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Toxicological Risk Assessment of Emerging Nanomaterials: Cytotoxicity, Cellular Uptake, Effects on Biogenesis and Cell Organelle Activity, Acute Toxicity and Biodistribution of Oxide Nanoparticles

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Lionel Maurizi, Anne-Laure Papa, Julien Boudon, Sruthi Sudhakaran, Benoist Pruvot, et al.. Toxicological Risk Assessment of Emerging Nanomaterials: Cytotoxicity, Cellular Uptake, Effects on Biogenesis and Cell Organelle Activity, Acute Toxicity and Biodistribution of Oxide Nanoparticles. Unraveling the Safety Profile of Nanoscale Particles and Materials-From Biomedical to Environmental Applications, InTech, 2018, 10.5772/intechopen.71833 . hal-02163546

HAL Id: hal-02163546

<https://hal.science/hal-02163546v1>

Submitted on 11 Mar 2021

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Toxicological Risk Assessment of Emerging Nanomaterials: Cytotoxicity, Cellular Uptake, Effects on Biogenesis and Cell Organelle Activity, Acute Toxicity and Biodistribution of Oxide Nanoparticles

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.71833>

Abstract

The lack of toxicological data on nanomaterials makes it difficult to assess the risk related to their exposure, and as a result further investigation is required. This chapter presents the synthesis of controlled oxide nanoparticles followed by the evaluation of their safety profile or toxicity (iron, titanium and zinc oxides). The controlled surface chemistry, dispersion in several media, morphology and surface charge of these nanoparticles are presented (transmission electron microscopy, dynamic light scattering, zeta potential, X-ray photoelectron spectroscopy). Classical cytotoxic and cellular uptake studies on different cancer cell lines from liver, prostate, heart, brain and spinal cord are discussed. The incidence of nanoparticles on biogenesis and activity of cell organelles is also highlighted, as well as their biodistribution in animal models. The acute toxicity on zebrafish embryo model is also presented. Finally, the stress is put on the influence and the necessity of controlling the protein corona, a layer of plasma proteins physically adsorbed at the surface of such nanoparticles as a result of their presence in the bloodstream (or relevant biological fluids).

Keywords: superparamagnetic iron oxide nanoparticles (SPIONs), titanate nanotubes (TiONts), zinc oxide, cytotoxicity, oxidative stress, cell organelle activity, zebrafish, cellular uptake, biodistribution, protein corona

1. Introduction

The development and production of nanomaterials are one of the fastest growing areas of advanced technologies, providing a wide range of novel applications in the electronic, health-care, cosmetic, agronomy, engineering and food industries. The nanotechnology era has increased nanoparticles concentration in the environment, causing continuous human exposure, with both uncontrolled contact by inhalation or through the skin, as well as exposure *via* oral administration or by drug injection.

The lack of toxicological data on nanomaterials makes it difficult to assess the risk due to their exposure. For all these reasons, there is an urgent need to develop rapid, accurate and effective testing strategies to assess the impact of these emerging materials on human health and the environment. Three nanoparticles of growing interest have been selected as key materials in this chapter: (1) **superparamagnetic iron oxide nanoparticles** (SPIONs) that are commonly developed as magnetic resonance imaging (MRI) contrast agents [1], (2) **titanate nanotubes** (TiONts) for their elongated morphology [2] and (3) **ZnO** nanoparticles, known for their potential hazards [3].

The major objective of this study concerns the detailed assessment of oxide nanoparticles toxicity or safety profile, through the development of pertinent bioassays. Cytotoxicity assays to check cellular homeostasis disruption, transmission electron microscopy (TEM) analysis and flow cytometry for particle uptake investigation, cell death evaluation, and the influence of nanoparticles on biogenesis and activity of cell organelles are described. Moreover, ecotoxicological monitoring was performed using zebrafish embryos as a model. Survival and hatching rates, and malformations were determined upon exposure to oxide nanoparticles. Finally, an understanding and factors to control the protein/nanomaterial interactions are further presented. These proteins influence the cellular interactions with the nanoparticles such as adhesion to the plasma membrane or uptake, but also their biodistribution.

2. Synthesis, purification and characterization tools for oxide nanoparticles toxicity control and profiling

To investigate the toxicological risk assessment of nanomaterials, it is necessary to jointly control their morphology, their size distribution, the nature of their interfaces (charges and chemistry) and their colloidal stability in several media. Indeed, many controversies in literature come from the lack of control of one of these parameters. In this part, the emphasis is placed on the synthesis as well as the characterization tools used to fully investigate nanoparticles and to control the parameters influencing their nanotoxicity.

2.1. Synthesis routes of oxide nanoparticles: the case of SPIONs and TiONts

2.1.1. Synthesis of SPIONs by soft chemistry as well as functionalization of their surface

SPIONs were prepared according to a method derived from the classical Massart protocol [4]. Briefly, a 1:2 molar ratio of ferrous and ferric chloride was added to a NaOH solution at 90°C under vigorous mechanical stirring. The product was magnetically settled down and washed

three times with 400 mL of 1 M HNO₃. Finally, the particle suspension was centrifuged at 450× g for 1 h to remove the biggest aggregates. The supernatant was dialyzed against an HNO₃ solution (pH 4.0) during 24 h [5].

In order to increase their colloidal stability for biological assays, bare SPIONs were subjected to 3-aminopropyltriethoxysilane (APTES) in an equivalent mass ratio into a 1:1 ethanol/water mixture, the pH of which was decreased to 4.0 by the addition of 1 M HCl prior to the APTES addition. The mixture was submitted to an ultrasonic treatment to afford a good particle dispersion leading to the polysiloxane coverage of individual particles rather than agglomerates. The mixture was then submitted to mechanical stirring during 48 h. Glycerol was then added followed by the evaporation of the ethanol/water mixture under reduced pressure to increase the polysiloxane condensation around SPIONs. Finally, glycerol was removed by acetone addition to the SPION suspension accompanied by a magnetic decantation. SPIONs were finally resuspended into ultrapure water yielding SPIONs-NH₂ and dialyzed 1 week against ultrapure water [6].

The surface of bare SPIONs or SPIONs-NH₂ was then functionalized with polyvinyl alcohol (PVA) or polyethylene glycol (PEG-COOH), respectively. Polymers of different molecular weights (from 2 to 30 kDa) and bearing different chemical groups were used. PEG chains were covalently grafted on the surface of SPIONs (EDC/NHS coupling), while PVA was linked *via* electrostatic interactions [7].

2.1.2. Synthesis of TiONts and functionalization of their surface

Titanate nanotubes were prepared by a classical hydrothermal method. Titanium dioxide rutile precursor powders (440 mg) were added to a NaOH aqueous solution (10 mol.L⁻¹, 110 mL) [2]. The mixture was subjected to ultrasound (15 min, 375 W) before being transferred into a sealed Teflon reactor. The temperature was set at 155°C for 36 h and the mixture was stirred by magnetic stirring (120 rpm). The resulting white product was isolated by centrifugation and washed with deionized water until pH 6.0 was reached. Finally, the powder was freeze-dried.

The biocompatibility of TiONts in biological systems has been improved through their surface modification with APTES, PEG or chitosan grafting. Bare TiONts were coated by APTES and PEG with protocols very similar to that used for SPIONs [8]. The method of chitosan (CT) grafting is based on electrostatic interactions between chitosan's amines and nanotubes' hydroxyl groups. Briefly, a mixture of TiONts and CT in a 1:2 molar ratio of TiONts(-OH)/CT(-NH₂) was prepared and the pH was adjusted to 7.0. The suspension was mixed up at 25°C under magnetic stirring during 24 h. The powder was washed several times with deionized water by an ultrafiltration device (300 kDa, regenerated cellulose) [9].

2.2. Purifications of bare nanoparticles and nanohybrids

Purification of nanoparticles is undoubtedly an important and challenging step in order to control both the chemistry of their surface and their dispersion in varied media. All the obtained nanohybrids were purified by ultrafiltration on 30 kDa membranes and/or dialyzed on 3.5 kDa membranes to remove ungrafted stabilizing molecules and remaining salts finally yielding nanoparticle suspensions at the pH and the conductivity of ultrapure water. Finally, a portion of the suspensions was freeze-dried for powder characterizations: X-ray diffraction

(XRD), infrared spectroscopy (IR), X-ray photoelectron spectroscopy (XPS) and thermogravimetric analysis (TGA) [8, 10, 11].

2.3. Importance of high-standard characterization tools for nanotoxicity evaluation and safety profile definition

It is well known that numerous parameters influence the toxicity of nanomaterials. This is the reason why each kind of nanoparticles (bare or coated-SPIONs, TiONts or ZnO) was thoroughly characterized by an array of analytical techniques, which allowed to keep track of the precise morphology, composition, agglomeration and surface chemistry (composition and charge). We ensured the highest standards of characterization of hybrid nanomaterials (**Figure 2**).

The size and morphology of the individual nanoparticles were determined by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The agglomeration state was investigated *via* dynamic light scattering (DLS) and specific surface area measurements (BET method). The colloidal stability was investigated by UV-visible spectroscopy in several media (e.g., PBS, MEM and albumin solutions): the UV absorbance evolution was recorded over time every 5 min during several hours. The faster the absorbance decreased, the less stable the particles were in suspension [9, 10].

The oxide nanoparticles structure was investigated by X-ray diffraction (XRD), high resolution TEM, selected area diffraction (SAD), Raman and FTIR spectroscopies. The nanoparticles chemistry was also analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (**Figure 2**) [6].

Moreover, the chemical composition of the nanoparticles surface was investigated through XPS, TGA, zeta potential measurements and FTIR spectroscopy. For instance, XPS was associated with TGA to quantify the molecule number grafted at the surface of nanohybrids [12].

3. Cytotoxicity, cellular uptake and biodistribution of oxide nanoparticles

The previously described metal oxide nanoparticles are excellent candidates for a variety of biomedical applications ranging from drug delivery to diagnostic aids, as well as implantable biomaterials [6, 8, 13, 14]. However, a thorough evaluation of particles interaction with the targeted cells, circulatory immune cells and tissues is the first prerequisite. Here, these interactions are presented from an internalization and cytotoxicity point of view, and the biodistribution of these particles in various *in vivo* models (healthy and tumor bearing rodents) is also highlighted.

3.1. Superparamagnetic iron oxide nanoparticles: toxicity, cellular interactions and biodistribution

3.1.1. Cytotoxicity of SPIONs

SPIONs are well developed for biomedical applications as a consequence of their easy and reproducible production. Another advantage of the SPIONs is their chemistry. They are in fact

made from one of the most abundant metals present in metabolism: iron. Despite the fact that iron could induce ROS generation in cells [15], working with this element clearly decreases the potential toxicity compared to other metal oxide nanoparticles during dissolution processes *in vitro* or *in vivo* [15]. Naked SPIONs tend to sediment and can precipitate at physiological conditions, leading to a severe toxicological hazard [16]. It is imperative to modify the surface of these nanoparticles to avoid any aggregation and the resultant risk of toxicity. Polyethylene glycol (PEG) is a polymer commonly used to increase the biocompatibility of the SPIONs as well as their stealthiness for specific targeting [17]. SPIONs grafted with PEG do not show any cytotoxicity *via* MTT assay at a concentration up to 270 $\mu\text{gFe/mL}$, 24 h after incubation with RAW 264.7 and HepG2 cell lines [5]. Polyvinyl alcohol (PVA) is also used to stabilize SPIONs and do not have any cytotoxic effects (MTS assay) for a concentration of 800 $\mu\text{gFe/mL}$ 24 h after incubation with RAW 264.7 cells [18]. Furthermore, PVA coated SPIONs do not significantly activate or influence human T helper cells and have a negligible influence on T cell apoptosis at a concentration of 100 $\mu\text{gSPIONs/mL}$ after 72 h [19]. Regarding PVA, the covalent binding of this polymer onto the SPION surface significantly decreases some inflammatory effects on same T helper cells [20]. Evaluating cytotoxicity of SPIONs is not a trivial operation. Many tests are measuring the evolution of absorbance and the nanomaterials are influencing the final value. It is then very important in order to avoid false positive or negative results, to carefully setup the experiments and the control to correct the absorbance [21].

As demonstrated in many studies, the addition of a biocompatible polymer layer on the SPION surface significantly improves their biocompatibility, which is a crucial step for the biological interactions targeted.

3.1.2. Cellular uptake and biodistribution of superparamagnetic iron oxide nanoparticles

First of all, the magnetic properties of SPIONs are very interesting to increase the cellular uptake rate of these nanoparticles with a magnet [22] and then to improve the labeling of cells for biomedical applications [23, 24]. The concentration and the charge play a significant role in the cellular internalization [25]. For instance, negatively charged fluorescently labeled SPIONs have a higher internalization in prostate-cancer PC-3 cell line as observed *via* confocal microscopy or flow cytometry, in comparison to positively charged SPIONs [26]. In the same way, neutrally charged SPIONs coated with PEG show less cellular interactions on RAW 264.7 cells by TEM and classical microscopy (labeled with Prussian blue) than negatively charged PEGylated-SPIONs [5]. On another side, positively charged PVA-SPIONs seem to have a better internalization by HeLa cells than neutral PVA-SPIONs with an additional influence of the culture medium used, especially depending on the presence of proteins [7]. Thus, the influence of the chemical coating is an important factor, however it seems that the nature of the medium used is much more critical.

Regarding their biodistribution, SPIONs usually show accumulation in the liver and spleen [1, 27]. SPION accumulation to the liver is delayed due to a coating of neutral PEG onto SPIONs, as observed by MRI and Prussian blue-based histology [5]. Their circulation time in the bloodstream is at least 3 h longer compared to negatively charged PEG-SPIONs [5]. The charge of PVA conjugated particles can also induce different *in vivo* behavior of SPIONs. When observed 15 min after injection into a rat model, 50% of the dose of positively charged PVA-SPIONs already accumulate in the liver when 90% of the neutral and the negatively charged PVA-SPIONs are still passing through the bloodstream [28].

Overall, SPIONs are well developed and tested for many biological applications. They do not show any dramatic toxicity and have interesting cellular and *in vivo* interactions, making them extremely attractive as theranostic agents [29].

3.2. Titanate nanotube *in vitro* toxicity and biodistribution testing

3.2.1. Cytotoxicity of TiONts: the surface chemistry matters

The cytotoxicity of titanate nanotubes made by hydrothermal treatment has been assessed in H596 human lung tumor cells [30], cardiomyocytes [31], SNB19 and U87-MG glioblastomas [32], Caco-2 cells [33], as well as 22Rv1 prostate cancer cells [8]. Interestingly, the degree of ion exchange via acid treatment, which partly or entirely substitutes sodium cations by hydrogen cations, is a key parameter that drives the cytotoxicity of titanate nanowires [30]. Titanate nanotubes that were not treated with acid did not induce significant cytotoxic effect in cardiomyocytes, as seen by MTT assay performed between 1 and 10 $\mu\text{g/mL}$ TiONts [31]. Similarly, TiONts concentration ranging up to 100 $\mu\text{g/mL}$ do not induce detectable cytotoxicity in glioblastoma cell lines [32]. In contrast, the viability of CHO cells significantly decreases to 66% viability after 24 h of incubation with 100 $\mu\text{g/mL}$ of bare TiONts; however, concentrations up to 20 $\mu\text{g/mL}$ were not found to be cytotoxic to these ovarian cells [10]. Additionally, PEGylation of TiONts did not modulate TiONts effect on cell viability up to 5 $\mu\text{g/mL}$ [10]. However, the subsequent surface functionalization of these nanotubes with Docetaxel reduces the drug availability and significantly increases Docetaxel IC₅₀ in 22Rv1 cells, compared to free drug control [8]. As described in the next section, *in vivo* studies have shown that TiONts acts like an anchor in the tumor, which prevents drug from leaching out of the cancerous cells, and as a result, the loss in drug potency was not detrimental in this specific case [8].

Beyond their cytotoxicity data, TiONts have been shown to arrest cell cycle in the G₂/M phase for both SH-B19 and U87-MG cell lines, as observed while investigating the origin of their radio sensitization effect in glioblastoma [32]. Indeed, an important intrinsic feature of these metal oxide nanotubes is their ability to potentiate gamma radiation effect on cells, making them an interesting candidate for combinatorial therapies on ongoing preclinical investigations (i.e., chemotherapy along with radiation therapy) [34].

3.2.2. Cellular uptake of nanotubes: the shape takes the lead

This chapter mainly focuses on metal oxide nanoparticles, however, beyond the surface chemistry of such materials, one of the key parameters to consider while studying nanoparticle interaction with cells and tissues is their shape. Indeed, due to their needle-like morphology, bare TiONts are internalized in cells not only by endocytosis, but also by diffusion across the plasma membrane, as observed by TEM analysis for cardiomyocytes [31], SNB19 and U87-MG glioblastoma cell lines [32]. Nanotubes display a significant higher specific surface compared to their spherical counterparts [2] and this potentially modifies their degree of interaction with plasma proteins and cells. Our group has observed that even by incubating 4 times more spherical TiO₂ than TiONts with cardiomyocyte cells to account for the difference in specific surface values, TiONts were internalized in much more cells than spherical TiO₂ [31]. Cell penetration via diffusion, along with their increased specific area, potentially makes

them an excellent candidate as a new nanomedicine platform after careful assessment of their cytotoxicity in each targeted cell model.

3.2.3. Biodistribution and “tumor retention effect” of titanate nanotubes

Nanotubes display unique behavior regarding their interaction with and internalization within cells, as well as distribution to tissues, compared to spherical nanoparticles. Indeed, the shape is a critical parameter governing circulation time and biodistribution for the same material. For example, the circulation time for tubular micelles in mice is 10 times longer than the one of spherical micelles [35]. Titanate nanotubes have been shown to transiently accumulate in the lungs before being quickly eliminated by the bladder more than 24 h following their IV injection in mice [36]. Lung accumulation has also been observed in the case of carbon nanotubes; however, they were still detected 3 months following injection [37]. Interestingly, single walled carbon nanotubes have been demonstrated to be uptaken in the bloodstream by a subset of monocytes that subsequently deliver them to the tumor [38]. Nanoparticles passively accumulate in tumor by enhanced permeability and retention effect (EPR effect), due to the poorly formed vasculature supporting the malignant cells, in combination with reduced clearance secondary to defective lymphatic drainage at site. While passive targeted delivery to tumor is estimated to deliver only a small fraction of the injected dose utilizing spherical nanoparticles, nanotubes are capable of reaching significantly greater accumulation than their spherical counterparts and also display greater surface area that potentially leads to greater effect [39]. Immune cells’ active delivery of tubular nanomaterials to the tumor [38], as well as the enhanced retention time of tubular-shaped nanomaterials into tumors [8] are attractive factors in using such particles for drug delivery. Indeed, our group has reported for the first time that prostate tumors retain more than 70% of docetaxel-functionalized titanate nanotubes up to 7 days following intratumoral injection, indirectly bypassing the well-known multidrug resistance effect [8] (**Figure 1-A**). Exploring the tubular shape of nanobiomaterials that can provide a solid “**tumor retention effect**” will be an important step forward in developing the next generations of drug delivery platforms in oncology.

3.3. Importance of the protein corona on biological interactions of nanomaterials

Understanding the *in vitro* and *in vivo* behavior of nanoparticles is one of the main objectives of current studies. It seems too simplistic today to draw conclusions about their behavior without taking into account the environment, especially the proteins present in the systems studied [40]. Nowadays, it is accepted that once nanoparticles are incubated in biological fluids such as blood, they will be covered by proteins [41]. Not only do these proteins interact with the chemical coatings of materials, but they mostly also modulate their biological fate [28, 42].

The nature of the coating, including resulting charge, surface chemistry and particle hydrodynamic size, influences the adsorption of proteins on the surface of nanoparticles: the protein corona [43, 44]. For example, we demonstrated that bare silica beads covered by either a gold or a titanium oxide layer have different preferential binding to proteins [43]. Once incubated 1 h at 37°C with fetal bovine serum (FBS), we showed that:

- naked silica nanoparticles have no interactions with neither plasminogen nor albumin (two of the most abundant proteins present in FBS),

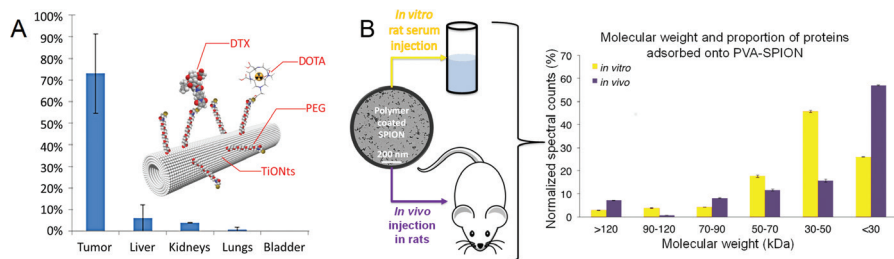


Figure 1. (A) Quantification of nanohybrid biodistribution via gamma counting 7 days post injection (mean of three mice \pm SD) (adapted with permission from [8], DTX: docetaxel). (B) Abundance of plasma proteins found at the surface of SPIONs after incubation with rat serum *in vitro* versus *in vivo* (adapted with permission from [28]).

- TiO_2 -coated silica nanoparticles interact only with albumin, and
- gold-coated silica nanoparticles interact with both the proteins.

For PVA-coated SPIONs, the charge of the polymers also influences the protein corona [45] and is different for the three types of PVA-SPIONs (neutral, positively and negatively charged) incubated in FBS for 1 h at 37°C . We also showed there were important differences between *in vitro* and *in vivo* protein coronas [28]. In the case of negatively charged PVA-SPIONs, for example, more than 60% of the adsorbed proteins from rat serum have sizes comprised between 30 and 50 kDa *in vitro* when the main proteins (more than 50%) are below 30 kDa *in vivo* (15 min post injection) (**Figure 1-B**). Literature regarding the protein corona of TiONts is very limited at present and our group aims to elucidate key aspects of the topic in the years to come. Interestingly, titanate nanotubes bind significantly less plasma proteins than spherical TiO_2 (Degussa P25) [46], even though they display a greater specific surface [2]. These proteins include albumin, Ig heavy chain (μ), Ig light chain, fibrinogen (α , β and γ chains) and complement C3.

The coatings of the nanoparticles influence the nature of their protein corona. The medium used is also important for the interactions between materials and proteins [47]. Thus, taking into account not only the physicochemical properties but also the biological environment, it is essential to understand cellular uptake and biodistribution of nanoparticles in order to better control their toxicological risks.

4. Influence of nanoparticles on the biogenesis and activity of cellular organelles

Organelles (mitochondria, peroxisome, lysosome, endoplasmic reticulum, and Golgi apparatus) are integral parts of the cells, essential for its proper functioning. Their dysfunctions can lead to serious consequences. For instance, mitochondrial alterations can go as far as to activate apoptosis [48], peroxisomal dysfunction affect the mitochondria, subsequently leading to oxidative stress and cell death [49, 50], alterations of the lysosome may have consequences on the induction of autophagy and apoptosis [51], endoplasmic reticulum damages can lead to reticulum stress which can trigger different forms of cell death in extreme cases

[52], and Golgi apparatus dysfunctions can disturb post-translational modifications and vesicular transport [53]. The incidence of the cytotoxicity of nanoparticles is often addressed in generalized terms such as induction of cell death, oxidative stress stimulation, inflammation activation and genotoxicity. The impact of nanoparticles on cell organelles is less known and must be taken into consideration as organelle dysfunctions affect general health in unexpected ways. As regards the peroxisome, whose dysfunctions can lead to severe neurodegenerative damage [54], there are currently no data on the effects of nanoparticles on this organelle.

It is therefore essential to understand the interaction of nanoparticles with cell organelles in terms of distribution and impact on their biogenesis and biological activities. This not only helps to prevent or optimize the toxic effects of nanoparticles depending on the intended purpose (cytoprotection or cell death induction), but also to use them specifically in nanomedicine without side effects.

4.1. Effect of nanoparticles on mitochondria

The interaction of nanoparticles with the mitochondria (as well as the other organelles) must be approached with the consideration that they are either the consequence of targeted interactions with specifically dedicated functionalized nanoparticles, or a random direct or indirect interaction which leads to unwanted side effects. This second aspect must be systematically taken into consideration, and integrated into a cytotoxic screening procedure which will permit to specify the biological activity of nanoparticles at the mitochondrial level. In order to understand the toxicological interactions of nanoparticles on biogenesis and mitochondrial metabolism, it is necessary to specify whether they interact physically with the mitochondria and accumulate at specific locations such as external membrane, mitochondrial space, internal membrane and cristae. In this context, it has been shown that Gadolinium oxide (Gd_2O_3) nanoparticles, which have a range of biomedical uses, induce mitochondrial apoptosis by acting on Bcl-2 and Bax [55]. Similarly, silver nanoparticles impair mitochondrial activity and decrease cell viability [56]. Nanoparticles interact with mitochondria in different manner, based on their physicochemical nature. TiO_2 nanoparticles, which are present in numerous manufactured products, induce loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) and an overproduction of superoxide anions in murine microglial BV-2 cells [57]. After exposure with a high concentration of ZnO nanoparticles [58], BV2 cells undergo an increase in mitochondrial transmembrane potential (**Figure 2**). In addition, MTT assays have highlighted that SPIONs and TiONts can also affect mitochondrial integrity depending on their concentrations (especially at high doses) and surface coating [10]. Since numerous types of nanoparticles are able to induce mitochondrial dysfunctions, which can have dramatic consequences on human health after chronic or acute exposures, a systematic evaluation of the impact of nanoparticles on the mitochondria is required.

4.2. Effect of nanoparticles on the peroxisome

Peroxisome has emerged as a key regulator in overall cellular lipid and reactive oxygen species metabolism. In mammals, these organelles have been recognized as important hubs in redox-, lipid-, inflammatory-, and innate immune-signaling networks. Peroxisomal dysfunctions are associated with important brain diseases [54]. To exert its activities, the peroxisome

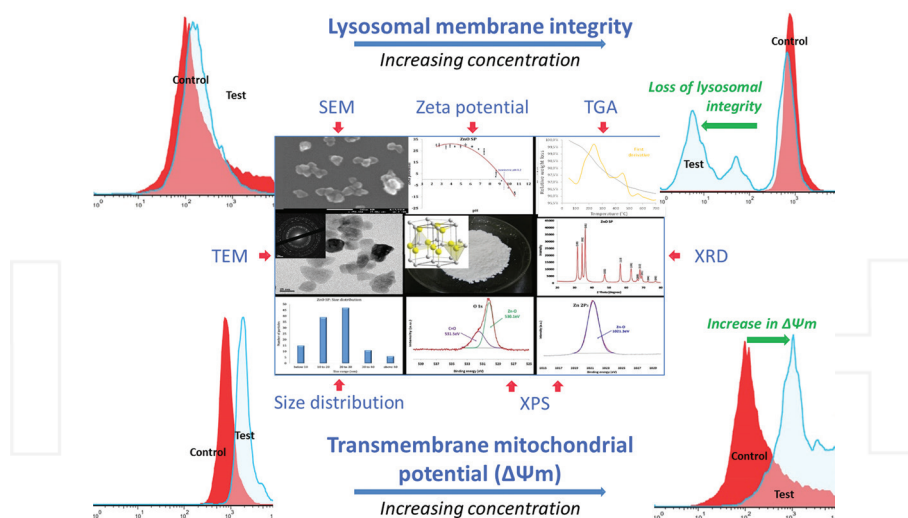


Figure 2. Interaction of ZnO nanoparticles with murine microglial (BV2) cells. Nanoparticles characterized for size, shape, surface charge, crystal structure, chemical composition and purity were exposed to BV2 cells for a maximum duration of 24 h. The ZnO nanoparticles exposure induced dose-dependent increase in transmembrane mitochondrial potential and loss of lysosomal membrane integrity as revealed by flow cytometry analysis using fluorescent probes DiOC6(3) and propidium iodide respectively.

must interact both functionally and physically with other cell organelles, mainly mitochondria and endoplasmic reticulum [59, 60]. It seems therefore important to precise the effects of nanoparticles on peroxisome. Numerous techniques such as fluorescent microscopy and flow cytometry are available to estimate the impact of molecules/nanoparticles at the peroxisomal level [61] nevertheless, no data are available concerning the impact of nanoparticles on this organelle.

4.3. Effect of nanoparticles on the lysosome

Endocytosis is the major uptake mechanism of particles by cells [62]. The nanoparticles entrapped in endosomes are eventually degraded by specific enzymes present in phagolysosomes, as the endosomes fuse with lysosomes. The function of lysosomes is to break down molecules and dispose unwanted materials [63]. This phenomenon can also limit the delivery of therapeutic nanoparticles to the intracellular target site. Nanoparticles depending on its physicochemical nature can alter the function of lysosome and subsequently favor the activation or the inhibition of autophagy [64–66]. For instance, we have observed that ZnO nanoparticles induce a loss of lysosome membrane integrity in BV2 cells at high-dose exposure (80 mg/mL) as seen by flow cytometry detection of acridine orange (**Figure 2**). Additionally, double-membrane vesicles closely resembling autophagosomes have been observed by TEM, following 6 h of cardiomyocyte incubation with TiONts [31]. As the lysosomal pathway may have beneficial or detrimental effects on cell activity, a panel of assays is required to define the influence of nanoparticles on this organelle and its potential consequences in major diseases (metabolic diseases, cancer and neurodegenerative diseases).

4.4. Effect of nanoparticles on the endoplasmic reticulum and Golgi apparatus

Currently limited data are available on the impact of nanoparticles on endoplasmic reticulum and Golgi apparatus. It has been reported that silica nanoparticles accumulate in the endoplasmic reticulum and triggers autophagy [67]. On the other hand, the intracellular accumulation of gold nanoparticles leads to inhibition of macropinocytosis and reduction of endoplasmic reticulum stress [68]. Thus, it appears that nanoparticles can have different effects on the endoplasmic reticulum. Consequently, their effects on this organelle must not be neglected.

There is evidence that some nanoparticles can be taken up by the Golgi apparatus for further processing; however, no additional information are available on the influence of nanoparticles on the activity of the Golgi apparatus [69, 70].

Among the most appropriated techniques available in nanotoxicology, observation of cells and tissues by TEM is well suited. This method permits quantitative and qualitative evaluation of modifications at the organelle level which are not easily detected with antigenic and functional changes. Various methods of flow cytometry with appropriate probes are also of interest to define the impact of nanoparticles on the biogenesis and activities of the organelles. These methods can be complemented with other methods of biochemistry, such as Western blot, PCR and RT-qPCR to study the nanobiointeraction at the molecular level. These methods make it possible to identify specific molecular targets and study the effects of nanoparticles on signaling pathways. The development of chip-based single-cell analysis is also of great interest for nanotoxicity assessment [71].

Overall, the beneficial or detrimental effects of nanoparticles on the organelles are difficult to predict. Systemic evaluation of nanoparticle interaction with organelles using simple techniques will help to minimize, if not to subdue, the biological risk associated with nanoparticles on human health, as well as with the environment.

5. Zebrafish as a model for testing the toxicity of SPIONs and TiONts

Due to the increase of nanotechnologies in an expanding range of applications in industrial and biomedical purposes, those new materials require ecotoxicological, biosafety and biocompatibility evaluation. While nanotoxicity can be rapidly assessed *in vitro*, results obtained do not reflect complex processes that happen in full organisms and ecosystems.

Various factors must indeed be taken into account, such as the route of administration (i.e., route of exposure), biodistribution, long-term exposure, induction of developmental defects or activation of the immune system [72, 73]. However, *in vivo* approaches using classical mammalian models have strict ethical considerations, are time consuming and are expensive. Most importantly, throughput approaches cannot be considered *via* those models.

Given its many advantages, zebrafish is now a recognized model for toxicological and biomedical assays [74–77]. The main advantages of this species are rapid development, external fertilization, easy observation of all developmental stages, small size, transparency of the

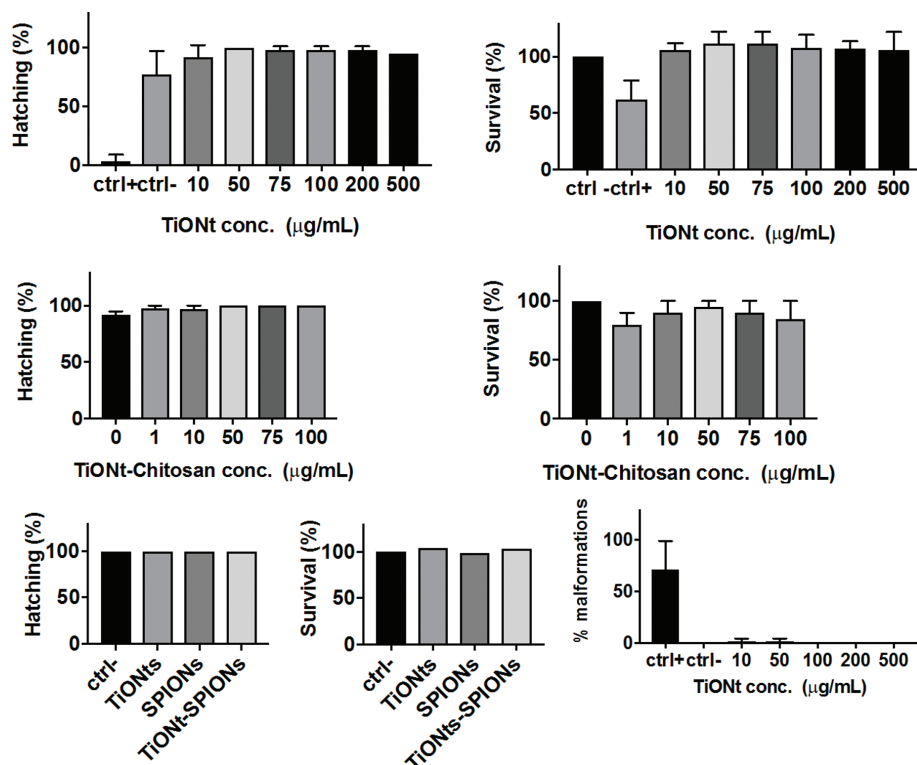


Figure 3. Toxicity evaluation of TIONts and chitosan modified TIONts, SPIONs and TIONt-SPIONs in zebrafish embryos. Unless noted, SPIONs, TIONts and SPION-TIONts concentration was 50 µg/mL; negative control: water; positive control: 4 µg/mL of 3,4-dichloroaniline.

embryos and larvae, large number of embryos, ease of maintenance and close contact with surrounding medium (water) allowing easy interface with materials. In addition, it is possible to take advantage of the sequenced and annotated genome *via* experimental and genetic tools such as fluorescent microscopy, time lapse, histology, transgenic organisms, microinjection and ectopic expression of specific genes. For all these reasons, small fish species represent one of the best choices to study pharmacological/toxicological effects and physiological alterations in vertebrates as a first screening step. Similarly, due to their small size, they are highly suitable for investigating alterations in vertebrate physiology under confined conditions [78]. Indeed, several zebrafish larvae can be placed into one well of 96 wells plates or one larva in a 384 wells plates. Finally, standardized fish embryo toxicity methods are recognized and can be applied to analyze nanomaterials' effects on vertebrates [79].

Recently, zebrafish models were used to evaluate the toxicity of various nanoparticles, including SPIONs and spherical TiO₂ [80–83]. Studies in zebrafish embryos point to toxicity using concentrations of iron oxide particles >10 mg/L resulting in increased mortality, hatching delay and malformations [81], showing the possibility of toxicity of SPIONs at elevated

concentrations. Another study revealed that SPIONs coated with cross-linked aminated dextran may cause acute brain toxicity in adult zebrafish [83]. Iron overload, changes of gene expression and inhibition of acetylcholinesterase were proposed as causes for the observed neurotoxicity. In another study, SPIONs, which were non-toxic *in vitro*, were lethal in zebrafish embryos when used at concentrations higher than 10 mg/mL [80]. Regarding TiO₂ nanotubes, they are reported to have an excellent biocompatibility [84]. However, another work using zebrafish as model organism showed that TiO₂ nanotubes at 1 mg/L may cause undesired tissue accumulation in injured animals and asymmetric and shorter regeneration after fin amputation [82]. We analyzed SPIONs as well as unmodified and modified TiONts in zebrafish embryos for toxicity (**Figure 3**). Embryos were incubated up to 96 h post fertilization with different amounts of these materials (500 µg/mL for TiONts, 50 µg/mL for SPIONs and TiONts-SPIONs). No lethality, developmental effects (no malformation) or delayed hatching were observed. Even if we used higher concentrations than Park et al. [82], we did not observe any toxicity. In contrast to Parker, we did not treat injured animals, and we used a higher number of animals in the experiments.

All together, we concluded that the nanomaterials we produced do not show any obvious toxicity, even at high concentrations. However, to get more precise information, the zebrafish embryos will be analyzed by qRT-PCR for detection of stress or inflammation related gene expression and with fluorescent markers for apoptosis events.

6. Conclusion

The outcomes presented in this chapter are the result of collaborations between chemists, physico-chemists, biologists and clinicians on the field of biomedical applications of nanoparticles. Such interdisciplinary collaborations are required to investigate nanotoxicity. Controlled nanoparticles, fully characterized and leading to stable suspensions in biological media, have to be prepared. Then, rapid, accurate and efficient testing strategies have to be developed to assess the effect of these emerging materials on the human health and the environment: *in vitro* assays but also *in vivo* evaluation (biodistribution, retention, elimination and ecotoxicity). All the skills (chemistry, physico-chemistry, nanomaterials engineering, toxicology, biology and medicine) are required to achieve this goal.

Acknowledgements

The authors would like to thank Dr. Vanessa Bellat, Yasmine Saïbi, Thomas Nury, Annette Luce, Fadoua Sallem, Alexis Loiseau, Dr. Guillaume Thomas, Dr. Rémi Chassagnon, colleagues from ICMUB laboratory, from the preclinical imaging platform of the CGFL, from the NVH Medicinal biotechnology company, from the CHU of Dijon. The authors are also indebted to the Université de Bourgogne, Inserm, CNRS, the Raman-Charpak Fellowship and the Conseil Régional de Bourgogne (Contrat d'Etude – CPER 2007–2013 and PARI Nano2Bio), the FEDER program and the Association Française de Cytométrie en Flux.

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