Chapter 13
Toxicity Assessment of Nanomaterials

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Abstract In the last decades, nanoscience had a spectacular evolution providing new, versatile engineered nanomaterials and nanotools with a plethora of applications in very diverse fields ranging from energy storage to medicine. Among the palette of nanomaterials, magnetic nanoparticles (in particular iron oxide-based) present unique physicochemical properties that are actively being exploited in the biomedical field. Currently, they are used for induced magnetic hyperthermia cancer treatments, as contrast agents for magnetic resonance imaging, as cell tracking elements, and for drug delivery modalities. In parallel to the growth of nanoscience and the ever-increasing applications of nanomaterials, concerns regarding the safety and toxicity of nanoparticles have arisen, both during and post-administration. In this chapter, we review key concepts related to nanotoxicology and to the fate of nanomaterials in the human body. A detailed description about the most accepted and practiced in vitro and in vivo methods used to evaluate the toxicity of nanomaterials is provided, with emphasis in magnetic nanomaterials for nanomedicine applications.

Keywords Nanotoxicity · Cell-nanomaterial interactions · In vitro and in vivo evaluation · 3D culture system · Animal model

Introduction

To begin, a foreword defines and delimits the scope of this chapter: Here, we deal with the toxicity of nanomaterials that are employed in biomedical applications and among those about nanomaterials used in nanomedicine. Therefore, toxicological effects related to biomaterials used in implants or for regenerative therapies, to nanoparticles

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naturally or anthropogenically present in the environment (ecotoxicity, occupational health, and safety), and to nanoparticles employed in the food, cosmetics, or clothing industry are out of the scope of this chapter. Furthermore, we will focus on the toxicity evaluation of magnetic nanomaterials, which are an extended and significant subgroup of the nanomaterials used in nanomedicine.

The chapter opens with an introduction that begins with a general description of nanomaterials for nanomedicines and their potential routes of uptake and administration in animals and humans. The nanomaterials' fate in the body and the associated physiological response to them are thereafter discussed depending on the exposure pathway. Fundamental mechanisms of cell response to the stress associated with cell interactions with the nanomaterial as well as prominent nanoparticle features responsible for nanotoxicological effects are subsequently described. Finally, examples of magnetic nanomaterials used in nanomedical applications are provided, with emphasis on iron oxide nanoparticles. Noteworthy, the introduction section does not intend to provide a comprehensive, thorough overview of the referred topics but rather a pertinent and concise description that will serve as a foundation to better comprehend the subjects dealt with in the subsequent sections.

In the following sections, a detailed description of the most accepted and reported methods to evaluate toxicity of magnetic nanomaterials is presented. In vitro cyto- and genotoxicity, 3D cultures, and in vivo methods are discussed and illustrative examples provided. In vitro methods with organoids are included due to their potential to provide more translatable, and possibly also more relevant, information from in vitro to in vivo assays.

Nanomaterial Definition and the "Nano" Hazard

The International Organization for Standardization (ISO) defines "nanomaterial" as a "material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale" and "nano object" as a "discrete piece of material with one, two or three external dimensions in the nanoscale", with nanoscale being in the 1–100 nm range (ISO/TS 80004-1 2015). Though it is generally accepted in the scientific community that nanomaterials are those whose main dimensions are below 100 nm, numerous initiatives exist within the framework of regulatory norms for better definitions to be adopted with the unequivocal aim to protect human health and the environment (Boverhof et al. 2015; Miernicki et al. 2019; Auffan et al. 2009). For some materials, nanoscale dimensions can drastically change material properties unveiling characteristics that could not be predicted from the large-scale features of those same materials, e.g., the size-dependent optical and electronic properties of quantum dots (Alivisatos 1996) or the size-dependent temperature transitions in ferromagnetic materials (Gangopadhyay et al. 1992). These unusual and novel attributes have propelled the design, production, and use of nanomaterials for a myriad of applications, ranging from food additives, cosmetics, pharmaceuticals, and flat-panel displays to sensitive biosensors, contrast agents in magnetic resonance imaging (MRI),

and nanomedicines. Noteworthy, for a vast range of materials, nanoscale dimensions do not impose a drastic alteration in material properties but a rather smooth, predictable, and continuous transition from the bulk materials (Donaldson and Poland 2013). The nanoscale feature, however, irremediably changes the proportion of atoms present at the surface compared to those in the core (or the surface-to-volume ratio), thereby leading to innate nanoscale attributes that will effectively differentiate these entities from their large-scale counterparts. Indeed, nanoparticles below 20–30 nm in size are characterized by their thermodynamic instability and enhanced surface reactivity; properties that may increase dissolution processes, redox reactions, or the generation of reactive oxygen species (ROS) (Auffan et al. 2009). These properties, together with other innate nanoparticle attributes, such as size, shape, or the presence of surface ligands, could constitute leading driving forces for undesirable cellular responses that may result in cellular and organ toxicity (Nel et al. 2006a; Sahu and Hayes 2017; Oberdörster et al. 2005; Xia et al. 2009). Compared to larger particles, nanoparticles of certain dimensions can enter the cells and have access to the intracellular milieu and its organelles, something that is precluded for larger particles and that could be potentially toxic (Pietroiusti et al. 2013; Oh and Park 2014; Shvedova et al. 2010). Though there appears to be a general consensus regarding the health and safety concerns associated with the biomedical use of nanoparticles, some argue that the mechanisms behind nanoparticle health hazard are already evident for larger particles, meaning that there is no evidence of novel "nanospecific hazard" (Donaldson and Poland 2013; Fubini et al. 2010). What appears then critical is the understanding of the mechanisms that are set up by the body during its interaction with nanoparticles, either naturally entering the body via inhalation, ingestion, or penetration through the skin or intentionally administered via intravenous or retroorbital, intrapulmonary, intraperitoneal, oral delivery or subcutaneous routes, and the possible consequences of those interactions onto cell and organ viability and function.

Nanomedicine

Differing from the commonly accepted understanding of nano-objects in nanotechnology areas having an upper size limit of 100 nm, in the drug delivery field the definition is extended to cover medicines in the size range from a few nanometers to <1000 nm in diameter. In practice, the useful range of nanomedicines normally falls within the range of 5–250 nm (Garnett and Kallinteri 2006). The sort of materials that could be called nanomedicines can include proteins, polymers, dendrimers, micelles, liposomes, emulsions, nanoparticles, and nanocapsules. Besides nanomaterials employed as drug delivery units, other nanomaterial types and applications exist that currently belong to the nanomedicine field. Magnetic nanomaterials are since long being used as contrast agents in magnetic resonance imaging, as therapeutic tools in hyperthermia for ablation of malignant cancer tissue, or as carriers for (image-guided) drug/gene delivery (Pankhurst et al. 2003; Banerjee et al. 2010; Wu et al.

2018; Kang et al. 2018). Recent developments in nanomaterials have produced small (SPIOs) and ultra-small superparamagnetic iron oxide particles (USPIOs) (Wu et al. 2018; Barry 2008; Bourrinet et al. 2006), as well as multimodal variants that combine magnetic nanomaterials with, for instance, near-infrared fluorescent quantum dots (Sitbon et al. 2014) or fluorochromes (Kircher et al. 2003) for double-imaging capacities. Further applications of magnetic nanomaterials in nanomedicine are described at the end of this section.

Exposure Pathways to Nanomaterials and Possible End Locations in the Body

Given their very small size, comparable to those of viruses and pathogens, engineered nanoparticles can naturally enter the human body: Some nanoparticles can penetrate lung, intestinal, or dermal skin barriers and translocate to the circulatory, lymphatic, and nervous systems, reaching most bodily tissues and organs including the brain, and potentially disrupting cellular processes and causing disease (Buzea et al. 2007; Ali and Rytting 2014; Kreyling et al. 2009; Elder et al. 2006; Oberdörster et al. 2004; Pietroiusti et al. 2013). Once in the body, they will come into contact with the immune system in charge of clearing them if potentially dangerous. Immune cells (and secreted biomolecules) present in the airway and digestive mucosae, as well as in the skin, are the first to intercept nanomaterials upon natural or accidental exposure. When nanomaterials are intentionally administered to the body, as with nanodrugs or nanomaterials for diagnostics and therapy, the immune cells present in blood, muscle tissue, liver, spleen, and kidney are those who will mainly interact with the nano-objects (Buzea et al. 2007; De Matteis 2017; Arami et al. 2015). Upon inhalation, specific sizes of nanoparticles are deposited by diffusional mechanisms in all regions of the respiratory tract, where they are uptaken by immune cells and translocating across epithelial and endothelial cells into the blood and lymph circulation to reach potentially sensitive organs, such as the bone marrow, lymph nodes, spleen, and heart (Oberdörster et al. 2005; Kreyling et al. 2009). Access to the central nervous system has also been reported (Elder et al. 2006; Oberdörster et al. 2004). Nanoparticles adsorbed through the skin distribute via uptake into lymphatic channels (Buzea et al. 2007). Nanomaterials ingested are primarily degraded in the gut, though if they survive the acidic environment of the gastrointestinal tract, they may reach the intestine and the bloodstream (this, if not eliminated in the liver) (Arami et al. 2015). Figure 1 depicts various pathways of human body exposure to nanoparticles, as well as the potentially affected organs and associated diseases.

Though hard to implement and standardize, cellular assays should reflect portal-of-entry toxicity in lungs, skin, and mucous membranes, as well as deleterious effects on target and non-target tissue, such as endothelium, blood cell elements, spleen, liver, nervous system, heart, and kidney (Nel et al. 2006b). Without such a broad interpretation of the nanotoxicity evaluation issue, results shall be deemed as incomplete,
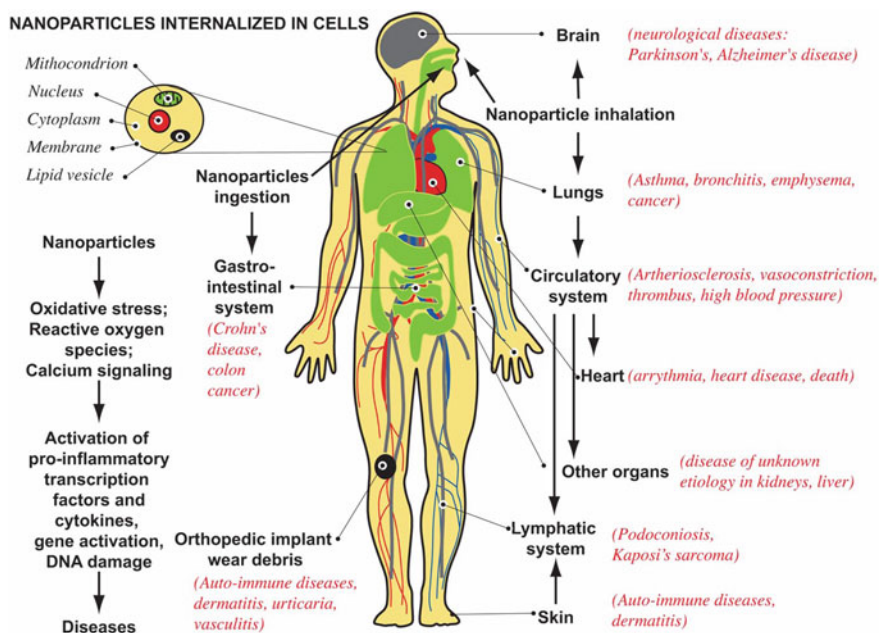
Fig. 1 Schematic representation of the various pathways of human body exposure to nanoparticles, with emphasis in various organs they could reach depending on the exposure pathway, as well as the potentially affected organs and associated diseases. Strategies to enable long-term nanoparticle circulation in the bloodstream together with immune system evasion have been reported. Such strategies are not depicted in this figure, though they constitute an alternative regarding the fate of these nanomaterials upon body exposure. Figure taken from Buzea et al. (2007). Reprinted with permission from AIP Publishing

partial representations of the short- and long-term toxicological hazard represented by nanomaterials.

The Immune Response and the Mononuclear Phagocytic System (MPS)

If the nanoparticles happen to elicit an immune response, a cascade of events is set in place to rapidly eliminate the nanoparticles via phagocytic, metabolic, and degradative processes in immune cells (e.g., white blood cells such as monocytes and residential tissue macrophages). An important part of the immune system, the mononuclear phagocytic system (MPS) also known classically as reticuloendothelial system (RES), has been defined as a family of cells comprising bone marrow progenitors, blood monocytes, and macrophages located in different organs, such as liver, spleen, lymph nodes, bone marrow, lung, and brain that play an active role in

defense reactions against certain microorganisms, interaction with lymphoid cells in immunity, disposal of cell debris, among others (Hume et al. 2019; Chow et al. 2011; Territo and Cline 1975). The major specialized tissue-resident macrophages are dermal macrophages and Langerhans cells in the skin, Kupffer cells in the liver, alveolar macrophages in the lungs, osteoclasts in bones, microglia in the brain, and histiocytes in interstitial connective tissues (Davies et al. 2013; Hirayama et al. 2017; Gordon and Martinez-Pomares 2017). Macrophages are a major cell population in most of the tissues in the body, and their numbers increase further in inflammation, wounding, and malignancy (Hume 2006). Tissue macrophages such as those found in the liver and spleen are the most critical cells in the clearance of NPs from blood circulation: They can eliminate nanoparticles from the bloodstream within seconds of intravenous administration, thereby imposing tremendous challenges to drug delivery nanomaterials or those designed to be site-specific (Arami et al. 2015; Gref et al. 1994). In particular for the liver, this is related to one of its physiologic functions that is to efficiently capture and eliminate particles in the ~10–20 nm size range for the clearance of viruses and other small particles (Choi et al. 2007; Longmire et al. 2008).

Opsonization and How to Evade It

Macrophages cannot directly identify the nanoparticles as such but rather recognize specific opsonin proteins bound to the surface of the nanoparticles following parental injection (Gustafson et al. 2015; Owens and Peppas 2006). Opsonins are blood serum components that adsorb to the surface of foreigner objects with the aim of making them visible to phagocytic cells. Once opsonization has occurred, phagocytes attach to the surface of the foreigner object and engulf it, typically by endocytosis. Endocytosed material will be eventually degraded by the phagocytes and if not, undigested material will either be removed by the renal system or sequestered and stored in one of the MPS organs (Gustafson et al. 2015; Owens and Peppas 2006; Aggarwal et al. 2009). Removal by the renal system occurs for molecules with a molecular weight of 5000 or less, but can be as high as 100,000 for more dense polymers such as dendrimers (Owens and Peppas 2006). For nanoparticles, the size threshold for renal clearance is <10 nm, meaning that nanoparticles of that size range are removed from blood and excreted through the urine (Arami et al. 2015). Noteworthy, if nanomaterials are intended to be used as nanomedicines, it is imperative to endow them with particular properties so that they could evade immune system recognition and elimination and persist in the organism sufficiently long time to reach their target and exert their beneficial effect. Surface modification of nanoparticles with antifouling molecules, such as PEG or zwitterionic polymers, has demonstrated utility at reducing opsonization and minimizing clearance by the MPS leading to improved pharmacokinetic properties (Li and Huang 2009; Guo and Huang 2011). Recent review articles provide a detailed and comprehensive overview of the various uptake pathways of nanoparticles and their fate for each uptake route, from their interactions

with biological barriers and elicited immune responses to biodistribution, pharmacokinetics, and clearance pathways (Sahu and Hayes 2017; Buzea et al. 2007; De Matteis 2017; Arami et al. 2015; Crisponi 2017).

From Extravasation of Nanoparticles to Uptake by Cells

As explained, parental injection of nanoparticles encounters tight immune defensive mechanisms arising from the MPS acting on opsonized nanoparticles, with the most likely result of vast nanoparticle clearance from circulation to the liver and spleen. If opsonization can be reduced and the MPS evaded, nanoparticles with increased blood circulation times still may need to trespass the vascular endothelium barrier, which has tight junctions of lower than 2 nm, to reach target organs. In the liver and spleen, the endothelium is fenestrated, thus allowing material up to 100 nm (liver) or higher than 150 nm (spleen) to pass from the endothelium to the underlying parenchymal cells, so exiting circulation (Garnett and Kallinteri 2006). Some NPs can pass through the endothelial cells by transcytosis mediated by caveolae. If the endothelium becomes leaky due to certain diseases or upon inflammation, nanomaterials can also exit circulation. In some cancers, the epithelium is not only leaky, but it also loses the lymphatics (Garnett and Kallinteri 2006; Greish 2010). For that reason, nanomaterials passing through the leaky epithelium can selectively accumulate in cancer tissue, a process named enhanced permeability and retention (EPR) effect.

Once out of the circulation, nanomaterials will be located in the extracellular matrix, composed of proteins, polysaccharides, and glycoproteins. Some macromolecules and nanoparticles can move through the extracellular matrix via its water channels. Nanomaterials in the extracellular matrix can be spontaneously internalized by cells. As partitioning across membranes is not possible for macromolecules or nanomaterials, entry into cells is largely governed by the mechanisms of endocytosis (Garnett and Kallinteri 2006; Foroozandeh and Aziz 2018). Large particles (0.25–10 µm) are uptaken by phagocytosis, performed by specialized cells such as macrophages and neutrophils, and a variety of other endocytic processes at a smaller scale (Garnett and Kallinteri 2006). Most of these endocytic routes end up in a degradative compartment of the cell, the lysosome, where materials are exposed to high concentrations of a wide variety of hydrolytic enzymes active on proteins, polysaccharides, and nucleic acids. Depending on the nature of the nanomaterial, the lysosomal compartment may be the final destination of the nanomaterial. Besides cell internalization of nanoparticles through endocytosis, positively charged nanomaterials can gain access to the cytoplasm. It is well known that positively charged polymers and polyelectrolyte DNA delivery systems can trigger cytotoxicity due to their interaction with cell membrane phospholipids that results in disruption of the cellular membrane structure (Foroozandeh and Aziz 2018; Gratton et al. 2008).

Consequences of Cell–Nanomaterial Interactions

Besides the description of general uptake pathways of nanoparticles, with their corresponding biodistribution, pharmacokinetics, and clearance routes from the body, it is of particular importance to describe the specific mechanisms set in place during cell–nanomaterial interactions and their dependence with the intrinsic properties of the designed nanomaterials as well as with the biologically modified forms of those nanomaterials when in contact with biological fluids. As noted, the increased surface area per mass compared with larger-size materials of the same chemistry renders nanoparticles thermodynamically unstable, more surface reactive, and more biologically active. This activity relates to a potential for inflammatory and pro-oxidant, but also antioxidant capacity (Oberdörster et al. 2005). Nanoparticle-mediated cytotoxicity mechanisms commonly proposed in the literature include oxidative stress (reactive oxygen and nitrogen species), inflammation, cell membrane damage, genotoxicity, immune system response, autophagy dysfunction, ultrastructural changes in cell or cell organelle morphology, lactate dehydrogenase release, inhibition of cell growth and cell death, among others. The first four mechanisms are described more extensively below.

1. *Reactive Oxygen Species*

    Reactive oxygen species (ROS) are a number of reactive molecules and free radicals derived from molecular oxygen, like superoxide anion, hydrogen peroxide, and nitric oxide. These molecules, produced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal-catalyzed oxidation, can be responsible for a series of deleterious events. As stated, ROS are continually produced during cell metabolic processes, though their generation is normally counterbalanced by the action of antioxidant enzymes and other redox (reduction–oxidation) molecules. An imbalance toward the pro-oxidative state is often referred to as "oxidative stress." Glutathione may be the most important intracellular non-enzymatic small molecule that acts in the defense against the damaging effects of reactive oxygen species. Reduced glutathione (GSH) is regenerated from its oxidized form (GSSG) by the action of an NADPH-dependent reductase. The ratio of the oxidized form of glutathione (GSSG) and the reduced form (GSH) is a dynamic indicator of the oxidative stress of an organism (Jones 2002). The generation of ROS and the related oxidative stress responses are frequent causes of nanoparticle toxicity. The production of ROS has been found in a diverse range of nanomaterials, including semiconductor nanocrystals, fullerenes, carbon nanotubes, and metal oxides (Nel et al. 2006a; De Matteis 2017; Sharifi et al. 2012; Malvindi et al. 2014; Huang et al. 2010). Oxidative stress induced by engineered nanoparticles is due to acellular factors such as particle surface, size, composition, and presence of metals, while cellular responses such as mitochondrial respiration, nanoparticle-cell interaction, and immune cell activation are responsible for ROS-mediated damage (Manke et al. 2013). Nanoparticle-induced oxidative stress responses are behind genotoxicity,

carcinogenesis, inflammation, and fibrosis effects, as demonstrated by activation of associated cell signaling pathways (Xia et al. 2009; Manke et al. 2013). Most of the metal-based (Fe, Si, Cu, Cr, Va) nanoparticles elicit oxidative stress via the generation of free radicals following Fenton-type reactions (Huang et al. 2010). Intracellularly produced free radicals have adverse effects on cell components, like proteins (protein oxidation), lipids (lipid peroxidation), and DNA (DNA strand break) and also alter mitochondrial membrane potential (Manke et al. 2013; Huang et al. 2010). Extremely toxic levels of oxidative stress result in mitochondrial dysfunction and membrane damage, which finally lead to cell death. For transition metal oxide nanoparticles, the physicochemical properties that determine toxicity include surface catalytic activity (e.g., metallic, semiconducting properties), nanoparticle uptake, and nanoparticle dissolution (Huang et al. 2010). In particular for iron oxide nanoparticles, their physical interactions with cellular structures involved in the catalysis of biological redox processes, as well as their dissolution in biological media, catalyze ROS generation and formation of OOH· and OH^- radicals from H_2O_2 via Fenton reaction (Ling and Hyeon 2013; Gaharwar et al. 2017). Indeed, zero-valent iron at the surface of iron oxide nanoparticles is oxidized by dissolved oxygen in aerobic organisms to give rise to Fe^{2+} and OH^-. Fe^{2+} can be further oxidized to Fe^{3+}. Redox-active iron may enhance the generation of more highly reactive and highly toxic hydroxyl radicals from less reactive hydrogen peroxide (a product of mitochondrial oxidative respiration) via Fenton chemistry: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH· + OH^-$ (Ling and Hyeon 2013). Therefore, by their involvement in catalytic redox reactions, zero-valent iron and iron oxide nanoparticles can be directly linked to the production of ROS, in particular of hydroxyl radicals. Protecting the surface of these nanoparticles from oxidation and dissolution in biological media, both intra- and extracellularly, is therefore a *sine qua non* condition for the translation of these nanomaterials to clinical trials.

1.1. *Mitochondrial iron homeostasis*: Mitochondrial iron homeostasis is indispensable for cellular iron management in particular by controlling the synthesis of heme and Fe–S clusters, functional groups that are essential to the functionality of numerous proteins, including those participating in energy production via the respiratory chain (Bresgen et al. 2015). About 0.2–5% of the total cellular iron is considered as transiently mobile, non-protein-bound low-molecular-weight redox-active iron which together with chelatable protein-bound iron defines the dynamic, intracellular "labile" iron pool (LIP) encompassing compartment-specific LIPs of the cytosol, the mitochondria, and the endo-/lysosomal compartment in total containing about 6–16 μM iron, mainly as Fe^{2+} (Bresgen et al. 2015). Iron is shuttled between these pools by distinct mechanisms and can also be directly delivered from endosomes to mitochondria via a "*kiss-and-run*" mechanism, characterized by the docking of iron-containing endosomes or vesicles to the outer mitochondrial membrane followed by the passing over of iron to mitoferrin (Bresgen et al. 2015). This mechanism enables a direct transfer of iron to

the mitochondria, which may be beneficial under physiologic conditions but can have deleterious effects under conditions of iron overload, as those observed upon administration of iron or iron oxide nanoparticles. Transfers involving free Fe^{2+} ions represent a constant hazard of Fenton reactions and their consequent generation of ROS and derived oxidative stress (Bresgen et al. 2015). The highly reactive hydroxyl radical can damage macromolecules within mitochondria, including lipids, proteins, and DNA, and generate genomic instability, organelle dysregulation, and cellular injury or apoptosis (Guo et al. 2013). Therefore, by disrupting the natural balance between generated ROS species, antioxidant defenses, and repair enzymes in mitochondria, zero-valent iron and iron oxide nanoparticles can lead to mitochondrial dysfunction and apoptosis through the transfer of free Fe^{2+} ions to the organelle.

1.2 *Sources of oxidative stress in SPIONs*. Superparamagnetic iron oxide nanoparticles (SPION), and the equivalent for iron oxide nanoparticles, have been associated with four primary sources of oxidative stress: (a) direct generation of ROS from the surface of the nanoparticle, (b) production of ROS via leaching of iron molecules from the surface due to enzymatic degradation, (c) altering mitochondrial and other organelle functions, and (d) induction of cell signaling pathways together with their consequent activation of inflammatory cells, which results in the generation of ROS and reactive nitrogen species (Buzea et al. 2007; Mahmoudi et al. 2012). Protein and lipid oxidation by SPIONS has also been reported (Stroh et al. 2004; Singh et al. 2010; Wei et al. 2016).

2. *Inflammation*

Inflammation is the major process through which the body repairs tissue damage and defends itself against foreign materials. Acute inflammation is typically caused by an external chemical, mechanical, or pathogenic influence, has a relatively short duration (hours to days), and is a necessary protection tool that removes foreign bodies and damaged tissue, preventing further damage (Stevenson et al. 2011). As explained previously, tissue-resident macrophages are involved in the primary immune response of tissues, producing various cytokines and immune regulators. Upon macrophage interaction with nanoparticles, macrophages can get polarized toward pro-inflammatory or anti-inflammatory phenotypes depending on the nanoparticle type (Miao et al. 2017; Reichel et al. 2019). The net effect of such a polarization may be the disruption of the stable equilibrium between macrophage-produced pro- and anti-inflammatory cytokines, a fact that has been linked to the pathogenesis of a number of inflammatory disorders, e.g., tumor necrosis factor-α (TNF-α) cytokine related to rheumatoid arthritis (RA) (Stevenson et al. 2011). Furthermore, oxidative stress also results in the release of cytokines. Indeed, several reports demonstrate that the exposure to nanomaterials is associated with inflammation, with various particle-related parameters, like size, playing a defining role (Kharazian et al. 2018; Manshian et al. 2018; Gojova et al. 2007; Deng et al. 2011).

3. *Cell membrane damage*

   Nanoparticles have been involved in cell and organelle membrane damage through various mechanisms, typically depending on nanoparticle attributes, such as size, surface charge, or hydrophilicity (Leroueil et al. 2007; Fischer et al. 2003; Contini et al. 2018; Chen et al. 2009a; Zhu et al. 2013a; Beddoes et al. 2015). Nanoparticles can cause cell membrane disruptions and, eventually, cell death (Ali and Rytting 2014; Pietroiusti 2012). Hemolysis, or the damage of red blood cell membrane, is one insightful parameter to measure nanoparticle-induced toxicity when interactions with the bloodstream are envisaged. Noteworthy, cell transfection and in vivo gene delivery applications rely upon nanostructures that can non-selectively cross the cell membrane and access the cytosol without inducing major cytotoxic effects. A vast literature supports the idea that polycationic organic polymers, such as PLL (polylactic acid), PEI (polyethyleneimine), and PAMAM (polyamidoamine) dendrimers, permeabilize the cell plasma membrane and, if sufficiently concentrated, result in cell lysis (Leroueil et al. 2007; Fischer et al. 2003; Hong et al. 2004, 2006). Regarding inorganic nanomaterials, examples abound too; for instance, copper metal (Cu; 100–200 nm size) and Cu–Zn (3.8 nm size) alloy nanoparticles induced significant membrane damage upon exposure to lung epithelial cells (A549), while CuO (20–50 nm) nanoparticles showed no such effect (Karlsson et al. 2013). Iron oxide nanoparticles coated with a copolymer of chitosan and PEG could pass the blood-brain barrier (Veiseh et al. 2009a), though other formulations could achieve the same result after magnetic field application and radiofrequency radiation to modulate blood-brain barrier integrity, thereby increasing its permeability (Busquets et al. 2015a). Nakamura and Watano have compiled information about organic and inorganic nanomaterials passing through the cell membrane, either by endocytosis or by direct penetration (Nakamura and Watano 2018). Whether such nanomaterial passages through the cell membrane elicit irreversible damage and nanotoxicity or not, that certainly depends on administered concentration and nanoparticle properties. Cell membrane damage can be determined by measuring the degree to which lactate dehydrogenase (LDH), a cytosolic enzyme present in many different cell types, leaks from the cells (Decker and Lohmann-Matthes 1988). The amount of enzyme that leaks into the extracellular medium can be quantified by an enzymatic reaction that colorimetrically indicates the amount of LDH present.

4. *Genotoxicity*

   Alterations in cell redox equilibrium between generated reactive oxygen species and antioxidant elements result in oxidation of critical cell biomolecules, like membrane lipids or nuclear DNA. DNA damage covers a wide range of DNA lesions, which include genome rearrangements, strand breaks, and the formation of modified DNA bases. These different types of DNA lesions can lead to chromosomal aberrations, gene mutations, apoptosis, carcinogenesis, or cellular senescence if left unrepaired (Singh 2017). Epidemiological, in vitro, and in vivo studies show that nanoparticles of various materials (diesel, carbon black, welding fumes, and transition metals) are genotoxic to humans and rats (Buzea et al. 2007). DNA damage was observed upon cell exposure to TiO_2 (Kansara et al.

2015), cobalt (Wan et al. 2017), silver (Ahamed et al. 2008), carbon nanotubes and graphite nanofibres (Lindberg et al. 2009), and iron oxide (Ahamed et al. 2013; Alarifi et al. 2014) nanoparticles. Furthermore, some nanomaterials, such as metal and metal oxide, quantum dots, fullerenes and fibrous nanomaterials, were found to induce chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts, and alterations in gene expression, sometimes even through cellular barriers (Singh et al. 2009; Bhabra et al. 2009).

5. *Others*

   Nanoparticles can cause negative physiological effects, such as inflammation, immune system response, autophagy dysfunction, ultrastructural changes in cell or cell organelle morphology, inhibition of cell growth and cell death, among others, that may partially or completely impair normal cell and organ functioning.

Autophagy and lysosomal dysfunction are proposed as emerging mechanisms of nanomaterial toxicity (Stern et al. 2012; Wang et al. 2018). Since most endocytosed nanoparticles accumulate within the lysosomal compartments without evident exit, the evaluation of the effects of nanoparticle accumulation in lysosomes, as well as in autophagy, a lysosomal degradative pathway, needs to be addressed. If the nanoparticles are taken up into the lysosomal compartment but are not biodegradable, they could potentially accumulate there and cause lysosomal dysfunction and hence cellular toxicity.

Nanoparticle Uptake by Cells

Cell–nanomaterial interactions also comprise cell internalization of nano-objects, unspecific adsorption of nanoparticles to the cell membrane, as well as induced cell responses when nanoparticles are functionalized with, e.g., targets of cell receptors or specific ligands that promote cell membrane permeabilization, as already discussed (Oh and Park 2014; Gratton et al. 2008; Beddoes et al. 2015; Salatin et al. 2015; Kurtz-Chalot et al. 2014; Pan et al. 2018). Nanoparticles adsorbed on cell membranes can block cellular ducts, cause structural changes to the membranes, or inhibit mobility and nutrient or ion intake and result in cell death (Kurtz-Chalot et al. 2014; Pan et al. 2018; Warren and Payne 2015). Nanoparticles have been functionalized with a variety of biomolecules that can bind to cell membrane receptors and promote nanoparticle internalization, e.g., endothelial growth factor (EGF)-functionalized nanoparticles to target EGF receptor (EGFR), folate-functionalized nanoparticles to target the folate receptor, RGD peptide, integrin receptor, among others (Xu et al. 2013).

When nanomaterials extravasate and leave circulation, they encounter the extra-cellular matrix (ECM), composed of proteins, polysaccharides, and glycoproteins. Through water channels present at the ECM, some biomolecules, but also nanoparticles, can pass through the ECM. As partitioning across membranes is not possible for macromolecules, entry into cells is largely governed by biological mechanisms

of endocytosis. These include the uptake of large particles (0.25–10 μm) by phago-
cytosis, performed by specialized cells such as macrophages and neutrophils, and a
variety of other endocytic processes at a smaller scale (Garnett and Kallinteri 2006).
Most of these endocytic routes end up in a degradative compartment of the cell, the
lysosome, where materials are exposed to acidic pH and high concentrations of a
wide variety of hydrolytic enzymes active on proteins, polysaccharides, and nucleic
acids (Garnett and Kallinteri 2006; Foroozandeh and Aziz 2018). The hostile lyso-
somal can degrade all but the most biopersistent of the nanomaterials. In addition to
the endolysosomal pathway, recent evidence suggests nanomaterials can also induce
autophagy (Stern et al. 2012).

Technologies based on cell/organ imaging, targeted drug delivery, or localized
therapy depend on nanoparticle uptake by cells. The safety of these techniques
depends, however, on cellular uptake of nanoparticles without affecting normal
cellular function, both at the target site and elsewhere in the body (Buzea et al.
2007).

A battery of cellular tests exists that can elucidate the actual mechanisms involved
in cellular toxicity, such as the MTT or MTS assays that assess cell metabolic activ-
ity relying on the presence of cellular oxidoreductases (NADPH) enzymes in the
cytosol, or the LDH assay that analyzes the activity of the cytoplasmic enzyme lac-
tate dehydrogenase when released to culture media upon cell membrane damage.
These methods will be described, and examples are provided in Sect. 13.2.

Nanomaterial Properties Affecting Cells and Organs

Nanomaterials properties are critical at defining possible cellular responses when
interaction takes place. It is generally believed that the interactions with biologi-
cal components (e.g., proteins, opsonins), cellular uptake, in vivo fate, and toxicity
of nanoparticles are strongly correlated with their physicochemical characteristics.
Some relevant properties include size, size uniformity, shape, composition, surface
area, surface charge, state of aggregation, degree of crystallinity, aspect ratio, surface
functionalization, aging in biological media, and the potential to generate ROS (Oh
and Park 2014; Buzea et al. 2007; Zhang et al. 2012a). Plentiful reviews provide
a comprehensive description of these factors and their implication in toxicological
responses (Shvedova et al. 2010; Buzea et al. 2007; Arami et al. 2015; Crisponi 2017;
Zhang et al. 2012a). For that reason, we are going to refer to some of them and to
focus on magnetic nanomaterials.

1. *Size*

Size is a significant property that can influence the toxicity of a material and its
distribution within the body and the cell (Nabiev et al. 2007). Numerous studies
have demonstrated size-dependent cell toxicity and organ biodistribution (Kim
et al. 2012; Vedantam et al. 2013; Feng et al. 2018a; Xie et al. 2016). In a recent
study, Xie et al. investigated the effect of iron oxide (Fe_3O_4) nanoparticle size

onto cytotoxicity in two human hepatoma cell lines, SK-Hep-1 and Hep3B. While 6-nm-size nanoparticles exhibited negligible cytotoxicity, 9-nm nanoparticles affected cytotoxicity through mitochondrial dysfunction and by inducing necrosis through mitochondria-dependent ROS generation. On the other hand, 14-nm nanoparticles were found cytotoxic by impairing the integrity of the plasma membrane and promoting massive lactate dehydrogenase leakage (Xie et al. 2016). In an in vivo trial with naked gold nanoparticles, those with sizes ranging from 8 to 37 nm induced severe sickness in mice, such as fatigue, loss of appetite, change of fur color, and weight loss. From day 14, mice in this group exhibited a camel-like back and crooked spine, and the majority of mice in these groups died within 21 days. However, researchers could not find sickness or lethality in mice injected with gold nanoparticles of 5 and 3 nm (Chen et al. 2009b). Iron oxide nanoparticles with a hydrodynamic diameter higher than 100 nm quickly accumulate in the liver and spleen through macrophage phagocytosis and entrapment in liver and spleen sinusoids, while nanoparticles below 10–15 nm (SPIOs) are likely to be eliminated through the kidneys (Arami et al. 2015; Amstad et al. 2011). Importantly, nanoparticle size is a relevant factor to promote the unspecific retention of nanoparticles in tumors through the enhanced permeation and retention mechanism (Barry 2008).

2. *Size uniformity, shape, composition, and surface charge*

Size uniformity of nanoparticles is a prerequisite for the proper evaluation of any type of nanomaterial to be employed in biological applications. Low polydispersity index nanomaterials are desirable for repeatable performance. Considering the shape, generally, one-dimensional nanostructures, such as polymer filaments, carbon nanotubes, and gold nanorods with a high length-to-width aspect ratio, have shown longer blood circulation times over the spherical counterparts (Arami et al. 2015). Short-rod mesoporous silica nanoparticles (MSNs) are easily trapped in the liver, while long-rod MSNs distribute in the spleen. MSNs are mainly excreted by urine and feces, and the clearance rate of MSNs is primarily dependent on the particle shape, where short-rod MSNs have a more rapid clearance rate than long-rod MSNs in both excretion routes (Huang et al. 2011a). Moreover, carbon nanotubes can interact directly with the cellular cytoskeleton, including the microtubule system during the formation of the mitotic spindle apparatus, leading to aberrant cell division (Sargent et al. 2009).

Composition is another factor that influences the toxicity of nanomaterials. Quantum dots (QD), for instance, may create a health hazard due to the presence of toxic heavy metal elements. It may, however, be possible to reduce the potential toxicity of nanomaterials such as QDs by adding a coating or nanoshell: Core–shell CdSe/CdS/ZnS QDs demonstrated acceptable cytotoxicity (as determined with the MTT test) after 48-h incubation with HeLa cells (Tasso et al. 2015). As stated in previous subsections, iron and iron oxide nanomaterials are, per se, biocompatible, so toxicity related to composition is not a concern, unless toxicity arises from the composition of surface functionalization.

Surface charge is another critical parameter that influences protein adsorption, the formation of the protein corona, and the subsequent biological behavior of the

nanoparticle. Positively charged SPIO nanoparticles interact with the negatively charged cell membrane more than their negatively charged or neutral counterparts (Thorek and Tsourkas 2008). Increased in vitro cytotoxicity and in vivo pulmonary toxicity have been observed for cationic polystyrene nanospheres when compared with anionic or neutral polystyrene (Xia et al. 2008).

3. *Surface functionalization and aging in biological media*

   Surface functionalization may contemplate both the addition of a shell around the nanoparticle core and the surface binding of (bio)macromolecules, polymers, short peptides or others, commonly referred to as ligands.

   3.1 *Shell*: Since pure metals, such as Fe, Co, and Ni and their metal alloys, are very sensitive to air, covering them with a shell could prevent oxidation and hence the release of metal ions to the biological environment. Coatings with inorganic components, like silica, carbon, precious metals (Au, Ag), or oxides, have been reported (Lu et al. 2007). Noteworthy, the shell can also serve the purpose of carrying a drug or a fluorescent label.

   3.2 *Ligand*: Without a ligand, nanoparticles would rapidly aggregate through interactions between themselves or with biological molecules and precipitate out of solution. Commercially available superparamagnetic iron oxide NPs are typically coated with sugars, such as dextran, or synthetic polymers such as silicone. These ligands have high molecular weights (>10 kDa) and poor affinity to the iron oxide core, which leads to the frequent binding of several particles. When shorter (molecular weight <10 kDa) ligands with a precise control over their structure are employed, higher reproducibility, nanoparticle stability, and narrower particle distributions are obtained. Rational design of ligands include the presence of (a) anchoring groups, (b) spacing groups, and (c) biofunctionalization groups (Amstad et al. 2011). Anchoring groups are especially relevant at ensuring a strong binding between the ligand and the nanoparticle surface, which in turns prevents ligand desorption from the surface or even its displacement by competitive species (e.g., proteins) present in biological media, as well as particle aggregation. This is particularly important in conditions of high dilution, as those employed in nanomedicine. Ligands that strongly bind to the nanoparticle surface and have a good surface coverage can overcome the attractive van der Waals and magnetic potentials imparting long-term colloidal stability under dilute conditions, high salt concentrations, and elevated temperatures (Amstad et al. 2011). Spacing groups are usually designed to contain protein-repelling units, such as PEG or zwitterions, to render the nanoparticle "stealth," i.e., able to prevent protein/biomolecule adsorption. Stealth nanoparticles are a *sine qua non* condition for in vivo applications and to increase circulation time by preventing the binding of opsonin proteins and therefore a prompt nanoparticle uptake by the RES system. Biofunctionalization groups are added to enable bioconjugation of targeting species, like folic acid to specifically direct nanoparticles to tumor tissue. Various types of iron oxide nanoparticles conjugated to folic acid were recently

employed for imaging and therapy of different cancer types. (Bonvin et al. 2017; Huang et al. 2017; Li 2015).

A novel approach to nanoparticle coating relies on the use of the membrane materials of the target cells, a strategy that could bypass immune system recognition and rapid clearance, as well as homologous targeting (Zhen et al. 2019). The red blood cell membrane-camouflaged nanoparticles are the first reported cell-membrane biomimetic system, and currently the most popular natural carriers in biomedical applications (Zhen et al. 2019).

3.3 *Aging in biological media*: Depending on the characteristics of the surface ligands, nanoparticles in contact with biological media may suffer from the unspecific adsorption of opsonins and other biomolecules that will form a so-called protein corona. The formation of this protein corona onto nanoparticles irremediably changes their innate physicochemical properties, such as size, surface charge, surface composition, biofunctionality if target-recognition species were added, hence giving nanoparticles a new biological identity (Corbo et al. 2016; Nguyen and Lee 2017; Lundqvist 2017; Yallapu et al. 2015). This nanoparticle–protein complex, not the bare nanoparticle, determines various biological responses such as fibrillation, cellular uptake, circulation time, bioavailability, and even toxicity (Nguyen and Lee 2017). The major proteins that form the protein corona are albumin, fibrinogen, apolipoprotein, and immunoglobulin G, though their relative composition in the protein corona varies with the physicochemical properties of the nanoparticles, as well as with the specific pool of proteins present at a given place (Aggarwal et al. 2009). In other words, the protein corona is dynamic. Nanoparticle hydrophobicity, size, and surface charge determine the number and types of adsorbed proteins (Aggarwal et al. 2009). As previously explained, the formation of a protein corona is a prelude for nanoparticle uptake by the RES system. Protein structural changes upon binding may also be a cause for macrophage uptake. Nanoparticles with bound albumin demonstrated increased circulation time and increased tumor permeating rates (Aggarwal et al. 2009; Nguyen and Lee 2017).

Besides the almost immediate formation of a protein corona upon nanoparticle interaction with biological fluids, long-term exposure can result in nanoparticle partial degradation and continuous modifications of its physicochemical properties, thereby altering the biological response. For instance, silver nanodots encapsulated in silica demonstrated a survival half-life of 2.6 ± 0.3 h in DMEM, a typical cell culture medium, prior degradation of the silica outer layer. Though silica nanoparticles showed extraordinary stability in PBS, a synergistic etching of silica by medium components, particularly the amino-rich compounds in cell culture medium, as well as blood, deteriorated the silica layers (Yang et al. 2018). Ligand destabilization, nanoparticle aggregation and dissolution, dynamic exchange of biomolecules onto the destabilized nanoparticle surface, catalytic processes, among others, are all consequences of long-term nanomaterials aging in biological conditions.

Magnetic Nanomaterials and Their Applications in Nanomedicine

1. *Magnetic nanomaterials*

    There exist a wide range of magnetic nanomaterials that include oxides (γ-Fe_2O_3, Fe_3O_4), pure metals (Fe, Co, Ni, etc.), ferromagnetic alloys ($CoPt_3$, FePt, FeNi, FeCo), or spinel-type ferromagnets ($MgFe_2O_4$, $MnFe_2O_4$, and $CoFe_2O_4$) (Pankhurst et al. 2003; Wu et al. 2018; Lu et al. 2007; Biehl 2018). Although pure metals are able to yield higher saturation magnetizations, they are not suitable for clinical use due to their high toxicity and oxidative properties that result in non-magnetic oxides (Wu et al. 2018; Biehl 2018). Similarly, since most of the ferromagnetic alloys contain toxic components, like Co or Ni, they have been excluded from biomedical applications.

    The shape, size, and composition of magnetic nanoparticles depend on parameters such as the type and concentration of salts used like chlorides, sulfates, and nitrates, ferrous and ferric ratio, pH and ionic strength of media, as well as on the specific synthesis method (Banerjee et al. 2010; Lu et al. 2007; Biehl 2018). For magnetic nanoparticles, nanoparticle stability and size have to be traded off against high saturation magnetization (Ms) values, a key property in MRI applications (Jun et al. 2008). Ms is one essential parameter that describes magnetic response: Higher MS enables easier magnetic separation and magnetic transport to other locations, as well as induce higher magnetic field gradients if dispersed in solutions and subjected to an external homogeneous magnetic field (Amstad et al. 2011). These magnetic field perturbations are responsible for changed relaxivities r2 of water molecules measured in MRI. Thus, the higher the Ms of magnetic nanoparticles with everything else being equal, the more effective they are as MR contrast agents. Ms of magnetic nanoparticles is always below that of the respective bulk materials and decreases with decreasing core size and with the presence of surface ligands (Amstad et al. 2011; Jun et al. 2008). Properties such as saturation magnetization, specific absorption rate (SAR), chemical stability, scalability of the synthetic route, non-toxic composition, and narrow size distribution are determinant of the potential for transfer of any of these materials to the biomedical field.

2. *Iron and iron oxide*

    Iron, one of the most abundant metallic elements in living organisms, is essential for various biological processes, such as oxygen transport by hemoglobin and cellular respiration by redox enzymes. Iron oxide nanoparticles are one of the few nanomaterials that can be injected into the body and incorporated into natural metabolic pathways of the humans (Ling and Hyeon 2013). Fe^{3+} ions resulting from iron oxide dissolution under acidic conditions can be fed into the natural iron storage pathway. Compared with many other nanoparticles, iron oxide nanoparticles are benign, non-toxic, and biologically tolerated. The most common biocompatible magnetic nanomaterials are pure iron oxides, such as maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4). For in vivo use, iron-based magnetic particles

have an attractive combination of high magnetization, well-described cellular metabolism, and relatively low toxicity (Barry 2008). Also, iron oxide particles under about 30 nm are superparamagnetic, with no magnetization in the absence of an externally applied magnetic field that can lead to aggregation. Iron oxide nanoparticles have been produced according to a variety of protocols resulting in nano-objects of various sizes, shell types, ligands, and diverse interactions with biological fluids, cells, and organs (Wu et al. 2018; Ling and Hyeon 2013; Amstad et al. 2011; Lu et al. 2007).

Although iron oxide nanoparticles are relatively biocompatible, naked iron oxide nanocrystals can contribute to in vitro cytotoxicity as a result of ROS generation. Similarly, iron oxide nanoparticles doped with magnetically susceptible elements (e.g., $MnFe_2O_4$ and $CoFe_2O_4$), and metal alloy nanoparticles (e.g., FeCo and FePt) are little employed in biomedical applications due to their potential toxicity and rapid oxidation, though their magnetism is stronger than for pure iron oxide nanoparticles. In spite of its biocompatibility, iron oxide nanoparticles are subjected to the release of iron from dissolved iron oxide nanoparticles: When in the bloodstream, the amount of dissolved iron is negligible if iron oxide nanoparticle concentrations in the $\mu g\,kg^{-1}$ body weight range are injected (Amstad et al. 2011); when accumulating locally, e.g., within the tumor tissue, the amount of dissolved iron can prove toxic, though.

3. *Applications of magnetic materials*

Magnetic nanomaterials have practical applications in the biomedical field, including magnetic cell labeling, separation, and tracking, for therapeutic purposes in hyperthermia and drug delivery, and for diagnostic purposes, e.g., as contrast agents for magnetic resonance imaging (MRI) or photodynamic therapy (PDT) (Banerjee et al. 2010; Amstad et al. 2011; Paul and Sharma 2010). Magnetic nanoparticles are also being tested for tissue engineering applications, for example, in the mechanical conditioning of cells growing in culture, and in magnetic biosensing, using, for instance, magnetic nanoparticles coupled to analyte-specific molecules to detect target molecules via nanoparticle aggregation upon target binding, and that monitored by changes in proton relaxation times on a bench-top nuclear magnetic resonance system (Pankhurst et al. 2003; Banerjee et al. 2010). For binding of nucleic acid molecules to nanomaterials and applying a magnetic field to preferentially locate those nano-objects at the target site with the posterior release of the genetic material, an application named gene therapy has also been proposed (Dobson 2006). Alternatively named magnetofection, this method has been used to site-specifically deliver SPIONs-PAA-PEI-pDNA complexes to murine B16F1 melanoma cells, while SPIO nanoparticles bound to siRNAs (targeting the HOTAIR sequence in human glioma) effectively mediated low expression of HOTAIR and inhibited the proliferation, invasion, and in vivo tumorigenicity of CD133^{+} human glioma stem cells (Fang et al. 2016; Prosen et al. 2013).

For imaging, iron oxide nanomaterials are advantageous since they are biocompatible, biodegradable, and have deeper imaging penetration in tissues than, for instance, fluorescent probes (quantum dots). Nevertheless, as previously

explained, they can be uptaken, opsonized, and rapidly cleared from the body by phagocytes. On the other hand, certain USPIO nanoparticles possess extended residence time in the bloodstream that make them well suited for vasculature imaging and also for imaging lesions where the particles can extravasate through leaky vasculature, as occured in cancer tissue. For the latter, USPIOs can provide valuable information on cancer stage and potential for metastasis, increased neovascularization, and vascular leakiness with tumor malignancy (Barry 2008). Furthermore, USPIOs, such as ferumoxytol and ferumoxtran-10, have shown excellent potential for brain tumor imaging because brain tumors often have an impaired blood-brain barrier (Neuwelt et al. 2009). Finally, multimodal variants of magnetic materials that include fluorescent nanoparticles for dual magnetic and fluorescence imaging (Mulder et al. 2007; Kwon et al. 2018), magnetic and photothermal (Nima et al. 2019), optoacoustic (Bell et al. 2019), or plasmonic (Stafford et al. 2018) modalities are flourishing these days and show great promise for enhanced diagnostics and therapy (Tomitaka et al. 2019). For further examples about magnetic nanoparticle applications, the reader is conveyed to a series of review articles (Pankhurst et al. 2003; Banerjee et al. 2010; Wu et al. 2018; Barry 2008; Lu et al. 2007; Huang et al. 2011b; Friedrich et al. 2007; Singamaneni et al. 2011).

Further Considerations

Though in vitro and in vivo assays are a fundamental part of the toxicological evaluation of nanomaterials, they shall remain a first approximation to the problem in as much as they do not consider aspects related to the long-term and whole-body impact of these nano-objects. Furthermore, in vitro assays should not only be restrained to the cell type that is the expected target of that nanomaterial (e.g., cancer cells for hyperthermia applications), but they should be widened to include all possible cell types that will have potential interactions with that nanomaterial, such as blood cells for systemic administration or the mucosa lining for inhalation of nanoparticles. Macrophages in the different organs that compose the mononuclear phagocytic system (MPS), as well as other cells representative of those organs, should also be considered. In vivo experiments would substantially increase our understanding of nanotoxicity if pharmacokinetic profiles and long-term toxicity of both, nanoparticles and their degradation products, could be clearly and systematically addressed. Systematic preclinical and clinical studies in relevant animal or human models should be performed in order to elucidate clearance mechanisms and residual biodistribution over several periods of time, e.g., from one day to months.

From the point of view of the nanomaterials, much is needed to foster material characterization in physiologically relevant biological conditions. Colloidal and chemical nanoparticle stability should be evaluated in blood serum and with suspensions mimicking the mucous lining. A better approximation than phosphate buffer

saline certainly consists in employing the serum-supplemented cell medium used for culturing cells. Colloidal stability and its relationship to nanoparticle aggregation and to potential toxicity or to an unintended nanoparticle removal from the body due to its higher-than-expected size should be carefully considered prior incurring in further in vivo tests. Similarly, chemical stability in different biologically relevant media, specially at high and low pH, e.g., in the presence of enzymes, should be systematically scrutinized to adopt strategies that limit nanoparticle degradation and to be able to separate nanoparticle degradation products from the nanoparticle characteristics themselves in toxicological examinations.
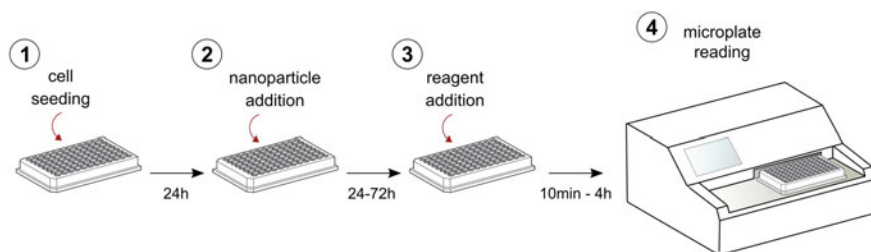
In Vitro Evaluation of Cytotoxicity

In vitro (toxicity) assays refer to experiments performed using subcellular systems (e.g., organelles), cellular systems (e.g., cell cultures, barrier systems), tissues, or whole organs. Within the context of nanomedicine, they are used to screen the adverse effects of potentially therapeutic nanomaterials before their in vivo evaluation. The toxicity of materials is frequently assessed first in vitro in order to ascertain suitability and dosage for further in vivo studies, thus reducing the use of research animals (Sutariya 2015; Mitjans et al. 2018).

Cytotoxicity is a broad and ill-defined term that refers to the potential of a compound (or treatment) to cause cell damage or death (Jain et al. 2018; Niles et al. 2008). Within this context, a material can be recognized as cytotoxic if alters cell growth rate, attachment, or morphology, or causes cell death (Horváth 1980). Usually, the cytotoxicity of a test nanomaterial is determined by evaluating the number of viable cells or some surrogate biomarker related to cell viability after a defined incubation time (Riss et al. 2011). Alternatively, the number of damaged/dead cells or parameters related to the degree of cell death, rather than cell viability, can be determined. The former and latter strategies constitute the basis for the so-called cell viability and cytotoxicity assays, respectively (Niles et al. 2008), though these terms are frequently used interchangeably.

There are several methods described in the literature to assess cell viability and cytotoxicity (Inglese 2010; Stoddart 2011; Gilbert and Friedrich 2017), ranging from assays that measure dye inclusion (e.g., neutral red assay) or exclusion (e.g., Trypan blue assay), metabolic activity (e.g., MTT assay), activity of released enzymes (e.g., LDH assay), apoptosis biomarkers (e.g., caspase 3/7 assay), the colony formation ability (i.e., clonogenic assay), among others. The instrumentation required for these assays is very diverse, implying the use of optical, fluorescence or confocal microscopy, flow cytometry, or microplate readers for absorbance, fluorescence, or luminescence measurements, depending on the case.

In this chapter section, we will focus on microplate-based cell viability/cytotoxicity assays. Compared to other methodologies, these cost-effective assays offer the possibility of assessing a large number of samples using simple equipment (frequently available in most laboratories) in short periods of time (Niles et al. 2008;

Fig. 2 Schematic representation of the general procedure followed in microplate-based cell viability/cytotoxicity assays

Riss et al. 2010). The general procedure for the evaluation of candidate nanomaterial toxicity using these methods is schematized in Fig. 2. Briefly, the cell line (or primary cell) of interest is seeded in a microplate and allowed to grow during a determined time (usually 24 h). After this, cells are exposed to varying concentrations of the nanomaterial under study for a given incubation time (usually 24–72 h). Then, the assay reagent, which measures a surrogate biomarker, is added to the microplates and allowed to react until the "response" is developed (usually in 10 min to 4 h). The response (absorbance, fluorescence, or luminescence) is measured in a microplate reader, and it is (ideally) proportional to the number of viable or damaged cells. When a clear dose–response curve is observed, quantitative parameters are derived, being the most important the inhibitory concentration 50 (IC_{50}) which, in cell viability assays, is the concentration that causes 50% of the measured response compared to control cells not exposed to the nanomaterial.

Table 1 summarizes the cell viability/cytotoxicity assays that will be explained in the following sections. They are based on the determination of cellular metabolic activity (tetrazolium-based, resazurin-based, and ATP–luciferase assays), dye-binding ability (neutral red and sulforhodamine B assays), or activity of released enzyme (LDH assay). In addition, Table 1 collects the main references that have applied these methods to study the cytotoxicity produced by (mostly iron oxide-based) magnetic nanomaterials.

Tetrazolium Salt-Based Assays

This group of colorimetric assays is based on the ability of metabolically viable cells to reduce tetrazolium salts into formazan products (Berridge et al. 2005). The first widely accepted assay to determine cell viability based on this kind of compounds was introduced by Mosmann in 1983 using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann 1983).

MTT is a yellow dye that upon (intracellular) reduction forms a purple formazan product (see Fig. 3a). The reduction is mainly catalyzed by dehydrogenases and oxidoreductases that use NADH and NADPH as cofactors (electron donors) and can

Table 1 Main cell viability/cytotoxicity assays used for the evaluation of magnetic nanomaterials

Assay	Principle	Response	References[a]
Tetrazolium salts	Metabolic activity (NADH dehydrogenase)	Absorbance	Arbab et al. (2003), Choi et al. (2009), Oliveira et al. (2013), Cai et al. (2013), Zhu et al. (2011), Xuan et al. (2011), Schleich et al. (2013), Halamoda Kenzaoui et al. (2012), Aranda et al. (2013), Kunzmann et al. (2011), Könczöl et al. (2011), Zou et al. (2010), Zhu et al. (2013b), Liu et al. (2011), Li et al. (2013), Hanot et al. (2015), Klein et al. (2012), Lee et al. (2009a), Hsiao et al. (2008), Mejías et al. (2013), Müller et al. (2007), Costa (2015), Sadeghi et al. (2015)
Resazurin	Metabolic activity (NADH dehydrogenase)	Fluorescence	Costa (2015), Kievit et al. (2011), Guarnieri et al. (2014), Narayanan et al. (2012), Boyer et al. (2010), Mok et al. (2010), Fernández-Bertólez et al. (2018a), Kiliç (2015), Kievit et al. (2009), Sun et al. (2008), Tse et al. (2015), Lartigue et al. (2012)
ATP–luciferase	Metabolic activity (ATP content)	Luminescence	Khandhar et al. (2012), Chu et al. (2013), Luo et al. (2015), Joris et al. (2016), Huth et al. (2004), Park (2014), Sharkey et al. (2017)

Table 1   (continued)

| Assay	Principle	Response	References[a]
Neutral red	Dye binding (lysosomes)	Absorbance Fluorescence	Könczöl et al. (2011), Müller et al. (2007), Costa (2015), Fernández-Bertólez et al. (2018a), Kiliç (2015), Granot and Shapiro (2011)
Sulforhodamine B	Dye binding (proteins)	Absorbance Fluorescence	Hanot et al. (2015), Wang et al. (2014), Shi et al. (2013), Zheng et al. (2018), Barick et al. (2009), Thu (2009), Shanavas et al. (2017), Fu et al. (2017), Guo et al. (2015)
Lactate dehydrogenase	Enzyme leakage (LDH activity)	Absorbance	Malvindi et al. (2014), Gaharwar et al. (2017), Ahamed et al. (2013), Choi et al. (2009), Sadeghi et al. (2015), Park (2014), Sharkey et al. (2017), Soenen et al. (2010), Hirsch et al. (2013), Alarifi et al. (2014), Han et al. (2011)

[a]Selected references accounting for the use of these cell viability/cytotoxicity assays to determine cytotoxicity of magnetic (mostly iron oxide-based) nanoparticles

take place at mitochondria, cytoplasm, and the plasma membrane (Berridge et al. 2005; Präbst et al. 2017). Since the formazan crystals are insoluble and intracellularly formed, cell lysis (detergent solutions) and solubilization (organic solvents like DMSO or isopropanol) steps are required prior absorbance measurement.

MTT assay can be used to determine viability of adherent or suspended cells from animal, plant, or fungal origin, offering good sensitivity and wide linear dynamic range (from 200–1000 to 50,000–100,000 cells, depending on the cell) (Präbst et al. 2017; Kupcsik 2011). In contrast to other well-established methods such as Trypan blue counting, ^{3}H-thymidine incorporation, fluorometric DNA assays, or flow cytometry-based techniques, MTT assay does not produce toxic or radioactive damage and is faster, relatively cheap, and easy to perform requiring only simple instrumentation (Sylvester 2011). Owing to these features, the assay has become popular in the screening of new drugs (Kupcsik 2011; Sylvester 2011).

In addition to MTT, other tetrazolium salts that produce water-soluble formazan products have been developed. Among them, MTS (Barltrop et al. 1991; Cory et al. 1991), XTT (Scudiero et al. 1988; Paull et al. 1988), WST-1 (Ishiyama et al. 1993),
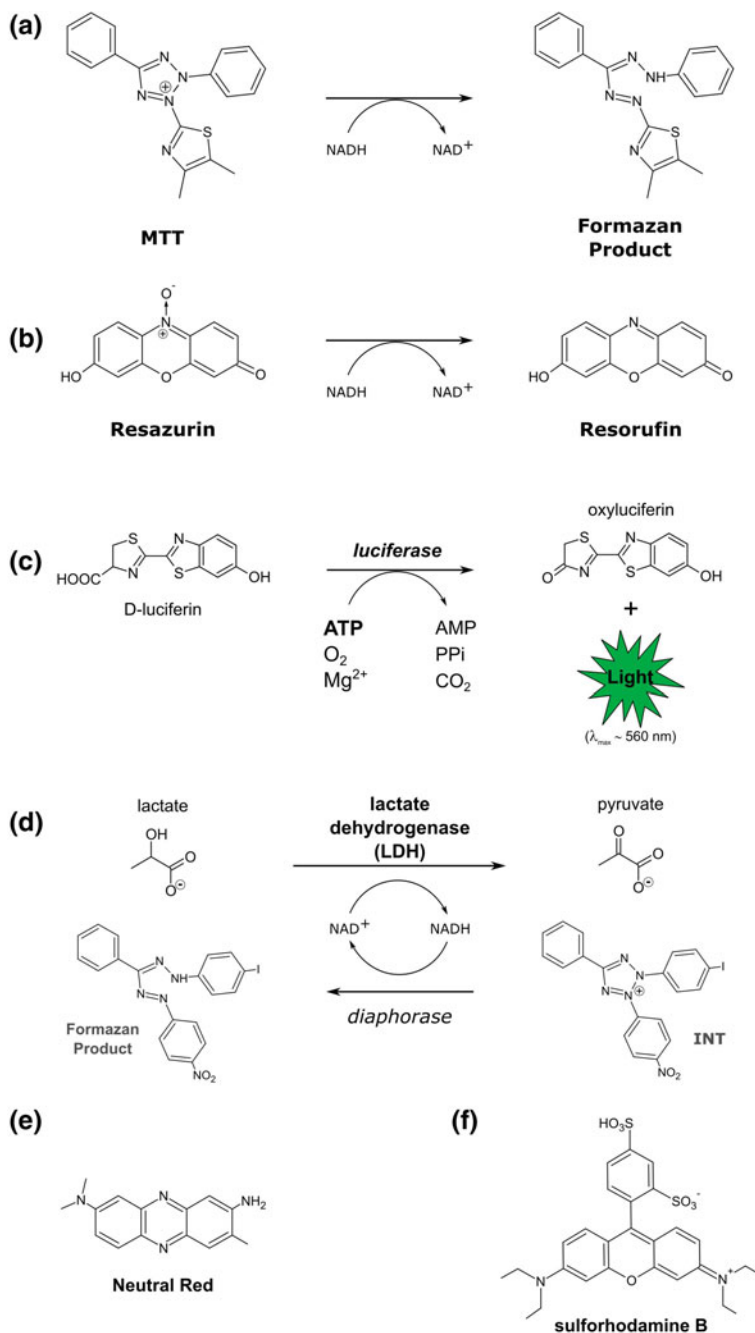
Fig. 3 Reagents and reactions involved in the cell viability/cytotoxicity assays explained in this section. **a–d** depict the reactions used in MTT, resazurin-based, ATP–luciferase, and LDH assays, respectively. **e** and **f** show the chemical structure of the dyes used in the neutral red and sulforhodamine B assays, respectively

and WST-8 (Tominaga et al. 1999) are the most widely used. In these compounds (and their derived formazans), the solubility is increased by decorating their phenyl moieties with sulfonates groups. Contrary to MTT, which is weakly cationic, these derivates are neutral or negatively charged and, as consequence, their passage through the cellular membrane is restricted. Therefore, the reduction of these molecules is mainly performed extracellularly, and intermediate electron acceptors, such as 5-methyl-phenazinium methyl sulfate (PMS) or 1-methoxy-5-methyl-phenazinium methyl sulfate (mPMS), are used to link the intracellular metabolism with the extracellular reduction (Berridge et al. 2005; Präbst et al. 2017; Cory et al. 1991).

The assays that use water-soluble formazans require fewer experimental steps (lysis and solubilization steps are no longer necessary) are more sensitive and present less cytotoxicity than MTT assay (Kupcsik 2011; Berridge et al. 1996; Bernas and Dobrucki 2002). Moreover, owing to the solubility of the reduced product and the absence of lysis and solubilization steps, they can be used in real-time assays (Berridge et al. 2005).

The practical procedure can be summarized as follows. For MTT assay, after incubation of cells with the test nanomaterial, the culture medium is removed and the cells washed with PBS. Then, the cells are incubated with MTT (dissolved in culture medium) for 1–4 h. The incubation time will depend on cell type, cell density, and incubation conditions among other variables. After this step, the medium is removed and the cells are incubated under shaking with the lysis solution (~10 min), followed by incubation with the solubilization solvent (~10 min). Depending on the commercial kit used, these steps may be combined into a unique one. Once solubilization is achieved, the absorbance is read at 570–590 nm.

In the cases of MTS, XTT, WST-1, and WST-8 assays, after incubation (1–4 h) with the tetrazolium compound, the absorbance is directly measured without performing any lysis or solubilization step. These soluble formazan products present maxima at around 450–500 nm, depending on the dye used (Berridge et al. 2005; Barltrop et al. 1991; Cory et al. 1991; Scudiero et al. 1988; Paull et al. 1988; Ishiyama et al. 1993; Tominaga et al. 1999).

Resazurin-Based Assay

Resazurin is a blue dye that upon reduction by metabolically active cells is converted to resorufin, a pink-colored dye which exhibits red fluorescence (see Fig. 3b). Discovered by Weselsky (Weselsky 1871) in the nineteenth century and used to assess metabolic activity since the late 1920s (for the evaluation of microbial contamination in milk) (Palmer et al. 1930), this redox indicator has been applied to study the growth/viability of different organisms, ranging from bacteria to mammalian cells (O'Brien et al. 2000).

The reduction of resazurin has been attributed to the enzymatic activity of dehydrogenases, oxidoreductases, and cytochromes that use NADH and NADPH as electron sources, located in the mitochondria, cytosol, and microsomes (O'Brien et al. 2000;

Gonzalez and Tarloff 2001; Candeias et al. 1998). Many of the enzymes that reduce resazurin have shown to reduce MTT as well (Niles et al. 2008; Gonzalez and Tarloff 2001). Both resazurin and its reduced form resorufin are water-soluble and able to diffuse through cell membrane. Resorufin can be further reduced to dihydroresorufin if cells are incubated for too long with the redox indicator or high cell density is used (Präbst et al. 2017; O'Brien et al. 2000; Twigg 1945; Nakayama et al. 1997). The latter is not fluorescent and toxic to cells, and its presence may lead to inaccurate results.

Considering that resazurin has an absorbance maximum at 605 nm (and is weakly fluorescent) and resorufin presents an absorption peak at 573 nm and fluoresces with excitation/emission maxima at 579/584 nm, the assay may be performed colorimetrically or fluorometrically, though the second option is preferred because it provides greater sensitivity (Präbst et al. 2017; Czekanska 2011).

The assay is simple, relatively rapid, cost-effective, and safe. If executed by measuring fluorescence, it is able to achieve remarkable sensitivity (better than tetrazolium assays) and linear dynamic range (200–50,000 cells, in 96-well plate, depending on cell type) (O'Brien et al. 2000; Czekanska 2011; Riss and Moravec 2006). Moreover, resazurin assays can be multiplexed with other methods (e.g., caspase assay, LDH assay, Hoechst stain) (Wu et al. 2009; Węsierska-Gądek et al. 2005) and, to a certain extent, used for high-throughput screening (Riss et al. 2010; Hamid et al. 2004).

The practical procedure can be summarized as follows. After incubation of cells with the test nanomaterial, the culture medium is removed and the cells washed. Then, the cells are incubated with resazurin reagent (dissolved in culture medium) for 1–4 h. As in the case of MTT, incubation time must be optimized for each cell type and incubation conditions. After incubation, the assay is read in a fluorescence plate reader using 560 and 590 nm as excitation and emission wavelength, respectively. Alternatively, absorbance can be measured (losing sensitive compared to fluorescence). In this case, both resazurin and resorufin contributions to the total absorbance should be considered.

ATP–Luciferase Assay

ATP (adenosine triphosphate) is the most important energy carrier molecule in cells, used to drive a variety of biochemical processes. In living animal cells, ATP is mainly produced by mitochondria, through the enzymes of the respiratory chain, and a lesser amount is generated by glycolysis in the cytosol (Alberts 2015). Dead cells lose the ability to synthesize new ATP, and the remaining ATP is rapidly depleted by endogenous ATPases. Owing to this, ATP is considered a well-founded marker of cell viability (Riss et al. 2006).

The assessment of cell viability using ATP quantification is performed by taking advantage of the firefly luciferase enzyme (Lundin et al. 1986). The luciferase is able to oxidize the pigment D-luciferin, in the presence of ATP (O_2 and Mg^{2+} are

also required), to oxyluciferin (see Fig. 3c). The reaction also produces ADP, PPi, CO_2, and the emission of light (luminescence). The emitted light is proportional to ATP concentration, and this latter is proportional to the number of viable cells. Since ATP is intracellularly located, a lysis step is performed before (or together with) the enzymatic reaction.

In its current versions, the ATP–luciferase assay presents outstanding features (Niles et al. 2008; Riss et al. 2011). The low background luminescence provides the assay with a high signal-to-noise ratio that makes it extremely sensitive, being able to detect even 4–5 cells in a well in 384-well plates. In addition, the assay possesses a wide linear dynamic range, typically from 50 to 50,000 cells (in 96-well plates) (Riss and Moravec 2006). On the other hand, it is really simple to perform, only having a few steps, and it is fast (results can be obtained in less than 30 min).

The practical procedure can be summarized as follows. After incubation of cells with the test nanomaterial, a reagent containing a detergent (to lyze cells), ATPase inhibitors (to stabilize the released ATP), the substrate luciferin and the luciferase, is added to cells and incubated for few minutes under shaking. Then the plates are left stand for 5–10 min (for luminescence stabilization). After this period, the luminescence is measured using a luminometer.

Neutral Red Uptake Assay (NRU Assay)

Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, see Fig. 3d) is a weakly cationic dye that at physiological pH presents almost no net charge, enabling it to penetrate the cell membrane by non-ionic diffusion. Inside the cell, it accumulates in lysosomes owing to a proton gradient that maintains the pH lower than that of the cytoplasm. In such condition, the dye becomes positively charged and remains retained in the lysosomes.

The ability of cells to uptake neutral red relies on their capacity to maintain pH gradients through the production of ATP. Dead cells, or those which cannot maintain the correct pH gradient, are not able to retain the dye. As a consequence, the amount of retained dye is proportional to the number of viable cells. Furthermore, the incorporation, binding, and cell retention of neutral red can be affected by alterations of lysosomal membrane or cell surface in viable cells.

The neutral red uptake assay presents many advantages. It is fast (3–4 h), cheap, relatively simple to perform, and very sensitive, and it can be used to test viability on most animal cells (primary cells and cell lines). Moreover, it is suitable for automation and high-throughput screening of test compounds (Bouhifd et al. 2012; Rodrigues et al. 2013). Compared to tetrazolium salt-based assays, the NRU assay is more sensitive, presents fewer interferences, uses more stable reagents, and is cheaper (Borenfreund et al. 1988), while it is simpler and cheaper than sulforhodamine B-based assay (Vichai and Kirtikara 2006). Perhaps one of its main drawbacks is that once started, it must be completed immediately without the possibility of pausing the sequence by adding a stop reagent or freezing the cells (Repetto et al. 2008).

The practical procedure can be summarized as follows. After incubation of cells with the test nanomaterial, the culture medium is removed and the cells washed with PBS. Then, the cells are incubated with neutral red containing culture medium for 1–3 h. After this step, the medium is removed and the cells are washed with PBS again. Thereafter, the incorporated neutral red is extracted from the cells using a destaining solution (usually containing ethanol and acetic acid) that is applied for 10–40 min under shaking until a homogeneous solution is formed. Finally, the absorbance of the neutral red extract is read at 540 nm. Alternatively, it is possible to use the fluorescence of neutral red to quantify the dye with higher sensitivity and fewer interferences. In this case, the measurement is performed using excitation and emission wavelengths of 530 nm and 645, respectively (Repetto et al. 2008).

Sulforhodamine B Assay (SRB Assay)

The SRB assay is based on the measurement of cellular protein content in order to determine cell density/viability. The assay was developed by Skehan et al. (1990), and since then it has become a popular method for cytotoxicity screening of test compounds (Vichai and Kirtikara 2006; Woolston and Martin 2011). Sulforhodamine B (SRB) is a bright pink aminoxanthene dye that possesses two sulfonic groups in its structure (see Fig. 3e). Owing to these groups, the dye binds to basic amino acid residues in proteins under mild acidic conditions and dissociates from them under basic condition. In the assay, after the binding of SRB to proteins of cells fixed with trichloroacetic acid (TCA), the dye is extracted and quantified spectrophotometrically. The amount of extracted dye is (ideally) proportional to cell mass/number.

As a result of the strong absorbance presented by SRB, the assay is very sensitive, showing higher sensitivity than other protein-staining colorimetric methods, such as Lowry and Bradford (Skehan et al. 1990), and comparable to fluorescent dye-staining methods like DAPI and Hoechst 33342 (McCaffrey et al. 1988). Moreover, it presents the advantage that TCA-fixed cells and SRB-stained cells can be stored indefinitely at room temperature. On the other hand, the multiple washing and drying steps may discourage some people from using the assay (Woolston and Martin 2011), which also makes the method hard to automate (Vichai and Kirtikara 2006).

It is important to mention that unlike cell viability assays based on metabolic activity (e.g., tetrazolium salt- or resazurin-based methods) that only detect viable cells, SRB assay does not distinguish between viable and live cells. In spite of this fact, the ability of SRB assay to evaluate cell viability and cytotoxic effects is not compromised. For instance, as demonstrated in the works by Rubinstein et al. (1990), Haselsberger et al. (1996), Perez et al. (1993), the results obtained with SRB assay correlate fairly well with those derived from MTT assay, being the main difference the slightly higher IC_{50} values estimated for the tested compound when SRB assay was used.

   The practical procedure can be summarized as follows. After incubation of cells with the test nanomaterial, the cells are fixed by the addition of a TCA aqueous solution and incubated at 4 °C for 1 h. If the tested material does not interfere with SRB, the later step can be performed without changing the incubation media. Then, the plates are washed with water (several times) and dried. At this point, the SRB solution (usually SRB in 1% v/v acetic acid) is added, the plates incubated for 10–30 min at room temperature, and then rinsed with acetic acid solution, to remove unbound dye, and dried thereafter. After this step, the solubilization of SRB is performed by adding a base solution (usually Tris Base solution; pH ~10.5). Finally, the absorbance of the SRB extract is measured at 564 nm. In the case of high absorbance values (above the linear range of the instrument), readings at suboptimal wavelengths (490–530 nm) are recommended. In addition, SRB amounts can be determined fluorometrically using 488 nm and 585 nm as excitation and emission wavelengths, respectively.

Lactate Dehydrogenase Assay (LDH Assay)

Lactate dehydrogenase is a tetrameric enzyme present in the cytosol of almost all living cells that catalyzes the conversion of pyruvate to lactate and, depending on the conditions, is able to catalyze the reverse reaction. Owing to the intracellular location of the enzyme, LDH assay has been considered a gold standard for assessing membrane integrity and has a long tradition in the evaluation of cell or tissue damage (Kroll et al. 2012; Koh and Choi 1987).

   In order to measure LDH activity, the medium containing the enzyme is incubated with lactate (substrate) and NAD+ (cofactor) which are converted to pyruvate and NADH, respectively. The produced NADH is used by a diaphorase to reduce a tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium; abbreviated as INT) yielding a cherry red formazan product that can be quantified by its absorbance at 492 nm and which is proportional to LDH activity (see Fig. 3f) (Nachlas et al. 1960).

   The LDH assay can be used to assess membrane damage/cell lysis (as a cytotoxicity assay) by measuring LDH activity on the culture supernatants after incubating the cells with the test compound for a determined time. In this case, the higher the LDH activity measured, the higher the number of damaged/dead cells. Alternatively, it can be used to estimate the total cell number (viable and non-viable) by incorporating a cell lysis step (to release LDH from non-damaged cells) prior determination of enzymatic activity (Riss and Moravec 2006). An aqueous solution of Triton X-100 is frequently used to perform the lysis step. In this case, the higher the detected LDH activity, the higher the cell number.

   The practical procedure can be summarized as follows. For assessing membrane damage (cytotoxicity), after incubation of cells with the test nanomaterial, aliquots from culture supernatants are transferred to a new microplate and incubated with the reagent used for measuring LDH activity (containing lactate, NAD+, diaphorase, and INT) for around 30 min at room temperature. Then, absorbance is read at ~490 nm.

On the other hand, to estimate total cell number, after incubation with the test nano-material and prior LDH activity measurement, the samples are incubated with a lysis solution (usually containing Triton X-100) for 45–60 min at 37 °C. Then, aliquots are transferred to a new microplate, and the procedure retaken as explained before.

In Vitro Evaluation of Genotoxicity

The term genotoxicity refers to the ability of a compound (or treatment) to induce damage in the genetic material (Doak et al. 2017; Kumar and Dhawan 2013). The damage may involve small lesions at DNA level (e.g., strand breaks, adducts, abasic sites, and point mutations) or abnormalities in chromosomes (e.g., alterations in the number of chromosomes, termed aneuploidy, and chromosome fragmentation, termed clastogenicity). The damage caused by genotoxic agents may or may not be transmitted to the next generation of cells. On the other hand, when an agent causes an alteration in the genetic material that is fixed and transmitted to daughter cells, it is considered a mutagen (Benigni and Bossa 2011).

Among the different methodologies used for the evaluation of genotoxicity (Sierra and Gaivão 2014; Dhawan and Bajpayee 2013; Graziano and Jacobson-Kram 2015), the comet and the micronucleus assays are the most popular tools for the screening of genotoxic potential of nanomaterials (Kumar et al. 2018; Azqueta et al. 2015; Magdolenova 2014) and will be described in the following subsections. The former is devoted to the detection of primary DNA damage, while the latter is performed to ascertain chromosomal aberrations in proliferating cells. Lesions detected by comet assay are potentially reversible, while those detected by micronucleus assay are irreversible. Table 2 summarizes the main concepts behind these two assays and collects the main references that applied them to study genotoxicity produced by (iron oxide-based) magnetic nanomaterials.

Comet Assay

The comet assay, also known as single-cell gel electrophoresis assay, is a widespread technique used to detect primary DNA damage at early stages, by measuring DNA strand breaks in individual cells. It was first introduced by Ostling and Johanson (neutral version of the assay) for the study of DNA damage induced by radiation in single cells (Ostling and Johanson 1984). Later, Singh and coworkers developed the alkaline version of the assay (Singh et al. 1988), which is more sensitive toward smaller amounts of DNA damage, being able to detect single and double DNA breaks, alkali-labile sites, among other damages (Singh et al. 1988; McKelvey-Martin et al. 1993; Fairbairn et al. 1995). This version is the recommended and most widely spread to identify agents with genotoxic activity (Tice et al. 2000), and in this subsection, we will only refer to it.

Table 2 Main assays used for the assessment of genotoxic potential of magnetic nanomaterials

Assay	Damage detected	Required equipment	References[a]
Comet assay	*DNA primary damage* (potentially reversible)	Gel electrophoresis equipment Fluorescence microscope	Malvindi et al. (2014), Ahamed et al. (2013), Könczöl et al. (2011), Fernández-Bertólez et al. (2018a), Kiliç (2015), Han et al. (2011), Karlsson et al. (2009), Bhattacharya et al. (2012), Kain et al. (2012), de Lima et al. (2013), Shaw et al. (2014), Gaharwar et al. (2017), Fernández-Bertólez et al. (2018b), Mishra et al. (2018), Turiel-Fernández et al. (2018), Abakumov et al. (2018), Sadiq et al. (2015), Zhang et al. (2012b), Buliaková (2017), Seo et al. (2017), Fernández-Bertólez et al. (2018c), Auffan et al. (2006), Seabra et al. (2014), Cowie et al. (2015), Harris et al. (2015), Magdolenova et al. (2015), Shydlovska et al. (2017)
Micronucleus assay	*Chromosome fragments Lagging chromosomes* (irreversible)	Optical or fluorescent microscope	Könczöl et al. (2011), Fernández-Bertólez et al. (2018a), Kiliç (2015), Sadiq et al. (2015), Zhang et al. (2012b), Buliaková (2017), Seo et al. (2017), Fernández-Bertólez et al. (2018c), Magdolenova et al. (2015), Evans et al. (2019), Shah et al. (2013), Pöttler et al. (2015)

[a]Selected references accounting for the use of the comet or micronucleus assays to determine genotoxic potential of magnetic (mostly iron oxide-based) nanoparticles

In the assay, after exposure to the tested nanomaterial, cells are embedded in agarose, placed in a microscope slide and lyzed by means of detergents and high concentration of salts. Then, the slide is incubated in alkaline electrophoresis buffer (pH >13), promoting the unwinding of the liberated DNA, and submitted to electrophoresis. Under the applied electric field, the fragmented DNA migrates out of the nucleoid body, toward the anode. After neutralization, DNA is stained with a fluorescent dye (e.g., ethidium bromide) for visualization. Cells with higher DNA damage display increased migration of chromosomal DNA and form an electrophoretic pattern that, under observation with a fluorescent microscope, resembles the shape of a comet where the nucleoid is the head and the migrated DNA the tail. Usually, the damage is quantified by image analysis measuring the tail length or estimating other metrics such as percentage of tail DNA and tail moment.

The procedure exhibits many advantages. Among them, it can be performed in almost any eukaryotic cell (proliferating and non-proliferating) as long as a cell suspension can be obtained, is very sensitive, requires low number of cells (<10,000 cells per slide), and is relatively fast and cost-effective.

Micronucleus Assay

The micronucleus assay is a popular method to detect chromosomal aberrations in proliferating cells, used for the evaluation of compounds with clastogenic (potential to break chromosomes) and aneuplodogenic activity (potential for lagging entire chromosomes) (Countryman and Heddle 1976; Bolognesi and Fenech 2013; Wolff and Müller 2006; Fenech and Morley 1985). Micronuclei are chromosome fragments or whole chromosomes that have failed to attach onto the spindle during anaphase and are not included in the main nucleus. During subsequent interphase, they condense to form characteristic small nuclei, much smaller than the principal nucleus (Zelazna et al. 2011).

For micronuclei to be generated, the cells must be undergoing mitosis, and consequently, they appear only in dividing cells. In order to improve the assay, many methods have been proposed to identify cells that have completed cellular division (Fenech and Morley 1985; Pincu et al. 1984; Fenech 2000). At present, the most widespread in vitro method used for the evaluation of micronuclei is the cytokinesis-block micronucleus (CBMN) assay, usually performed using (human) lymphocytes (Bolognesi and Fenech 2013; Fenech 2000). In CBMN assay, the cytokinesis is blocked using cytochalasin B, and cells that have completed one cell cycle appear binucleated being easily distinguished from undivided cells. These micronuclei are identified by microscopic observation of Giemsa-stained samples.

CBMN assay is sensitive, and its performance and result analysis is easier than the classical chromosomal aberration analysis performed in metaphasic cells. Furthermore, combined with fluorescence in situ hybridization (FISH) technique (using centromeric probes), it is able to discriminate between clastogenic and aneuplodogenic activity (Marshall et al. 1996; Eastmond and Pinkel 1990).

It is important to mention that cytochalasin B also inhibits endocytosis and, as a consequence, might interfere with the proper evaluation of nanoparticle genotoxicity (Doak et al. 2009; Gonzalez et al. 2011). Owing to this, it is important to treat the cells with nanoparticles and without cytochalasin B before the CBMN assay is performed.

The Use of 3D Cultures

Nanoparticle toxicity is initially evaluated in 2D cell culture as described in the previous section. The usual next step is to contrast those results in an entire animal, but oftentimes results obtained in cell culture do not translate in the same in vivo outcome. The limitations of cell culture in a monolayer to recreate three-dimensional morphologies of cells in tissues, as well as the dynamics and complex interactions of cells with their supporting extracellular materials, could explain those differences.

As demonstrated in multiple in vivo delivery tests and also discussed in the introductory section, nanoparticles have to overcome numerous biological barriers in order to reach target organs. In their review, Blanco et al. (2015) summarize those obstacles and propose different strategies to succeed. Those impediments include opsonization of nanoparticles with posterior sequestration by macrophages, what in turn leads nanoparticles to organs with filtration capability where nanoparticles can exert toxic effects.

In 2D cell culture, nanoparticles administered in a bulk solution reach cell membranes without impediments. In contrast, nanoparticles delivered in vivo face a hindrance, such as the extracellular matrix (ECM), in which nanoparticles could be retained. Furthermore, depending on their surface properties, NP can interact with charged ECM components (Goodman et al. 2007; Ruponen et al. 1999).

Another factor that can explain differences in toxicity observed between 2D culture and animal studies is that cell culture assays do not take into account the hydrostatic and osmotic pressures in tissues. In particular for tumors, poor lymphatic drainage, extensive fibrosis, and a dense ECM result in particularly elevated interstitial fluid pressures (Heldin et al. 2004). This high intratumoral pressure prevents extravasation of nanoparticles to distal regions, resulting in lower toxicity compared to a monolayer, where studies are performed under static conditions. Furthermore, 2D cell monolayer cultures are generally exposed to a uniform environment, whereas cells in tissues such as solid tumors are exposed to pH and concentration gradients that can affect nanoparticle internalization.

For the reasons mentioned above, there is an increasing interest in utilizing 3D culture systems in order to mimic more accurately the in vivo situation and ultimately the human clinical setting (Kenny et al. 2007). Test toxicity of nanoparticles in vitro 3D systems will allow obtaining more predictable results, not replacing in vivo assays but making it in a more efficient way and shortening the gap in translation from cell culture to animal studies and clinical trials.

3D Culture Systems to Test Magnetic Nanoparticle Toxicity

There are different types of 3D culture formats that can be used in order to answer specific biological questions. In their reviews, Shamir et al. and Goodman et al. make a comprehensive analysis of the different culture systems (Goodman et al. 2008; Shamir and Ewald 2014). Here, we are going to focus on the most relevant toxicity studies for magnetic nanoparticle.

1. *Hydrogels and 2.5D cultures*

    The use of hydrogels with no cells is the simplest model for assessing nanoparticle interaction with ECM components. In the case of magnetic nanoparticles, the same biophysical characteristics that limit the application of traditional therapies can be exploited to improve biodistribution. Magnetic NPs can be specifically localized within a fluid-filled cavity to the site of a lesion or target tissue by an external magnetic field. Kuhn et al. calculated the average velocity of different size and surface coating superparamagnetic NP in hydrogels (Kuhn et al. 2006). Although the usefulness of this systems is limited as it does not provide any information of cell toxicity, it offers valuable information about the effect of size, shape, and surface chemistry on the mobility of vectors through the ECM.

    The addition of ECM proteins to the medium in 2D cultures is sufficient to induce tissue-specific differentiation of diverse epithelial cells, including mammary (Streuli et al. 1991), kidney (O'Brien et al. 2001), and lung (Yu et al. 2007). This a very important feature since the expression of cell membrane receptors can change in the presence of ECM proteins, thus affecting nanoparticle uptake. This system is known as 2.5D culture. Even though this system does not perfectly resemble the in vivo environment, as cells are still in contact with a large fluid reservoir, it induces cells to form a more physiological tissue architecture than 2D assays and the cells remain accessible for molecular analysis (Debnath et al. 2003).

2. *Multicellular spheroids*

    In this culture system, cells form spherical clusters. There are different ways to form spheroids: culturing cells in spinner flasks, in agar-coated culture plates, or the hanging drop method (Nederman and Twentyman 1984; Timmins and Nielsen 2007). Under these conditions, spheroids can survive for weeks and can reach sizes of up to several millimeters in diameter. Large spheroids are characterized by an external proliferating zone, an internal quiescent zone caused by the limited distribution of oxygen, nutrients, and metabolites, and a necrotic core (Vinci et al. 2012) resembling the cellular heterogeneity of solid in vivo tumors (Mueller-Klieser 1987, 2017). As spheroids are relatively easy to handle, they are amenable to confocal analysis, cryosectioning, and commonly used fixing methods. They can also be disaggregated by trypsin treatment and individual cells collected for cytotoxic assays like LHD and MTT, or flow cytometry analysis. These properties make spheroids a very useful and widely used model for investigating nanoparticle–tissue interactions. Although it is not the aim of this section to make a detailed description of cell culture types, it is important to

differentiate multicellular tumor spheroids from tumorospheres. Both are characterized by their well-rounded shape, the presence of cancer cells, and their capacity to be maintained as free-floating cultures, but tumorosphere is a type of culture that allows the selection of cancer stem cell subpopulation.

3. *Multilayer cell cultures*

Multilayer cultures are most commonly formed by growing a monolayer culture beyond confluence in transwell cell culture dishes, composed by a microporous filter membrane insert within a chamber. The transwell dishes produce isolation between the apical and basal layer of the multilayer, so that any compound delivered to one side must go through the cell layers to reach the opposing side. This allows for easy assessment of transcellular nanoparticle delivery by incubating drugs on one side of the growing culture and collecting media on the opposite side to assess the amount of nanoparticles that passed through the cell layer.

Transwell inserts are also commonly used to reproduce the blood-brain barrier (BBB). This barrier is built up by the brain capillary endothelial cells connected by tight junctions and supporting pericytes and astrocytic end-feet. Polar molecules and small ions are almost totally excluded by the tightly closed intercellular cleft. Although they are well suited for magnetic resonance imaging (MRI), magnetic nanoparticles employed as contrast agents are not suited to cross biological barriers like the BBB (Veiseh et al. 2009b). Thus, in vivo application of magnetic nanoparticles as MRI contrast agents for brain imaging is still limited. For these reasons, there are several research groups designing new strategies to overcome this barrier, utilizing the multilayer cell culture in transwell to perform in vitro assays (Qiao et al. 2012; Shi et al. 2016; Busquets et al. 2015b). For example, Qiao et al. tested a brain delivery probe based on the PEG-coated Fe_3O_4 nanoparticles in an in vitro BBB model based on primary porcine brain capillary endothelial cells (PBCECs) cultured over transwells (Qiao et al. 2012).

4. *Organ-on-a-chip*

This system consists of a device for culturing living cells in continuously perfused, micrometer-sized chambers in order to model physiological functions of tissues and organs. The goal is not to build a whole living organ but rather to synthesize minimal functional units that recapitulate tissue and organ-level functions. The simplest system is a single, perfused microfluidic chamber containing one kind of cultured cell that exhibits functions of one tissue type. In more complex designs, two or more microchannels are connected by porous membranes, lined on opposite sides by different cell types, to recreate interfaces between different tissues (e.g., lung alveolar-capillary interface or BBB) (Bhatia and Ingber 2014). Similar analyses can be conducted with chips lined by cells from different organs that are linked fluidically, to mimic physiological interactions between different organs or to study drug distribution in vitro (Bhatia and Ingber 2014).

Differing form 3D static cultures, in this system, many cell parameters can be controlled, such as tissue barrier integrity (Douville et al. 2010), cell migration (Nguyen et al. 2013), and fluid pressure (Liu et al. 2013).

Organ-on-a-chip platforms are of great relevance to evaluate the potential hazards of nanoparticles for human health, as they have the advantage of using human cell lines—a more similar context to an in vivo environment. For example, there are several works testing the toxicity of nanoparticles in lung-on-a-chip. Since inhalation is a potential route of administration, it is fundamental to have a device that recreates the alveolar–blood barrier (Zhang et al. 2018).

In the same way, models to accurately predict transvascular permeation of nanoparticles to the brain are highly valuable. Kim et al. have probed nanoparticle translocation across the endothelium in a model that mimics the BBB (Kim et al. 2014).

Magnetic Nanoparticle Toxicity in 3D Culture

As mentioned before, 3D culture is a useful tool for the study of nanoparticle toxicity since it gives different information from monolayer cell culture. It has been reported that nanoparticle uptake and cell viability are significantly lower when cells are cultured in 3D.

In this subsection, we are going to describe methods found in the literature for the study of cell toxicity in multicellular spheroids, the most widely used model to asses nanotoxicity in 3D culture. Some of those methods will be briefly mentioned as they were fully described in the previous subsection.

1. *Nanoparticle uptake*

   1.1 *Prussian blue*: To verify whether magnetic nanoparticles are endocytosed, the Prussian blue (PB) method (Perls' acid ferrocyanide) can be employed to detect iron within the cell cultures. The PB method reduces ferric iron to the ferrous state with the formation of a blue precipitate (Neri et al. 2007). This method can be utilized both in 2D and in 3D culture. Wang et al. (2012) used PB to detect PLL-modified γ-Fe2O3 nanoparticles within glioblastoma spheres, and observed that the staining is preserved even when the spheres grow and cells become differentiated.

   1.2 *Confocal microscopy*: Magnetic nanoparticles can also be labeled with a dye and be tracked inside the spheroid. To achieve it, different cell structures (e.g., endosomes, nucleus) can be stained in order to study the subcellular localization of the NPs. In their work, Theumer et al. (2015) measured NP uptake in spheroids covering the magnetic core of coated SPION with a green lipophilic dye. Taking images in confocal microscopy in several z-stacks allowed them to calculate spatial location within the middle cross section of the spheroid (see Fig. 4).

   1.3 *Transmission electron microscopy*: Spheroids can be cryo-sectioned or fixed, inserted in resin and cut into ultrathin sections to observe them with
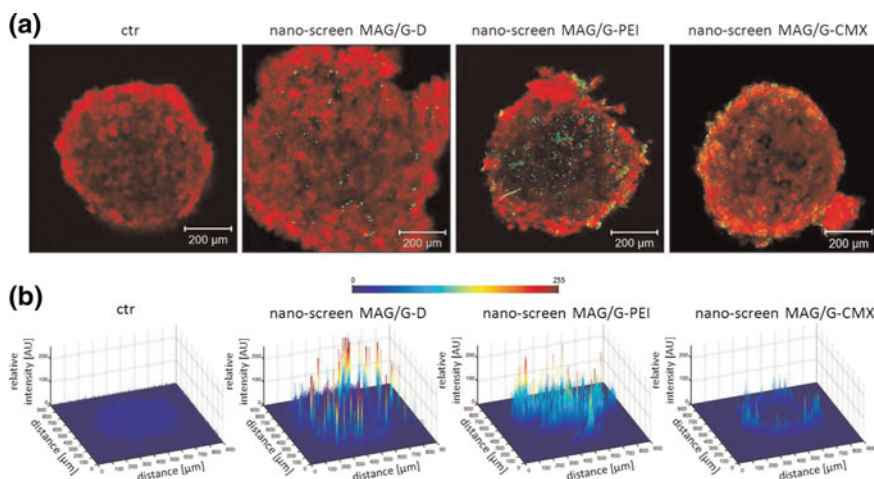
Fig. 4 Spatial nanoparticle distribution in human brain microvascular endothelial cell spheroids. Spheroids were treated with NPs with a core made of iron oxide and a shell of differently charged polymers (green) in a concentration of 25 mg/cm^2 for 3 h. F-actin of fixed and permeabilized cells was stained using Alexa Fluor 633 phalloidin (red). NP distribution was analyzed by confocal laser scanning microscopy. **a** 3D overlays of spheroids' z-stacks recorded at a magnification of 100. Scale bars indicate 200 µm. **b** Relative fluorescence intensity of labeled NP was plotted according to their spatial location within the middle cross section of the spheroid. Dark blue color indicates no fluorescence; red color indicates high fluorescence. Figure taken from Theumer et al. (2015). Reprinted with permission from Elsevier

TEM. Child et al. (2011) demonstrated by TEM that magnetic NPs penetrate spheroids following application of a magnetic field.

2. *Cytotoxicity assays*

    Assessing NP toxicity on 3D-cultured cells is usually performed using common viability assays originally designed for drug studies in monolayers. Since most of them are fully described in the previous section, here we are going to make a brief account of the most utilized methods focusing on particularities of their application to 3D cultures.

2.1 *MTT and LDH*: The MTT assay measures the amount of enzymatically reduced MTT by viable cells. In the case of spheroids, due to their composition of proliferating, quiescent, and dead cells, a lower mitochondrial activity is measured compared to 2D culture when a similar number of cells are tested.

    Lactate dehydrogenase (LDH) assay detects the amount of LDH that leaks out through the plasma membrane of damaged cells. This extracellular protein assay protocol is carried out identically in both 2D and spheroid cultures. In their work, Lee et al. (2009b) analyze nanoparticle cytotoxicity in spheroids utilizing this assay for gold nanoparticles toxicity testing.

2.2 *Acid phosphatase assay*: A modified acid phosphatase (APH) assay, which is based on quantification of cytosolic acid phosphatase activity, was validated for determining cell viability in spheroids. Intracellular acid phosphatases in viable cells hydrolyze *p*-nitrophenyl phosphate to *p*-nitrophenol. Its absorption at 405 nm is directly proportional to the cell number in the range of 10^3–10^5 monolayer cells. In the case of spheroids, they are centrifuged, supernatant is discarded, and the pellet is washed with PBS. Then, the assay performed similar to the one developed for monolayer cell culture. For a full description of the method adapted for spheroids, we refer the readers to Friedrich et al. (2009)

2.3 *Live/Dead staining*: This assay is based in staining cells with colored or fluorescent dyes that differentially label live and dead cells. For example, Wang et al. (2012) use PI and FDA double-staining protocol (Kristensen et al. 2003) to detect the cellular viability of the magnetically labeled glioblastoma spheres and their differentiated progenies, as well as to determine if cell death occurred after magnetic labeling. PI is a DNA-binding fluorescent dye that only enters dead or dying cells with damaged or leaky membranes. On the other hand, FDA, which stains cells with intact membranes, produces a bright green fluorescence. Cells then can be seen in a microscope or analyzed by flow cytometry.

Trypan blue stains dead cells since the colorant is not able to go through the membrane in viable cells. However, it is able to penetrate dead cells, in which the cell membrane is comprised. In their work, De Simone et al. (2018) utilize Trypan blue to asses cell toxicity in spheroids treated with iron oxide nanoparticles.

There are commercial kits used to test cell viability, as used by Theumer et al. (2015) They determined the ratio of living to dead cells with a kit based on two fluorescent dyes: calcein (excitation: 494 nm, emission: 517 nm) and ethidium homodimer-1 (excitation: 528 nm, emission 617 nm) to establish the effects of iron oxide NP onto the viability of spheroids. Stocke et al. (2017) also used a commercial dye to evaluate the toxicity that magnetic hyperthermia induced by magnetic NPs exerted in breast cancer spheroids. This dye is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes and can be excited with argon 406 lasers, making it a useful indicator of dead cells within a population.

Newer commercial kits specially designed for determining cell viability in 3D microtissue spheroids are also used. In their work, Zanoni et al. (2016) compare two of these commercial kits to common cell viability staining protocols. These kits allow the reagent to penetrate large spheroids and have increased lytic capacity for more accurate determination of viability.

2.4 *Spheroid morphology*: As spheroids can be cultured for long periods of time, morphological parameters are important factors linked to cell viability. For example, spheroid size is related to cell proliferation according to Gompertz law:

$$\ln\left(\ln\frac{V}{V0}\right) = \alpha t + \frac{V\max}{V0}$$

where V is a measure of spheroid size, V_0 the initial size, $V_{\max}$ the final size, and α is the cell proliferation rate (Helmlinger et al. 1997).

Variations in spheroid shape were also accompanied by changes in the dimension of the inner core and in the thickness of the surrounding shell that consists of proliferative, actively dividing cells. Zanoni et al. (2016) demonstrated by a luminescence metabolic assay that spherical spheroids showed a significantly reduced viability compared to irregular-shaped ones. This correlates with Mina Bissell's group work, in which an association between distinct morphologies and tumor cell invasiveness is demonstrated (Kenny et al. 2007).

To conclude, we consider of great importance taking into account the cell environment in magnetic nanoparticles toxicity testing. This will lead not only to produce more accurate results that can be more easily translated to the clinic, but also to diminish the number of animals in high-throughput screenings.

In Vivo Evaluation of Toxicity

Relevance of in Vivo Assays

In vitro assays for toxicity evaluation are limited due to incomplete consideration of the number of actors involved and the interactions between them. Among leading restrictions, we can highlight: (1) Only in limited cases, target cells are evaluated in the presence of cells from other origins, such as immune system cells. For example, nanoparticles (NP) uptake was found to be enhanced in cocultures of epithelial, macrophages and dendritic cells compared to monocultures (Rivera Gil et al. 2010); (2) the relevance of biological fluid hydrodynamics (e.g., blood, biliary, and lymphatic transport) is usually not contemplated. To overcome these limitations, cocultures that take into account spatial organization of tissues and heterogeneity of cell interactions (Costa et al. 2013; Müller et al. 2018), and microdevices that mimic vascular networks (Rosano et al. 2009; Prabhakarpandian et al. 2008) have been developed, but their degree of similarity to in vivo processes is controversial (Sayes et al. 2007). In the same line, some reactions that alter the toxic properties of NP can only occur inside a living organism. Metabolic processing of NP can alter their shape, coating, composition, size, and rugosity, which are properties relevant to their toxicity degree (Chouly et al. 1996). On the other hand, NP surfaces are subject to catalytic and oxidation reactions that can also modify their cytotoxicity (Nel et al. 2006b). Currently, toxicokinetic studies and unequivocal identification of NP target tissues can only be accomplished by using in vivo models (Sahu and Casciano 2009).

In this section, we do not intend to present an exhaustive list of the strategies used to perform toxicological short-term in vivo analysis but only to highlight contemporary procedures prevailing in the literature.

Animal Models to Test in Vivo Magnetic NP Toxicity

NP attempting to enter the biomedical field will have to go through animal preclinical trials almost exclusively, although a part of the scientific community is making big efforts to find novel human-specific approaches which could model human diseases with lower costs, higher predictive power, and absence of ethical concerns (Langley et al. 2015). Particularly in the magnetic NP (MNP) research area, rodent models such as mice and rats have been the most frequently selected to test in vivo toxicology, and therefore, methods to evaluate toxicity in them will be further detailed. Mice and rats are representative of the two types of mammalian spleens (mice have non-sinusoidal spleens, whereas rats, like humans, have sinusoidal spleens). Both have different microcirculatory pathways and distinct mechanisms of blood clearance (Adiseshaiah et al. 2010). Toxicity has been shown to be dependent on the species tested. Studies with the dextran-coated USPIO Ferumoxtran-10, proposed for MRI differentiation of metastatic lymph nodes and recently discontinued, demonstrated a clear difference in the kinetics of blood clearance among different animal species (Bourrinet et al. 2006). The plasma clearance was much faster in rats compared to monkeys, a difference that is probably accountable to alterations in RES organ distribution and activity across animal species.

In vivo toxicity of MNP has also been evaluated on zebrafish (*Danio rerio*), since the community working with this organism has proposed its use as an intermediate screening step between cell culture assays and studies in rodents (Caro et al. 2019). To evaluate toxicity, authors usually quantify the proportion of alive or dead larvae after a certain period of treatment, e.g., in a study where ferrite or manganese ferrite oxide MNP was used, and survival rate was evaluated (Caro et al. 2019). Moreover, the zebrafish's pattern of development is similar to higher vertebrates (Grunwald and Eisen 2002), making it a good model to study toxicity associated with alterations in embryogenesis. For instance, in one study, uncoated, flavin mononucleotide or guanosine monophosphate-coated USPIO was applied to fertilized eggs, and development of the zebrafish embryos was evaluated by optical microscopy up to 168 h post-fertilization (Rizzo et al. 2013).

With less frequency, other organisms like *Caenorhabditis elegans* (Gonzalez-Moragas 2017) and *Daphnia magna* (Kumar et al. 2017) have also been used.

Administration Routes and Fate of NP In Vivo

A comprehensive assessment of NP suitability for medical purposes should include the evaluation of its pharmacokinetics (PK). PK research includes the examination of absorption, distribution, metabolism, and excretion of a drug, which are characteristics that determine the appropriate duration and concentration for drug administration. Regarding absorption, NP can enter the organism through six principal routes: intravenous, dermal, subcutaneous, inhalation, intraperitoneal, and oral (Fischer and Chan 2007). Other ways of administration for specific purposes have been developed, like intratracheal instillation (NP administration via incision of trachea) and pharyngeal/laryngeal aspiration for research on pulmonary toxicity due to environmental hazards (Fadeel 2015). Depending on the administration route, different organs could suffer cytotoxicity. In one study in rats, intratracheally instilled ferric oxide NP produced acute lung injury due to accumulation in alveolar macrophages and epithelial cells (Zhu et al. 2008). Concerning MNP, intravenous injection is currently among the most studied routes, due to their interest in nanomedicine (Choi and Frangioni 2010); inhalation and intratracheal instillation have also been used (Sahu and Casciano 2009).

Once NP comes into biological fluids, they will interact with proteins, such as complement (Reddy et al. 2007) and immunoglobulins (Walkey and Chan 2012), during a process called opsonization (Moghimi et al. 2011). This novel structure, formed by the original nanoparticle covered by proteins termed corona, may influence subsequent interactions of the particles with biological systems and consequently affect their in vivo distribution and cytotoxicity (Liu et al. 2016; Lundqvist et al. 2008). It has been shown, for example, that bare or coated SPIONs can induce irreversible conformational changes in iron-saturated human transferrin, producing the release of iron (Mahmoudi et al. 2011). Although with limitations, among which enzymatically plasma inactivation and depletion of coagulation factors from serum outstand, different ex situ toxicity assays have been performed to emulate in vivo conditions considering NP opsonization (Walkey and Chan 2012). Most of them are based on isolated serum or plasma usage (Shi et al. 2012; Chonn et al. 1992).

In the distribution process, NPs can remain unaltered or suffer modifications (Borm et al. 2006). Researchers must carefully select the administration procedure to maximize targeting to selected organs since NP could encounter obstacles in the form of body barriers (skin, placenta, gastrointestinal epithelial barrier, blood-brain barrier), preventing them to freely distribute through all biological systems (Pietroiusti et al. 2013). To track MNP inside an alive or dead organism, the most frequent techniques are radiolabeling (Same et al. 2016), inductively coupled plasma mass spectrometry (ICP-MS) (Al Faraj et al. 2014), and magnetic resonance imaging (MRI) (Liu et al. 2010). Complementary, two other strategies have also been proposed: conjugation of NP with NIR fluorophores to be measured by near-infrared fluorescence (Lee 2010), though penetration depth is typically lower, and electron spin resonance spectroscopy (Chertok et al. 2010). Most of existing reports, heterogeneous in administration doses and selected time points for observation, focused

on describing the relationship between physicochemical properties of NP and their distribution. Although targeting and circulation half-life are highly dependent on the NP coating, size, and surface charge, the liver appears to be the major accumulation site via uptake by liver-resident macrophages termed Kupffer cells, followed by the spleen (Sahu and Casciano 2009). These two organs, together with lymph nodes and bone marrow, form the reticuloendothelial system (RES), outstanding for its large phagocytic monocytes and macrophages population. This is also the case for MNP, where studies conducted in rats showed that after SPIO-alginate intravenous injection, NP was eliminated rapidly from serum with a half-life of 0.27 h and accumulated dominantly in liver and spleen with a total percentage of more than 90% of dose (Ma et al. 2008). It has been suggested for iron oxide MNP (IONPs) that larger ones are more quickly taken up by the liver and spleen and have shorter circulation time in blood, whereas smaller have increased access to organs such as the lymph nodes and longer circulation time (Almeida et al. 2011). However, both are usually cleared from blood circulation relatively fast compared to other NP, normally within 36 h post-administration.

According to their properties and target tissues, NP could be metabolized in different ways, or not metabolized at all. In the case of superparamagnetic iron oxide nanostructures (SPIONs) for MRI contrast agents, they are shown to degrade (Briley-Saebo et al. 2004). Lysosomal degradation within Kupffer cells and transference to the spleen has been described as the metabolic pathway chosen by IONPs for their final breakdown (Levy et al. 2011).

Lastly, excretion routes also depend on the type and size of the NP. Among existing mechanism, elimination through exhalation, urination (via the kidneys), defecation (via the biliary duct), perspiration, salivation; and mammary and seminal fluids can be named (Sutariya and Pathak 2014). For MNP, renal (Bourrinet et al. 2006) and hepatobiliary system (Pham 2018) have been described as two important clearance pathways. Depending on their characteristics, excretion time can be quite long, a property not usually considered in the short-termed studies prevailing in the literature. This was the case of silica-coated magnetic nanoparticles containing rhodamine B isothiocyanate, which were still found in several organs of mice such as liver, lungs, kidney, and spleen 4 weeks after injection (Kim et al. 2006). It has been proposed that excretion time is particularly slow for IONPs because iron would be incorporated to the body's iron pool upon degradation of the iron oxide core, the reason for which MNPs are classified as biocompatibles (Almeida et al. 2011).

Methods for In Vivo Toxicity Evaluation

There are at least four parameters that need to be measured to determine biocompatibility of NP: cytotoxicity, hemocompatibility, immune response, and acute toxicity (Sutariya and Pathak 2014). Cytotoxicity and hemocompatibility (induction of red blood cells lysis and coagulation studies) are usually assessed in vitro or ex vivo. Host's immune response may be evaluated through immunohistochemistry

or enzyme-linked immunosorbent assay (ELISA). Acute toxicity can be determined by histological evaluation, protein tissue extraction, gene expression, and measurement of damage markers in plasma (see Fig. 5). It should be noticed that compared to the in vitro research area, there is a clear lack of in vivo studies characterizing NP genotoxic effects, and they are mainly restricted to rodents. Among the most frequent methods used are comet assay in liver, lung, colon, bone marrow; and micronucleus test in blood or bone marrow (Bourrinet et al. 2006). The protocols to perform these techniques do not substantially differ when in vivo or in vitro models are used and therefore will not be further described. Readers are referred to the previous section.

1. *Blood analysis*

   Blood is often used to determine the presence of markers related to damage. Depending on the organs expected to be affected, a wide variety of molecules can be analyzed. Jia and colleagues injected neuropilin-1-targeted exosomes containing SPIONs and curcumin in BALB/c nude mice carrying gliomas (Jia 2018). Toxicity was evaluated by measuring the level of markers of cardiac (creatine kinase MB isoenzyme), hepatic (aspartate aminotransferase), and renal (serum creatinine) damage in serum 15 days after the treatment. In another study, IONPs coated with PEG or PEI were injected intravenously, and aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, total bilirubin, and creatinine levels were quantified in blood 7 days post-administration to evaluate hepatic and renal function (Feng et al. 2018b). Most values were in the normal range except for alanine aminotransferase, which returned to normality 14 days post-injection.
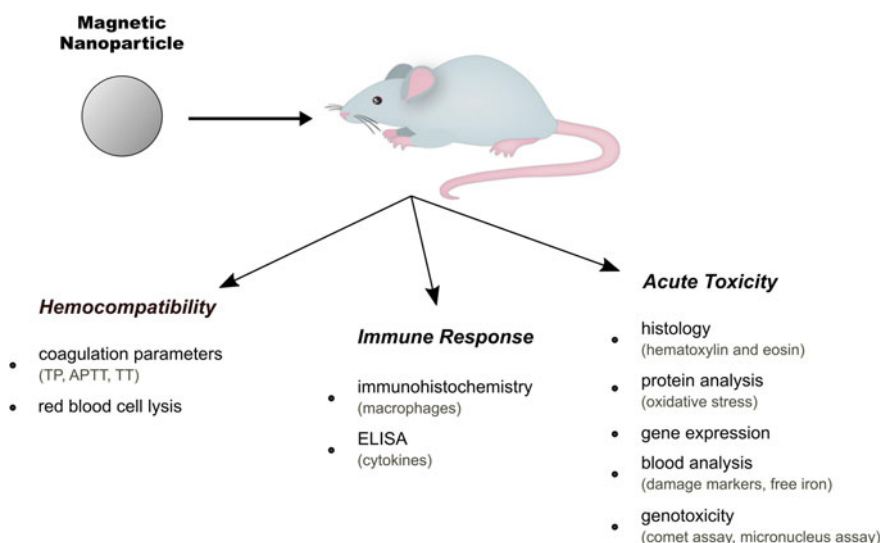
Fig. 5 Frequent assays and parameters evaluated when in vivo toxicity studies of nanoparticles are performed

Excessive tissue accumulation of free iron is known to cause toxicity (Weir et al. 1984; Barnham and Bush 2008). Quantification of iron in blood can be performed using ICP-MS. In one of these studies, iron concentration was measured 24 and 48 h following intravenous injection of citrate-stabilized IONPs in male athymic nude mice (Sharma et al. 2018). In this case, authors wanted to evaluate the effect of negatively (carboxymethyl dextran) or positively (PEG-PEI) coatings on biodistribution and concluded that nanoparticle clearance from blood circulation occurred within 24 h. On the contrary, in the study from Jain and colleagues, rats needed more than 3 weeks to recover normal content of iron in serum after oleic acid-Pluronic-coated IONP intravenous injection (Jain et al. 2008). In this case, a colorimetric method based on Fe(III) to Fe(II) reduction to measure iron content in serum was used. Once again, results appear to be highly dependent on NP physicochemical properties, biological model, and experimental design.

Plasma can also be separated from blood by centrifugation at 1500 g for 10 min to determine if coagulation parameters, typically prothrombin time, activated partial thromboplastin time, and fibrinogen, are affected (Zhu et al. 2008).

2. *Tissue analysis through histology and immunohistochemistry*

Histopathological examination has been used in tissue exposed to NP, such as lung, eyes, brain, liver, kidneys, heart, and spleen (Kumar et al. 2017). To perform this method, the animals are sacrificed after NP administration and organs are removed and fixed using 4% paraformaldehyde solution or 10% formalin. After dehydration by increasing alcohol concentration immersion, they are paraffin embedded and 4 μm thickness sectioned for hematoxylin and eosin (H&E) staining (Fischer et al. 2008) to visualize cellular morphology (see Fig. 6) or Prussian blue staining to detect ferric iron ions (Liu et al. 2010).

To perform H&E staining, the sections must be deparaffinized on a heating block followed by washing of xylene and rehydrated by immersing them successively for 5 min with agitation in xylene, 100% ethanol, and 70% ethanol, followed by 1 min in an appropriate buffer or water. Samples are then stained with a solution containing one or more cationic aluminum–hematein metal complex, subsequently termed hemalum. Hematein is generated by oxidation from hematoxylin, a colorless compound, and binds to chromatin conferring the characteristic blue color to cell nuclei. The nuclear staining is followed by counterstaining with an anionic solution of eosin Y dye, which colors eosinophilic structures in various shades of red, pink, and orange. Finally, samples are washed and dehydrated in 95 and 100% alcohol. Before visualization, alcohol can be extracted with xylene, and samples can be mounted. H&E staining is probably the most popular staining method used by pathologist in their daily work. However, important drawbacks of this technique include insufficient cytoplasmic differentiation and poor contrast between cytoplasmic and extracellular structures (Wittekind 2003).

Immunohistochemistry analysis can be also conducted to analyze colocalization of NP with immune system cells. If necessary, tissue must be deparaffinized. Positively charged slides must then be dehydrated in 100, 95, and 70% ethanol
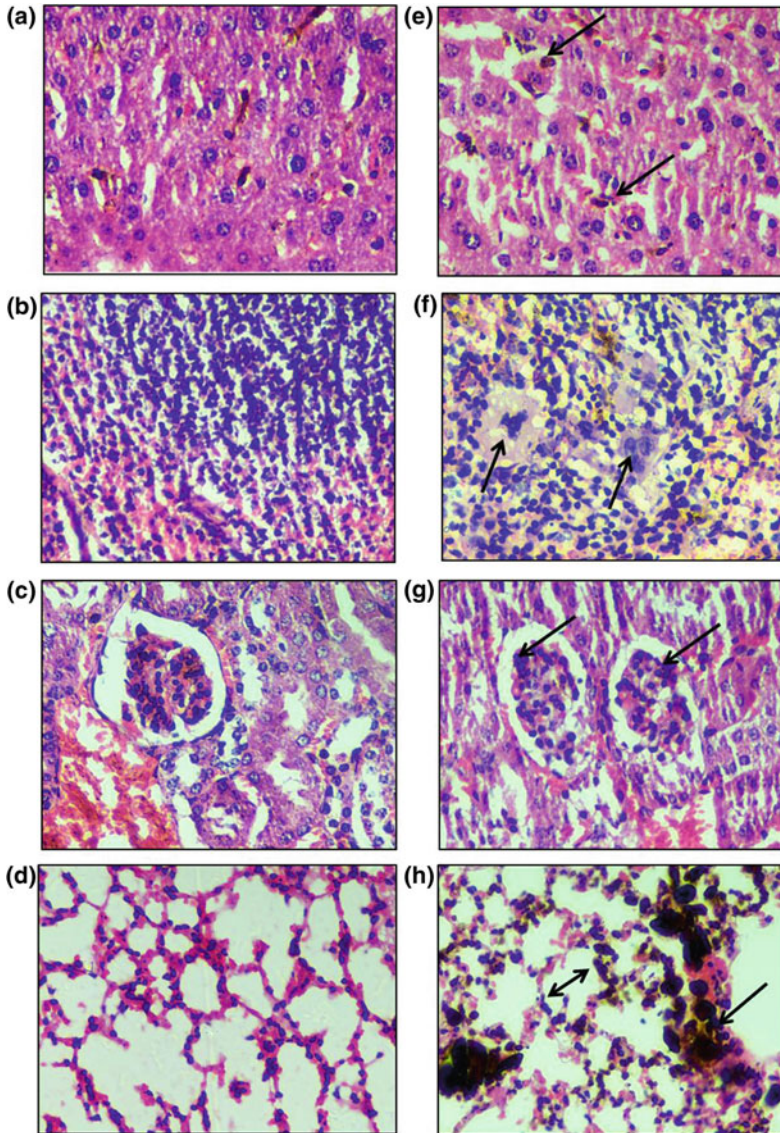
Fig. 6 Representative histological photomicrographs showing the untreated group of animals depicting normal liver (**a**), spleen (**b**), kidney (**c**), and lung (**d**). The pathology of the group of animals treated with PEGylated SPIONs at a dose of 500 mg/kg showing **e** section of the liver showing granular pigmentation in the kupffer cells (black arrow); **f** megakaryocyte hyperplasia in spleen (black arrow) and increased cellularity; **g** section of the kidney showing congestion in the glomerulus (black arrow); and **h** section of lung showing pigmentation in the alveolar sacks and widening of the interstitial walls (black arrow). Figure taken from Prabhu et al. (2015). Reprinted with permission of Springer Nature

followed by water. Then, heat-induced antigen retrieval must be performed, and samples must be incubated with an antibody targeting macrophages or monocytes (e.g., IBA-1, CD68, or F4/80 antibody). Finally, after incubation with secondary antibody, the slides must be developed with a peroxidase substrate, typically 3,3′-Diaminobenzidine (DAB). A counterstain with hematoxylin can also be carried out. Samples are visualized and photographed with bright-field microscopy.

Limitations of staining methods are associated with variation in results depending on fixatives, fixation temperature, and fixation time.

3. *Analysis of immune response through ELISA*

It is well established that organisms facing allergens may develop an anaphylactic reaction. Depending on the chemical nature of the molecule, hosts will produce antibodies or inflammatory responses. Studies in animals showed that immune responses can have phenotypical and behavioral manifestations, such as a hunched position, piloerection, slowing of movement, bronchospasm, respiratory arrest, and anaphylaxis (Gamboa and Leong 2013). Several parameters, like antibody titers and cytokine release, can also be measured in blood by ELISA (Chen et al. 2010). There are multiple ways to perform ELISA assays but all are based on the use of an enzyme bound to an immune reactant to specifically recognize a desired molecule (Schuurs and Van Weemen 1980). In the simplest strategy, named direct ELISA, the protein sample binds to the plate through absorption and an enzyme-conjugated antibody is added to detect the expected antigen by spectrophotometry. Another way to perform this method, termed sandwich ELISA, involves coating the plates with the antibodies to measure the amount of a specific antigen in the plasma (Wottrich et al. 2004). After sample addition, a secondary antibody is used to detect the captured molecule, and then a third enzyme-conjugated immunoglobulin is used to perform quantification. This is the case when cytokines, proteins that regulate function of immune cells and increase in response to inflammation, are measured. Typically detected proteins include IL1β, IL-8, tumor necrosis factor-a (TNF-a), and IL-6 (Wang et al. 2015).

Among common difficulties outstand false positives by inefficient blocking and inhibition of detection by non-specific serum factors.

4. *Detection of upregulation of stress-related molecules through microarrays*

Analysis of gene expression inside a tissue is one of the most sensitive methods for detecting molecules associated with cellular damage. This is done by lysing tissue samples, obtaining and purifying RNA, and performing PCR or microarrays. Microarrays consist of an arranged series of thousands of microscopic spots of oligonucleotides, each containing picomoles of a specific sequence used as a probe to assay. Through hybridization under high-stringency conditions, the presence of a complementary sequence in the sample can be measured (Sahu and Casciano 2009). Hybridization is usually detected and quantified by fluorescence-based detection of a fluorophore-labeled target to determine the relative abundance of each sequence in the sample. In one of these studies, Teeguarden and colleagues used a microarray to evaluate transcriptional regulation of inflammation markers in BALB/c mice exposed to SPIO by inhalation (Teeguarden et al.

2014). They found that NP led to an inflammatory response in the alveolar region characterized by interstitial inflammation, macrophage infiltration, and increased expression of CXCL2 and CCL3.

Microarrays have proven especially useful when expression of large number of genes needs to be analyzed at the same time, but results of special interest must be validated by real-time PCR. It should also be taken into consideration that variation in transcripts abundance will not necessarily have a direct translation into protein levels due to diverse mechanisms of regulation. Therefore, microarrays must be complemented with techniques measuring toxicity at protein level.

5. *Measurement of protein related to damage in target tissues*

Oxidative stress caused by MNP is considered to be one of the most important mechanisms of toxicity. This could be due to induction of reactive oxygen species and reactive nitrogen species, which react with biological molecules causing DNA damage, protein oxidation, lipids peroxidation, and impairing redox internal balance (Khanna et al. 2015). If repair mechanism cannot compensate, the final outcome for the cell will be necrosis or apoptosis. ROS-associated damage protein analysis can be done by sacrificing the animal, removing the organ of interest, and homogenizing the tissue to obtain proteins whose concentration or activity could be determined by different techniques. The classical procedure to identify and determine relative amounts of proteins, Western blotting, is not the first choice when tissue extracts must be analyzed, probably due to difficulties that antibody recognition has in complex samples. Rather, enzymatic reactions, usually coupled to colorimetric assays, are selected. This is the case for total glutathione, reduced glutathione, and oxidized disulfide, as well for activity of glutathione peroxidase, superoxide dismutase, and nitric oxide synthase (Zhu et al. 2008; Wang et al. 2009).

Conclusion and Future Perspectives

Research area on MNP has spread due to their attractiveness as theragnostic agents. They are generally associated with low toxicity and present high potential use for biomedical applications.

Although many reports evaluating in vivo toxicity of MNP can be found, there is still much to be standardized regarding proper routes and doses of administration, methodologies to follow, and relevant tissues to analyzed. Also, the temporal window in which toxicity should be examined in each metabolic step has not been clearly established, and a systematic approach to address these issues is missing. One of the reasons that interfere with reaching a common criterion regarding lines of action is that NPs are usually tailor-made. Consequently, studies differ in NP composition, chemical properties and biological models assayed, hindering comparisons. Another factor obscuring the possibility of drawing firm conclusions is that not all MNPs under the same name are identical; formulations can vary in purity, reactivity, surface

chemistry, and porosity. In this context, a common agreement regarding essential techniques for MNP characterization is urgently needed.

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