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Magnetic nanoparticles for theragnostics

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Abstract

Engineered magnetic nanoparticles (MNPs) represent a cutting-edge tool in medicine because they can be simultaneously functionalized and guided by a magnetic field. Use of MNPs has advanced magnetic resonance imaging (MRI), guided drug and gene delivery, magnetic hyperthermia cancer therapy, tissue engineering, cell tracking and bioseparation. Integrative therapeutic and diagnostic (i.e., theragnostic) applications have emerged with MNP use, such as MRI-guided cell replacement therapy or MRI-based imaging of cancer-specific gene delivery. However, mounting evidence suggests that certain properties of nanoparticles (e.g., enhanced reactive area, ability to cross cell and tissue barriers, resistance to biodegradation) amplify their cytotoxic potential relative to molecular or bulk counterparts. Oxidative stress, a 3-tier paradigm of nanotoxicity, manifests in activation of reactive oxygen species (ROS) (tier I), followed by a pro-inflammatory response (tier II) and DNA damage leading to cellular apoptosis and mutagenesis (tier III). In vivo administered MNPs are quickly challenged by macrophages of the reticuloendothelial system (RES), resulting in not only neutralization of potential MNP toxicity but also reduced circulation time necessary for MNP efficacy. We discuss the role of MNP size, composition and surface chemistry in their intracellular uptake, biodistribution, macrophage recognition and cytotoxicity, and review current studies on MNP toxicity, caveats of nanotoxicity assessments and engineering strategies to optimize MNPs for biomedical use.

Keywords

magnetic nanoparticles; iron oxide; nanotoxicity; oxidative stress; macrophages; ROS; surface chemistry; DMSA

Introduction

Nanotechnology is at the leading edge of the rapidly developing new therapeutic and diagnostic concepts in all areas of medicine [1]. Magnetic nanoparticles (MNPs) are a class nanoparticles (i.e., engineered particulate materials of < 100 nm) that can be manipulated under the influence

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of an external magnetic field. MNPs are commonly composed of magnetic elements, such as iron, nickel, cobalt and their oxides.

The unique ability of MNPs to be guided by an external magnetic field has been utilized for magnetic resonance imaging (MRI), targeted drug and gene delivery, tissue engineering, cell tracking and bioseparation [2–5]. When further "functionalized" with drugs and bioactive agents, such as peptides and nucleic acids, MNPs form distinct particulate systems that penetrate cell and tissue barriers and offer organ-specific therapeutic and diagnostic modalities [4]. The ability of MNPs to be functionalized and concurrently respond to a magnetic field has made them a useful tool for theragnostics - the fusion of therapeutic and diagnostic technologies that targets to individualize medicine [6]. Through multilayered functionalization, MNPs can simultaneously act as diagnostic molecular imaging agents [7,8] and drug carriers. MNP-based MRI imaging has been already combined with cell replacement therapy [9], organ-specific gene delivery [10], intra-operative tumor removal surgery [11,12] and other applications discussed below. However, the resulting variety of MNP composition, shape, size, surface chemistry and state of dispersion each may influence their biodistribution and toxic potential [3].

The increased toxic potential of nano-sized materials relative to their bulk and molecular counterparts is owed to their largely enhanced reactive surface area, ability to cross cell and tissue barriers, and resistance to biodegradation [13]. Activation of oxidative stress and inflammatory signaling leading to apoptosis and genotoxicity are the key paradigm(s) of nanotoxicity [14–18]. In an *in vivo* setting, macrophages of the defense reticuloendothelial system (RES) quickly challenge and internalize MNPs, neutralizing their cytotoxic potential [5]. But in order to promote their circulation time, engineering strategies to modify MNP surface chemistry are used to allow for evasion of macrophages [5]. Therefore, an integrative approach to advancing MNP designs and understanding their interface with specific organ systems, with regards to their application and safety, are imperative to advancing nanomedicine [14,16–18].

Several recent reviews have discussed engineering designs, physiochemical characteristics [19,20] and biomedical applications of MNP [3–5]. Here, we will review current studies of MNP toxicity and issues relevant to the development of the discipline of magnetic nanotoxicology.

Formulations of MNPs for biomedical applications

Iron oxide MNPs, such as magnetite Fe_3O_4 or its oxidized and more stable form of maghemite γ -Fe₂O₃, are superior to other metal oxide nanoparticles for their biocompatibility and stability and are, by far, the most commonly employed MNPs for biomedical applications [2–4,21, 22]. Thus, we here refer to iron oxide MNPs as "MNPs", unless otherwise specified. Typically, magnetic nanoparticles are synthesized and dispersed into homogenous suspensions, called ferrofluids, composed of a large number of engineered composite nanoparticles. Each MNP consists of a magnetic core and a non-magnetic coating of different surface chemistry.

Thermal energy, quantum size effects and the large surface area of individual MNPs are responsible for superparamagnetic phenomena of ferrofluids [23]. Hydrodynamic particle sizes range from superparamagnetic (50–500 nm) and ultrasmall superparamagnetic (< 50 nm), and influence their magnetization values, dispersibility, stability in solution, and determine their biomedical modalities [2,19]. Based on the biokinetics of particles, the sizes of 10–100 nm are optimal for *in vivo* delivery, as they escape rapid renal clearance (< 10 nm) and sequestering by the reticuloendothelial system (RES) of the spleen and liver (> 200 nm) [2]. Numerous engineering approaches aimed at achieving uniformity of MNP size, shape and composition

(e.g., types of salts used, Fe^{2+} and Fe^{3+} ratio, pH and ionic strength of the media), and each may influence MNP size and magnetization properties [3,19].

MNP shapes and structure designs range considerably from particle suspensions, sheets, tubes, shells and arrays. An emerging theme in MNP biomedical research is to influence function and magnetic properties in biological systems by control of shape. For example, increased *in vivo* circulation time for up to 48 h and effective tumor targeting was achieved through the use of magnetic nanoworms, representing elongated assemblies of dextran-coated iron oxide MNPs [24]. Surface chemistry is another critical determinant that regulates physiochemical characteristics of MNPs, including their size, solubility, state of dispersion and magnetization values. Because surface chemistry greatly influences MNP fate in the biological system, including the mechanisms of their cell recognition, biodistribution and immune response [3, 25] it presents a specific focus for advancing engineering strategies to minimize potential nanotoxicity.

Surface chemistry and biocompatibility

Without a coating, MNPs have hydrophobic surfaces with large surface area to volume ratios and a propensity to agglomerate [19]. A proper surface coating allows iron oxide MNPs to be dispersed into homogenous ferrofluids and improve MNP stability. Several groups of coating materials are used to modify MNP surface chemistry:

- **a.** organic polymers, such as dextran, chitosan, polyethylene glycol, polysorbate, polyaniline
- b. organic surfactants, such as sodium oleate and dodecylamine
- c. inorganic metals, such as gold
- d. inorganic oxides, such as silica and carbon
- e. bioactive molecules and structures, such as liposomes, peptides and ligands/receptors

Biodegradable polymers

Clinical preparations of MNPs have traditionally relied upon organic biodegradable dextran and carbohydrate derivatives because of their common use as plasma expanders and high affinity to iron oxides [26]. Several such formulations are now available, including Ferridex, Resovist, Combidex, and AMI-288/ferumoxytol, successfully utilized as MRI contrast agents [4]. Through creating steric repulsion, polymers stabilize MNPs in suspension. But polymercoated MNPs are unstable at high temperatures and are not suitable to protect reactive MNPs due to poor air stability and susceptibility to leaching in acidic conditions [19]. Poor functionalization and conjugation capacity of polymer-coated MNPs [4] limit their use for more complex, integrative applications. However, amination or carboxylation of dextran resulting in cross-linked iron oxide MNPs (CLIO) improves their conjugation capacity. For example, CLIO conjugated to fluorescent probes are successfully used as reporters to $\alpha_{v}\beta_{3}$ integrin, cathepsin B, E-selectin, VCAM-1, macrophage-specific and other ligands for molecular MRI imaging [4,8,27]. Polyethylene glycol (PEG) is a polymer widely used for its hydrophilicity and low antigenicity. In addition to steric stabilization of MNPs, PEG prevents their plasma opsonization and uptake by macrophages, increasing MNP circulation in vivo [28]. PEG-coated MNPs are efficiently internalized by cells via fluid-phase endocytosis and through amphiphilic affinity to lipid bilayers on plasma membranes [28]. These qualities, however, increase their potential to overload cells with iron and become toxic [3]. Polyvinyl alcohol (PVA) has excellent film-forming, emulsifying and adhesive properties [3]. While PVA use for intravenous (i.v.) delivery is limited due to poor persistence and agglomeration parameters in a ferrofluid [3], when cross-linked to form a magnetic gel, PVA can be used as

a vitreous eye substitute [29]. Potentially, this property can be utilized in targeted drug delivery, tissue engineering and biosensor technology [3]. *Chitosan* provides a natural, biocompatible, cationic and hydrophilic polymer coating, suitable for affinity purification of proteins and magnetic bioseparation [3]. Interestingly, labeling of fibroblasts with chitosan-coated MNPs improved their invasive potential under magnetic force, showing promise for tissue engineering [30].

Precious metals, such as gold, have been used to protect iron oxide cores against oxidation, as they form highly stable particles of low reactivity [31,32]. Figure 1, B-C depicts gold-coated MNPs. Nanogold is known for its superior optical properties, biocompatibility and outstanding capacity for functionalization [31,32]. These properties make gold particularly attractive surface chemistry for MNPs. One limitation is that gold coating can weaken magnetic properties of iron oxide MNPs [20] and is considered difficult to achieve due to the dissimilar nature of the two surfaces [19,32]. When achieved, gold-coated MNPs are stable under neutral and acidic conditions [33,34]. Engineering a continuous gold shell over an MNP core is a challenge, but it can provide an extremely effective barrier against oxidizing agents [3].

An inert silica, shell of different thicknesses is used to tune the MNP core to different extents of dipolar coupling (i.e., direct magnetic dipole-dipole interaction) and cooperative magnetic switching [19]. Based on the negative charge of the silica shell, silica-coated MNPs are redispersible and stable in aqueous conditions [19]. They provide good control of interparticle interactions both in solutions and within structures through variations of the shell thickness [19]. Depending on the protocol for their synthesis, silica-coated iron oxide MNP range in size from 1–2 nm [35] to 150 nm [36], but the development of monodispersed solutions often proves difficult. Silica-coated MNPs have longer circulation times and their hydrophilic negatively charged surface provides ideal anchorage for covalent binding to ligands presenting an excellent platform for drug delivery [3]. Figure 1, D depicts a 100 nm silica-coated iron oxide (magnetite) MNP.

Dimercaptosuccinic acid (DMSA) is a negatively charged sulfur-containing chelating agent. DMSA- conjugated iron oxide MNPs are referred to as anionic MNPs (AMNPs, see Figure 1, A). DMSA prevents MNPs from aggregation and interacts strongly with the positively charged regions of the plasma membrane due to its negative charge. AMNPs are suitable for highefficiency cellular uptake via adsorptive endocytosis [37–40]. Because of the scattered positioning of cationic sites on the plasma membrane, AMNPs cluster at the cell surface prior to their endocytosis; this clustering phenomenon is believed to contribute to their high efficiency uptake [26, 37]. Cellular internalization of AMNPs can be reduced with dextran coating that is known to limit efficiency of intracellular MNP uptake [2, 37]. A very wide range of cells have shown rapid, uniform and efficient labeling with AMNPs [41]. However, due to the efficient intracellular uptake, considerations for functional effects of iron accumulation and concentration-dependent cytotoxicity should be given [42].

Functionalized MNPs

Surface functionalization, a process of chemical conjugation of functional groups to the surface, is now integral to MNP designs. As we already mentioned, certain coating surface chemistries have inherently superior functionalization capabilities, such as silica and gold [3]. Both coated and uncoated MNPs have been functionalized with peptides, nucleic acids, small molecules and sometimes antibodies. Antibodies are used as conjugates for magnetic beads in cell sorting and imaging, but because they are as large in size as MNP cores, they often suffer from steric hindrance, poor cell internalization and display low affinity constants [4]. Both the HIV-derived TAT peptide [12,43–45] and transferrin, a plasma protein for iron delivery, have been effectively used as MNP delivery systems [46,47]. MNP functionalization with the RGD (arginine-glycine-aspartic acid) peptide allowed for tumor detection of integrins

and imaging of tumor vascularization [48]. Surface chemistry of MNPs has functional effect, e.g., albumin conjugation significantly promoted proliferation of primary human fibroblasts, whereas uncoated and dextran-coated MNPs inhibited their proliferation [49]. Numerous successful functionalization protocols have been developed, some of which are described under specific biomedical applications. Other types of MNP surface chemistries and protocols of their synthesis applicable to biomedical use have been reviewed elsewhere [3,4].

MNPs for theragnostic platforms

Theragnostics (the fusion of therapeutic and diagnostic approaches) aims to personalize and advance medicine [6]. MNPs represent a particularly appropriate tool based on their ability to be simultaneously functionalized and guided by an external magnetic field. Novel designs have focused on sophisticated multilayering of MNPs with the goal to develop controlled nanodelivery systems. Already, functionalized MNPs have been used in combination theragnostic approaches for gene delivery with selective tumor imaging [10], MRI-guided therapeutic cell replacement [9] and MRI-assisted diagnostic and surgeries using a single MNP formulation [11,12]. After removal of the magnetic field MNPs do not retain magnetism [21], providing additional versatility. We here touch upon MNP-based therapeutic applications (e.g. magnetic hyperthermia, drug/gene delivery and tissues engineering) and diagnostic imaging or biosensor platforms, depicting integrative approaches that capitalize on the MNPs' dual magnetic and functionalization modalities.

Magnetic hyperthermia

Hyperthermia is a cancer therapy that relies on the localized heating of tumors above 43°C for about 30 minutes [50]. MNPs can generate heat under alternating magnetic fields due to energy losses in the traversing of the magnetic hysteresis loop [20]. Generation of different degrees of heat depends on the magnetization properties of specific MNP formulations and magnetic field parameters [2]. Selectivity to tumors was considerably improved through the use of silane coatings [51,52] and through functionalization approaches. For example, MNPs conjugated with antibodies to cancer-specific antigens improved selectivity of MNP uptake by tumors during hyperthermia therapy [22]. Magnetic hyperthermia using magnetic cationic liposomes has been used in a combination approach with TNF- α gene therapy and stress-inducible gadd153 promoter, resulting in a dramatic arrest in tumor growth [53].

Magnetic resonance imaging (MRI)

MNP-enhanced MRI is based on the superior superparamagnetic qualities of iron oxide MNPs [22]. Several dextran-coated MNP formulations have been approved for clinical use as MRI contrast agents, including ferumoxides, ferumoxtran and ferucarbotran [7]. Experimental studies show that MNP-enhanced MRI is vastly superior to other noninvasive methods of identifying lymph node metastases from solid tumors and histologically positive lymph nodes outside of the usual field of resection [54]. MNP application in delineating primary tumors and detecting metastases, in imaging angiogenesis and mapping vascular supply to primary tumors has a realistic translational potential [4,7]. Macrophage-specific MNP labeling protocols are used to image inflammatory pathologies, including atherosclerosis, multiple sclerosis and rheumatoid arthritis [4,7,55]. Long-term MRI-guided *in vivo* cell tracking of CNS regeneration became possible with the use of grafted MNP-labeled stem cells [9] and neuroprotective glia (Schwann cells and olfactory ensheathing cells) [56]. FITC-conjugated MNPs have been used by surgeons to delineate gliomas both preoperatively, based on their MRI modality, and intraoperatively, based on fluorescent tag [11,12]. This is particularly valuable since certain tumors, such as gliomas, can shift position during surgery.

MRI has advanced to *cellular and molecular* sensitivities, with nanofunctionalized MRI contrast agent libraries now available [7,8]. For example, the HIV-derived TAT peptide conjugated to ultrasmall iron oxide MNP allows efficient T cell labeling within 5 minutes [57]. A number of studies demonstrate the immense potential of MNP-based molecular MRI as a combination imaging and drug/gene-delivery strategy. For example, monocrystalline iron oxide MNPs (3 nm core) sterically protected by a layer of low-molecular weight dextran and covalently conjugated to holo-transferrin, promotes overexpression of engineered transferrin receptor (ETR) to selectively visualize tumors *in vivo* in real time at exceptionally high spatial resolution [10].

Bioseparation and biosensors

In vitro streptavidin-coated magnetic beads are used for phenotypic selection in different cell sorting protocols, including stem cells [58], sensory neurons [59] and others [3,4]. Conjugated to monoclonal antibodies, MNPs could be used to decontaminate blood from infective agents [60]. The principles of MNP-based magnetic bioseparation have been applied to integrative biosensor technologies. For example, ultrasensitive bio-barcodes can detect protein analytes using specific MNP-conjugated antibodies, followed by dehybridization of the co-tagged oligonucleotides [61].

Targeted drug delivery

The development of drug delivery systems with selectivity to pathologic sites is an ambitious goal. The principles of magnetic guidance of MNP-conjugated drugs have been applied experimentally, and have reached clinical trials as a cancer therapy [5]. Following i.v. delivery of MNPs, an external magnetic field is used to concentrate MNPs at a specific target site; this procedure has been well tolerated in cancer patients [5,50]. Nanoparticle-based drug and gene delivery systems may solve the insurmountable obstacle of treating neurological diseases: delivery across the blood-brain barrier [62,63]. But issues of potential embolization with MNP aggregates in capillaries and the necessity for large distances between the pathological site and external magnetic field still present a challenge [3,5]. When considering the MNP surface chemistry for drug delivery, it is favored that MNPs retain sufficient hydrophilicity and, with coating, do not exceed 100 nm in size to avoid rapid clearance by RES [64,65]. Overall, the smaller, more neutral and more hydrophilic the nanoparticle surface, the longer its plasma half-life [5].

Magnetic transfections

When functionalized with DNA vectors, MNPs could be used as effective gene transfection systems under external magnetic field, termed magnetofections (MF) [2]. Direct comparison of state-of-the-art magnetic polycation polyethylenimine-coated MNP and corresponding standard gene vectors demonstrated a profound increase in transfection efficiency for both non-viral and viral vectors in permissive and non-permissive cells [66]. In endothelial cells, MF increased the efficiency of the luciferase reporter by 360-fold [67] and effective antisense oligonucleotide delivery *in vivo* and *in vitro* [68]. An example of magnetic nonviral gene transfer protocol includes steps of MNP synthesis, testing intact DNA binding with MNP core, preparation of magnetic lipoplexes and polyplexes, magnetofection and data processing [69].

Tissue engineering

Iron oxide MNPs have several distinct applications in tissue engineering. They have been used in stem cell replacement therapy for cell labeling, sorting, monitoring, engraftment and targeted *in vivo* delivery [9]. MNPs can be also used to weld the joining tissue surfaces under high temperatures, a process typically accompanied by protein denaturation followed by repolymerization of adjacent protein chains [2,3]. With regard to nanomagnetic welding, gold

and silica coating are expected to increase MNP sensitivity and robustness to light absorption [70,71] and allow for selection of light sources and wavelengths with minimal damage to tissues [2]. Magnetic nanoparticles have been used for construction and harvesting of multilayered keratinocyte sheet-like 3-D constructs [22]. Self-assembled magnetic nanowire arrays [72] could potentially be used in tissue engineering.

Iron detection and chelation therapy

Excess metal deposits are associated with virtually every neurodegenerative disease, including multiple sclerosis, Friedreich's ataxia, Alzheimer's, Parkinson's, and Huntington's diseases [73]. Advanced technologies of nano-sized iron detection in neuronal tissues, such as by superconducting quantum interference device (SQUID) magnetometry, have found use as a diagnostic strategy in identifying iron deposits in the brains of Alzheimer's [74] and neuroferritinopathy patients [75]. As experimental therapeutics, MNPs may be used as iron chelators for neurodegenerative diseases [76], potentially presenting another theragnostic application.

MNP biodistribution, clearance and toxicity

MNP metabolism

Typically, upon their intracellular internalization via endocytosis, MNPs are clustered within lysosomes where, presumably, they are degraded into iron ions by an array of hydrolyzing enzymes at low pH according to endogenous iron metabolism pathways [3]. MNP size, charge, surface chemistry and route of delivery each influence their circulation time and biodistribution patterns in the body [5]. Large (> 200 nm) particles are usually sequestered by the spleen via mechanical filtration followed by phagocytosis, whereas smaller < 10 nm particles are rapidly removed through extravasation and renal clearance, with particles 10-100 nm believed to be optimal for i.v. administration [2]. The typical final biodistribution of particles is 80–90% in liver, 5–8% in spleen and 1–2% in bone marrow [5]. Surface chemistry dictates the efficiency and the mechanism of MNP internalization by cells as well as their overall biodistribution in organ systems [2,77], metabolism and potential toxicity [13-15]. Depending on the routes of their delivery/exposure, MNP surfaces may interact with extracellular matrix components and the plasma cell membranes of macrophages, endothelial cells, skin epithelium, and respiratory or gastrointestinal tracts [14,78–80]. A point worth noting is that MNPs accumulate in the brain, liver, spleen and lungs after their inhalation [81], demonstrating their ability to cross the blood-brain-barrier. MNPs can also penetrate the hair follicles and stratum corneum, reaching viable skin epidermis [82].

Macrophages in nanoparticle clearance: a double-edged sword

Upon their *in vivo* administration, within minutes nano-sized particles are challenged by macrophages of the RES [5]. The efficacy of particle clearance by the RES depends upon the size, chemical composition, cumulative projected area and surface chemistry of the MNPs and can influence mechanisms of macrophage activation and particle internalization [83,84].

Multiple mechanisms of intracellular internalization of nanoparticles have been described, including phagocytosis (mediated by mannose, complement, Fcγ and scavenger receptors) and endocytosis (clatrin- and calveolin-mediated, fluid-phase) [25]. MNPs have been reported to be internalized via phagocytosis, scavenger receptor-mediated endocytosis, fluid-phase endocytosis and diffusion [77]. Depending on surface hydrophobicity and charge, nanoparticles can get opsonized by plasma proteins (e.g., albumin, apolipoprotein, immunoglobulins, complement, fibrinogen), which promotes their recognition and clearance by cells of RES [25,55]. Opsonization can change the mechanisms of particle uptake by cells of RES. For example, iron oxide MNP binding to plasma fibronectin and vitronectin changed

from receptor-mediated to fluid-phase endocytosis [85]. Efforts are made to engineer MNPs of size and surface chemistry that will minimize their opsonization and macrophage-mediated clearance and thus increase their circulation time [77]. For example, surface coating with amphiphilic polymeric surfactants, such as polyethylene glycol (PEG), significantly reduces MNP interactions with plasma proteins, minimizing their internalization and clearance by macrophages [5,86–88]. But because PEG-coated MNP uptake is highly efficient [28], it is anticipated that their cytotoxic potential in non-immune cells will be increased [3]. It is likely that cytotoxic de-differentiation and cell death after exposure to anionic DMSA-coated MNPs (AMNPs) relates to the very efficiency of uptake, resulting in dose-dependent intracellular iron overload [42]. *In vitro*, AMNPs are internalized by macrophages [37,89] and neuron-like PC12 cells [42] and other cells. In fact, their rapid and efficient internalization was noted to be uniform between 14 different cell types *in vitro*, including hepatocytes, fibroblasts, smooth muscle cells, stem and progenitor cells and immune cells [41]. MNPs internalization by macrophages is mediated via integrin MAC-1 (also known as CD11b, CD18) [90] and scavenger receptors SR-A [91].

It is not yet clear whether *in vivo* AMNPs are internalized uniformly between the cells, particularly immune or non-immune tissue cells. Potency of macrophages to "sense", migrate towards and phagocytose nanoparticles depends highly on MNP surface chemistry, and occurs typically 6-12 h after their in vivo exposure [79]. In addition, inherent immunocompetence of organ and tissue systems is likely to play an important role. For example, immunocompetence of the nervous system is not homogenous. Peripheral nerve is capable of recruiting highly potent macrophages that, by clearing degenerating debris, creates a permissive environment for neurite growth [92]. This immunocompetence is believed to be promote propensity of peripheral nerve for regeneration, in great contrast to the central nervous system (CNS), where macrophages are few and ineffective [92]. We found AMNPs to stimulate macrophage recruitment into peripheral nerve 24 h after their injection, where they seem to predominantly label infiltrating macrophages (Fig. 2). Thus, MNP microinjection into peripheral nerve may present an advantageous experimental model for studying the MNP-macrophage interface within a mammalian nervous system, preventing MNP clearance issues characteristic of intravenous delivery. Understanding the molecular interactions at the macrophagenanoparticle interface has lead to new approaches for selective MR imaging of cardiovascular diseases, multiple sclerosis, stroke and other diseases [8,93–96].

Macrophages are critical elements in the body's defense system against diseases, and introduction of nanoparticles into the body may affect macrophage defensive function. It is known that nanoparticles promote activation as well as phagocytotic, cytoskeletal and cytokine-releasing functions of macrophages [25,97-99]. Dextran-coated MNPs induce differentiation of monocytes into macrophages. While proinflammatory signaling many manifest macrophage activation, it may mediate MNP-induced macrophage cytotoxicity. Given that particle clearance by macrophages is size-dependent, ultrasmall nanoparticles can escape phagocytosis and lavage within the macrophages [79], promoting activation of oxidative stress and ROS-mediated redox-sensitive transcription via NF-kB and AP-1 [100], proinflammatory TNF- α /p38 signaling and apoptosis [99]. In fact, these properties have translated into therapeutic «macrophage-suicide» approaches of nanocarrier systems for macrophage-driven diseases of bacterial infection, atherosclerosis, rheumatoid arthritis and neuroinflammation [55,80]. However, the limited selectivity of such designs to macrophages due to often similar cell surface composition in other immunocompetent cells, such as Schwann cells and microglia in the nervous system, as well as the adverse effects of macrophage destruction on local and central immunity, should be considered in the overall scheme of nanotoxicity. Inhibition of p38 MAPK by SB-239063 reduces uptake of MNPs by aortic macrophages after their i.v. administration [101], providing a potential strategy to modulate MNP toxicity in vivo.

Based on the multi-factorial relationship of MNPs with macrophages, engineering strategies to increase the ability of MNPs to evade macrophages have to be accompanied by thorough evaluation of their toxic potential associated with relevant routes of delivery and identification of target cells of their internalization. Intrinsic to their enhanced reactive surface area, propensity to cross tissue and cell barriers, and ability to lavage within cells, nano-sized materials can compromise functions of cell membranes, mitochondria, nuclear membranes and DNA [14,78].

Oxidative stress: A paradigm for nanotoxicity

Iron oxide MNPs are believed to induce redox cycling and catalytic chemistry via The Fenton Reaction $[H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^*]$ [14], the most prevalent source of reactive oxygen species (ROS) in biological systems. In fact, uncoated magnetite nanoparticles are significantly cytotoxic [28]. But the mechanism of cytotoxicity of the fully oxidized (Fe₂O₃) maghemite MNPs is not directly explained by the Fenton chemistry. Furthermore, ROS generation leading to oxidative stress (OS) is associated with the nanotoxicity of non-metal nanoparticles as well [13–15,78,102]. While the mechanisms by which nanoparticles generate ROS are still largely unknown, it is hypothesized that disruption of the well-structured electronic configuration of the nano-sized material surface creates reactive electron donor or acceptor sites, leading to the formation of superoxide radicals [14]. As a multistage process, nanoparticle-induced ROS activation induces defense anti-oxidant response elements by the transcription factor Nrf-2, leading to increased expression of over 200 phase II antioxidant enzymes, such as heme oxygenase 1 (HO-1), superoxide dismutase, etc. (tier I OS) [99]. If damage proceeds, protective systems are superseded by mitogen-activated protein kinase (MAPK) and NF-kB-activated intracellular signaling, resulting in pro-inflammatory cytokine, chemokine and matrix metalloproteinase (MMP) release (tier II OS), leading to apoptosis (tier III OS) [14]. Current studies on mechanisms of nanotoxicity overwhelmingly point to OSmediated activation of proinflammatory cytokines and MAPK signaling pathways in respiratory and gastrointestinal tracts, blood cells, skin and the nervous system, as carefully reviewed [14,77,79,103]. A continuum of molecular events from tier I to III OS manifested through increased HO-1 and JNK-mediated TNF-α production leading to apoptosis [99]. Studies into the effects of NP size, shape and state of dispersion indicate a clear correlation between these physiochemical characteristics with cytotoxicity [104] and the cellular proinflammatory response [105].

Tier II OS can manifest in vivo through immune cell infiltration, mediated by the actions of proinflammatory cytokines and MMPs [106]. For example, ROS controls MMP activity by two distinct mechanisms: MAPK-induced overexpression of MMP gene and directly through structural oxidative modification of thiol residues on inactive pro-MMPs, resulting in release of the zinc-binding domain and MMP activation [107]. In Figure 3 we summarize our hypothesis of ROS-induced MMP activity in the nervous system leading to increased bloodbrain-barrier (BBB) permeability and neuronal damage [108–110]. In support of this hypothesis, MMPs are proposed to mediate (and present a highly sensitive measure for) macrophage activation in response to nanoparticles [55]. Internalization of chitosan-coated MNPs promote invasive potential of cells into the 3-D-porous scaffolds via MMP action [30]. MNPs are believed to promote cytotoxicity of human fibroblasts through the action of proinflammatory cytokines and MMPs [111], which are considered to be useful biomarkers for metal toxicity [112]. In the context of AMNP-induced macrophage recruitment into the nerve, depicted in Figure 2, it is worth noting that cytokines and MMPs are important modulators of macrophage recruitment into the nerve [113,114]. Together, these studies support a model in which MNPs induce ROS to promote MMP-mediated degradation of the blood-brain and blood-nerve barriers to promote recruitment of macrophages into the nervous system (Figure 3).

Metals and MNPs in neurodegeneration

While iron oxide is considered safe, imbalance in its homeostasis has known toxic implications to many organ systems [115]. As the abundant, redox-active metal, iron facilitates the generation of free radicals in the brain [116,117] and excess iron is associated with multiple neurodegenerative disorders, including multiple sclerosis, Alzheimer's and Parkinson's diseases [73]. We have mentioned that nano-scale iron oxide deposits have been identified in neurological tissues using SQUID magnetometry in the brains of Alzheimer's patients [74] and basal ganglia of neuroferritinopathy patients [75]. These iron nanoparticles are believed to be biogenic in nature and linked to ferritin, a large (12 nm) natural ferric oxide phosphate storage protein [15,74,118]. However, evidence of brain accumulation of MNPs after passive exposure through inhalation [81] calls for confirmation of the source of MNP deposits in the brains of neurodegeneration patients. It is particularly important due to the ability of MNPs to penetrate through skin [82] and cross blood-brain barrier after exposure through inhalation [81] and by intraperitoneal delivery [119]. Studies on the neurotoxicity of MNPs and identifying their safe formulations are required prior to their therapeutic use as an iron chelating therapy for neurodegenerative disorders [76] and drug conjugates (e.g., glial derived neurotrophic factor) as a therapy for drug addiction [120].

Current studies of MNP toxicity

Given the variety of MNP formulations and their biomedical applications, the limited number of studies evaluating their toxicity is astounding. The findings on iron oxide range from cytotoxicity *in vitro* [121–123] to transient and acute *in vivo* toxicity [119,124–126] to unremarkable changes *in vivo* [27,56,119,127]. In relevance to the journal's Theme Issue of nanoparticle toxicity, in this section we refer to toxic properties of iron oxide and other metal and metal oxide MNPs.

MNP cytotoxicity to non-immunogenic cells in vitro is increasingly being noted, in addition to that in macrophages reviewed above. Several metal oxide nanoparticles of 20-45 nm produced dose-dependent apoptosis and membrane function in mouse neuro-2A cells that were measured by membrane leakage of lactate dehydrogenase and MTT reduction [128]. In studying the capacity of AMNPs to extend neurite outgrowth under magnetic force, we observed de-differentiation and cell death of neuron-like PC12 cells with increasing concentration of iron oxide [42]. Although DMSA itself (used for coating) did not cause toxicity and is a known non-toxic BBB-crossing therapeutic chelator of metal [129], it presents an efficient cellular internalization system of iron-oxide MNPs [37–39], resulting in intracellular overload with iron. Because target intracellular compartments of iron oxide MNPs include mitochondria and nucleus, increase in oxidative stress-related changes and gene expression can occur. In fact, MNPs of various compositions, sizes and even at mild exposures can activate pro-inflammatory and ROS-induced cell signaling, as well as interference with mitochondrial energy production in cultured cells [105,111,122,123,128,130,131]. However, of several MNPs comparatively tested, iron oxide MNPs were shown to be the safest, producing cytotoxic changes at levels of 100 µg/ml or higher [122,128,131]. Reduced cytotoxicity of oxide nanoparticles is associated with increased particle solubility and modification of surface chemistry [121].

In vivo toxicity data on MNPs is hopeful, demonstrating no long-term implications of their use when administered at clinically relevant concentrations via relevant routes. Several reports found iron to accumulate in tissues, but with unremarkable histological changes in vital organs, concluding safety of the respective formulations [27,56,119,127]. Although MNP deposits were detectable in the prostates of prostate cancer patients after a year of magnetic hyperthermia therapy, no signs of systemic toxicity were found [132]. The median lethal dose (LD_{50}) of dextran-coated magnetite MNPs is very high at 400 mg/kg in rats, with cytotoxic effects on

peritoneal cells, lymphocytes and neutrophils [133,134]. Hemangiectasia and leukocyte infiltration observed after single subcutaneous administration of dextran-coated MNPs almost disappeared 72 h after their administration [125]. Signs of oxidative stress, evident in spleen and to a lesser degree kidney and liver of rats after intravenous administration of oleic acid-pluronic-coated iron oxide MNPs, gradually declined after 3 days of administration [126]. As mentioned, MNP surface chemistry that ensures high efficiency of intracellular MNP uptake or effective evasion of the RES is likely to increase their toxic potential [3].

Although the studies on MNP toxicity are limited in number, they point at the influence of MNP composition, size, dispersibility, surface chemistry and the regimens of their administration in the toxic outcomes, the factors expected to influence nanotoxicity [14]. Next, we will outline the influences of testing and study design on nanotoxicity data.

Intricacies of nanotoxicity testing and data analyses

The necessity, purpose and directions in the formation of the new discipline of nanotoxicology have been discussed in detail [14,15], and MNPs bear no exception. Physiochemical characteristics of nanoparticles (size, shape, surface chemistry, solubility) each influence their toxic potential [104]. Particokinetics and dosimetry approaches to nanotoxicity assessment encompass measures for dose of exposure (mass administered, media mass, surface area), delivered dose (number per cell or cm³), and cellular dose (internalized mass) [135]. With many parameters to assess, the demand for toxicity evaluation exceeds current capabilities of the research field. Thus, prioritization of the physicochemical parameters of clinical relevance to the target organ groups is imperative. E.g., for skin, that includes NP dissolution, size and partition coefficient, whereas for brain, hydrophobicity and surface chemistry, relating to possibility to cross barriers, size and shape and chemical composition are more relevant [136].

It has been suggested that study designs center only on clinically relevant target cells and include in-depth mechanistic analyses of the hierarchical process of oxidative stress (OS) [14]. For tier I OS, ROS species generation and measures of anti-oxidative HO-1 have been proposed as useful measures of OS-specific nanotoxicity [99]. As mentioned already, activation of proinflammatory signaling and MMPs have been adopted by many studies and represent reliable measures of tier II OS. It has been shown, however, that measures of MMP-9 provide higher sensitivity when cytokine measures fail [55]. Overall, the use of multiple assays is important to avoid false-positive or false-negative results [137]. Tier III OS can be identified by activation of pro-apoptotic pathways [14,99]. Thus, whether or not activation of tier I OS would indicate ROS-induced changes of nanotoxicity, determination of cell apoptosis of tier III OS will help understand the extent of cytotoxicity. In vivo, the cell source for specific changes could be identified by dual-labeling of OS-specific antigens and cell specific markers using confocal microscopy. Caution in the interpretation of mechanistic studies is important and a consideration that low-level ROS signaling contributes to normal cellular redox signaling [138] and cytokine-protease activation may relate to the body's defense reaction in immobilizing immune cells.

For *in vivo* nanotoxicity assessment, cell viability and mechanistic OS measures should be supplemented by evaluation of organ-specific toxicity, such as renal filtration or blood-brain barrier breakdown [139,140]. The biokinetics of nanoparticles through confirmed and potential routes of exposure involves multiple pathways of their translocation prior to clearance [15], particularly as nanoparticle binding to proteins generates complexes that are more mobile and pervasive through tissues normally inaccessible [14]. The time-course of nanotoxicity measures may help reconcile studies demonstrating safety of MNPs [27,56,119] with those of transient and acute *in vivo* toxicity [119,124]. Direct comparison of *in vivo* and *in vitro* pulmonary nanotoxicity using the same assays showed little correlation [141], as NP

biodistribution and RES clearance both factor in their *in vivo* toxicity. Toxicity of MNPs in normal and pathologic tissues may also differ due to the differences in their cell composition and phenotypic status (e.g., availability of specific receptors to mediate MNP endocytosis), and should be addressed independently.

Some toxicity data may not be specific to nanoparticles *per se*, but relate to the increased substance quantities and accumulation, which could be adjusted with adoption of appropriate concentration standards [136]. Since most substances become toxic at high doses, it is important that nanotoxicity studies incorporate doses at the anticipated human exposures [78]. Toxicity could also be qualitatively different based on size, surface chemistry or specific interaction [136]. When nanotoxicity is observed, the role of each component of the composite structure should be evaluated as control factors, if possible (e.g., its vehicle or coating material). For example, polyakylcyaniacrylate-coated MNPs (220 nm, with 10–20 nm cores) showed an LD_{50} of 245 mg/kg, however, a similar LD_{50} was observed in polyakylcyaniacrylate (not MNP-based) particles [142].

Conclusions

Potential benefits of nanotechnology in medicine are inimitable owing to refined, highly targeted, blood-brain barrier-crossing drug delivery and imaging platforms, unique transfection, labeling, bioseparation, as well as analytical and tissue engineering approaches. The versatility of superparamagnetic iron oxide MNPs is owed to their capability to respond to an external magnetic field and be functionalized with bioactive agents at the same time and/ or differentially. Challenges to the rapidly advancing field of magnetic nanotechnology include: the reduced magnetization modalities with certain advanced surface modifications; the distances between the target site and magnetic field; the potential dissociation of the functionalized domain (e.g., therapeutic peptide) with the magnetic core; and the agglomeration of particles in magnetic fields, promoting embolization or other tissue toxicities [3]. Capitalizing on the agglomeration properties of MNPs in tissues has led to therapeutic strategies of localized cytotoxicity, such as tumor targeting and macrophage-suicide approaches. However, potentially toxic implications for MNP overload to healthy tissues should be considered. So should engineering approaches to increase MNP ability to evade macrophages. Increasingly surfacing data on MNP toxicity should not be ignored. A rising chorus of government, industry, academia and environmentalists is calling for studies on toxicity of nanoparticles [13,14,78,143] that are already abundantly used in consumer areas that do not require rigorous toxicity assessments, such as cosmetics [144]. Complex issues of regulatory legislature is highly dependent upon the availability of relevant toxicity studies [80]. As the demand for nanotoxicity assessment increases, clinical relevance and mechanistic approaches should be prioritized [14,79,136]. The coalescence of bioengineering, biomedical and toxicology disciplines will foster development of relevant strategies to engineer advanced magnetic nanoparticle formulations and nanodevices with biocompatible interfaces.

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Figure 1. Examples of MNPs of different size, composition and surface chemistry

Transmission electron microscopy (TEM) of **A**, maghemite (Fe₂O₃) nanoparticles coated with anionic DMSA forming ~ 8-nm-thick round-shaped AMNPs. **B**, magnetite (Fe₃O₄) of ~8-nm-think core coated with ~2-nm-thick gold (Au), forming core-shell nanoparticles. **C**, magnification x 50 of B. (**D**). Silica coated Fe₃O₄ nanoparticles of ~ 100 nm, synthesized by sol gel method.



Figure 2. Anionic DMSA-coated MNPs promote macrophage infiltration in nerve

Plastic-embedded transverse rat sciatic nerve sections after intrafascicular injection of DMSAcoated MNPs (B) or DMSA (A). Stained with Methylene blue Azure II. **A.** Uniform axonal morphology typical of uninjured nerve and no evidence of infiltrating immune cells is seen after DMSA (control coating) injection. **B.** Increased incidence of infiltrating macrophages (yellow arrows), particularly in the perivascular spaces, is noted after DMSA-coated MNP injection. Note pixilated MNPs phagocytosed within macrophages and axonal myelin splitting (red asterisks) at the interface with MNP-filled macrophages. Objective magnification x 100 (scale bar = $25 \mu m$). *Legend: a, myelinated axon; v, vessel; red asterisks, myelin splitting; yellow arrow, infiltrating macrophages*.



Figure 3. A proposed mechanism of MNP-induced macrophage recruitment into neuronal tissues (1) Exposure to cytotoxic MNPs stimulated the formation of ROS in resident cells. (2) ROS promotes expression and release of proinflammatory cytokines, such as TNF- α . Through its two receptors (TNFR), TNF- α activates p38 and ERK mitogen-activated protein kinases pathways to (3) induce the expression of matrix metalloproteinases (MMPs) in its inactive, pro-MMP form. In addition, (4) ROS can directly promote MMP activation from pro-form. MMPs are the only enzymes in the body capable of degrading blood-brain and blood-nerve barriers (BBB/BNB), which (5) promotes infiltration of circulating macrophages (m Φ) into neuronal tissues. MNP size and surface chemistry determines the mechanisms and the target cells of MNP internalization, as well as extent of neurotoxicity of MNPs.

Key abbreviations used in the article

AMNP	anionic (DMSA-coated) MNP
Au-MNP	gold-coated MNP
BBB	blood-brain barrier
BNB	blood-nerve barrier
BrdU	5-bromo-2-deoxyuridine
CNS	central nervous system
DMSA	dimercaptosuccinic acid
HO-1	heme-oxygenase 1
i.v.	intravenous injection
MMP	matrix metalloproteinase
MNP	magnetic nanoparticles
NP	nanoparticle
OS	oxidative stress
RES	reticuloendothelial system
ROS	reactive oxygen species
SQUID	superconducting quantum interference device
TEM	transmission electron microscopy
TNF-α	tumor necrosis factor alpha

Table 1