNA cross-links. According to Singh et al. [24], all of the aforementioned damages have the potential to initiate and promote carcinogenesis. Once the DNA molecule has been damaged, several cellular processes can be triggered, such as cell cycle arrest, apoptosis, or DNA repair [24].

Apart from the oxidative stress-related lesions, other genotoxic effects may contribute significantly to the promotion of genetic instability, as nanomaterials can cross the pores of the nuclear envelope and interact directly with the genome of the cell and/or with nuclear proteins [25]. Under these conditions, Louro et al. [25] reported that some nanomaterials induce the formation of intranuclear protein aggregates, inhibiting the processes of cell replication, transcription, and proliferation. When the nanomaterials are not able to cross the nuclear envelope, there is still the possibility of interaction with the DNA molecule and nuclear proteins during the mitotic process, which can cause aneuploidies [24, 25].

Depending on the organism exposed to the nanomaterials, different interactions can be evidenced. Bielmeyer-Fraser et al. [26] demonstrated that nanoparticles of ZnO, AgO, and CuO were able to induce toxicity to algae bioindicator in a similar way to the respective solubilized metals. However, these researchers noted the metals accumulated in different regions of the cells, and the nanoparticles were retained mainly in the cell wall, while the metals were observed mainly in the organelles, as fragments of the endoplasmic reticulum. However, in both ways, the
authors point out that the accumulated metal could be transferred by the trophic chain and carried to other organisms.

When a nanomaterial enters a living organism, several components can adhere to its surface, drastically modifying its interaction with cellular structures. Proteins are molecules that can adhere to the nanomaterial and form a type of coating, called biomolecular corona [27]. This corona may alter the ability of a nanomaterial to cross physiological barriers, influencing its toxic potential [7].

According to Drasler et al. [22], in vitro assays performed with cell culture can indicate the biological fate of nanomaterials at the cellular and multicellular level, even in an excluding mode according to the cell type (i.e., to determine which cell type is actually affected by a certain type of nanomaterial).

5. Methods for evaluating the toxicity of nanomaterials

Within nanotoxicology there is an impasse on how best to assess the possible adverse effects of nanomaterials, both for human health and environmental monitoring. Toxicity tests may be performed employing live (in vivo) organisms, such as microcrustaceans, fishes, rodents, and other animals and/or cell cultures (in vitro). Several standardized toxicological tests are available to measure the biological response of an organism to a chemical. However, there is no standardization for the evaluation of the toxicity of nanomaterials, which hampers the comparison of results and the consensus about their toxicity. Most of the studies performed so far are adaptations of the standard procedures used for other substances [3]. Although some minimal combinations of assays have been proposed, Drasler et al. [22] have described that there is no standard evaluation protocol due to the wide range of physicochemical properties that nanomaterials can present.

Animal tests are more predictive for human effects but have limitations, mainly because of physiological and biochemical differences between the species. In addition, there is a growing public and legal demand that ethically supports the substitution of animal testing for alternatives not based on in vivo testing. New concepts of experimentation have been based on strategies with primary culture of human cells and permanent cultures of well-established cell lines, since they present efficient, cheap, and reliable results [22].

Understanding the demand for orientation and applicability, this chapter will address some of the main evaluation methods, developed both in vivo and in vitro, to better characterize the toxicity of nanomaterials.

5.1 In vivo methods

In vitro evaluations have increased considerably, but in vivo validation still is necessary to understand and interpret its results. Furthermore, animal experimentation was also part of the NanoTEST project, whose purpose was to understand the effects on the physiology of organisms tested. Currently, the OECD presents some test guidelines on which biomarkers should be used for each test organism [10].

In general, there are more researches on human toxicity, using rodent models, whereas few in vivo studies addressing the ecotoxicity of nanomaterials are available. Furthermore, most of those found in the literature consider the impact of nanomaterials on aquatic organisms, since the continental and marine waters end up being the main receiving compartment. Some scarce trials address the toxicity of nanomaterials in soil and in atmosphere, commonly as suspended particles. In general, bacteria (e.g., Aliivibrio fischeri), algae (e.g., Raphidocelis subcapitata),
nematodes (e.g., *Caenorhabditis elegans*), microcrustaceans (e.g., *Daphnia magna*, *D. pulex*, *Ceriodaphnia dubia*), mollusks (e.g., *Lymnaea stagnalis*), fish (e.g., *Danio rerio*), and rodents (Wistar rat and mice) are the most used test organisms for the evaluation of acute toxicity (Table 2).

### 5.2 In vitro methods

According to Drasler et al. [22], assays can be performed with primary cultures or eternal cell lines. According to these authors, cell lines are preferably chosen because they present great homogeneity and stability, which favors reliability in the results, especially in initial tests. For more specific tests, these same researchers recommend the use of 3D co-cultures, to better understand the mechanisms of action of nanomaterials on tissues.

For the nanomaterial toxicity evaluation, the use of epithelial cell lines (skin, gastrointestinal tract, or lung) is usually indicated as these cells present characteristics of real barriers against harmful agents and are therefore the first to suffer the influence of these compounds [37]. However, it is important to note that some strains may not be responsive to the effects of nanomaterials and, in this case, primary cultures may be more indicated [22].

Aiming at the reproducibility of in vitro assays with culture of cell lines, it is necessary to record details that are generally missing from the publications. The origin of the cells, the number of the passage, the detailed method of cell culture, the brand of plastics, and reagents used during the cultivation/exposure, besides the description of the morphology, growth, and cell differentiation, before and after the test, are the information that should be included in the results’ publication [22]. Among the in vitro assays, those performed with mammalian cells are considered to be more important than those performed with other cell types [13].

For the in vitro comet assay with mammalian cell culture, Collins et al. [23] make some recommendations: (1) use non-cytotoxic concentrations (less than 20% of cell viability loss; if the nanomaterial is not cytotoxic, concentrations below 100–150 μg/mL are recommended); (2) choose the cell type according to the exposure scenario (based on exposure route and target organ); (3) determine both short (2–3 h) and long (24 h) tests to obtain a better understanding of the mode of action of the nanomaterial; and (4) determine if the genotoxic damage evidenced is a result of the direct effect with the DNA or due to the oxidation of the DNA. According to Drasler et al. [22], the exposure period is one of the main factors related to contradictory toxicity results for identical nanomaterials, as this involves transformations and the aging of their components.

In vitro assays can cover specific endpoints, such as dermal absorption, skin and eye irritation, endocrine disruption, and genotoxicity, among others. Among the tests, most nanomaterial evaluation protocols align the main routes of exposure, being dermal, oral, and inhalation [22].

According to Catalán et al. [13], the relevance and limitations of genotoxicity/mutagenicity assays should be taken into account when choosing the most appropriate monitoring method. According to these authors, the tests considered in the evaluation should be based on three categories, following the importance order: (1) gene mutation, (2) chromosomal damage, and (3) DNA damage. DNA damage is considered a mild effect because of the possibility of repair, while chromosomal damage and gene mutation are considered to be severe effects because they are irreparable changes.

Regarding the mutagenic potential of nanomaterials to humans, the effects observed in vivo should be considered more relevant than those observed in vitro,
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| Nanomaterial         | Mean diameter of the particles (nm) | Test organism                                                                 | Main results                                                                 | References               |
|----------------------|--------------------------------------|-------------------------------------------------------------------------------|------------------------------------------------------------------------------|--------------------------|
| Ag                   | 13–17 nm                             | *Lymnaea stagnalis* (Mollusca)                                               | Growth alteration and bioaccumulation                                        | Croteau et al. [28]      |
| ZnO, TiO₂             | 15–30 nm                             | *Skeletonema marinai* (Diatom—Skeletomataceae), *Thalassiosira pseudonana* (Diatom—Thalassiosiraceae), *Dunaliella tertiolecta* (Algae—Dunaliellaceae), *Isochrysis galbana* (Algae—Isochrysoidaceae) | Only nanoparticles of ZnO have decreased growth rate of diatom and algae population | Miller et al. [29]      |
| Graphene family nanoparticles | —                                    | *Caenorhabditis elegans* (Nematoda)                                          | Decreased reproduction rates                                                  | Chatterjee et al. [30]   |
| ZnO, CuO, AgO        | ZnO: 20–30 nm, CuO: 20–100 nm, AgO: 20–70 nm | *Thalassiosira weissflogii* (Diatom—Thalassiosiraceae)                         | Decreased diatom population growth in similar way to respective dissolved metals. Bioaccumulation of nanoparticles in cell wall and possible transfer through trophic chain | Bielmeyer-Fraser et al. [26] |
| ZnO, Al₂O₃, TiO₂     | —                                    | *Danio rerio* (Chordata)                                                     | Metal oxide nanoparticles induced different toxic effects in zebrafish development according to each metal. ZnO delayed larvae and embryo development and also induced serious ulceration in larvae | Zhu et al. [31]          |
| TiO₂                 | ~43 nm                               | *Pimephales promelas* (Chordata)                                             | Fish immunotoxicity and gene expression alteration                           | Jovanović et al. [32]   |
| TiO₂                 | 5, 10, and 32 nm                     | *Xenopus laevis* (Chordata)                                                  | Significantly affected tadpole growth. The highest concentration caused mortality, suppressed tadpole body length, and delayed animal development | Zhang et al. [33]        |
| Nanomaterial | Mean diameter of the particles (nm) | Test organism | Main results | References |
|--------------|-----------------------------------|---------------|--------------|------------|
| TiO₂         | —                                 | Daphnia similis (Crustacea) | The highest concentration (100 mg L⁻¹) did not induce toxic effects under experimental conditions. A mixture of TiO₂ forms induced toxic effects by ROS generation when exposed to UVA light | Marcone et al. [34] |
| TiO₂         | TiO₂: 25–70 nm                    | Vibrio fischeri (Gammaproteobacteria), Daphnia magna (Crustacea), Thamnocephalus platyurus (Crustacea) | Suspensions of nano- and bulk TiO₂ were not toxic. A nano-ZnO formulation was very toxic to V. fischeri, D. magna, and T. platyurus. Cu compound also showed toxicity; however, for Daphnia magna were less bioavailable than for bacteria | Heinlaan et al. [35] |
| ZnO          | ZnO: 30 nm                        | Raphidocelis subcapitata (Algae—Selenastraceae), Ceriodaphnia dubia (Crustacea), Daphnia pulex (Crustacea), Danio rerio (Chordata) | Nanometals caused acute toxicity in multiple aquatic organisms, but the effect was different according to the metal particle and the species used. Since R. subcapitata, C. dubia, and D. pulex were susceptible to nanometals, trophic chain could be compromised | Griffitt et al. [19] |
| CuO          | CuO: 30 nm                        |                            |              |            |

Metallic nanoparticles of Ag, Cu, Al, Co, Ni and TiO₂

| Ag (20–30 nm) | Cu (15–45 nm) | Al (51 nm) | Co (10–20 nm) | Ni (5–20 nm) | and TiO₂ (30 nm) | Raphidocelis subcapitata (Algae—Selenastraceae), Ceriodaphnia dubia (Crustacea), Daphnia pulex (Crustacea), Danio rerio (Chordata) | Nanometals caused acute toxicity in multiple aquatic organisms, but the effect was different according to the metal particle and the species used. Since R. subcapitata, C. dubia, and D. pulex were susceptible to nanometals, trophic chain could be compromised | Griffitt et al. [19] |

| Ag (15 nm) | ZnO | TiO₂ (34–42 nm) | CeO₂ (10–23 nm) | Cu (10–33 nm) | Cu (76 nm) | Raphidocelis subcapitata (Algae—Selenastraceae), Daphnia magna (Crustacea), Danio rerio (Chordata) | Ag and Cu nanoparticles affected all organisms; ZnO was toxic to algae and daphnids; TiO₂ and CeO₂ were toxic only to algae | Hund-Rinke et al. [36] |

**Table 2.**

*Experimental conditions and obtained results through in vivo tests.*
since the first allow the detection of inflammation and, therefore, secondary genotoxic effects [13]. Although more predictive for human effects, animal tests still have limitations, mainly because of the physiological and biochemical differences between species. Also, there is a trend to substitute animal testing for suitable alternatives that do not promote pain and suffering [22].

Among the tests recognized by the scientific community, those with certified guidelines for nanomaterial assessment have greater “weight” than others that have not been validated in the determination of genotoxicity/mutagenicity [13]. Although they cannot be used to determine mutagenicity, the remaining assays can be used to demonstrate the genotoxic potential of nanomaterials.

There are several recommended tests to assess nanomaterials, especially those described by the OECD. In accordance with the OECD guidelines [38], in order to select a test and evaluate the genotoxicity of a nanoform, exposure, absorption, solubility, metabolites, and other derivatives should be considered, as well as possible side effects (e.g., generation of ROS).

Comparing the genotoxicity tests for chemical substances, the comet assay and the micronucleus test are also the most indicated and used by the researchers [13]. The comet assay (single cell gel electrophoresis) is a common method of DNA damage evaluation, which can be performed with very diverse cell types. Briefly, a suspension of individualized cells is mixed with agarose and placed on a pre-glutaralized slide. Then, cell lysis on Triton X-100 removes membranes and soluble cellular components, while NaCl removes the histones from the DNA, promoting a superadhesion of this material to a matrix, forming a structure known as a nucleoid. When there are breaks in DNA strands (single or double), the fragments tend to move toward the anode during electrophoresis. When there is damage and it is observed by fluorescence microscopy, a comet-like image is noted. The percentage of DNA in the tail is proportional to the frequency of breaks, that is, the damage inferred to the genetic material [23].

As described by Collins et al. [23], several nanomaterials (e.g., TiO₂, ZnO, Au, Ag, Co₃O₄, Fe₃O₄, SiO₂, ZrO₂, and others) have already been evaluated by variations of the comet assay with specific endonucleases for some lesions, which increase the power of this tool. Among these enzymes, formamidopyrimidine DNA glycosylase (FPG) recognizes lesions of the 8-oxo-7,8-dihydroguanine (8-oxoG) and formamidopyrimidine type (open-ring purines) and is therefore widely used to estimate oxidative damages to DNA caused by nanomaterials [23].

However, other famous trials are not recommended, such as the Ames test [13]. Catalán et al. [13] discourage the use of this test to evaluate nanomaterials, since some compounds are unable to cross the bacterial wall, while others have bactericidal effect.

6. Final considerations

Undoubtedly, nanoscience and nanotechnology offer the prospect of great advances to the most different sectors of industry and medicine. However, as any area of technology that makes intensive use of new materials/structures, it brings some risks to the health of organisms and the environment. Generally, toxicological studies involving nanomaterials are still scarce, with results often controversial when compared to each other, mainly due to incipient standardization. In this context, the combination of in vitro and in vivo methods in a battery of tests is still the best way to assess the toxicity of nanomaterials [22, 23].
One of the major concerns is the choice of dose/concentration range of nanomaterials to be tested. The inclusion of excessively high doses/concentrations may generate false positives, while excessively low doses may prevent detection or may underestimate the genotoxic potential [23]. Drasler et al. [22] provide all guidelines to be considered in evaluating the toxicity of nanomaterials by cell culture, but in vivo evaluation must not be overlooked. Paschoalino et al. [3] state that the environmental risk analysis of nanomaterials depends mainly on the regulatory structure, which involves the generation of protocols, which must be based on a multidisciplinary interaction, in order to obtain a more risk assessment possible.

As demonstrated by Valsami-Jones and Lynch [18], harmonization of methods and approaches could benefit this young science, as there is still no consensus on basic assessment protocols. Current protocols involve specific techniques and methods to collect and analyze data sufficient to quantitatively describe the release, destination, transport, transformation, exposure, and toxicity of chemicals. Furthermore, in order to be more precise about the toxicity and mechanism of action of nanomaterials on living organisms, the physicochemical characteristics must be sufficiently detailed. So far, a great effort has been made by the OECD to try to standardize test methods that can correctly evidence the risk of nanomaterials. There are a number of internationally accepted test guidelines that are used for toxicity assessment involving trials with organisms for aquatic, soil, and sediment monitoring. Since 2013, experts from all over the world hold strategic meetings to determine what directions the OECD should take regarding the assessment of nanomaterials, as explored in the Petersen et al. [39].

Also, there is a lot of potential in computational models to help elucidate the possible effects of nanomaterials on humans and the environment. Currently, the quantitative structure-activity relationship (QSAR) model seems to be quite adequate because it can relate the structural, physical, and chemical characteristics to the behavior that some nanomaterial can present. To date, the combination of field, laboratory, and computational work still is the most promising technique to ensure reliable responses to the issues involved with nanomaterial toxicity.

Conflict of interest

The authors declare no conflict of interest.

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