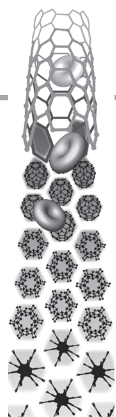

Assessing iron oxide nanoparticle toxicity *in vitro*: current status and future prospects

The *in vitro* labeling of stem or therapeutic cells with engineered nanoparticles with the aim of transplanting these cells into live animals and, for example, noninvasively monitoring their migration is a hot topic in nanomedicine research. It is of crucial importance that cell–nanoparticle interactions are studied in depth in order to exclude any negative effects of the cell labeling procedure. To date, many disparate results can be found in literature regarding nanoparticle toxicity due to the great versatility in different parameters investigated. In the present work, an overview is presented of different types of nanomaterials, focusing mostly on iron oxide nanoparticles, developed for biomedical research. The difficulties in assessing nanoparticle-mediated toxicity are discussed, an overview of some of the problems encountered using commercial (dextran-coated) iron oxide nanoparticles is presented, several key parameters are highlighted and novel methods suggested – emphasizing the importance of intracellular nanoparticle degradation and linking toxicity data to functional (i.e., cell-associated) nanoparticle levels, which could help to advance any progress in this highly important research topic.

KEYWORDS: **biomedical materials** ▪ **cell labeling** ▪ **cytotoxicity** ▪ **iron oxide nanoparticle** ▪ **magnetoliposome**

Stefaan JH Soenen[1,2] & Marcel De Cuyper[1†]

[1]Interdisciplinary Research Centre, Laboratory of BioNanoColloids, K.U. Leuven – Campus Kortrijk, E. Sabbelaan 53, B-8500 Kortrijk, Belgium
[2]Faculty of Pharmaceutical Sciences, Laboratory of General Biochemistry & Physical Pharmacy, University of Gent, Harelbekestraat 72, B-9000 Gent, Belgium
†Author for correspondence:
Tel.: +32 5624 6221
Fax: +32 5624 6997
Marcel.DeCuyper@kuleuven-kortrijk.be

In the past two decades, improvements in physical and material sciences have revolutionized the area of nanotechnology. A practical definition of nanotechnology without any direct size limitations has been proposed by Bawa *et al.* [1] as "the design, characterization, production and application of structures, devices and systems by controlled manipulations of size and shape at the nanometer scale (atomic, molecular and macromolecular scale) that produces structures, devices, and systems with at least one novel/superior characteristic or property". Nanotechnology has provided fascinating new tools that also boosted further knowledge and discoveries in biology and medicine [2–4]. The great developments in both fields were conjoined, where nanotechnological advances were applied for medical purposes, as such inaugurating a new scientific domain at the cutting-edge of science: 'nanomedicine'. Nanomedicine is generally defined as "the use of materials, of which at least one of their dimensions that affects their function is in the scale range of one to several hundreds of nanometers, for a specific diagnostic or therapeutic purpose" [5]. In the past decade, nanomedicine has developed into a well-established scientific field that encompasses numerous applications, such as:

- Controlling and modulating stem cell differentiation and proliferation by altering the topography of the culture dishes [6];
- Engineering materials to be used as scaffolds for artificial tissues or even whole organs [7];
- Coupling of nanodevices to the nervous system in order to restore vision and hearing [8];
- The development of new or the improvement of already existing multimodal nanoparticles (NPs) that can be used for biomedical imaging, drug or gene delivery, and cancer therapy [9].

Nanomaterials for cell labeling

Nanoparticle delivery systems can generally be classified in three different groups, based on their morphological and functional properties [10]:

- Nanocapsules: vesicular systems where compounds of interest are confined in a central cavity enclosed by a polymer or liposomal membrane;
- Nanospheres: matrix systems in which the compounds of interest are embedded within small cavities of the matrix and can even be uniformly dispersed;
- NPs: solid colloidal particles consisting of macromolecular substances of submicrometric sizes [11].

Depending on the preparation method, nanocapsules, nanospheres and NPs can be designed with tightly controlled properties to optimize

future medicine part of fsg

encapsulation and delivery, and to reduce cytotoxicity [10]. Among the many types of NPs used, quantum dots (QDs), carbon nanotubes (CNT), gold and iron oxide NPs are the most widely studied.

When nanomaterials reach ultrasmall dimensions (a few nanometers), the electronic, optical and mechanical properties will differ from the bulk material. For QDs, which are nearly spherical semiconductor nanocrystals composed of elements from periodic groups II–VI or III–V ranging in size between 2 and 10 nm, quantum confinement occurs and the emission wavelength of QDs following excitation is tunable by the size of the crystal [11]. Together with a high brightness and efficient multiplexing, these particles have gained a lot of interest as potential biomedical research tools [12].

Carbon nanotubes are a new form of carbon, which can configurationally be seen as single or multiple graphene sheets (single versus multiwalled CNTs) rolled into a tube with a diameter of several nanometers, but up to 1 mm in length [13]. For medical purposes the key advantages of CNTs are their potential to effectively cross biological barriers according to the nanosyringe model and their excellent conductivity, which enables electrical stimulation to neuronal pathways [14].

Nanometer-sized gold NPs are small compared with the wavelength of visible light, and when the light has a frequency close to that of the surface plasmon (the natural oscillation of an electron gas inside a given nanosphere), then the surface plasmon will absorb energy. As such, these particles can be used for biomedical purposes, especially in the field of cancer research for tumor detection and laser-induced therapy [15,16].

Author Proof

Iron oxide NPs

The typical magnetic characteristics of iron-containing NPs depend on the nature of the iron oxide core (e.g., magnetite: Fe_3O_4 or FeCoxOy) and the size thereof. A ferromagnetic material, such as iron oxide, consists of atoms with strong magnetic dipoles, where the individual magnetic moments tend to be coupled, leading to subdomain structures with a single magnetic moment (Weiss domain) [17]. In the absence of an external field, the individual Weiss domains of a material are ordered randomly, leading to zero net magnetization. When an external magnetic field is applied, the magnetic moments of the Weiss domains will gradually align along this field, leading to a single large magnetic domain with a high net magnetization. In contrast to both dia- and para-magnetic materials, removal of the magnetic field will not lead to a loss of magnetism, but the particles will still exhibit a remnant magnetisation as the large Weiss domains are still coupled along the original field. Although ferromagnetic materials generally exhibit among the highest magnetic susceptibilities, their remnant magnetisation often hinders their application in biomedicine. When ferromagnetic particles are decreased in size, into the nanometer-range, their individual diameters will become equal to or smaller than the diameter of Weiss domains. As such, these particles still exhibit high magnetic susceptibility, but as a single particle consists of only a single Weiss domain, no magnetic coupling of the domains can occur and there will be no remnant magnetisation [17]. These types of particles are termed superparamagnetic and are the most widely applied in biomedical research. In general, a distinction between two groups of these particles is made based on their diameter: ultrasmall superparamagnetic iron oxide particles (USPIOs) are smaller than 50 nm, superparamagnetic iron oxide particles (SPIOs) are larger than 50 nm [18]. In literature, however, this distinction is not always clearly made and the terms are not always correctly used.

The magnetic characteristics displayed by small iron oxide NPs have bestowed these particles with a wide range of biomedical applications as contrast agents for MRI [18], magnetically guided drug or gene delivery [19], magnetic hyperthermia [20] or combinations of multiple applications, both diagnostic and therapeutic (FIGURE 1) [21]. More information on these topics and an overview of the biomedical applications of a special type of iron oxide NP: the magnetoliposome (ML; i.e., 14 nm diameter iron oxide cores each individually coated by a [phospho]lipid bilayer [22]) can be found in elsewhere [23]. In order to render iron oxide nanocores usable for biomedical purposes, a suitable coating must be applied to stabilize the iron oxide cores in an aqueous environment by steric hindrance and/or electrostatic repulsion (see the next section for an overview of frequently used iron oxide formulations in biomedical research). The most widely applied type of iron oxide NPs are the dextran-coated iron oxide NPs, which are clinically approved as liver contrast agents for MRI and are also routinely used in biomedical research.

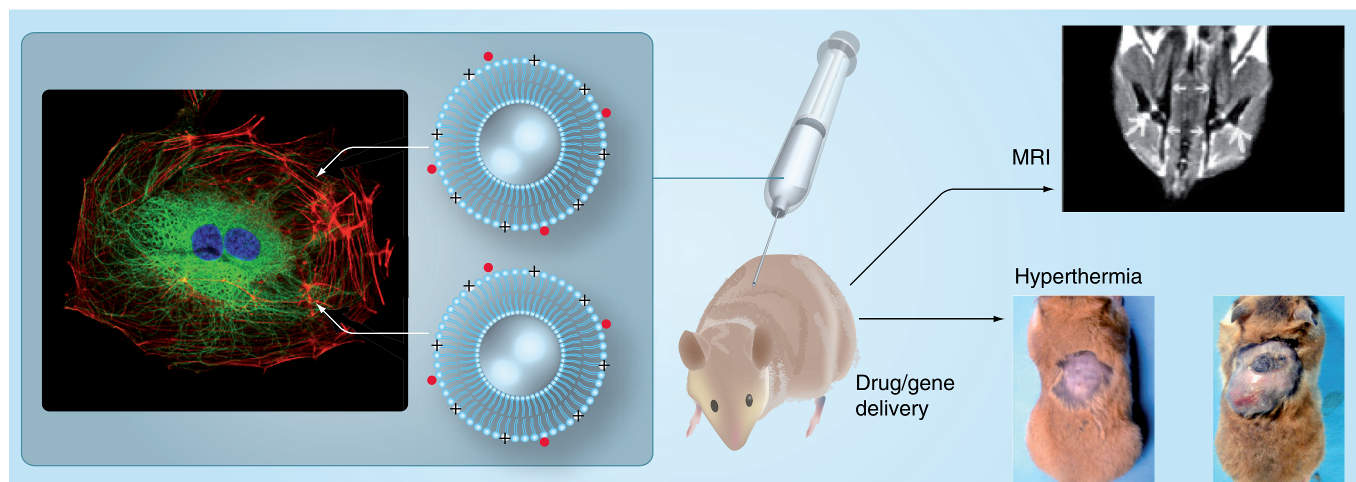

Figure 1. The use of magnetic nanoparticles in biomedical applications involving (*in vitro*) cell labeling. Cultured cells (indicated on the left) are human blood outgrowth endothelial cells stained for F-actin (red), α-tubulin (green) and 4',6-diamidino-2-phenylindole (blue), and can be labeled with specially designed magnetic nanoparticles, such as fluorescently tagged, cationic magnetoliposomes. These labeled cells can then be injected in an animal model and tracked by MRI, used for hyperthermia treatment or enhance gene or drug delivery by magnetic targeting.
MRI image reproduced with permission from [79].
Hyperthermia images reproduced with permission from [80]. See [101].

Different iron oxide particles for cell labeling

As indicated in Table 1, a great variety of different iron oxide NP formulations for biomedical research can be encountered in literature, varying in:

- Size of the iron oxide core
- Nature of the coating material
- Thickness of the coating
- The presence of any other imaging modalities or therapeutic compounds

Cell–NP interactions

The application of any of the particles previously mentioned in biomedical research automatically indicates that the particles will be exposed to biological cells, tissues and live animals. Therefore, it is of crucial importance that the effect(s) any of these particles may exert on biological entities is thoroughly characterized [24]. In view of this, the increase in the importance of nanomedicine was conjoined with the establishment of a new area of scientific research: nanotoxicology. The assessment of potential toxic effects of NPs has proven to be a difficult task and, so far, no straightforward answer has been found to the question of whether NPs are safe to be used in the clinic. Numerous studies can be found that, quite often, report on seemingly contradicting findings. A summary of several reports on *in vitro* toxicity of various iron oxide formulations can be found in Table 2, emphasizing some of the contradicting findings for identical iron oxide NPs (IONPs), which have been described in literature. A full overview of the described cytotoxic effects of IONPs can be retrieved in the review by Pisanic *et al.* [25]. The evaluation of toxic effects of NPs is being made difficult due to various reasons, which are discussed in here.

The many different compositions of particles, each with their specific features, can all alternatively affect cell physiology and exert cytotoxic effects that are specific for the type of NP used; for example, the intrinsic toxicity of heavy metals as present in most QDs (CdSe) [26]. In the study by Brunner *et al.* [27], it was observed that the toxic effects of IONPs were observed at concentrations that were approximately 40-times lower than the chemical toxicity of iron ions. Furthermore, it has been shown that the solubility of the particles is an important feature. Degradable NPs require solid testing for acute cytotoxic effects, whereas slowly degradable NPs should be assed more for their long-term effects [27]. For IONPs, this presents a particular problem as the presence of the coating will impede intracellular degradation but, generally, will not completely block it. This renders both short- and long-term cytotoxicity studies highly important where 'acute' effects, which generally only occur when free iron is present, may only be detectable at later time points, after intracellular degradation of the IONPs [28,29].

Table 1. Overview of commonly used and more recently developed iron oxide nanoparticle formulations.

Number	Coating	CD (nm)	Other functions	Common name	Ref.
1	Dextran	4[†]	–	Endorem	Guerbet SA, Villepinte, France
2	Lipid	14	Optional	ML	[81]
3	Carboxydextran	4[†]	–	Resovist	Bayer Schering Pharma AG, Berlin, Germany.
4	Citrate	4	FITC	VSOP	[70]
5	Carboxy-dendrimer	7–8	–	MD-100	[43]
6	Dimercapto-succinic acid	8	–	AMNP	[82]
7	Polystyrene/divinylbenzene	~1000	Dragon Green	MPIO	[83]
8	Bifunctional PEG–silane	5–10	–	–	[84]
9	Pullulan	13.6	–	–	[85]
10	Amine-functionalized PEG	5–8	Chlorotoxin Methotrexate	–	[86]
11	Silica	6.5	FITC	–	[87]
12	Lactose-functionalized silane	ND	–	–	[62]
13	Polyvinylpyrrolidone	6.9	–	–	[88]
14	Poly(acrylic acid)	8	NIR dye Taxol® Folate	–	[9]
15	Polystyrene	ND	Qdot800	–	[89]
16	PEG–gallol	9	Biotin Anti-VCAM-1 antibody	–	[51]
17	Gold	26	Adenovirus vector	GoldMaN	[90]

[†]These particles are beads (i.e., several nanometer range cores are embedded within a single matrix).
AMNP: Anionic magnetic nanoparticle; CD: Core diameter; FITC: Fluorescein isothiocyanate; ML: Magnetoliposome; MPIO: Micrometer-sized iron oxide; NIR: Near-infrared; ND: Not disclosed; PEG: Polyethylene glycol; VCAM: Vascular cell adhesion molecule; VSOP: Very small iron oxide paramagnetic particle.

The miniaturization of materials into the nanometer range (equivalent to the size of proteins) can induce novel toxic features that are absent in the bulk material, purely as an effect of the small size of the particles. For instance, gold, which is considered to be a safe and biocompatible inert material, has been found to be highly toxic when colloidal gold particles with diameters of only a few nanometers were used [30]. The miniaturization further leads to a high surface area for the NPs, which is a key parameter in nanotoxicology [31]. In the study by Soenen *et al.,* it was theoretically calculated that when IONPs of 250 and 10 nm diameter were taken up by cells, reaching identical intracellular iron levels, the respective surface area for the 10-nm particles was 25.025-times larger than for the 250-nm IONPs [32]. This increases the potential interaction of the NPs with cellular microstructures, and when present in degradative endosomes the greater surface will also increase the rate of IONP degradation.

The different synthesis protocols that are available for every type of particle can have an effect on the toxicity of these NPs; for example, cetyltrimethylammonium bromide, which is used as a surfactant to stabilize gold NPs in some syntheses, has been described to cause toxic effects [33]. In addition, the intrinsic toxicity of cationic lipids can also induce cellular toxicity when these molecules are used as coating agents [34,35]. The great importance of IONP surface characteristics on cytotoxicity was recently proven in the study by de la Fuente *et al.* [36]. It was shown that minute changes in the IONP coating consisting of maltose, lactose or glucose led to drastically different effects on the morphology of human fibroblasts and only the maltose-functionalized IONPs significantly decreased cell viability. These data were further confirmed in a recent study, showing that in IONPs coated with polyethylenoxide (PEO) triblock copolymers, the length of the PEO tail was critical in terms of toxic effects [37]. For human umbilical vein endothelial cells, retinal pigment epithelial cells and prostate cancer cells, the shortest (0.75 kDa) PEO tails were most toxic and the longest (15 kDa) PEO tails were the least toxic. Furthermore, the wide variety in NP properties, relating to surface charge and coating molecules, can all contribute to varying physicochemical characteristics, which in turn lead to alternate interactions with cellular components [38]. The combination of a nanocore with coating molecules leads to a new entity, which displays features that are not intrinsic for either the core or the coating molecules by themselves; for example, dimercaptosuccinic acid stabilized iron oxide

Table 2. An overview of several historically important and some very recent toxicology-related findings for bare, dimercaptosuccinic acid-coated and carboxy-dextran-coated iron oxide nanoparticles.

Cell type	Important findings	Ref.
Bare nanoparticle		
Human dermal fibroblasts	Cell morphology affected at 48 h post-NP uptake	[91]
Human hepatoma and lung adenocarcinoma cell lines	No effects on cell viability could be noted up to 100 µg Fe/ml	[48]
A549 human alveolar type II-like cell line	Only very low toxic effects were noted in terms of cell viability, DNA or mitochondrial damage. No differences between nano- or micro-meter sized particles	[92]
Human mesothelioma, rat fibroblasts	Rodent fibroblasts were relatively insensitive to IONPs whereas human meosthelioma showed greatly reduced viability	[27]
Murine alveolar macrophage, human macrophage and epithelial cell lines	Significant reductions in cell viability for all three cell types tested. Results were comparable with those observed when exposing cells to asbestos	[66]
Human aortic endothelial cells	No induction of inflammatory responses at concentrations up to 50 µg Fe/ml	[68]
DMSA-coated particles		
14 different cell lines, including adult, progenitor, immune and tumor cells	High uptake of DMSA-coated particles without any effects on cell viability and functionality. Endothelial progenitor cell migration and tube formation is unaffected upon labeling	[52]
Human fibroblasts	Cell viability and mitochondrial activity were decreased at higher concentrations	[93]
Human melanoma	No effects on cell viability and morphology, in contrast to citrate or lauric acid-coated particles	[65]
Human cervical carcinoma cell line	Low uptake but no cytotoxic effects in contrast to heparin-coated particles, which induced abnormal mitotic spindle formation	[94]
Rat pheochromocytoma cell line	Dose-dependent reduction in cell viability, cell adhesion and NGF-induced neurite outgrowth	[39]
(Carboxy)dextran-coated particles		
Human dermal fibroblasts	48 h post-IONP uptake, cell morphology, viability, proliferation and migration were decreased to a greater extent than uncoated IONPs	[91]
Rat skeletal myoblasts	Induction of free radicals, decreased cell proliferation and cell viability	[95]
Human mesenchymal stem cells	Endorem with poly-L-lysine did not affect cell viability, proliferation, osteogenic or adipogenic differentiation but impeded chondrogenic differentiation	[53]
Swine endothelial progenitor cells	Viability and proliferation of ferucarbotran-labeled cells was not affected, but adhesion capacity was increased and migration impeded in a dose-dependent manner	[96]
Human blood outgrowth endothelial cells, murine C17.2 neural progenitor cells	High intracellular concentrations of ferucarbotran, ferumoxides or lipid-coated IONPs impeded cell proliferation, affected cell spreading, focal adhesion maturation and focal adhesion kinase signaling	[60]
Murine C17.2 neural progenitor cells, rat pheochromocytoma cells	Ferucarbotran and ferumoxide-labeled cells displayed intracellular degradation of the iron oxide core, resulting in induction of free radicals, increased expression of transferrin receptor 1, and impeded NGF-induced neurite outgrowth. Effects were most outspoken for citrate-coated particles	[28]
Human mesenchymal stem cells	Ferucarbotran did not affect cell viability, induce free radicals or affect mitochondrial membrane potential and did not impede differentiation. At later time points, lysosomal degradation was evident	[97]
Rat mesenchymal stem cells	Ferucarbotran without transfection agent results in an increased expression of transferrin receptor 1	[58]
Human mesenchymal stem cells	Ferucarbotran possesses intrinsic peroxidase-like activity and diminishes intracellular reactive oxygen species. Upon degradation, free iron stimulates cell growth and accelerates cell cycle progression	[59]
Human mesenchymal stem cells	Ferucarbotran inhibits osteogenic differentiation in a dose-dependent manner and activates cellular signaling molecules. All effects were the result of free iron as they could be impeded by the use of the iron chelator desferrioxamine	[29]

DMSA: Dimercaptosucccinic acid ; IONP: Iron oxide nanoparticle; NGF: Nerve growth factor; NP: Nanoparticle.

Author Proof

nanocores lead to high toxic effects at concentrations where neither dimercaptosuccinic acid or iron oxide alone displayed any toxicity [39].

Investigation of toxic effects usually occurs in a limited number of cell types per study, which can lead to great variances in observed toxicity of a certain particle as different cell types will interact with the same particle in different ways [40]. The latter was also evidenced in the study by Brunner *et al.* [27] who found that the viability of human mesothelioma was greatly affected by exposure to bare IONPs, whereas rat fibroblasts did not show any clear effects. The authors postulated that this may be due to the higher metabolic activity of the mesothelioma, which more avidly took up the IONPs. The conditions of incubation described in literature also vary greatly, having incubation times from a few hours to approximately 1 week [41,42] and NP concentrations varying between a few µg to a few mg per ml incubation medium [41–43]. In the study by Díaz *et al.* [44] where a wide variety of cell types, including lymphoid, myeloid and epithelia cell lines and peripheral blood-derived cells, were exposed to different IONPs and other particles, the effects on cell viability and induction of reactive oxygen species (ROS) was influenced by the number of cells tested and, resulting from this, the number of particles per cell may influence the outcome of common assays. Furthermore, the presence or absence of additional factors, such as high concentrations of serum, supplemented transfection agent, or differences in cell culture conditions, such as 2D or 3D culture models [45].

For animal studies, toxic effects of NPs can also be highly dependent on their site of accumulation; for example, CNT, which were nontoxic after intravenous administration, display asbestos-like toxicity when present in lung epithelium [46,47].

Assessing NP toxicity frequently occurs using commonly used toxicity assays, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or lactate dehydrogenase (LDH) assays. These assays are used in drug studies, but can lead to aberrant results when using NPs as these can sometimes interfere with the assay components or the readout [32]. Some particles show absorbance at the same wavelength of the analysis [44], which can be dealt with by centrifugation of the cell media and removal of the IONPs [48]. In addition, the induction of ROS can affect the cellular redox equilibrium state and hereby influence mitochondrial activity and, thus, alter the results of an MTT assay, for instance [35]. In the case of a LDH assay, release of LDH can be impeded by the presence of the IONPs, as the latter are prone to protein adsorption [32], and may thus bind LDH and impede its release into the extracellular medium. This can be verified by comparing the LDH-release of lysed cells in the absence and presence of the particles that should be identical in case no interference of the IONPs occurs [32]. Furthermore, NPs can lead to specific effects, for example alterations of actin cytoskeleton, which are not typical in drug studies, and therefore require new and adjusted methods for analysis [49,50].

Potential hazards of dextran-coated SPIOs for cell labeling

Dextran-coated iron oxide cores (Endorem, Feridex® in the USA) have been US FDA approved and are routinely used in clinical settings as liver contrast agents. For almost two decades, they have also been used in cell labeling studies, an application for which they were not designed and are intrinsically not optimally suited. Indeed, to date, there is a great lack of knowledge regarding the effects of these (U) SPIOs on cell viability and functionality, and several reports have stated that these particles can in fact exert drastic effects on the cell's well-being (TABLE 2).

Dextran itself is a large macromolecule that continually undergoes conformational changes and is even found to completely desorb from the particle surface. This results in a NP with noncontrollable physicochemical properties that hardens a controlled and similar presentation of the NPs to the cells [51]. The relative weak attraction of dextran towards iron oxide further drastically impairs an efficient functionalization of the dextran coat by chemical manipulations [18].

The particles themselves are very inefficiently internalized as low-molecular weight dextran is typically taken up by fluid phase-mediated endocytosis, which is a rather inefficient uptake process [52].

Increasing NP uptake can occur by complexing it with commonly used (cationic) transfection agents, although the latter molecules are described to elicit toxic effects and when associated with dextran-coated SPIOs leads to complexes with noncontrollable surface features, which have been reported to affect cellular functionality, for example impairing chondrogenic differentiation of mesenchymal stem cells [52,53]. It is still an issue of debate whether the dextran-coated particles, the transfection agent used or

Author Proof

the combination of the two are responsible for the reported effects as when these particles were combined with protamine sulphate, no inhibition of stem cell differentiation could be noted [54]. Alternatively, the different findings might be due to intrinsic problems related to stem cell differentiation and immunohistochemical detection (i.e., long time span, high rate of cell death and no quantitative data).

When the particles are taken up by the cells and reside in endosomal compartments, they have been described to affect the normal endocytic lifecycle, delaying the maturation of late endosomes into lysosomes [55]. When present in acidic organelles, it has further been found that the dextran coat is easily degraded, leaving only a naked iron oxide core in the degradative lysosomal environment, resulting in a rapid degradation of the whole nanocrystal, which is accompanied by the induction of reactive oxygen intermediates produced by the Fenton or Haber–Weiss reaction [56,57].

Upon metabolization of the iron oxide core, free iron can be shuttled out of the endocytic compartment into the normal cellular iron pool. These altered cytoplasmic iron concentrations can then affect cellular functionality (e.g., by altering the level of transferrin receptor expression) and can affect cellular proliferation capacity by altering the expression of cyclins and cyclin-dependent kinases [58,59].

Next to cell-related properties, dextran-coated particles were soon deemed as inefficient mediators for magnetic hyperthermia [41]. In order to get an equal heating effect of magnetic NPs, uniformly sized iron oxide cores are preferred. As Endorem® is built up of multiple 4 nm diameter iron oxide cores encapsulated in a dextran corona leading to an overall diameter of 70–150 nm, this leads to a high variety in iron oxide present in each individual particle cluster. All these data highlight the great need for a thorough analysis of cell–NP interactions in order to gain more insight regarding any possible negative effects of iron oxide NPs on cellular homeostasis and functionality. This information is crucial for optimizing any future biomedical research applications and to allow any applications to move from bench to clinic.

Key points for future *in vitro* assessment of NP safety

Intracellular NP concentration & toxicity

One important aspect that has not received adequate attention to date is the important link between toxic effects and the intracellular concentration of the NPs. Several studies have shown that high intracellular amounts of NPs can lead to drastic effects on, for example, cellular proliferation [60,61]. As with any study regarding the assessment of drug safety, the concentration range wherein these drugs are safe to use is of primordial importance. The same is true for any NP type, where for iron oxide NPs, which are used in cell labeling studies, the effective amount of particles is the amount actually internalized by the cell and/or specifically associated to the plasma membrane. However, to date, most studies link any cytotoxicity data to the concentration of particles present in the incubation medium [62–64]. Any such findings can therefore not be well interpreted, as the reduced toxicity of a novel type of iron oxide formulation might simply be due to a reduced internalization efficiency. As such, these particles, although nontoxic under the conditions used, might not lead to sufficient intracellular amounts to enable efficient MR contrast or hyperthermia cancer treatment. It is therefore of crucial importance to simultaneously study uptake efficiency and cytotoxicity of iron oxide NPs at the same time and to correlate any data obtained. Next to denoting the IONPs in terms of mass of Fe/cell, more insightful data could be retrieved by describing the toxic effects in terms of total surface areas of the internalized IONPs. Since smaller particles will have a much larger total surface area than the larger ones for an identical amount of iron, this will also increase the potential interactions with cellular microstructures [32]. For targeted IONPs, the density of surface ligands would also be of great interest as this will determine the number of interactions of the IONPs with cellular components [31]. Other factors that should be taken into account are the zeta potential of the particles in physiological medium, the hydrodynamic diameter, lipophilicity of the particle surface, the adsorption of any proteins and potential aggregation, as all these factors will further govern the degree of cell–IONP interactions.

Standardized protocols

To date, there is a great variety in protocols used for cell labeling, which hardens making any comparison between different studies. The differences relate to, but are not limited to, first, the use of a single cell type to be studied where the cell type under investigation can be any type of cell, ranging from human mesenchymal stem cells [53] to rodent neural cells [39], cancerous

Author Proof

cells [65] or immune cells [66]. As cell–NP interactions have been shown to be highly dependent on the specific cell type used [27], the application of multiple cell types, preferably both primary cells and established cell lines as well as normal cells and stem or progenitor cells, would be required to prove whether the particles are 'universally' safe. Cancer cells themselves display several specific features that are different from healthy cells and could potentially lead to aberrant results [67], and should therefore only be used in cases where cancer cells are the envisaged target. Second, the differences also relate to NP concentrations varying from less than 1 µg Fe/ml up to over several mg Fe/ml [50,68] and incubation times ranging from a couple of hours to nearly a week [41,42]. To this end, it would be optimal if several key parameters could be standardized, such as particle concentration and incubation times. Preferably, the use of multiple concentrations would be optimal as they would allow determination of the potential toxic dose of NPs [69]. As a potential useful concentration range, IONPs could be incubated at 0 (= control), 20, 50, 75, 100, 150, 200, 250, 300, 400 and 500 µg Fe/ml. Several of these can, of course, be omitted, but the closer the differences between the concentration ranges used, the more precise the toxic threshold level can be determined. For most IONPs, levels below 20 µg Fe/ml are generally not advisable as in that case, the overall internalization of the IONPs would be too low for most applications. Conversely, higher concentrations of above 200 µg Fe/ml are generally not necessary either for efficient internalization and, therefore, most studies employ concentrations of 50, 100 or 200 µg Fe/ml. Furthermore, multiple time points would allow full assessment of both the short- and long-term effects as toxicity effects can often be quite variable in time [34]. When investigating IONP toxicity, incubation times of 2, 4, 8 and 24 h would allow to assess any acute cytotoxic effect, by means of an MTT or an LDH assay. If desirable, the IONPs can also be incubated for longer time points, although 24–48 h is generally more than sufficient to reach adequate intracellular levels of IONPs [34,52]. To address secondary toxicity or long-term effects, the IONPs can be removed from the incubation medium and the cells can be kept in culture for several more days, which would allow to analyze cell proliferation, intracellular degradation of the IONPs, dilution of the particles and the expression of cell surface receptors. The preferred timeframe would depend on several factors, such as the cell doubling time and the stability of the IONPs for intracellular degradation, but generally the assessment of six to eight cell doublings and 1 week of further culture allows investigation of proliferation and intracellular IONP degradation, respectively. Furthermore, when novel particles are being tested, the inclusion of commonly used iron oxide formulations, such as Endorem, would be a highly valuable tool for comparison.

Use of generally applicable, specialized cell model systems

In order to test the effects of cell labeling on cellular homeostasis and functionality and to obtain results that can easily be compared between different research groups, a specialized model system, which is optimally suited to address several issues regarding NP-mediated toxicity, would be highly valuable. In this regard, Pisanic *et al.* proposed the use of the rat pheochromocytoma (PC12) cell line [25]. One of the key features of PC12 cells is their ability to rapidly respond to their putative biological cue NGF by the induction of neurite outgrowth and the establishment of intercellular contacts between neurites [39]. The rapid response (assays can be limited to 48 h) makes this cell type well suited to address cell functionality. This fast response contrasts with differentiation assays for commonly used cell types, which can take up to 14 days and typically yield a high level of apoptosis by itself, hardening the evaluation of the effect of NPs on toxicity and cellular function [32]. Pisanic *et al.* furthermore demonstrated that neurite outgrowth and the formation of intercellular contacts can easily be quantified, allowing a clear comparison between different studies [39]. Figure 2 shows an example of PC12 cells treated with Endorem and citrate-coated iron oxide particles, where it is shown that citrate-coated iron oxide particles reduce neurite outgrowth far more than Endorem at similar intracellular iron concentrations (Figures 2A, C & D). When the values are expressed in terms of identical concentrations in the incubation medium, the effect of citrate-coated iron oxide particles is greatly impeded (Figures 2A, C & D), due to the lower uptake efficiency of the latter particles. This again highlights the importance of intracellular NP concentrations in assessing toxicity. In terms of general applicability, the rapid response, which allows quantitative data on cell functionality to be obtained within 2 days, is a major benefit in comparison with common stem cells where differentiation assays can take

Author Proof

up to 3 weeks and typically yield high numbers of cell death by itself [32]. Furthermore, the small PC12 cells have been proven to be quite sensitive to IONPs, as toxic effects of citrate-coated IONPs were more pronounced in PC12 cells than in C17.2 neural progenitor cells, although the former cells contained much less iron [28]. A wide variety of IONPs, including DMSA-, citrate-, dextran-, carboxydextran- and lipid-coated IONPs have already been evaluated using this assay, indicating the broad range of IONP types, which can be evaluated. Despite all the advantages, the possibility still remains that some IONPs would not be toxic to PC12 cells, although they present high toxic effects to some other cell types. Therefore, it is of great importance to use multiple cell types, as suggested earlier. The main contribution of the PC12 cell model system would be to allow an efficient analysis of a few extra parameters, such as cell functionality and to serve as a first, sensitive model system to screen various IONPs in regard of their toxic effects.

Assessing intracellular stability of the NPs

One important aspect that has not been given much attention to date is the intracellular stability of the particles and their possible degradation when exposed to the lysosomal environment. Of crucial importance in this regard is the understanding of how the particles are endocytosed

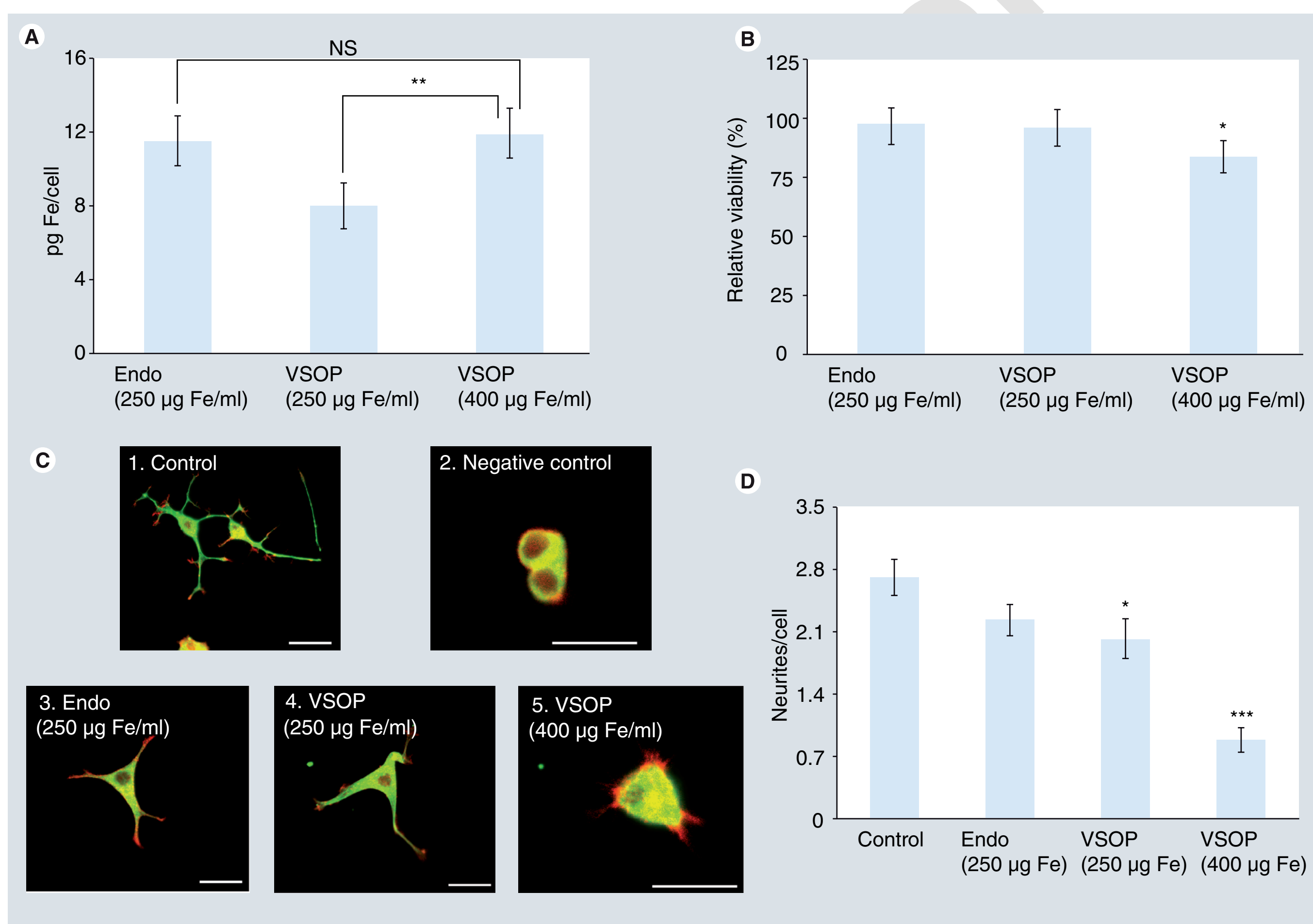

Figure 2. The effect of Endorem and very small iron oxide paramagnetic particle on the functionality of PC12 cells. PC12 cells were incubated for 24 h with Endo (250 µg Fe/ml) or VSOP (250 or 400 µg Fe/ml) and supplemented with 5 µl/ml Lipofectamine 2000 to improve cellular uptake. **(A)** Cellular iron concentrations determined as described previously [28] showing similar iron loads for Endorem at 250 µg Fe/ml and VSOP at 400 µg Fe/ml and **(B)** relative viability as assessed by an lactate dehydrogenase assay. **(C & D)** The induction of neurites when PC12 cells were exposed to 100 ng/ml of NGF for 48 h **(C1)**, in the absence of NGF **(C2)** or after labeling with Endorem at 250 µg Fe/ml **(C3)**, or VSOP at 250 or 400 µg Fe/ml (**C4**, **C5**, respectively). Cells were stained for F-actin (red) and α-tubulin (green) as described previously [28]; scale bars: 25 µm. **(D)** Quantitative data on the number of neurites per cell for control cells and cells incubated with Endorem or VSOP at 250 µg Fe/ml and VSOP at 400 µg Fe/ml. For every condition, 172 cells were considered to allow statistical analysis. Data are expressed as mean ± standard error of the mean. When appropriate, the level of significance is indicated (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).
Endo: Endorem; VSOP: Very small iron oxide paramagnetic particle.

and, in a second step, where they finally reside after uptake and intracellular routing. Typically, the particles end up in lysosomes and as a result of the degradative environment they can be completely degraded [56]. This will lead to the generation of free ferric ions, which due to the Fenton reaction or Haber–Weiss reactions, induces ROS, which can be detected by nitroblue tetrazolium salt or 2′,7′-dichlorodihydrofluorescein diacetate [35]. Furthermore, free ferric ions can bind to low molecular weight molecules, such as citric acid, and be shuttled out of the endosomes into the labile iron pool of the cytoplasm. This can then lead to an altered expression of the transferrin receptor [58] or influence cell cycle progression [59]. These effects can be tested using flow cytometry analysis of cell surface receptors and by monitoring cell cycle progression by means of cell counting or using cell cycle-specific stainings, such as 5-bromo-2-deoxyuridine or 5-ethynyl-2′-deoxyuridine. Although the induction of ROS by IONPs has been described for various types of IONPs, including dextran- or citrate-coated ones [28,70], the intracellular degradation of the IONPs and the possible effect thereof on cell homeostasis has not been adequately dealt with thus far. In 2005, Arbab *et al.* [55] displayed clear evidence for the degradation of Endorem particles located in acidic endosomes of mesenchymal stem cells. Idee *et al.* [56] followed up on that study, demonstrating that within 1 week, Sinerem (dextran-coated USPIOs) particles were completely degraded in the acidic compartments of macrophages. Recently, Huang *et al.* [59] demonstrated that intracellular degradation of ferucarbotran (carboxydextran-coated IONPs) altered the cytoplasmic labile iron pool and thereby affected the cell cycle progression of human mesenchymal stem cells, a process which is known to be sensitive to free iron. Chen *et al.* [29] also used ferucarbotran to label mesenchymal stem cells, finding a dose-dependent inhibition of osteogenic differentiation and altered cell migration. As all effects could be suppressed by using desferrioxamine, an iron chelator, this indicated that free iron that was generated upon IONP degradation was responsible for these effects. Various IONPs (citrate-, dextran-, carboxydextran- and lipid-coated ones) were recently compared in terms of their intracellular stability [28]. It was found that citrate-coated particles were most prone to degradation, followed by carboxydextran- and dextran-coated IONPs, and lipid-coated IONPs being the most stable ones. The degradation of the IONPs resulted in acute cytotoxicity, induction of ROS (where citrate-coated ones led to a higher maximal ROS level and a faster induction of ROS) and inhibition of PC12 neurite outgrowth when stimulated with NGF.

Next to the potential adverse effect on cellular homeostasis and functionality, the degradation of the iron oxide particles will also restrict their use in biomedical applications. Arbab *et al.* [55] set up a lysosomal model system to evaluate the effect of the pH *in vitro*, demonstrating that when Endorem particles were exposed to a lower pH (4.5), this resulted in their degradation, and concomitantly significantly decreased their effect on transversal relaxation, making them less effective T_2 contrast agents in MRI. Making use of this model system, the resistance of various particles to pH-dependent degradation can be easily tested and quantified. These tests should always be carried out together with cell-based assays, such as determination of ROS levels, transferrin receptor expression and cell cycle progression.

Effect of long-lived particles on cell homeostasis

In line with the previous topic concerning the intracellular fate of the particles, the long-term effects of internalized NPs are poorly understood and only rarely investigated. *In vivo*, NPs are generally removed out of the biological system by renal or hepatic clearance [71], but in cultured cells, the fate of the ingested NPs is rather unclear. In general, NPs can either be actively exocytosed [72], completely degraded [56] or they will remain in the cells 'indefinitely'. The latter proposition has to be taken into consideration whilst also considering that the cells which have taken up the NPs will eventually die and release the NPs, or that by continued cell divisions the number of NPs per cell will eventually dilute to near zero. In this respect, slow-dividing cells or cells with a limited lifespan in number of cell divisions, such as many stem cell types used for therapy, will keep high doses of ingested NPs for a long time. The effect thereof on cell metabolism and homeostasis should be carefully analyzed by comparing labeled and unlabeled cells for the whole of their lifespan or until the particles have diluted to nondetectable levels. In terms of NP synthesis, the diameter of the iron oxide cores and the coating that is used to stabilize the particles will play an important role [73]. For example, the effect of the coating is twofold, where initially the coating will influence the stability of the IONPs in physiologically relevant media and thus govern the degree of possible aggregation that may

Author Proof

occur [44]. For example, in the study by Diaz *et al.* [44] Polyethylene glycol (PEG)ylated silica-encapsulated IONPs were found to be stable in 10% fetal calf serum-containing medium, whereas non-PEGylated, graphite encapsulated or aluminosilicate encapsulated IONPs displayed extensive aggregation. The availability of particles to be internalized by cells also seems to strongly depend on the size and morphology of the aggregates [44]. Due to a decrease of available particles to interact with cellular microstructures, aggregation was also described to reduce any cytotoxicity [74]. Next to stabilization in physiological media, the coating will also determine the intensity of interactions with cellular structures and will also determine the degradability of the NPs. This has been shown in a recent study by Soenen *et al.* [28] where it was observed that citrate-coated IONPs were much more prone to pH-dependent degradation than dextran or lipid-coated ones. In line with this, the citrate-coated particles also induced the highest level of ROS, the greatest increase in transferrin receptor 1 upregulation and impeded cell functionality most gravely when all particles were incubated under conditions leading to similar intracellular iron concentrations. From these data, it would appear that lipid coatings or nondegradable polymers would be best suited as an IONP coating for a particle with a total diameter of approximately 30–80 nm. The greater diameter reduces cell surface area and possible degradation, whereas the size must be kept quite low to allow sufficient cellular internalization [38].

Several important aspects regarding common toxicity assays

The evaluation of NP toxicity usually occurs by means of common toxicity assays, such as MTT, LDH and calcein acetoxymethyl ester assays. These tests were originally developed for testing drug-related toxicity effects and may not be well suited to address NP-mediated toxicity assays [75]. The MTT assay, for example, has been described to lead to aberrant results due to the presence of free amine groups on the NP surface [76] or in case high levels of ROS were generated that disturbed the natural redox equilibrium state of the cell [35]. The LDH assay can be influenced by potential binding of lactate dehydrogenase to the NPs, impeding its release into the extracellular medium [77]. Interpretation of calcein stainings can be rendered impossible when high levels of NPs are used, especially when they are prone to aggregation, as in the case of Resovist® combined with transfection agents [58], as they will shield any excitation or emission light. To address these issues, appropriate controls are required, for example by comparing the deliberate lysis of NP-treated cells and untreated cells in terms of LDH release. Furthermore, as all these tests generally determine cytotoxicity in terms of a single parameter (plasma membrane permeability, cellular esterase or mitochondrial activity) it would be highly recommended to use multiple assays [44,50]. This would at the same time enable to more specifically investigate the cause of toxicity, and the potential interference of the NPs with a single assay would become more readily detectable. Furthermore, a more broad spectrum of potential interferences of NPs with cellular components should be investigated, even if any effects appear to be rather unlikely. For example, Mahmoudi *et al.* [78] studied the effect of bare and polyvinylalcohol-coated IONPs on murine fibroblasts and observed that bare IONPs did not affect cell viability, whereas polyvinylalcohol-coated ones decreased cell viability at higher concentrations (400 mM Fe). It was found that the coated particles induced apoptosis and cell cycle arrest in G1 phase, which was possibly due to irreversible DNA damage and repair of oxidative DNA lesions. In the paper by Pisanic *et al.* [39] it was observed that the uptake of higher levels of DMSA-coated IONPs by PC12 cells impeded the expression of growth-associated protein-43, which was hypothetically linked to the high levels of IONP-containing endosomes in the proximal perinuclear region, which may impede transcriptional regulation and protein synthesis. To assess the possible effects of IONPs on DNA damage or altered gene expression levels, a gene toxicity panel could be conducted, including several assays, such as the AMES test which evaluates the mutagenic potential of compounds, a chromosome aberration test which assesses the occurrence of double DNA strand breaks, the Comet assay which analyzes double or single DNA strand breaks or measuring unscheduled DNA synthesis (DNA synthesis outside the S phase of the cell cycle in an attempt to repair the damaged DNA).

Another interesting aspect which to date, has received too little attention is the effect of an applied magnetic field on cell homeostasis. For an alternating magnetic field, the heating effect that accompanies it has been well-studied and is exploited in cancer hyperthermia [20]. Alternatively, exposure to a strong constant

Author Proof

magnetic field (1.5 or 3 T), such as those used in clinical MR scanners, could also influence biochemical processes. It would be interesting to see whether any labeled cells exposed to such field display any aberrant behavior to unlabeled cells which are also exposed to the magnetic field, or controls cells, which are not exposed.

Conclusion & future perspective

In this perspective, several assays are proposed that might aid researchers in determining the suitability of iron oxide formulations for biomedical applications. To date, the great variety in types of NPs, cells and incubation protocols have rendered it impossible to make any conclusions regarding the safety of iron oxide NPs for cell labeling. In order to enable any progress in this field, there is a great need for more standardized protocols, which would allow comparison of results obtained by different groups. Several recent studies, of which some are highlighted in the present perspective, have put forward suitable model systems to address these important issues and have dealt with the possible complications involved in assessing the safety of iron oxide NPs. Furthermore, next to the standard viability tests, other parameters need to be evaluated as well, such as the intracellular stability of the NPs, the potential degradation and the effect this has on cell functionality and the usefulness of the applied particles for long-term biomedical applications. For future studies, the use of standardized protocols where IONP toxicity is investigated in relation to their intracellular levels and where intracellular stability of the particles is investigated could greatly advance any progress in this field. In this regard, there is a great need for a multidisciplinary approach, where material scientists and cell biologists have to collaborate and think together in order to fully assess all aspects involved in the broad field of cell–NP interactions. Only then will the tremendous effort that has been put into developing and optimizing iron oxide formulations specifically destined to be used in biomedical research pay off as more applications will become possible once the interactions of these particles with biological systems have been carefully analyzed. These studies would also improve the transition of any biomedical application involving iron oxide NPs from the scientist's bench to a clinical setting.

Financial & competing interests disclosure

Stefaan JH Soenen is a post-doctoral fellow from the FWO-Vlaanderen. MDC is a recipient of an IWT grant (StrategischBasisOnderzoek), sponsoring project nr 80017 entitled Integrated magnetic nanoparticle-enabled imaging of therapeutic cells - 'iMAGiNe'. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Iron oxide nanoparticles

- Over the past 25 years, magnetic iron oxide nanoparticles (NPs) have been widely used for biomedical applications, as MRI contrast agents, vectors for magnetic hyperthermia cancer treatment or as tools to enhance drug or gene delivery. Multiple studies, particularly *in vitro*, have recently indicated several potential adverse effects of these particles upon cell labeling.

Cell–NP interactions

- Biomedical applications of iron oxide NPs requires a detailed analysis of cell–NP interactions to assess whether the particles are completely free of hazards.
- To date, many disparate results have been reported due to the great variety in types of particles, cells and incubation protocols, hampering any efficient analysis.

Described adverse effects of dextran-coated iron oxide NPs

- Dextran-coated iron oxide cores have been the most widely used iron oxide NPs in biomedical research and have historically been considered biocompatible and nontoxic, but several recent studies have called this claim into question, emphasizing the importance of in-depth cell–NP interaction studies.

Future assessment of NP safety

- To more efficiently assess the safety of iron oxide NPs, several changes can be made, which could already significantly improve our understanding of all aspects involved in cell labeling. Several key aspects highlighted in the present article are:
  - Linking toxicity data to functional (intracellular) NP concentrations.
  - The need for standardized protocols for cell labeling and the use of reference material, such as dextran-coated particles.
  - The use of a single-cell model system to efficiently compare the effects of various iron oxide formulations.
  - Assessing the intracellular fate of the particles, related to potential degradation and functionality of the particle itself and of the labeled cells.
  - A more cautious and in-depth analysis of cell–NP interactions, combining several assays to study as many features as possible.

Bibliography

Papers of special note have been highlighted as:
▪ of interest
▪▪ of considerable interest

1 Bawa R: Patents and nanomedicine. *Nanomedicine* 2, 351–374 (2007).

2 Karoutsos V: Scanning probe microscopy: instrumentation and applications on thin films and magnetic multilayers. *J. Nanosci. Nanotechnol.* 9, 6783–6798 (2009).

3 Andrews RJ: Nanotechnology and neurosurgery. *J. Nanosci. Nanotechnol.* 9, 5008–5013 (2009).

4 Sosnik A, Carcaboso AM, Glisoni RJ, Moretton MA, Chiappetta DA: New old challenges in tuberculosis: potentially effective nanotechnologies in drug delivery. *Adv. Drug Deliv. Rev.* 62, 547–559 (2010).

5 Kostarelos K: The emergence of nanomedicine: a field in the making. *Nanomedicine* 1, 1–3 (2006).

6 Lipski AM, Pino CJ, Haselton FR, Chen IW, Shastri VP: The effect of silica nanoparticle-modified surfaces on cell morphology, cytoskeletal organization and function. *Biomaterials* 29, 3836–3846 (2008).

7 Bueno EM, Glowacki J: Cell-free and cell-based approaches for bone regeneration. *Nature Rev. Rheumatol.* 5, 685–697 (2009).

8 Kotov NA, Winter JA, Clements IP *et al.*: Nanomaterials for neural interfaces. *Adv. Mater.* 21, 3970–4004 (2009).

9 Santra S, Kaittanis C, Grimm J, Perez JM: Drug/dye-loaded, multifunctional iron oxide nanoparticles for combined targeted cancer therapy and dual optical/magnetic resonance imaging. *Small* 5, 1862–1868 (2009).

10 Singh R, Lillard JW Jr: Nanoparticle-based targeted drug delivery. *Exp. Mol. Pathol.* 86, 215–223 (2009).

11 Xing Y, Xia Z, Rao J: Semiconductor quantum dots for biosensing and *in vivo* imaging. *IEEE Trans. Nanobioscience* 8, 4–12 (2009).

12 Bruchez M Jr, Moronne M, Gin P, Weiss S, Alivisatos AP: Semiconductor nanocrystals as fluorescent biological labels. *Science* 281, 2013–2016 (1998).

13 Kostarelos K, Lacerda L, Pastorin G *et al.*: Cellular uptake of functionalized carbon nanotubes is independent of functional group and cell type. *Nat. Nanotechnol.* 2, 108–113 (2007).

14 Gaillard C, Cellot G, Li S *et al.*: Carbon nanotubes carrying cell-adhesion peptides do not interfere with neuronal functionality. *Adv. Mater.* 21, 2903–2908 (2009).

15 Qian X, Peng X-H, Ansari DO *et al.*: *In vivo* tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags. *Nat. Biotechnol.* 26, 83–90 (2008).

16 Troutman TS, Leung SJ, Romanowski M: Light-induced content release from plasmon-resonant liposomes. *Adv. Mater.* 21, 2334–2338 (2009).

17 Gupta AK, Gupta M: Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* 26, 3995–4021 (2005).

▪▪ **A great in-depth review emphasizing the importance of surface topology and physicochemical characteristics of iron oxide nanoparticles and the influence of these factors on use of these particles in biomedical applications.**

18 Mornet S, Vasseur S, Grasset F, Duguet E: Magnetic nanoparticle design for medical diagnosis and therapy. *J. Mater. Chem.* 14, 2161–2175 (2004).

19 Mykhaylyk O, Zelphati O, Hammerschmid E, Anton M, Rosenecker J, Plank C: Recent advances in magnetofection and its potential to deliver siRNAs *in vitro*. *Methods Mol. Biol.* 487, 111–146 (2009).

20 Gazeau F, Lévy M, Wilhelm C: Optimizing magnetic nanoparticle design for nanothermotherapy. *Nanomedicine* 3, 831–844 (2008).

21 Weinstein JS, Varallyay CG, Dosa E *et al.*: Superparamagnetic iron oxide nanoparticles: diagnostic magnetic resonance imaging and potential therapeutic applications in neurooncology and central nervous system inflammatory pathologies, a review. *J. Cereb. Blood Flow Metab.* 30, 15–35 (2010).

22 De Cuyper M, Soenen SJ: Cationic magnetoliposomes. *Methods Mol. Biol.* 605, 97–111 (2009).

23 Soenen SJ, Hodenius M, De Cuyper M: Magnetoliposomes: versatile innovative nanocolloids for use in biotechnology and biomedicine. *Nanomedicine* 4, 177–191 (2009).

24 Kunzmann A, Andersson B, Thurnherr T, Krug H, Scheynius A, Fadeel B: Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation. *Biochim. Biophys. Acta* DOI 10.1016/j.bbagen.2010.04.007 (2010) (Epub ahead of print).

25 Pisanic TR 2nd, Jin S, Shubayev VI: Iron oxide magnetic nanoparticle nanotoxicity: incidence and mechanisms. In: *Nanotoxicity*. Sahu S, Casciano D (Eds). Wiley-VCH, Weinheim, Germany, 397–425 (2009).

26 Tang M, Wang M, Xing T, Zeng J, Wang H, Ruan DY: Mechanisms of unmodified CdSe quantum dot-induced elevation of cytoplasmic calcium levels in primary cultures of rat hippocampal neurons. *Biomaterials* 29, 4383–4391 (2008).

27 Brunner TJ, Wick P, Manser P *et al.*: *In vitro* cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. *Environ. Sci. Technol.* 40, 4374–4381 (2006).

28 Soenen SJ, Himmelreich U, Nuytten N, Pisanic TR II, Ferrari A, De Cuyper M: Intracellular nanoparticle coating stability determines nanoparticle diagnostics efficacy and cell functionality. *Small* 6(19), 2136–2145 (2010).

29 Chen YC, Hsiao JK, Liu HM *et al.*: The inhibitory effect of superparamagnetic iron oxide nanoparticle (Ferucarbotran) on osteogenic differentiation and its signaling mechanism in human mesenchymal stem cells. *Toxicol. Appl. Pharmacol.* 245, 272–279 (2010).

30 Pan Y, Neuss S, Leifert A *et al.*: Size-dependent cytotoxicity of gold nanoparticles. *Small* 3, 1941–1949 (2007).

31 Verma A, Stellacci F: Effects of surface properties on nanoparticle–cell interactions. *Small* 6, 12–21 (2010).

32 Soenen SJ, De Cuyper M: Assessing cytotoxicity of (iron oxide-based) nanoparticles: an overview of different methods to exemplified with cationic magnetoliposomes. *Contrast Media Mol. Imaging* 4, 207–219 (2009).

33 Alkilany AM, Nagaria PK, Hexel CR, Shaw TJ, Murphy CJ, Wyatt MD: Cellular uptake and cytotoxicity of gold nanorods: molecular origin of cytotoxicity and surface effects. *Small* 5, 701–708 (2009).

34 Soenen SJ, Baert J, De Cuyper M: Optimal conditions for labelling of 3T3 fibroblasts with magnetoliposomes without affecting cellular viability. *Chembiochem* 8, 2067–2077 (2007).

35 Soenen SJ, Brisson AR, De Cuyper M: Addressing the problem of cationic lipid-mediated toxicity: the magnetoliposome model. *Biomaterials* 30, 3691–3701 (2009).

36 de la Fuente JM, Alcantara D, Penades S: Cell response to magnetic glyconanoparticles: does the carbohydrate matter? *IEEE Trans. Nanobiosci.* 6, 275–281 (2007).

37 Häfeli UO, Riffle JS, Harris-Shekhawat L *et al.*: Cell uptake and *in vitro* toxicity of magnetic nanoparticles suitable for drug delivery. *Mol. Pharm.* 6, 1417–1428 (2009).

38 Jiang W, Kim BY, Rutka JT, Chan WC: Nanoparticle-mediated cellular response is size-dependent. *Nat. Nanotechnol.* 3, 145–150 (2008).

39 Pisanic TR 2nd, Blackwell JD, Shubayev VI, Fiñones RR, Jin S: Nanotoxicity of iron oxide

Author Proof

nanoparticle internalization in growing neurons. *Biomaterials* 28, 2572–2581 (2007).

- **Good paper introducing the use of PC12 cells as a model system to study toxic effects of nanoparticles.**

40 Barua S, Rege K: Cancer-cell-phenotype-dependent differential intracellular trafficking of unconjugated quantum dots. *Small* 5, 370–376 (2009).

- **In-depth study showing clear differences in the intracellular routing and localization of quantum dots in three highly similar prostate cancer cell types.**

41 Jordan A, Scholz R, Wust P *et al.*: Endocytosis of dextran and silan-coated magnetite nanoparticles and the effect of intracellular hyperthermia on human mammary carcinoma cells *in vitro*. *J. Magn. Magn. Mater.* 194, 185–196 (1999).

42 Metz S, Bonaterra G, Rudelius M, Settles M, Rummeny EJ, Daldrup-Link HE: Capacity of human monocytes to phagocytose approved iron oxide MR contrast agents *in vitro*. *Eur. Radiol.* 14, 1851–1858 (2004).

43 Bulte JWM, Douglas T, Witwer B *et al.*: Magnetodendrimers allow endosomal magnetic labeling and *in vivo* tracking of stem cells. *Nat. Biotechnol.* 19, 1141–1147 (2001).

44 Diaz B, Sanchez-Espinel C, Arruebo M *et al.*: Assessing methods for blood cell cytotoxic responses to inorganic nanoparticles and nanoparticle aggregates. *Small* 4, 2025–2034 (2008).

45 Lee J, Lilly GD, Doty RC, Podsiadlo P, Kotov NA: *In vitro* toxicity testing of nanoparticles in 3D cell culture. *Small* 5, 1213–1221 (2009).

46 Schipper ML, Nakayama-Ratchford N, Davis CR *et al.*: A pilot toxicology study of single-walled carbon nanotubes in a small sample of mice. *Nat. Nanotechnol.* 3, 216–221 (2008).

47 Poland CA, Duffin R, Kinloch I *et al.*: Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenic behaviour in a pilot study. *Nat. Nanotechnol.* 3, 423–428 (2008).

48 Liu SY, Long L, Yuan Z, Yin LP, Liu R: Effect and intracellular uptake of pure magnetic Fe_3O_4 nanoparticles in the cells and organs of lung and liver. *Chin. Med. J. (Engl.)* 122, 1821–1825 (2009).

49 Pernodet N, Fang X, Sun Y *et al.*: Adverse effects of citrate/gold nanoparticles on human dermal fibroblasts. *Small* 2, 766–773 (2006).

50 Soenen SJ, Illyes E, Vercauteren D *et al.*: The role of nanoparticle concentration-dependent induction of cellular stress in the internalization of non-toxic cationic magnetoliposomes. *Biomaterials* 30, 6803–6813 (2009).

51 Amstad E, Zurcher S, Mashagi A, Wong YJ, Textor M, Reimhult E: Surface functionalization of single superparamagnetic iron oxide nanoparticles for targeted magnetic resonance imaging. *Small* 5, 1334–1342 (2009).

52 Wilhelm C, Gazeau F: Universal cell labeling with anionic magnetic nanoparticles. *Biomaterials* 29, 3161–3174 (2008).

53 Kostura L, Kraitchman DL, Mackay AM, Pittenger MF, Bulte JW: Feridex labeling of mesenchymal stem cells inhibits chondrogenesis but not adipogenesis or osteogenesis. *NMR Biomed*. 17, 513–517 (2004).

54 Arbab AS, Yocum GT, Rad AM *et al.*: Labeling of cells with ferumoxides-protamine sulfate complexes does not inhibit function or differentiation capacity of hematopoietic or mesenchymal stem cells. *NMR Biomed*. 18, 553–559 (2005).

55 Arbab A, Wilson L, Ashari P, Jordan E, Lewis B, Frank J: A model of lysosomal metabolism of dextran coated superparamagnetic iron oxide (SPIO) nanoparticles: implications for cellular magnetic resonance imaging. *NMR Biomed*. 18, 383–389 (2005).

56 Idee JM, Port M, Raynal I *et al.*: Superparamagnetic nanoparticles of iron oxides for magnetic resonance imaging applications. In: *Nanotechnologies for the Life Sciences* (*Volume 10*). Kumar CSSR (Ed.). Wiley-VCH, Weinheim, Germany, 51–84 (2007).

57 Arbab AS, Bashaw LA, Miller BR *et al.*: Characterization of biophysical and metabolic properties of cells labeled with superparamagnetic iron oxide nanoparticles and transfection agent for cellular MR imaging. *Radiology* 229, 838–846 (2003).

58 Schäfer R, Kehlbach R, Wiskirchen J *et al.*: Transferrin receptor upregulation: *in vitro* labeling of rat mesenchymal stem cells with superparamagnetic iron oxide. *Radiology* 244, 514–523 (2007).

59 Huang DM, Hsiao JK, Chen YC *et al.*: The promotion of human mesenchymal stem cell proliferation by superparamagnetic iron oxide nanoparticles. *Biomaterials* 30, 3645–3651 (2009).

60 Soenen SJ, Nuytten N, De Meyer SF, De Smedt SC, De Cuyper M: High intracellular iron oxide nanoparticle concentrations affect cellular cytoskeleton and focal adhesion kinase-mediated signaling. *Small* 6, 832–842 (2010).

- **Describes the effects of high intracellular nanoparticle concentrations on cell cytoskeleton and its associated signaling and its relation with cell proliferation.**

61 Hu F, Neoh K, Cen L, Kang E: Cellular response to magnetic nanoparticles PEGylated via surface-initiated atom transfer radical polymerization. *Biomacromolecules* 7, 809–816 (2006).

62 Huang G, Diakur J, Xu Z, Wiebe LI: Asialoglycoprotein receptor-targeted superparamagnetic iron oxide nanoparticles. *Int. J. Pharm*. 360, 197–203 (2008).

63 Mahmoudi M, Simchi A, Milani AS, Stroeve P: Cell toxicity of superparamagnetic iron oxide nanoparticles. *J. Colloid Interface Sci*. 336, 510–518 (2009).

64 Choi JY, Lee SH, Na HB, An K, Hyeon T, Seo TS: *In vitro* cytotoxicity screening of water-dispersible metal oxide nanoparticles in human cell lines. *Bioprocess. Biosyst. Eng.* 33, 21–30 (2010).

65 de Freitas ERL, Soares PRO, Santos RD *et al.*: *In vitro* biological activities of anionic γ-Fe_2O_3 nanoparticles on human melanoma cells. *J. Nanosci. Nanotechnol.* 8, 2385–2391 (2008).

66 Soto K, Garza KM, Murr LE: Cytotoxic effects of aggregated nanomaterials. *Acta Biomater.* 3, 351–358 (2007).

67 Delehanty JB, Mattoussi H, Medintz IL: Delivering quantum dots into cells: strategies, progress and remaining issues. *Anal. Bioanal. Chem*. 393, 1091–1105 (2009).

68 Kennedy IM, Wilson D, Barakat AI; HEI Health Review Committee: Uptake and inflammatory effects of nanoparticles in a human vascular endothelial cell line. *Res. Rep. Health Eff. Inst.* 136, 3–32 (2009).

69 Soenen SJ, Vercauteren D, Braeckmans K, Noppe W, De Smedt S, De Cuyper M: Stable long-term intracellular labelling with fluorescently tagged cationic magnetoliposomes. *Chembiochem* 10, 257–267 (2009).

70 Stroh A, Zimmer C, Gutzeit C *et al.*: Iron oxide particles for molecular magnetic resonance imaging cause transient oxidative stress in rat macrophages. *Free Radic. Biol. Med*. 36, 976–984 (2004).

71 Longmire M, Choyke PL, Kobayashi H: Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. *Nanomedicine* 3, 703–717 (2008).

72 Jin H, Heller DA, Strano MS: Single-particle tracking of endocytosis and exocytosis of single-walled carbon nanotubes in NIH-3T3 cells. *Nano Lett.* 8, 1577–1585 (2008).

73 Lin MM, Kim HH, Kim H, Dobson J, Kim do K: Surface activation and targeting strategies of superparamagnetic iron oxide nanoparticles in cancer-oriented diagnosis

Author Proof

and therapy. *Nanomedicine* 2010 5, 109–133 (2010).

74 Brown SC, Kamal M, Nasreen N *et al.*: Talc pleuradesis: a particulate analysis. *Adv. Powder Technol.* 18, 739–750 (2007).

75 Monteiro-Riviere NA, Inman AO, Zhang LW: Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. *Toxicol. Appl. Pharmacol.* 234, 222–235 (2009).

■■ **Very clear paper discussing the use and possible complications of a range of common toxicity assays in the evaluation of different nanoparticle types.**

76 Hoshino A, Fujioka K, Oku T *et al.*: Physicochemical properties and cellular toxicity of nanocrystal quantum dots depend on their surface modification. *Nano Lett.* 4, 2163–2169 (2004).

77 Sohn O-J, Kim C-K, Rhee JI: Immobilization of glucose oxidase and lactate dehydrogenase onto magnetic nanoparticles for bioprocess monitoring system. *Biotechnol. Bioprocess Eng.* 13, 716–723 (2008).

78 Mahmoudi M, Simchi A, Imani M: Cytotoxicity of uncoated and polyvinyl alcohol coated superparamagnetic iron oxide nanoparticles, *J. Phys. Chem. C* 113, 9573–9580 (2009).

79 Bulte JWM, De Cuyper M, Despres D, Frank JA: Short- vs. long-circulating magnetoliposomes as bone marrow-seeking MR contrast agents. *J. Magn. Reson. Imaging* 9, 329–335 (1999).

80 Matsuoka F, Shinkai M, Honda H, Kubo T, Sugita T, Kobayashi T: Hyperthermia using magnetite cationic liposomes for hamster osteosarcoma. *Biomagn. Res. Technol.* 2, 3 (2004).

81 De Cuyper M, Joniau M: Magnetoliposomes: formation and structural characterization. *Eur. Biophys. J.* 15, 311–319 (1988).

82 Wilhelm C, Billotey C, Roger J, Pons JN, Bacri JC, Gazeau F: Intracellular uptake of anionic superparamagnetic nanoparticles as a function of their surface coating. *Biomaterials* 24, 1001–1011 (2003).

■ **Good paper regarding the uptake of anionic maghemite particles where the particles were compared with dextran-coated ones and the influence of protein-absorption was also investigated.**

83 Shapiro EM, Skrtic S, Sharer K, Hill J, Dunbar C, Koretsky A: MRI detection of single particles for cellular imaging. *Proc. Natl Acad. Sci. USA* 101, 10901–10906 (2004).

84 Kohler N, Fryxell GE, Zhang M: A bifunctional poly(ethylene glycol) silane immobilized on metallic oxide-based nanoparticles for conjugation with cell targeting agents. *J. Am. Chem. Soc.* 126, 7206–7211 (2004).

85 Gupta AK, Gupta M: Cytotoxicity suppression and cellular uptake enhancement of surface modified magnetic nanoparticles. *Biomaterials* 26, 1565–1573 (2005).

86 Sun C, Fang C, Stephen Z *et al.*: Tumor-targeted drug delivery and MRI contrast enhancement by chlorotoxin-conjugated iron oxide nanoparticles. *Nanomedicine* 3, 495–505 (2008).

87 Heitsch AT, Smith DK, Patel RN, Ress D, Korgel BA: Multifunctional particles: magnetic nanocrystals and gold nanorods coated with fluorescent dye-doped silica shells. *J. Solid State Chem.* 181, 1590–1599 (2008).

88 Huang H, Xie Q, Kang M *et al.*: Labeling transplanted mice islet with polyvinylpyrrolidone coated superparamagnetic iron oxide nanoparticles for *in vivo* detection by magnetic resonance imaging. *Nanotechnol.* 20, 365603 (2009).

89 Shi D, Cho HS, Chen Y *et al.*: Fluorescent polystyrene-Fe_3O_4 composite nanospheres for *in vivo* imaging and hyperthermia. *Adv. Mater.* 21, 2170–2173 (2009).

90 Kamei K, Mukai Y, Kojima H *et al.*: Direct cell entry of gold/iron-oxide magnetic nanoparticles in adenovirus mediated gene delivery. *Biomaterials* 30, 1809–1814 (2009).

91 Berry CC, Wells S, Charles S, Aitchison G, Curtis AS: Cell response to dextran-derivatised iron oxide nanoparticles post internalization. *Biomaterials* 25, 5404–5413 (2004).

92 Karlsson HL, Gustafsson J, Cronholm P, Möller L: Size-dependent toxicity of metal oxide particles – a comparison between nano- and micrometer size. *Toxicol. Lett.* 188, 112–118 (2009).

93 Auffan M, Decome L, Rose J *et al.*: *In vitro* interactions between DMSA-coated maghemite nanoparticles and human fibroblasts: a physicochemical and cyto-genotoxical study. *Environ. Sci. Technol.* 40, 4367–4373 (2006).

94 Villanueva A, Cañete M, Roca AG *et al.*: The influence of surface functionalization on the enhanced internalization of magnetic nanoparticles in cancer cells. *Nanotechnology* 20, 115103 (2009).

95 van den Bos EJ, Wagner A, Mahrholdt H *et al.*: Improved efficacy of stem cell labeling for magnetic resonance imaging studies by the use of cationic liposomes. *Cell Transplant.* 12, 743–756 (2003).

96 Yang JX, Tang WL, Wang XX: Superparamagnetic iron oxide nanoparticles may affect endothelial progenitor cell migration ability and adhesion capacity. *Cytotherapy* 12, 251–259 (2010).

97 Yang CY, Hsiao JK, Tai MF *et al.*: Direct labeling of hMSC with SPIO: the long-term influence on toxicity, chondrogenic differentiation capacity, and intracellular distribution. *Mol. Imaging Biol.* DOI 10.1007/s11307–010–0360–7 (2010) (Epub ahead of print).

■ Website

101 Biomagnetic Research and Technology www.biomagres.com/content/2/1/3

Author Proof