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REVIEW

Cellular toxicity of inorganic nanoparticles: Common aspects and guidelines for improved nanotoxicity evaluation

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Summary The safe use of inorganic nanoparticles (NPs) in biomedical applications remains an unresolved issue. The present review presents an overview of the cytotoxic effects of commonly used inorganic NPs: quantum dots, gold and iron oxide nanoparticles. The main focus is on presenting recent findings and identifying similar cytotoxic effects which appear common to all these NPs. Next, several considerations are provided for optimizing cell—NP interaction studies which could aid in improving our understanding of NP toxicity. Finally, several critical NP parameters are discussed and suggestions are made on how to optimize NP design in view of minimal cytotoxicity.

Introduction

The biomedical use of inorganic nanoparticles (NPs) has enjoyed an increasing interest over the past decade owing to the numerous abilities they possess such as enabling non-invasive and long-term imaging of the whole body, potential treatment of cancer as currently being studied in clinical trials for magnetite and gold particles, or shedding light on the complex cellular environment [1—4]. As a result, cells are frequently exposed to a wide array of inorganic NPs. The frequency of resulting cell—NP interactions necessitates a more profound knowledge of nanoparticle effects on cells. To date, this question is far from answered, as many ambiguous findings have been reported in the literature, mostly based on experiments with cultured cells [5,6]. The assessment of NP safety has been complicated due to a great variety in: (1) types of NPs [5], (2) stabilizing coating agents [7,8], (3) physicochemical parameters of the NPs (diameter, surface charge, surface topography, surface area) [9], (4) incubation conditions (time and concentration) [10], (5) type of cells used [11], (6) type of assay used [12] or (7) possible interference of the NPs with the assay readout [13]. Since most papers describe a limited number of effects

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of a single NP type on one particular cell type for a specific incubation protocol [14—16], a direct comparison of results between different studies is near impossible. As such, the safety of nanomaterials for biomedical applications and their exposure to (cultured) cells remains unclear.

In the last couple of years, the interest in nanotoxicology has increased and more data regarding the cytotoxic properties of NPs have been reported. Several reviews present an overview of the most important findings on this topic [17—20]. Some recent findings, such as the intracellular degradability of NPs [21] or the close correlation between intracellular localization and local NP concentration and their cytotoxic effect [22,23] have provided novel insights in understanding the effect of NPs on cells. The present work addresses these novel findings and focuses on the cellular toxicity of the NPs, as many biomedical applications require the *in vitro* exposure of cultured cells to NPs prior to *in vivo* translation. To date, the majority of NP research is therefore also focusing on *in vitro* setups.

In this review we aim at providing an update of the most recent findings rather than giving a complete overview of all the literature reports. The main goal of the present work is to address the general question whether inorganic NPs are safe to be used for biomedical applications. To this end, this review focuses on common aspects involved in the cytotoxic effects of frequently used inorganic NPs (iron oxide NPs (IONPs), gold NPs and quantum dots (QDs)). Based on literature data and our own experience, several parameters are then defined which appear to be inherent to inorganic NPs. Furthermore, several important considerations are given which could provide a basis to carefully assess the safety of inorganic NPs and aid to optimize cellular toxicity studies. In the final section, some key parameters of the inorganic NPs are discussed and suggestions are given on how to optimize these in order to limit cytotoxicity of novel NPs which should aid in the future design of safe NPs for biomedical use.

Inorganic NPs

IONPs

Iron oxide is a ferromagnetic material, which responds to an external magnetic field by a resulting magnetic moment along the field lines of the external field [24]. This process occurs by changes in the ordering of the magnetic subdomains (Weiss domains) of the iron oxide particles. Upon removal of the external field, the magnetic domains remain coupled to some extent, leading to a remnant magnetization. For biomedical use, this remnant magnetization could pose some problems. As such, small IONPs are preferably employed (Fig. 1a , left panel), of which the diameter of the iron oxide core is smaller than the superparamagnetic limit (approximately 12—15 nm [25]), resulting in NPs which consist of only a single Weiss domain. Owing to this small size, these particles become superparamagnetic as no remnant magnetization remains in the absence of an external magnetic field, whereas the magnetic susceptibility is still about as high as for the ferromagnetic bulk material. The small size of IONPs is also advantageous for many biomedical applications, where IONPs are used as contrast agents for magnetic resonance imaging (MRI), mediators in magnetic cancer hyperthermia or magnetically enhanced and targeted drug or gene delivery (Fig. 1c, left panel) [26—28].

IONPs have been used for biomedical applications for over 2 decades, where some dextran-coated formulations have been FDA-approved as MRI contrast agents for the detection of liver tumors. Typically, IONPs are spherical particles with a diameter of 10—100 nm, but they can also be several hundreds of nm in size (Fig. 1b, left panel) [29]. A large variety of coating agents are used, including synthetic and natural polymers, lipids or small molecules [30]. With regard to cell physiology, iron is an important molecule which plays key roles in several intracellular signaling pathways such as cell cycle progression. Ferric iron is normally transported by means of transferrin, which can bind the cell-surface localized transferrin receptor. Within the cell cytoplasm, the majority of the cytoplasmic iron pool is stored in specialized proteins called ferritin. Due to the physiological relevance of iron, IONPs were initially considered to be non-cytotoxic. IONPs can naturally be broken down resulting in the release of ferric iron which can then participate in the normal iron metabolism. It has, however, been recognized that the small size of IONPs might pose an additional hazard as the particles can reach high local concentrations within the cells and are generally more difficult to be efficiently cleared from the body [4,31].

QDs

QDs are nanosized semiconductor nanocrystals which possess size-tunable optical and electrical properties (Fig. 1a and b, right panel) [32]. Compared to organic fluorophores, QDs have a high fluorescence emission intensity, high photostability, a narrow emission spectrum and a broad excitation spectrum [32]. Excitation of QDs occurs through absorption of energy, which causes an electronic transition from the ground state to an excited state. Absorption is followed by the release of energy, partly in the form of a photon when the QD system relaxes back to the ground state. For biomedical applications, their excellent optical properties make them highly attractive probes for long-term intracellular and *in vivo* optical imaging (Fig. 1c, right panel) [33,34]. In terms of triggered cancer therapy, photosensitizing QDs have been developed which generate free radicals upon exposure to visible light [35].

The optical properties of QDs are determined by their chemical composition as they consist of a semiconductor core with a narrow bandgap made up of elements from groups 12 and 16 (CdSe, CdTe, ZnS) or groups 13 and 15 (InP, GaN; Fig. 1a, right panel). QDs can either be synthesized directly in aqueous solution or in organic solvent, which offers higher flexibility in synthesis. Hydrophobic QDs are then encapsulated by a protective coating material which renders the QDs water-soluble, as described in Pellegrino et al. [36]. Core—shell particles, where the core material is surrounded by a shell of semiconductor material with a larger bandgap, are more frequently used than core-only QDs as the shell passivates the core which enhances the fluorescence quantum yield and reduces photobleaching [33]. As QDs are composed of heavy metals like Cd^{2+} or In^{3+}, they

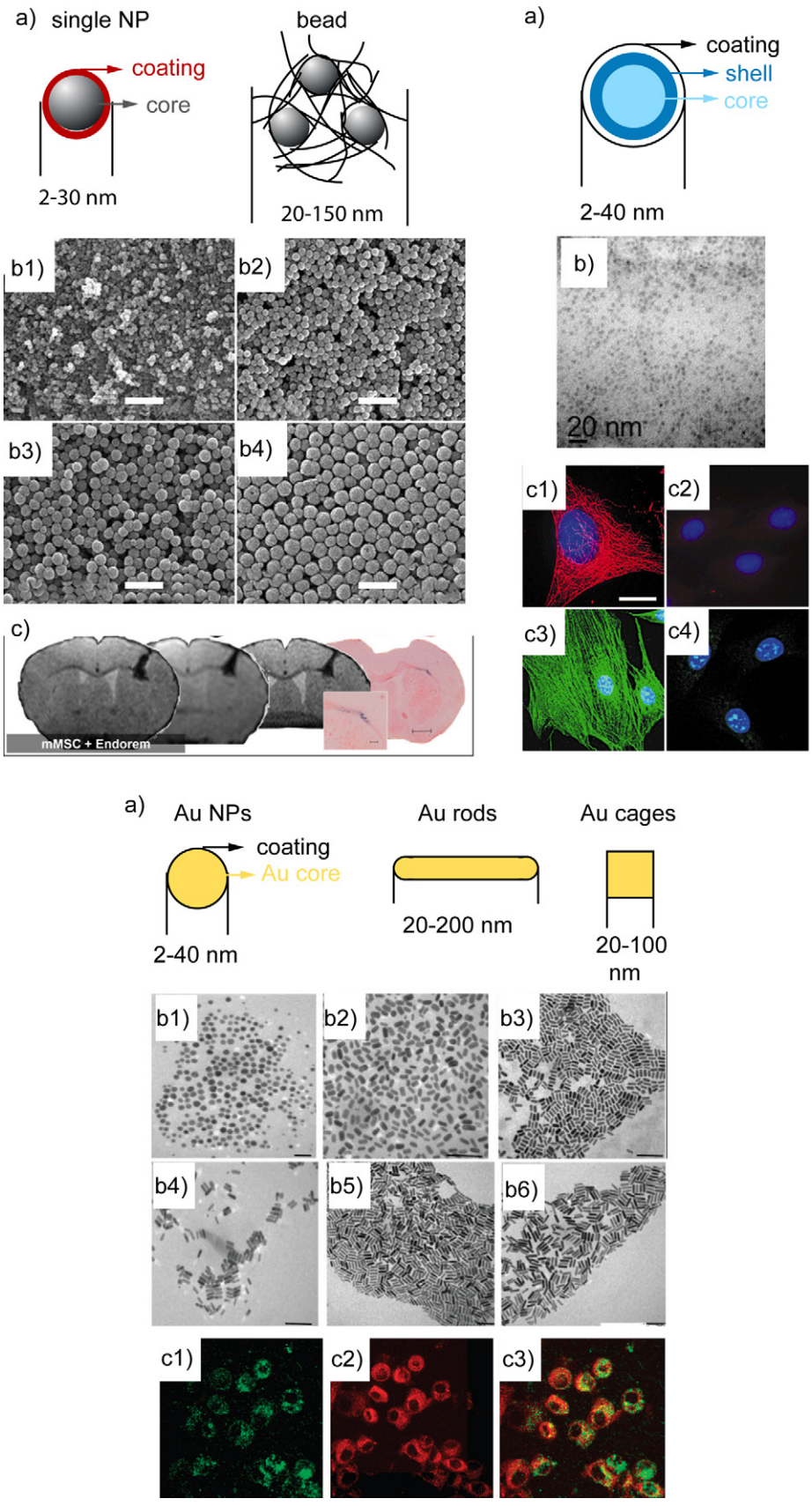

Figure 1 Left: (a) Schematic overview of the two main types of IONPs, being single NPs and beads, where multiple cores are embedded in a single matrix. (b) Scanning electron micrographs of citrate coated IONPs of different sizes. Scale bar: 1 μm. Reprinted with permission from [148], ©Wiley-VCH. (c) Representative 3D T_2* MR images of mice brain after 1, 3 and 4 weeks after injection

have been used in biomedical applications with great caution due to expected cytotoxic effects.

Gold NPs

Gold nanomaterials have unique optical features which make them well suited for a number of biomedical applications such as drug or gene delivery, cancer treatment and biological imaging (Fig. 1c, bottom panel) [37,38]. Depending on the size and shape of gold nanomaterials, they can strongly adsorb or scatter incident light at a certain resonance wavelength, a phenomenon called localized surface plasmon resonance (LSPR; Fig. 1b and c, bottom panel) [39]. For biomedical applications such as whole body imaging, the latter feature is highly advantageous as the LSPR peaks can be tuned into the near infrared region (800—1100 nm) which is the optically transparent window for soft tissues [40]. Gold NPs can also convert near infrared light into heat via the photothermal effect, providing an interesting platform for cancer therapy [41].

Gold NPs are produced in a variety of shapes, depending on the desired application, going from spherical NPs to gold nanorods with high aspect ratios and gold nanocages with a hollow interior and porous walls (Fig. 1b and c, bottom panel) [39]. The size of the particles can vary from a single nanometer to several tens of nanometer. A variety of coating agents can also be used, including small molecules such as citrate, surfactants such as cetyltrimethylammonium bromide (CTAB) or polymers [42—44]. As bulk gold is a noble metal and thus chemically inert, gold NPs were also expected not to evoke any cytotoxic effects. However, at very small sizes (below 4—5 nm) Au NPs have been described to potentially induce toxicity by penetrating the nuclear compartment and binding to DNA. As gold is the most electronegative metal it is easily attracted to DNA grooves which have a negative environment. Furthermore, NPs of about 1.4 nm diameter almost perfectly match with the size of the major DNA groove, leading to strong potential toxic effects of Au NPs, especially for those in the smaller size range [4].

Common mechanisms of cytotoxicity

Regardless of the intrinsic differences between the various inorganic NPs, the ''nano''-factor itself appears to cause several adverse effects. As the NPs approach the size of natural proteins, the particles can reach places where larger particles cannot enter, such as — in some cases — the nucleus or, in case of *in vivo* settings, NPs can transfer across the placental barrier from pregnant mice to pups [45,46]. The high surface area over volume ratio of NPs augments the NP surface available for interaction with cellular components [47]. Furthermore, the confinement of NPs in subcellular structures such as endosomes can lead to very high local concentrations which cannot be achieved by free ions and which can locally exceed the LD_{50} (lethal dose 50, i.e. the dose applied by which 50% of animals or cells dies). The size and physicochemical properties dictated by the surface of NPs greatly determine the extent of cellular interactions, their endocytic routing and uptake efficiency [9,48]. Due to these common features for inorganic NPs, it is expected that NPs exert several similar mechanisms by which they affect cell homeostasis, apart from some NP-type specific aspects. In the following paragraphs, several of such mechanisms which have been reported for different types of inorganic NPs are discussed.

Reactive oxygen species

The generation of reactive oxygen species (ROS) by cultured cells upon exposure to NPs is quite a common phenomenon. When cells are exposed to environmental stress such as pathogens or heat, they can generate chemically active oxygen-containing molecules. These ROS species can be subdivided into two different types, being radical ROS (nitric oxide or hydroxide radicals) and non-radical ROS (hydrogen peroxide). Most cells have defense mechanisms such as the glutathione redox system, which can buffer a certain amount of ROS. When the increase in ROS species is too high, cells are prone to undergo various negative effects. The link between ROS levels and the induction of toxic effects is however cell type-dependent and not very well defined [49]. In general, small and transient increases in ROS can be tolerated by most cell types, whereas higher levels which persist over a longer time period are more likely to result in cell damage.

For NPs, which can be seen as foreign materials by the cells, the generation of ROS as a reaction to these foreign species is quite standard [50,51]. The large surface area of the NPs and reactive surface molecules gives rise to massive oxidizing capabilities. NPs have been described to possibly generate ROS by different mechanisms [52]: (1) Direct generation of ROS as a result of exposure to an acidic

of Endorem-labeled mesenchymal stem cells in the striatum. Prussian blue staining after 4 weeks confirms the presence of iron labeled cells. Reprinted with permission from [149], ©Wiley-VCH. Right: (a) Schematic overview of a core/shell type quantum dot. (b) Representative transmission electron micrograph (TEM) of well dispersed quantum dots with average diameter of approximately 5 nm. Reprinted with permission from [150], ©Elsevier. (c) Typical confocal micrographs of 3T3 mouse fibroblasts stained with secondary QDs against (c1) primary antibodies for tubulin and (c3) phalloidin (actin filaments-specific). (c2 and c4) Controls without primary antibody or phalloidin, respectively. Nuclei were counterstained with Hoechst 33342 blue dye. Scale bar: 10 μm for (c1), 24 μm for (c2) through (c4). Reprinted with permission from [151], ©Nature Publishing Group. Bottom: Schematic overview of the most common types of gold nanoparticles, being: nanospheres, nanorods and nanocages. (b) TEM images of gold nanorods with different aspect ratios (b1: 1; b2: 2.1; b3: 2.6; b4: 2.9; b5: 3.4; b6: 4.1). Scale bars: 100 nm. Reprinted with permission from [42], ©Wiley-VCH. (c) Confocal images of the epidermal growth factor receptor (EGFR)-expressing U87MG cells after incubation for 3 h with 0.02 nM of anti-EGFR Au nanocages and 5 μg/mL of FM4-64 dye: (c1) photoluminescence from Au nanocages; (c2) red fluorescence from FM4-64; and (c3) superimposition. Reprinted with permission from [39], ©American Chemical Society.

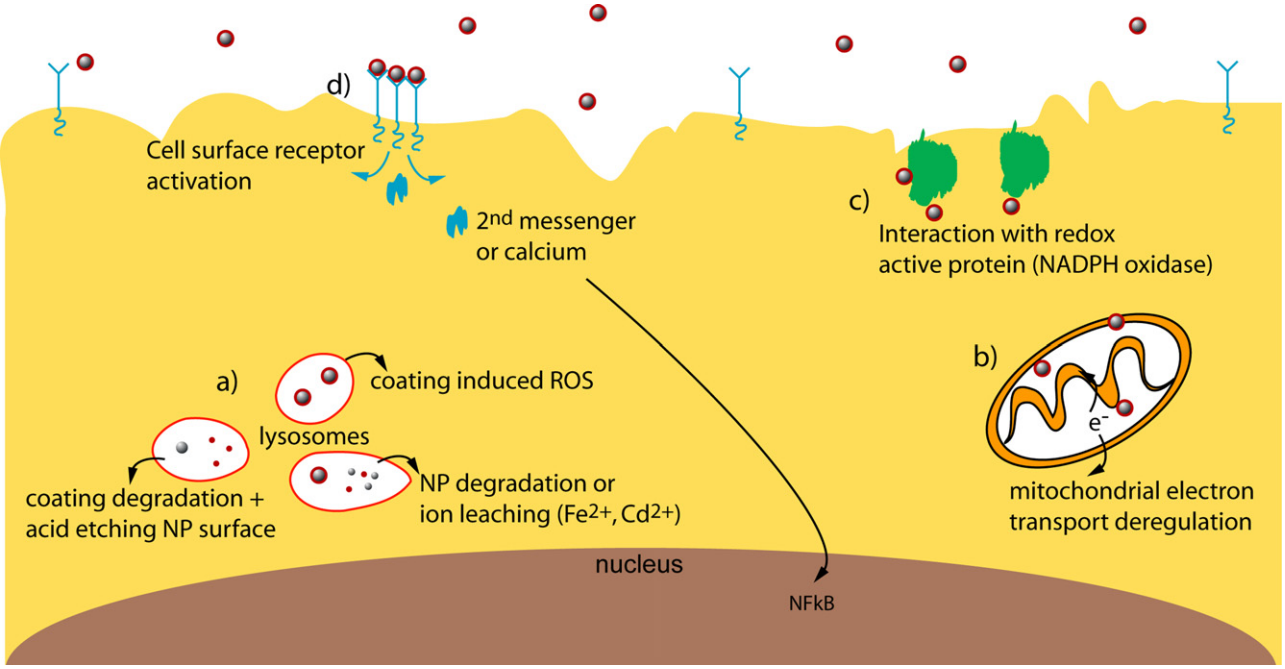

Figure 2 Schematic overview of the different pathways by which nanoparticles can induce oxidative stress. (a) Nanomaterial present in the acidic environment of lysosomes can induce ROS by direct reactivity of their surface coating, degradation of the coating and direct interaction of the acidic media on the metal surface or degradation of the whole nanoparticle and production of ions (Fe^{2+}, Cd^{2+}) which can induce ROS species by various chemical reactions. (b) Nanomaterial can also directly interact with oxidative organelles such as the mitochondria by destabilizing the outer membrane, deregulating the mitochondrial membrane potential and hereby disrupting the electron transport chain of the oxidative phosphorylation. (c) Nanoparticles can directly interact with redox active proteins such as NADPH oxidase and hereby stimulate large ROS production in cells of the immune system. (d) Interaction of nanoparticles with surface located receptors can lead to receptor activation and triggering of intracellular signaling cascades (activation of second messenger or calcium waves), finally resulting in expression of stress response genes which can upregulate ROS.

environment, such as the lysosomes, either from the surface of the NPs or from leached ions (Fig. 2a) [53,54]. (2) Interaction of the NPs with cellular organelles such as mitochondria which can affect the function of the latter (Fig. 2b) [55]. (3) Interaction of NPs with redox active proteins such as NADPH oxidase (Fig. 2c) [52]. (4) Interaction of NPs with cell surface receptors and activation of intracellular signaling pathways (Fig. 2d) [52].

For IONPs, the induction of ROS is typically a transient effect, which reaches maximal levels after approximately 24 h after cell internalization and then decreases to near control levels over a period of about 72 h [56]. The kinetics depend on the stability of the coating of the IONPs, where citrate-coated particles have a much faster maximal ROS induction (4 h) compared to most other formulations such as dextran- or lipid-coated IONPs [21,53]. The level of induced ROS relates to the total amount of IONPs which have been internalized by the cells and are greatly affected by the total surface area of the IONPs and the stability of the coating against intracellular degradation. In contrast, Gao et al. [57] have recently shown that magnetic nanoparticles possess an intrinsic peroxidase-like activity which can actually diminish cellular ROS levels, as long as the particle remains intact and is not degraded. Based on these findings, Huang et al. [58] reported that dextran-coated iron oxide cores indeed diminish intracellular H_2O_2 which promoted cellular proliferation rather than impeding it. These data show that although the induction of ROS has been generally accepted as being one of the major potential problems associated with IONPs for cell labeling [59], this issue is still a matter of concern as the relation between ROS levels and the occurrence of cytotoxic effects is still unclear.

For QDs, the induction of ROS has also been frequently reported [60]. Generally, the same mechanisms as described for IONPs are valid, where ROS can be induced by the reactivity of surface-located transition metals [61], leaching of free Cd^{2+} ions [62] or by direct interaction of QDs with mitochondria [61,63]. However, the cytotoxicity of these particles cannot be attributed solely to the toxic effect of Cd^{2+} ions. Although release of Cd^{2+} ions is generally considered as being the main cause of QD-induced cellular toxicity, the use of stabilizing coatings, passivating shell layers and the development of cadmium-free QDs are all being investigated in order to reduce any Cd^{2+} release and thereby promote further biomedical use of QDs. Despite Cd^{2+} release being the primary cause of toxicity, other effects such as ROS may not be neglected and must also be looked into as they can lead to various toxicological effects different than those induced by Cd^{2+} release and furthermore, these other factors may also play an important role in the toxic effects of cadmium-free QDs. QDs can be activated with light and generate ROS, which cause cytotoxicity due to photo-oxidative processes

involving singlet oxygen or electron transfer from excited QDs to oxygen. For instance, Tang et al. [64] found that unmodified CdSe QDs elevated induced ROS which lead to elevated cytoplasmic calcium levels. Of particular interest for QDs, it has been described that core–shell type QDs which showed only reduced effects compared to core-only QDs, emphasizing the important protective role of the QD shell layer [63,65], although some effects will still prevail as the shell layers likely do not provide 100% coverage [66]. The coating of the QDs also plays an important role, as was observed for organic, carboxyl and amine-containing polyethylene glycol (PEG)-coated particles which interacted with J774.A1 macrophages in a different way according to their specific surface properties [7]. The latter findings likely relate to differences in intracellular concentrations of the QDs as these coating agents do not differ much in their protective capacity against degradation. Rather the different physicochemical properties of the QDs might lead to changes in their level of internalization where higher levels of ROS would then likely be observed for the particles which are most avidly taken up by the cells.

For gold NPs, the induction of oxidative stress upon cell labeling has not been clearly established. As Au is a noble and inert metal which has been described to exhibit some therapeutic and even medicinal value, Au NPs were long considered to be non-toxic [67]. A recent study by Li et al. [68] has, however, indicated that Au NPs caused oxidative stress in human lung fibroblasts after internalization. The induction of ROS resulted in lipid peroxidation and malondialdehyde protein adducts which are indicative of oxidative damage. Qiu et al. [14] have confirmed these findings, showing that accumulation of Au NPs in human breast adenocarcinoma (MCF-7) cells resulted in mitochondrial depolarization and swelling, indicative of outer mitochondrial membrane rupture.

As ROS has been claimed to be of major importance in the toxicological profile of NPs [59], the possible evaluation of elevated ROS levels is of crucial importance. Commonly, fluorescent probes such as dichlorodihydrofluorescein and its derivatives are used to assess ROS levels. It is important to use multiple time points to accurately define the maximal ROS levels, as the kinetics of ROS induction can largely differ between various NPs and it is also important to know whether the effects are transient or more long-lasting. As the link between elevated ROS levels and cytotoxicity is unclear, it is also important to further investigate any possible secondary effects in case significant ROS induction is observed. Useful parameters to study would be mitochondrial metabolism (using fluorescent probes such as JC-1), lipid or protein peroxidation, cytoplasmic calcium levels, cytoplasmic redox state (by measuring glutathione levels) or DNA defects.

Cell morphology and cytoskeleton defects

As NPs have certain physical dimensions, the intracellular volume they occupy can lead to alterations in cellular morphology or affect the structure of the cellular cytoskeleton network [69,70]. The latter effects can also be due to the high demands the NPs pose on the cellular endocytic machinery [71].

For IONPs, intracellular localization of the particles has been found to be associated with the disruption of the cell cytoskeleton network [71,72]. Gupta and Gupta [71] described different effects in cytoskeleton disorganization depending on the coating of the IONPs. Wu et al. [73] showed that IONPs greatly disrupted actin fibers and tubulin network of human umbilical vein endothelial cells (HUVECs) and also impeded the maturation of focal adhesion complexes, which link the cytoskeleton network to the extracellular matrix. These cytoskeletal deformations also decreased the capacity of HUVECs for vascular network formation. Buyukhatipoglu and Clyne [16] found that bare IONPs also affected the cell cytoskeleton, leading to cell elongation and an increase in actin stress fiber formation. Soenen et al. [69] also found cytoskeleton disorganization caused by lipid-coated IONPs, which otherwise did not evoke any direct cytotoxic effects. In a follow-up study by the same group [70], it was observed that a variety of IONPs, including lipid-, dextran- and citrate-coated ones, induced actin and tubulin network deformations when reaching high intracellular levels in C17.2 neural progenitor cells and primary human blood outgrowth endothelial cells (Fig. 3a). It was hypothesized that the mere physical presence of high amounts of IONPs enclosed in large and bulky lysosomal structures typically located in the perinuclear region, sterically hindered the cytoskeleton network and hereby induced the remodeling of the actin network.

For QDs, the effects of cell labeling on deformation of the cell cytoskeleton have only scarcely been addressed. Initially, Prasad et al. [74] found that rat pheochromocytoma cells (PC12) did not show any differences in cellular morphology after being incubated with CdTe QDs for 72 h. Recently, however, Mahto et al. [75] described significant structural changes in actin and tubulin networks of 3T3 fibroblasts after incubation with CdSe/ZnSe QDs (Fig. 3b). The authors further found that, similar to the work of Gupta and Gupta [71] on IONPs, different surface modifications led to various degrees of cellular effects.

Gold NPs have been described to have a profound effect on the morphology of several cell types, such as A549 human carcinoma lung cells [76]. Gold NPs have also been described to have a concentration-dependent effect on the actin fibrils of human dermal fibroblasts (Fig. 3c) [43]. Mironava et al. [10] further showed the cytoskeleton filaments to be disrupted as a function of Au NP exposure time, concentration and size of the NPs although actin or β-tubulin protein expression levels were not affected.

The effects of NPs on cellular morphology and cytoskeleton have only recently received more attention and the underlying mechanism and forthcoming consequences have not been investigated in depth. In this regard, it is important for all novel NP types to evaluate their endocytic uptake pathway and intracellular localization as a function of time. For different types of NPs, the effects have been described to be dependent on intracellular NP concentration and to be transient, where after recurrent cell divisions, the intracellular NP concentrations decrease exponentially and the effects are no longer observed. Also, possible endosomal escape of the NPs must be assessed. As cytoskeleton defects have been described to be clearly dependent on NP concentrations, a wide concentration range of particles should be tested in order to try and assess the maximal cellular

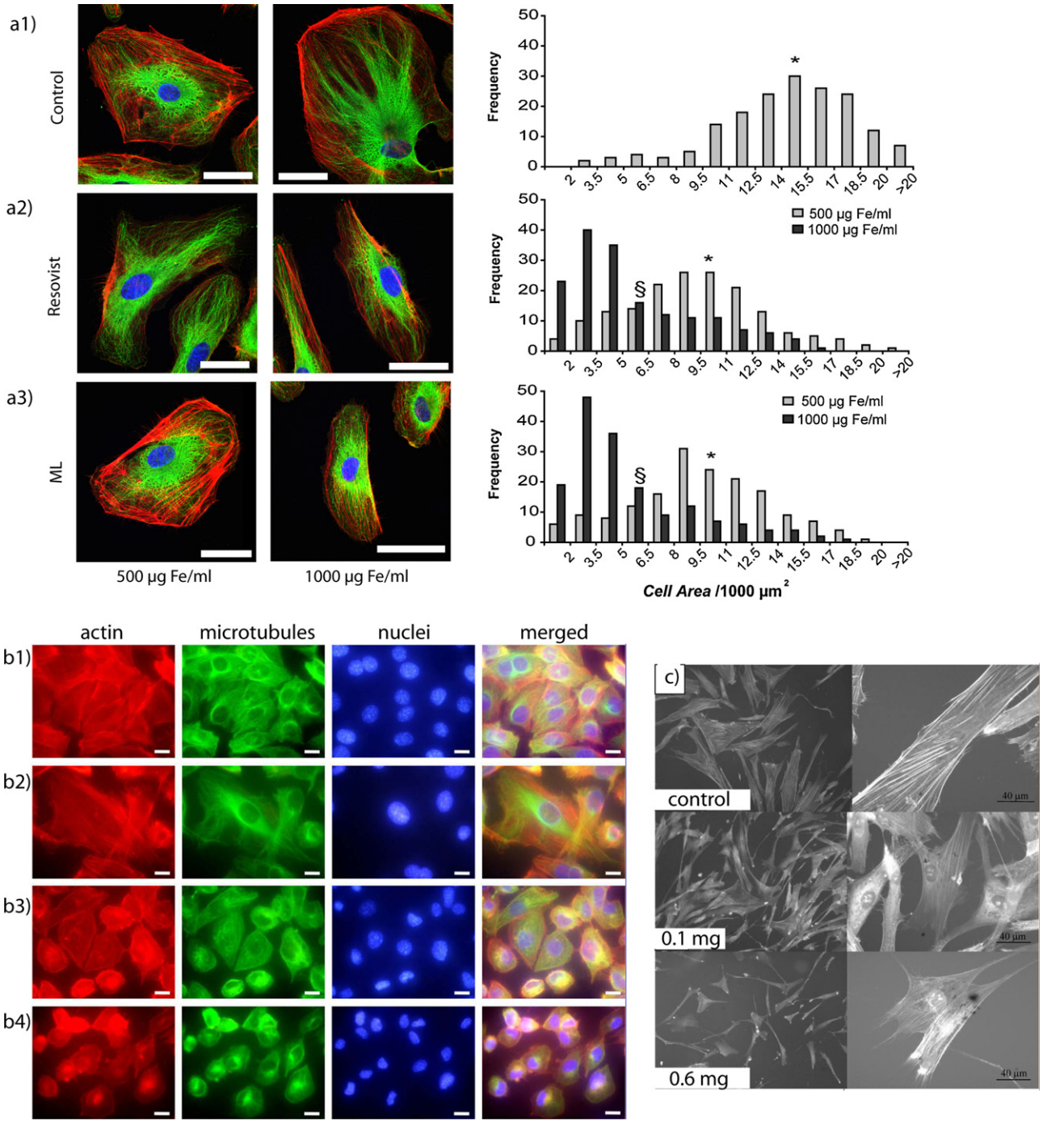

Figure 3 (a) Representative confocal images of human blood outgrowth endothelial cells (BOECs) at 3 days post labeling of (a1) control cells or cells incubated with (a2) carboxydextran-coated Resovist or (a3) lipid-coated magnetoliposomes (MLs) at 500 (1st column) or 1000 µg Fe/mL (2nd column) depicting the actin cytoskeleton (red), α-tubulin (green) and DAPI-stained nuclei (blue); scale bars: 50 µm. The third column shows histograms representing the cell surface areas of (a1) control cells or cells incubated with the respective particles (a2 and a3) at 500 (light grey) or 1000 µg Fe/mL (dark grey).The average cell area is indicated by an asterisk (*) for control cells or particles at 500 µg Fe/mL and by (§) for particles at 1000 µg Fe/mL. Reprinted with permission from [70], © Wiley-VCH. (b) Representative fluorescence images showing structural changes in cytoskeleton and nuclei of 3T3 fibroblasts of (b1) control cells; and cells treated with (b2) 1 nM mercaptopropionic acid (MPA)-coated QDs, (b3) 0.746 nM gum arabic/tri-n-octyl phosphine oxide (GA/TOPO)-QDs, and (b4) 50 µM $CdCl_2$ for 6 h. Scale bars: 10 µm. Reprinted with permission from [75], © Elsevier. (c) Dermal fibroblasts imaged with an Hg lamp after six days for the control and for cells exposed to 13 nm diameter gold NPs at concentrations of 0.1 and 0.6 mg/mL. Reprinted with permission from [43], © Wiley-VCH.

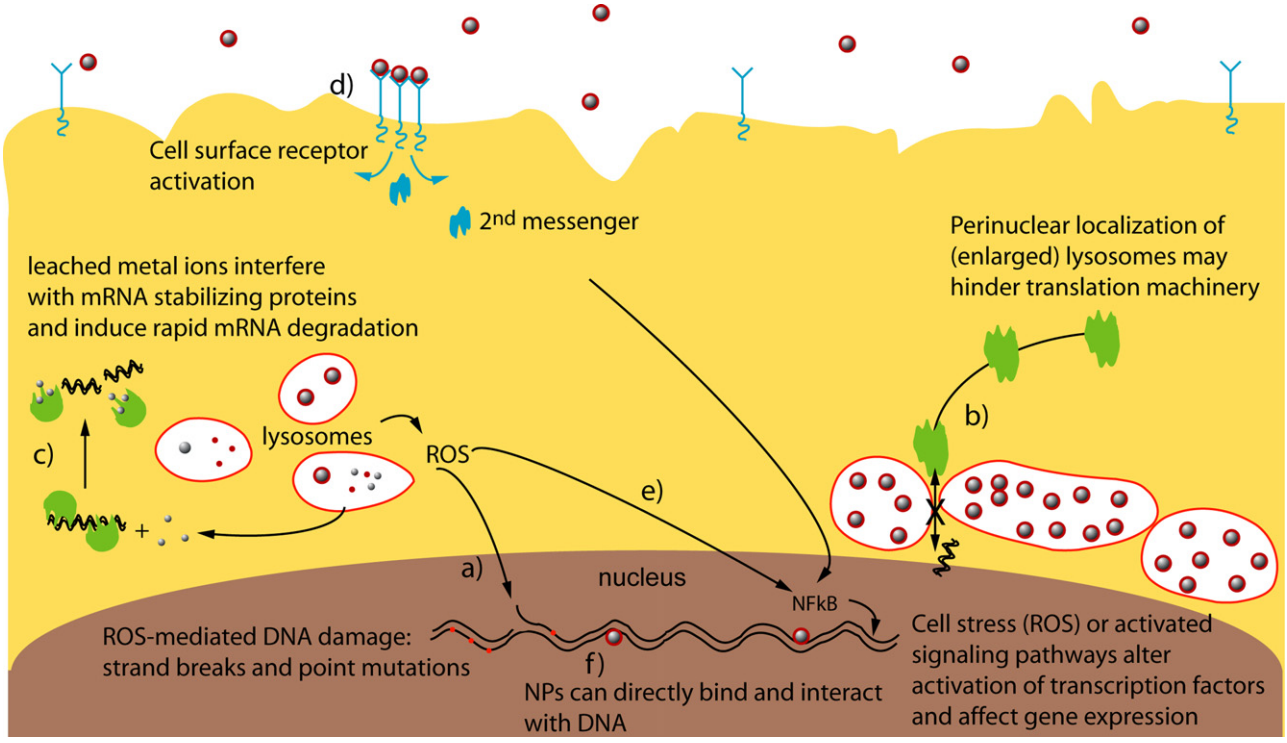

Figure 4 Schematic overview of the different mechanisms by which NPs can induce genotoxic effects or affect intracellular signaling pathways. (a) High levels of induced ROS by NPs localized in lysosomes can directly induce DNA point mutations or lead to single or double strand breaks. (b) The proximal perinuclear localization of large numbers of NP-loaded lysosomes can hinder the cellular transcription and translation machinery and hereby affect global protein synthesis. (c) Leached metal ions from lysosomal located NPs can transfer to the cell cytoplasm via specialized complexes (e.g. divalent metal transporter) where it can then interact with mRNA stabilizing proteins which contain metal responsive domains; resulting in the release and degradation of the mRNA (e.g. mRNA of transferrin receptor in response to ferric ions). (d) Interaction of nanoparticles with surface located receptors can lead to receptor activation and triggering of intracellular signaling cascades (activation of second messenger or calcium waves). (e) NP-mediated ROS induction and associated protein and lipid peroxidation can also indirectly affect gene expression patterns by activation of stress response or repair genes. (f) Nanosized particles (such as Au NPs) can penetrate the nucleus and bond to and interact with DNA directly.

loading capacity without any effects. Furthermore, as the cytoskeleton is also involved in many intracellular signaling pathways, it remains to be investigated whether the NP-induced cytoskeletal disruption leads to secondary effects.

Intracellular signaling pathways and genotoxicity

Inorganic NPs can interfere with the delicate balance of cellular homeostasis and hereby alter complex intracellular signaling pathways, resulting in a cascade of possible effects. These interactions can occur by several mechanisms, such as: (1) genotoxic effects caused by high levels of ROS (Fig. 4a) [77], (2) altered protein or gene expression due to the perinuclear localization of the particles which may hinder the functioning of the transcription and translation machinery (Fig. 4b) [47], (3) altered protein or gene expression levels due to leaching of free metal ions (Fig. 4c) [21], (4) altered activation status of proteins by interfering with stimulating factors such as cell-surface receptors (Fig. 4d) [78] or (5) altered gene expression levels in response to the cellular stress that the NPs induce (Fig. 4e) [79]. To date, the effect of inorganic nanomaterials on protein or gene expression levels has only scarcely been investigated and more data needs to be generated in order to get a better idea to what extent NPs can cause alterations to intracellular signaling pathways. As intracellular signaling pathways and genotoxic effects cover a very broad area, these parameters are difficult to study in a straightforward manner and many ambiguous results have been obtained. Here, we mainly focus on reported DNA damaging effects and altered gene expression levels.

For IONPs, QDs and Au NPs, several studies have described no effect on stem cell differentiation [80], no genotoxic effects [81], and almost no effects on gene expression patterns [82,83]. On the contrary, several others studies have described severe effects, which highlight the need to further address this topic. For IONPs, several studies have shown an inhibition of stem cell differentiation [84,85], upregulation of genes involved in lysosomal function and detoxification [79], reduction in protein synthesis [86] or an impeded functionality of PC12 cells by DMSA-coated IONPs [47] or citrate- and dextran-coated IONPs [21]. It has been hypothesized that the proximal perinuclear localization of IONP-containing endosomes might drastically impede transcriptional regulation and protein synthesis. Alternatively,

IONP-induced actin network deformations may affect focal adhesion complexes and focal adhesion kinase-mediated signaling mechanisms in a concentration-dependent manner. Many of these effects can be ascribed to either high intracellular IONP concentrations (mostly due to cytoskeleton deformations), ROS generation or leaching of ferric ions. Such leached ions can be released from the lysosomes, enter the labile cytoplasmic iron pool and affect the regulation of cyclin-dependent kinases (controlling proliferation) [58] or alter the levels of transferrin receptor-1 [21,87]. As transferrin receptor-1 is involved in controlling cellular iron levels, its mRNA has an iron regulatory motif which will lead to the destabilization of the mRNA and thus lower protein expression levels when the cytoplasmic iron pool increases. As this is a normal physiological response of the cell to higher iron levels, the toxicological relevance is still unclear and must be further studied. Interestingly, this cellular response does provide a sensitive and easy parameter to investigate the rate of intracellular IONP degradation and associated release of ferric ions [21].

In the case of QDs, their high oxidative nature can result in DNA damaging effects. The mere presence of CdSe/ZnS QDs has been described to induce strand nicking of plasmid DNA [88], an effect which is even more pronounced for photoactivated QDs [89]. Intracellular QDs have also been described to induce apoptotic DNA fragmentation [90], increase DNA strand breaks and expression of heat shock proteins [91] and activate p53-associated signaling [92]. Au NPs have been less frequently linked to genotoxic effects or altered gene expression patterns. For particles of 3 nm or smaller, however, nuclear penetration has been observed which greatly increases the potential DNA-damaging effects of the particles [45]. Oxidative DNA damage has also been found, associated with a downregulation of DNA repair genes [93], an increased secretion of the proinflammatory cytokine tumor necrosis factor (TNFα) [22] and distinct disturbances in the expression pattern of genes involved in cell cycle regulation, energy metabolism and cytoskeleton organization [94].

The genotoxic effects of NPs have only rarely been investigated to date and more data is required to evaluate the possible genotoxic potential of the NPs used. Studying gene expression levels and protein synthesis allows to evaluate whether the cell displays any signs of stress and which signaling pathways are affected by the intracellular presence of the NPs. It is important to note that several changes (such as for genes involved in endocytosis regulation) will automatically be associated with NP uptake without indicating any cytotoxic effect. Especially genes involved in cell viability, cellular stress, oxidative damage or cell cycle progression are worth studying. For any type of particle, a rapid but transient effect will likely be seen, especially in cellular stress genes, which would indicate the normal response of a cell to a foreign object being ingested. Only when the effects are noticeable over a certain time period (e.g. several days), this would indicate actual NP-induced toxicity.

Intracellular NP degradability

As practically all NPs are internalized by cultured cells through endocytic mechanisms, their surrounding pH will shift from 7.4 in the extracellular medium over 6.0 (early endosomes) to 4.5 (lysosomes). As NPs often have a high surface charge density, the local pH at the NP surface can often be even lower or higher than the global value of their surroundings [95]. Along with the pH differences, NPs will also be exposed to various degradative enzymes, such as cathepsin L [96] which has recently been suggested to be able to degraded nearly all bioconjugated particles [97]. Upon degradation of the coating molecules, the acidic environment of the endosomes can lead to acid etching of the NPs, resulting in the generation of free ions from the NP surface and gradually decrease the NP core diameter. Apart from a complete loss of NP functionality (e.g. loss of magnetic moment for IONPs or loss of fluorescence properties for QDs), the leached metal ions can potentially also affect cell homeostasis.

Dextran-coated IONPs were shown to degrade in time in a pH-dependent manner, resulting in the generation of free ferric iron and a complete dissolution of the iron oxide core when particles were exposed to a sodium citrate buffer of pH 4.5 [98]. These results agree well with those obtained by Levy et al. [99] who showed that particles with different surface coatings exhibited different dissolution kinetics. Interestingly, it was shown that only a part of the NPs degraded which is in line with a theoretical model for any NP suspensions containing both rapidly dissolving NPs and NPs which remain intact for longer times. Soenen et al. [21] confirmed these findings and found that the nature of the coating molecules greatly influenced the degradation rate of the particles (Fig. 5a). Intracellular IONP degradation has further been described to induce apoptosis and inflammation [100], impede neurite outgrowth of PC12 cells (Fig. 5b) [21] or inhibit osteogenic differentiation of human mesenchymal stem cells [85].

The pH-sensitivity of QDs has also been described, where under acidic (pH < 4) or alkaline (pH > 10) conditions, rapid destabilization of the QDs occurs, resulting in the release of high levels of toxic cadmium ions [101]. This destabilization of QDs has been found to occur by means of acid etching in the presence of physiologically relevant concentrations of hypochlorous acid and hydrogen peroxide, two compounds known to be generated in professional phagocytes in the body (Fig. 5c) [102]. Alternatively, Gagne et al. [91] described a time-dependent destabilization of QDs where release of free Cd^{2+} was found to increase with aging of the QDs. In the intracellular microenvironment, QDs were found to be altered after internalization, showing a distinct blueshifting of the fluorescence peaks, indicative of the QDs becoming smaller through surface erosion [103] and a decrease in fluorescence intensity of intracellular QDs as a function of time [104]. Released Cd^{2+} results in a decreased cell viability [75] where the extent of Cd^{2+} release depends on the coating agent used and the presence of stabilizing shell layers [66]. As can also be seen in Fig. 3b4, when cells are exposed to free Cd and Cd-containing QDs, toxic effects are more outspoken in case of free Cd^{2+}. This can be explained as only a low amount of QDs will be taken up and intracellular QD degradation has a rather slow kinetics, making the amount of Cd^{2+} available to the cells a lot less than in the case of free Cd^{2+} being administered. For QDs, the release of highly toxic Cd^{2+} ions is considered to be the most significant cause of cellular toxicity and therefore, QD

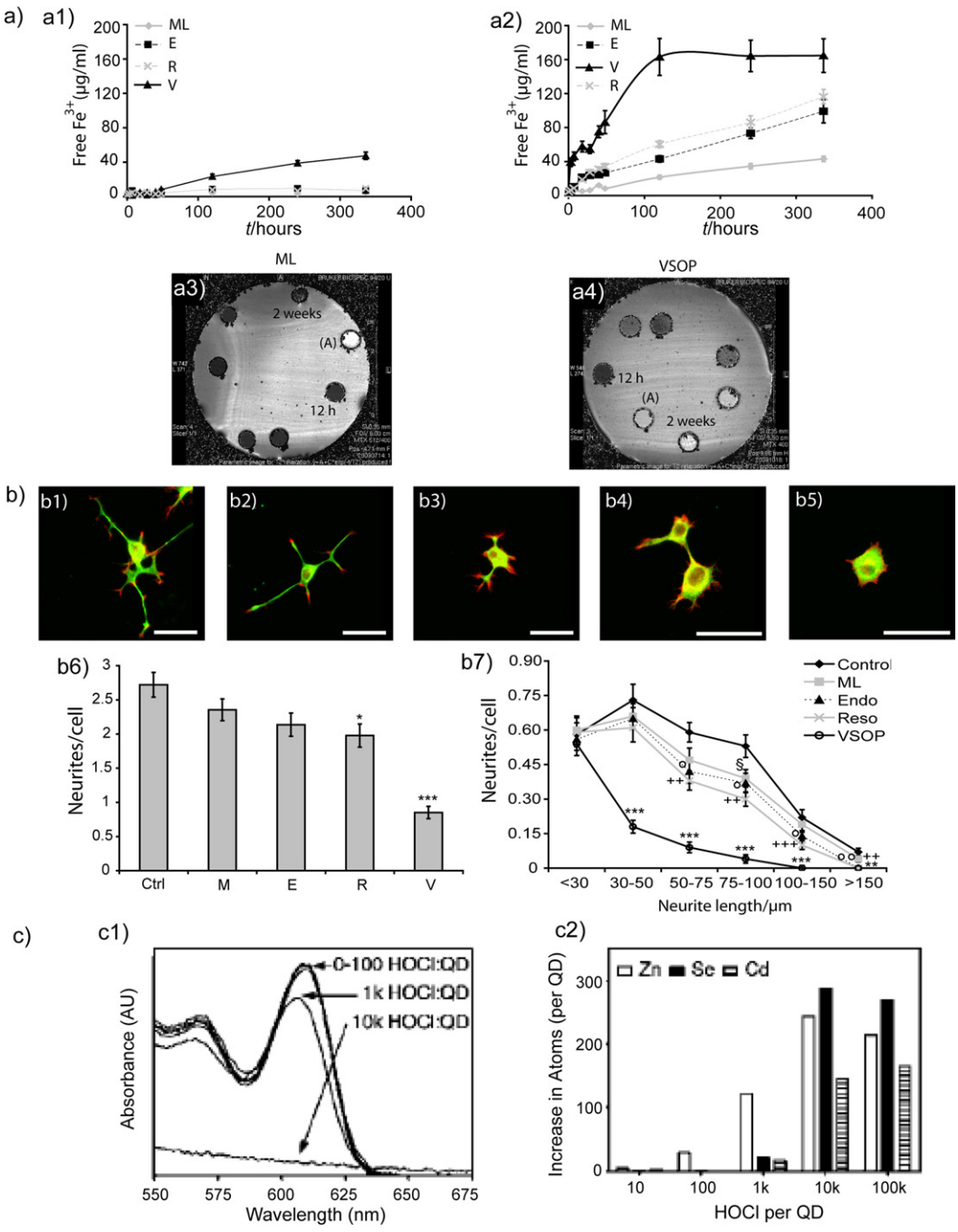

Figure 5 (a) pH effect on IONP degradation and MR signal intensities. The amount of free ferric iron measured as a function of time for lipid-coated MLs, dextran-coated Endorem (E), carboxydextran-coated Resovist (R) and citrate-coated very small iron oxide particles (VSOP: V) at (a1) pH 7.0 and (a2) pH 4.5. (a3 and a4) Representative T_2* maps measured for (a3) MLs and (a4) VSOP at pH 4.5 indicating the typical darkening of MR images caused by IONPs. Samples were collected after 12 h, 24 h, 48 h, 72 h, 1 week, and 2 weeks incubation. (A) Pure agar, without particles. From the images, it can clearly be noted that upon degradation of the VSOPs, the particles no longer give a nice MR contrast whereas MLs, which are less prone to degradation, give an MR contrast which persists well in time. Reprinted with permission from [21], © Wiley-VCH. (b) Effects of intracellular IONP degradation on cell functionality. (b1—b5) Representative microscopy images of PC12 cells after 2 days of exposure to nerve growth factor (NGF) showing α-tubulin (green) and g-actin (red) staining. Scale bars = 50 μm. (b1) Untreated control cells, (b2—b5) cells incubated with (b2) MLs, (b3) Endorem, (b4) Resovist or (b5) VSOP. (b6) The number of neurites per cell and (b7) the number of neurites of a certain length per cell after 2 days of NGF exposure. When appropriate, the degree of significance when compared with untreated controls cells is indicated (*$p < 0.05$; *$p < 0.01$; ***$p < 0.001$); for clarity, the degree of significance is indicated with different symbols for every type of NP in (b6): ML (§), Endorem (°), Resovist (+), and VSOP (*). Reprinted with permission from [21], © Wiley-VCH. (c) (c1) UV—vis absorption spectra and (c2) ICP-MS elemental analysis data showing chemical degradation of 50 nM QDs (polymer encapsulated CdSe/CdS/ZnS) after exposure to hypochlorous acid. Reprinted with permission from [102], © American Chemical Society.

degradation must be minimized and preferably completely avoided. The use of cadmium-free QDs or application of passivating shell layers and stabilizing coatings are therefore of great importance to avoid these effects (see also Section 'Reactive oxygen species').

On the other hand, gold NPs are highly stable and degradation has not yet been reported for the studies carried out in cells [105]. In these cases, the toxicity of the particles is usually associated to the stabilizing coating [42] or to the nanoparticle size, with particles of diameter $\leq$3 nm showing a higher toxicity but also showing a dependence on the stabilizer [106]. For *in vivo* situations, this high stability usually leads to accumulation in tissues and organs [107]. For *in vivo* studies in rats, it is hypothesized that the NPs can only be excreted from the body if the hydrodynamic diameter is $\leq$5.5 nm, whereas the bigger particles remain in the body [108]. For *in vitro* setups, cellular excretion is a topic only rarely studied, with exocytosis being the main process reported [109]. More data on *in vitro* setups are needed to try and define whether cellular exocytosis also has a diameter-dependent limitation and what the fate of the bigger Au NPs may be when residing within the cells for longer time periods.

For many biomedical applications, NPs need to be cell-internalized and have to remain in the cell interior for a relatively long time span, during which the NPs must remain functional (e.g. remain magnetic which allows to track the cells by MRI). Also, even when the NPs are no longer needed, their intracellular presence should not induce any effects on the cell. As QDs and IONPs have been described to degrade when present in lysosomes, the degradation products formed must also be safe for the cell. In case of IONPs, slowing down the degradation by adding a stabilizing coating should be enough, whereas for cadmium-containing QDs, any degradation should be strictly inhibited to avoid the release of toxic Cd^{2+}. It is important to study the kinetics and extent of intracellular NP degradation and to quantify the release of any (toxic) ions. If degradation is observed, acute cytotoxicity and the induction of ROS should be studied as well.

Interaction with biological molecules

The small size of the NPs results in distinct properties from the bulk form of the same materials. The high surface area and high local charge densities generate a large area which can interact with surrounding biological molecules. When the NPs are subjected to physiologically relevant conditions, the surface charges will favor binding of available serum proteins, leading to a so-called protein corona [110]. The mere presence of serum proteins in the surrounding media of NPs will automatically lead to a tight association of both entities [111], unless a protective NP coating could be employed which inhibits protein attachment. Using fluorescence correlation spectroscopy, Rocker et al. [112] quantitatively studied the binding of human serum albumin to CdSe/ZnS QDs, showing the formation of a 3.3 nm thick protein monolayer on all particles. The binding itself was a dynamic phenomenon where proteins generally resided on the QDs for approximately 100 s. Maiorano et al. [113] found that the type of cell culture medium used had profound effects on the kinetics and constitution of the adsorbed protein layer. The authors further observed that the average composition of the proteins does not reflect their relative abundance in serum. When combined, these results indicate that although NP surface properties may be well controlled, the effective surface with which the particles will present themselves to cultured cells can be highly dependent on temporal and environmental factors [114]. Using polystyrene nanospheres as model particles it was recently reported that both the size of the NPs and their surface properties play very significant roles in determining the formation of the protein corona [115].

In terms of possible biological/toxicological effects, it was further observed by Lacerda et al. [116] or Mahmoudi et al. [117] that the proteins interacting with NPs undergo conformational changes. These conformational changes could have profound effects on cellular well-being since our immune system may then not recognize these proteins as native but rather as foreign objects and may try to eliminate them, inducing then autoimmunity. Binding of serum proteins to NPs may also directly affect their endocytic route and degree of cytotoxicity, as was shown by Yen et al. [118] who linked differences in uptake mechanism and toxicity between gold and silver NPs to variations in serum protein attachment to the NPs.

Next to proteins, NPs can also interact directly with other biological molecules, such as lipids. Lin et al. [119] recently showed that depending on the surface charge density, Au NPs can directly adhere to lipid membranes and induce structural defects, leading to cellular penetration of the particles. This direct penetration into the cell opens a path for intracellular delivery while bypassing the endocytic machinery, but could also induce specific toxic effects.

Effect of NP illumination or magnetic field exposure during live cell experiments

Biological cells are generally exposed to NPs for a specific (biomedical) purpose, such as non-invasive imaging or advanced drug delivery. This requires the NPs to possess certain properties which can be exploited by researchers for the desired applications. Quite often, these traits (fluorescence quantum yield, magnetic moment) are linked with the NP structure and surface properties and will therefore be greatly affected by NP degradation, protein binding or alterations to the immediate NP environment. Furthermore, in order to fully exploit the properties of the NPs, external stimuli are often required (e.g. exposure to a magnetic field or light) in order to put the NPs to full use. However, this external factor can (1) induce alterations in the NPs which lead to potential toxic effects or (2) directly influence cell homeostasis.

For IONPs, alternating magnetic fields lead to local heating of the NPs, which can be used for killing tumor cells [120]. As magnetically guided drug delivery or MRI employs a constant magnetic field gradient, no direct effect on the cells were expected. However, the increased internalization of IONPs due to a magnetic field gradient can induce toxic effects [121]. Additionally, Schafer et al. [121] observed that mesenchymal stem cells exposed to a constant magnetic field at a clinically relevant field strength of 3.0 T in

the absence of IONPs resulted in affected gene expression profiles.

QDs are generally used for biological imaging, where their high absorption in the whole UV range makes them easily excitable with common UV lasers. This is however also associated with possible dangers as UV illumination of QDs can also be employed for photodynamic therapy [122]. Samia et al. [123] further showed that QDs can also interact directly with oxygen, resulting in ROS formation. Recently, several groups confirmed that UV illumination of QDs enhances their cytotoxic effects [15,124]. The photostability of QDs largely depends on surface passivation which is determined by the QD coating molecules. Nida et al. [125] found that amphiphilic polymers provided the best protection of QDs against photo-oxidation.

Gold NPs and especially nanorods hold great promise for thermal cancer therapy since their absorbance peak can be finely tuned by the size and shape of the nanorods [126]. As both heating and optical imaging of nanorods occur primarily by irradiation with near-infrared light [127], possible heating effects of the particles and cells during optical imaging cannot be excluded. Recently, Huang et al. [128] have furthermore shown that extracellular localized Au nanorods can also lead to cell death upon irradiation with near-infrared lasers, indicating that the mere presence of Au NPs, even when not directly cell-associated can already affect cell viability upon irradiation.

Key aspects for improving NP toxicity studies

To date, the field of nanotoxicology is steadily gaining importance, leading to the generation of more and more data. Unfortunately, most data is generated from stand-alone studies where the interactions between a certain type of NP and a certain cell type are investigated by studying a few parameters only. This rarely gives a complete toxicological profile of the NP type and the wide variety in incubation conditions also hinders a direct comparison between obtained results from different studies. In the following sections, several issues are pointed out which could help to optimize nanotoxicology studies.

Nanoparticle characterization

In order to provide more comparative and reliable data on the cytotoxic profile of engineered NPs, a thorough characterization of the NPs is required [129]. A great number of physicochemical properties must be defined for every specific NP type, such as: chemical composition of the NP core and coating, size of the core and surface topography of the NPs (Fig. 6a, left). All these factors will influence the extent of interaction of NPs with biological components to different degrees and are therefore of great importance in understanding any observed effects [130].

Whereas a full characterization of NPs in dry state is indispensable, NP properties in solution (colloidal stability, surface potential, hydrodynamic diameter) must also be carefully characterized (Fig. 6a, middle). For cell labeling purposes, it is furthermore imperative not only to study NP behavior in aqueous media, but to also investigate the behavior of NPs under physiologically relevant conditions, such as serum containing saline (Fig. 6a, right). The presence of salt already causes many NPs to agglomerate, mainly due to electrostatic screening of the NP charge which is essential for colloidal stability. In addition many particles are prone to be covered by proteins, which can alter the interaction of particles with cells [112,114] or induce NP aggregation and lead to extensive covering of the cell surface by NP aggregates [66].

It should be noted that data regarding the behavior of NPs in serum containing media are quite rare as monitoring the stability of NPs in the presence of serum is technically challenging. There is a great need for novel techniques which allow to accurately monitor NP stability as a function of time. Recently, Braeckmans et al. [131] have reported the use of fluorescence single particle tracking to monitor the aggregation state of fluorescent NPs in undiluted biological fluids. Using this method it was, for instance, observed that aggregation of NPs in whole blood was far more outspoken than in full serum, indicating the importance to characterize NP properties directly in the medium that the particles will be applied to.

Standardization of cytotoxicity measurements

Currently, most NP-related cytotoxicity data are generated by individual studies, where a few specific parameters have been monitored for a certain type of NP together with a certain cell type. In order to enhance the comparison of generated data and to enhance our understanding of NP induced cytotoxicity, there is an urgent need for standardization of the protocols used (Fig. 6b). For a specific type of NP, the range of concentrations and incubation times used should always be precisely defined and should preferably be the same for all studies. The cell type used is also of great importance as different cell types, even when closely related, can react quite differently for the same type of nanomaterial [132]. Preferably, those cell types which are most involved in the (future) biomedical applications of the NPs should be tested (e.g. epithelial, endothelial cells), or multiple cells which are derived from the different germ layers (see also the following section). We note that, when investigating cytotoxic effects, the use of cancer cell types should be minimized, as these can lead to aberrant results [5]. Cancer cells have several specific characteristics and altered intracellular signaling pathways which are destined to upregulate proliferation and maintain cell viability, which will make them less prone to some NP-mediated cytotoxic effects.

Standardization is also necessary in terms of the assays used. Cell viability is quite a general term and can be investigated by numerous assays which determine one or more cellular parameters, such as: (1) WST or MTT assays (mitochondrial activity), (2) lactate dehydrogenase (LDH), trypan blue or propidium iodine assay (cell membrane permeability), (3) calcein AM (intracellular esterase activity), (4) fluorescent Annexin V or caspase substrates (apoptosis indicators). These assays are generally suited to measure acute toxic effects of cultured cells and although they can be used to investigate cell viability, the results from one assay cannot be compared directly with the other as they measure different parameters [133]. The nanotoxicity field,

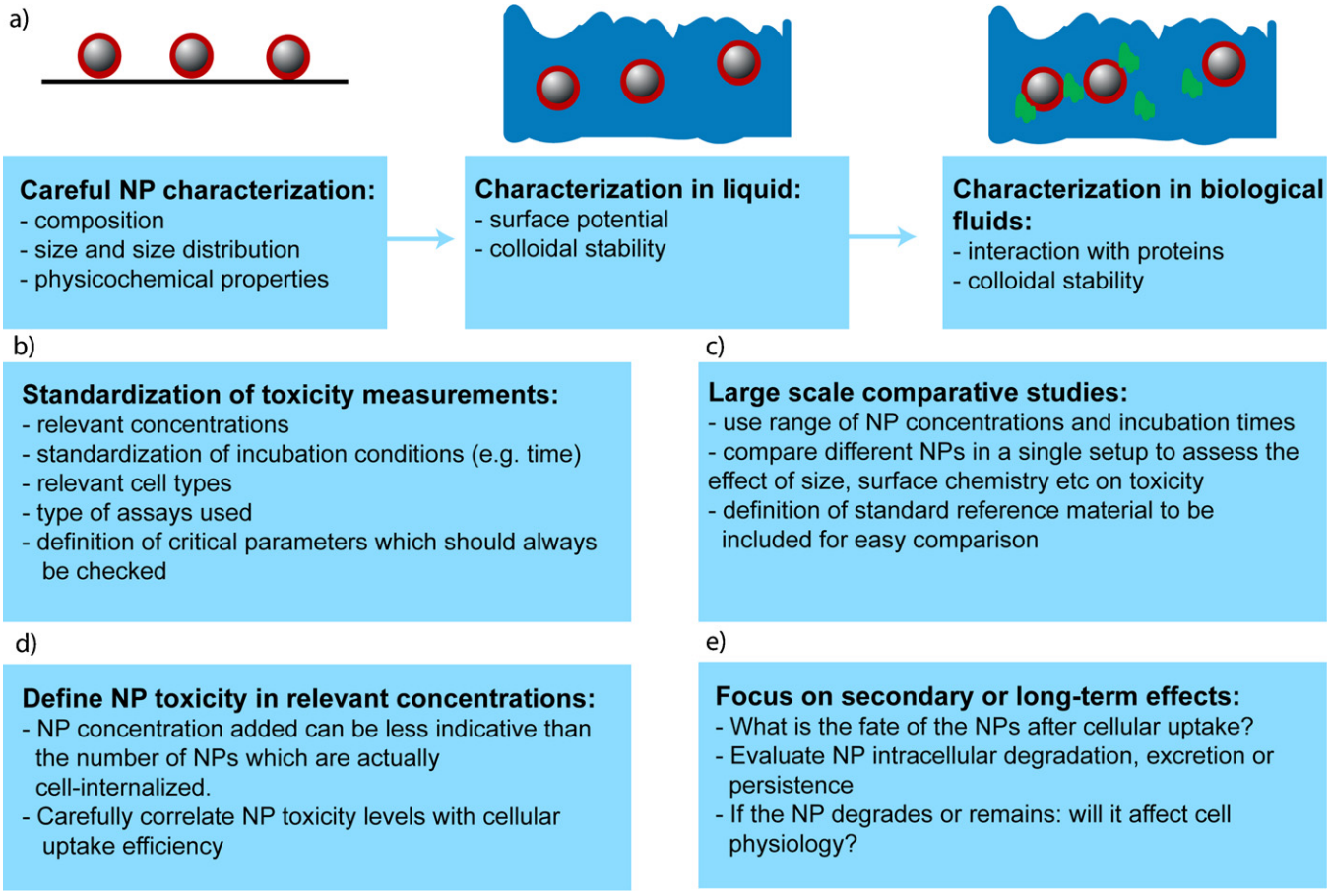

Figure 6 Schematic overview of the key parameters involved in evaluating NP toxicity: (a) NP characterization in dry state (left), in liquid (middle) and in biological fluids (right). (b) standardization of toxicity measurements, (c) use of large scale comparative studies, (d) defining NP toxicity in function of relevant concentration and e) focus on secondary and long-term effects.

therefore, would strongly benefit if the assays used and the parameters which are investigated should be more uniformly defined between different studies. Next to acute cytotoxicity, more uniformity is also needed in terms of the parameters which have to be investigated. NPs can lead to various effects and interact with biological components in numerous ways, making it nearly impossible to cover the whole scale of cell—NP interactions in a single study. Therefore, critical parameters must be selected, for instance: ROS, cellular morphology and cytoskeleton integrity, cell functionality and genotoxicity. Also, the assays should be carried out with great care and adequate controls have to be included as NPs can interact with the assay components or interfere with the readout [12]. For example, fluorescence-based assays can be confounded when evaluating QDs. NP-induced ROS can affect mitochondrial enzyme activity and disturb MTT assays, while LDH assays can be disturbed by NPs that can bind LDH and impede its release into the extracellular medium [133,134]. Furthermore, there is a need for the definition of a reference type of material (e.g. the most frequently studied NP types: dextran-coated IONPs (Endorem®), polymer-coated QDs or citrate-coated gold NPs), either for all NPs in general or for every specific type of NP separately. When novel NP formulations are then being tested, these should be compared to the reference material in order to enhance the transparency of all obtained results.

Set up of large scale comparative studies

In order to adequately address all questions regarding NP-mediated cytotoxicity, standardization of the assays and incubation conditions is not sufficient in itself. A next step should comprise large scale comparative studies, where a range of concentrations and incubation times are used in order to efficiently assess the LD_{50} of the NPs and the effect of time (Fig. 6c). The latter factor is also quite important as several effects have been reported to sometimes be only transient [70], or sometimes the effect takes several hours or days before it is observed [21]. Also, the use of multiple cell types, preferably both primary cells and established cell lines would be needed to generate data which is more representative for a variety of cells. For NPs which are to be intravenously injected, their effect on endothelial cells, macrophages and liver or immune cells would be very interesting as these will be most representative for future *in vivo* applications.

Based on such studies, for instance the effect of size, surface charge and surface topography of the NPs could be

more rigorously investigated by simply including a variety of NPs with slightly different properties [11]. However, the concept of individual cell exposure must be considered, i.e. evaluation of probabilistic effect on individual cells rather than the average effect on the cell population since the classical biological tests cannot measure a biological effect by NPs at not toxic (very low) concentrations.

Defining toxic NP concentrations in terms of different parameters

Nanoparticle toxicity issues are commonly linked to concentration-dependent effects, where a higher number of NPs will logically augment the risk for any toxic effects. One problem with toxicity studies is the applied definition of ''concentration'', which can be used to signify either the mass or the number of particles per unit of volume. Currently, many researchers report their findings in a mass-concentration (μg/mL) to indicate the amount of nanomaterial with which the cells were incubated. However, depending on the particle size, the particle number and the total surface area can differ by several orders of magnitude [135]. As NP-based toxic effects have been associated with the available surface area [136], this can drastically alter the so-called intrinsic toxicity of a certain type of NP. From a toxicity perspective, expressing the obtained result in terms of the total number of particles with their size (distribution) would therefore be much more relevant, since the total surface area and mass readily follows from that.

Furthermore, most studies link cytotoxic effects to the number of particles which were added to the medium in which cells are cultured. However, similar to pharmaceutical components, the functional amount of product is more relevant than the amount of product added. For NPs, their use often depends on cellular internalization, in which case internalized and surface-attached NPs are functionally relevant whereas non-cell associated NPs are irrelevant (Fig. 6d). Many cytotoxic effects are also dependent more on the number of internalized NPs rather than the number of NPs added to the cell medium, although the latter can also induce toxic effects, for instance, by severe aggregation and covering of the cell surface or leaching of metal ions. Taking the internalized fraction of particles into account is for example essential when comparing two NPs with identical cores but different coatings. Consider for instance NPs A and B, each with an identical core but a different surface coating. NP A results in 2000 NPs/cell and induces 30% toxicity, while NP B results in 500 NPs/cell and induces 20% toxicity when incubated under the same conditions. Based on these data, NP A could be considered as the most toxic one and therefore the least interesting NP type for the envisioned application. However, NP A is taken up 4 times more avidly, while only causing 10% additional toxicity. It is therefore quite likely that when altering the incubation conditions, more NP A particles will be cell-internalized than NP B particles when both reaching 20% toxicity (1333 vs. 500, when making the assumption that the relationship between concentration and toxicity is linear). Or to put it differently, in case both reach 2000 NPs/cell, NP B will be far more toxic (e.g. 30% vs. 80% toxicity). For any biomedical application requiring higher doses of NPs, NP A would therefore be the most suited one, whereas NP B is more suited in case cellular uptake is not essential. In conclusion, it is important to link cytotoxicity data both with the amount of NPs added as well as with the internalized number of NPs over time. Important in this regard is also the development of methods which allow to accurately assess the intracellular NP concentration as most methods currently used (inductively coupled plasma, fluorescence intensities, spectrophotometric determinations) do not allow to discriminate between cell-internalized and surface-bound NPs.

Focus on secondary or long term effects

One key issue which still remains in nanotoxicology is the long term effect these NPs may have on biological organisms (Fig. 6e). Acute cytotoxicity can be easily assessed in a straightforward manner, in particular when high (and often unrealistic) doses are used. However secondary or long term effects can be more diverse and less outspoken [70]. More data are required where the effect of the NPs on cell homeostasis is evaluated after several days and even weeks. Important in this regard is the intracellular stability of the NPs. If the NPs would undergo dramatic structural changes or even completely degrade as a result of their intracellular localization, their effects on cell viability and functionality could be dramatically changed [21,136]. Alternatively, the high stability and persistence of Au NPs may also activate cellular defense mechanisms such as autophagy [68] or lead to particle excretion. Therefore, more methods are required which allow to efficiently monitor the intracellular localization and structure of NPs as a function of time.

Possible setup for studying cellular NP toxicity

The wide versatility in NP toxicity studies makes it rather difficult to try and define the optimal method to study NP cytotoxicity. Based on the key aspects to improve NP toxicity studies and the common effects of NPs on cultured cells, we propose the following scheme (Fig. 7) as a possible blueprint for any cellular NP toxicity studies based on a model system described in previous studies [5,30].

Several questions which are essential to be answered are indicated on this scheme. First, NP properties must be carefully characterized both in dry state as well as in physiologically relevant media. Next, a choice must be made in terms of which cell types are to be used in the study. Various cells with different physiology will generate a more global overview of the possible NP effects. Cellular uptake of NPs must be evaluated and preferably quantified. In case the NPs are well-internalized, acute toxicity can be assessed, preferably by multiple assays (e.g. MTT, LDH and calcein assay). It is important to get comparable readouts for the different assays and by doing this, exclude any interference of the NPs with one of the assay components.

If non-toxic concentrations of the NPs can be found, the induction of ROS, effects on cell morphology and functionality and NP degradability must all be evaluated. For cell functionality, the use of cell models, such as the PC12 cells [5,21,30] which allow a rapid and quantitative determination of any effects are highly beneficial compared to the more typical cell differentiation protocols which often

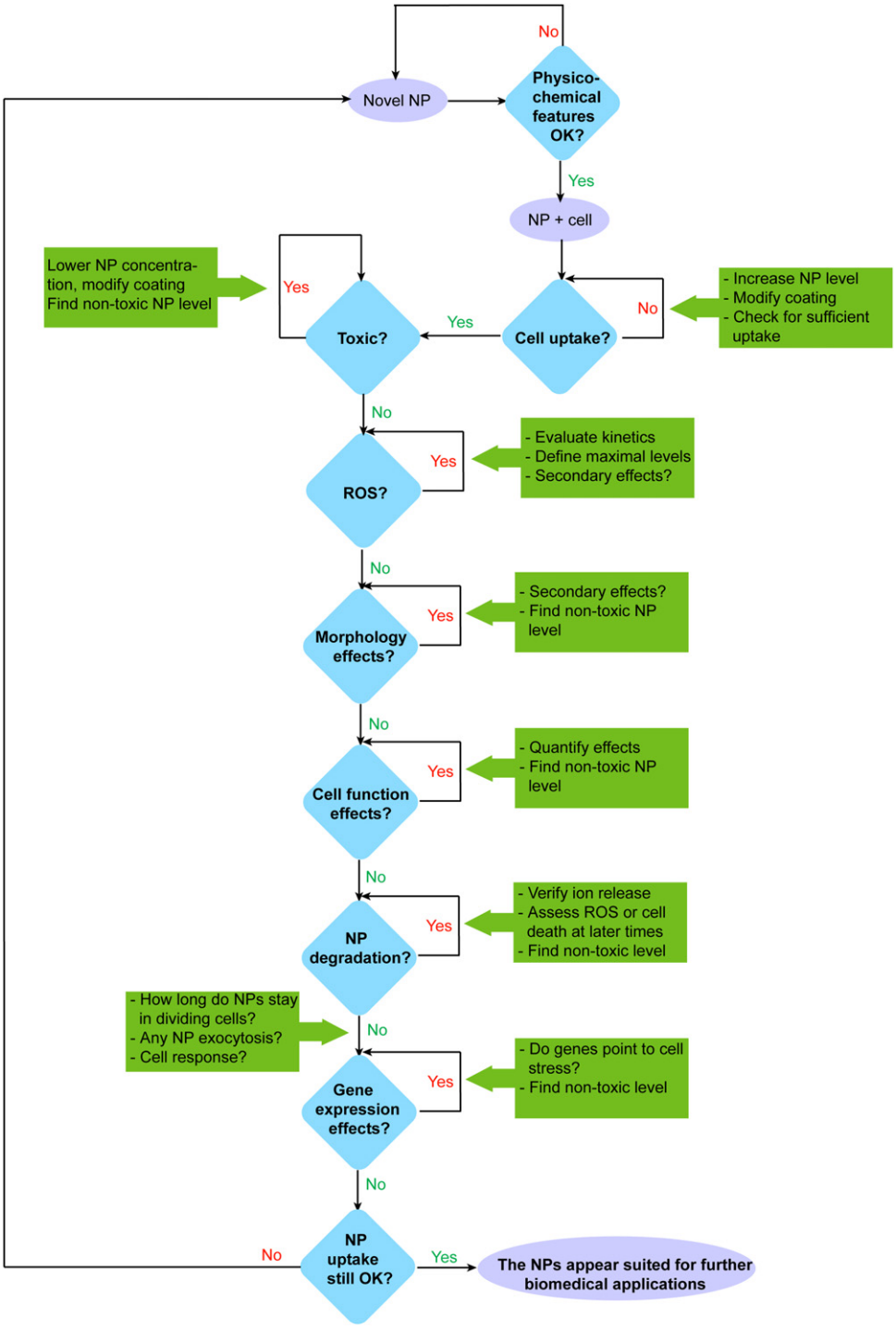

Figure 7 Schematic overview of a possible workflow in the design of optimized cellular NP toxicity studies. Please see the main text for more details.

take several days, are associated with high number of cell death and often rely on less quantitative immunostaining for cellular markers [133]. For studying NP degradation, lysosomal model systems can be used rather than actual cells as this will allow to study this issue more easily [21]. Only in case conditions (concentrations, incubation times, etc.) can be found at which no effects can be observed caused by the NPs, these particles may be further tested for safe biomedical use. It is however important to first verify whether at the incubation conditions required for a complete absence of toxicity, the NPs are still sufficiently internalized to concentrations which still allow to perform the envisioned biomedical application. In case the NPs lead to one or more toxic effects, the incubation conditions must be altered (e.g. lower concentrations) in order to find suitable non-toxic conditions. In case no such conditions can

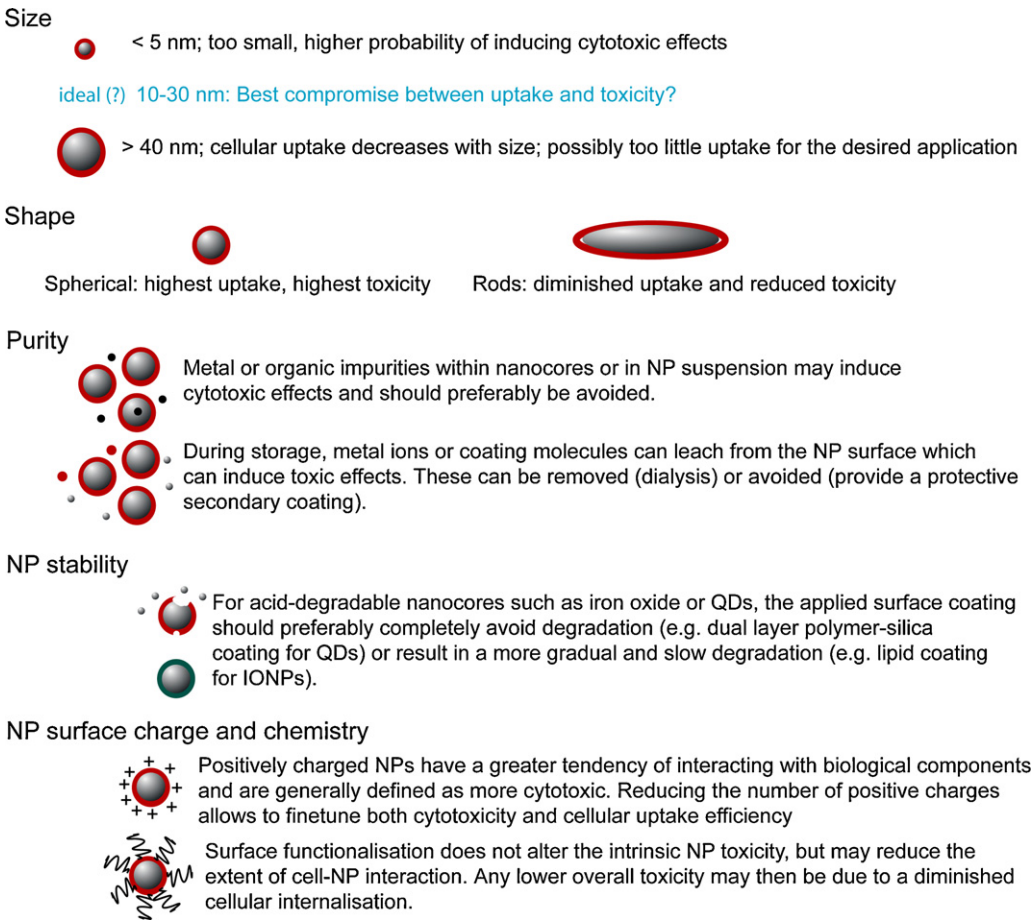

Figure 8 Schematic overview of the relevant NP characteristics which can be controlled in order to improve biocompatibility: size, shape, purity, intracellular stability and NP surface charge and chemistry. Please see the main text for further details and interpretations.

be found or when these lead to greatly reduced NP uptake, these NPs would not seem to be well suited in biomedicine and should first require some optimization (e.g. application of a new protective coating layer) prior to further use.

Optimizing NP characteristics to diminish toxicity

The number of NPs which are being developed for biomedical applications is steadily increasing. From nanotoxicology studies, it is possible to distillate several parameters which may be influenced during the production process in order to generate particles with improved biocompatibility. The following paragraphs outline several issues which can be considered during the development of novel nanomaterials.

NP size and shape

NP size is a very important parameter in both cellular internalization efficiency and cytotoxicity (Fig. 8). This has been shown in numerous publications, indicating, for instance, the differences in subcellular distribution and toxicity between red and green emitting QDs [137] or differences in cell death pathway initiated by Au NPs of 1.4 nm (rapid necrosis) and 1.2 nm diameter (apoptosis) [138]. In general, NPs below 5 nm diameter can be considered as being the most hazardous due to possible nuclear penetration and very high surface area over volume ratios and very small sizes should therefore preferably be avoided. IONPs and Au NPs can be made with bigger cores, whereas for QDs, a dense stabilizing coating on top of a protective shell layer can also increase the NP diameter. According to one study, an increasing diameter (over 40 nm) is accompanied with a diminished internalization efficiency which is accompanied by less pronounced cytotoxic effects [133]. Although further studies are needed here, this could indicate that NPs of 10—30 nm diameter seem to be an optimal choice, depending on the desired NP characteristics.

Furthermore, the NP population should preferably be rather monodisperse in order to get representative and reproducible data. More controlled synthesis protocols are required in order to allow such high level of uniformity. The surface properties of the NPs should then also be tailored very carefully so as to completely avoid any particle aggregation. NP surfaces should also be produced to minimize protein adsorption, or alternatively, lead to a well-controlled and reproducible protein corona of certain composition and thickness.

Less attention has been paid to the effect of NP shape, arguably because not all particles are easily prepared in a variety of shapes. Fe_3O_4 NPs, for instance, are typically spherical NPs with some rare exceptions. The same is true for QDs and Au NPs, although these NPs can also be manufactured in more rod-like structures with different aspect ratios. QDs with higher aspect ratios were found to diffuse much slower in the plasma membrane than spherical NPs [139]. Similar data have been observed for Au NPs, where nanorods have been show to be less toxic than spherical NPs [14,140]. Hauck et al. [141] confirmed these results and reported that Au nanorods induced only negligible cell death even at high incubation concentrations. As the cellular internalization efficiency of Au nanorods also diminishes for greater aspect ratios [14], it must still be evaluated whether the lower toxicity is due to the particle itself or to a decrease in cell internalization.

NP purity

During preparation of the NPs, it is imperative that the final product is as pure as possible when applied to the cells. Any impurities such as metal ions or organic stabilizers can diminish cell viability even when the NPs themselves would be 100% biocompatible. Post-production processing can remove most of these residual ions, but in some cases up to 15% residual metal were found in purified samples [77]. Further purification can overcome this problem, but the purification strategies as such can also induce novel contaminants or affect the NP stability [77]. The effect of time must also be considered as during storage of the NPs stabilizers or metal ions can be leached from the NP surface. For instance, Alkilany et al. [42] have shown that CTAB, which is used to stabilize Au NPs, induces strong cytotoxicity when it detaches from the NP surface. A full characterization of the NPs and any contaminants are highly important in order to interpret any cytotoxic effects correctly. For most NP types, leached ions and stabilizers can be largely removed by means of dialysis against physiological buffer as an efficient purification strategy shortly before applying the NPs to cells.

Surface properties

NP stability

The intracellular stability of the NPs is typically determined by their inherent core-properties. Iron oxide NPs have been described to break down relatively easily when subjected to endosomal conditions [21], whereas QDs break down more slowly [102] and Au NPs appear to be highly stable [105]. The kinetics of degradation depend to a large extent on the available surface area (the larger the exposed surface area to the cellular microenvironment, the faster the degradation) and the type of coating applied. For Au NPs, the high persistence of the core itself does not require any special coatings in this regard. Iron oxide NPs will generate Fe^{2+} upon degradation, which can be well tolerated by cells up to certain levels. In order to impede degradation and promote a more slow generation of ferric ions, larger NPs (10—30 nm diameter) coated with lipid systems, polymers or silica shells are preferred compared to small molecules (e.g. citrate coatings) [21]. For QDs, phototoxicity or acid etching of the QD surface resulting in leaching of Cd^{2+} ions must be avoided at all costs considering the high toxicity of cadmium ions. In this regard, amphiphilic polymers or silica coatings have been described to provide a decent protection against light and oxygen exposure [125]. Recently, Hu and Gao [142] have demonstrated that silica—polymer dual-layer encapsulated QDs are highly resistant against acid degradation, offering a lot of potential in diminishing QD cytotoxicity.

NP surface charge and chemistry

The protective coating further greatly determines the physicochemical properties of the NPs, such as the total size and surface charge. The charge itself is of great importance for determining the colloidal stability of the NPs and to avoid aggregation. Furthermore, NP surface charge will also directly influence the extent of cell—NP interactions and toxic potential of the NPs. In general, cationic particles have been described to be the least stable and exert the greatest cytotoxic effects [77,143]. For IONPs, it has been shown that by carefully controlling the number of positive charges on the NP surface, both cellular uptake and cytotoxicity could be well-controlled and optimal results were obtained with IONPs containing only 3% positive charges on their surface [144]. Of course, the reduction in positive charges might accompany a diminished cellular internalization, but it is important to find the optimal balance between a lack of toxicity and internalization efficiency.

Further NP functionalisation with peptides or pharmaceutical agents can have profound effects on the physicochemical properties of these NPs. For QDs, it has been shown that the functionalising group which was introduced at the NP surface has a great effect on NP toxicity with organic coatings resulting in the most toxic NPs and carboxyl coatings being the best tolerated by cells [90]. Chang et al. have furthermore shown that the addition of PEG to the QD surface does not alter inherent QD toxicity, but results in a decreased intracellular uptake, which is then associated with a diminished cytotoxic response [145], which again emphasized the need to interpret cytotoxic responses in terms of cellular uptake levels.

Conclusions and perspectives

To date, the question remains whether inorganic NPs such as QDs, IONPs or Au NPs are safe to be used for biomedical purposes. More and more data are becoming available regarding NP toxicity, but a lot of effort is still required in order to truly advance our knowledge in this field. Most data are derived from stand-alone studies, where a single type of NP with specific physicochemical properties is delivered to a certain cell type under arbitrarily selected conditions. As discussed in the present work, standardization of incubation conditions, careful characterization of NPs in their biologically relevant environment and large scale comparative studies could be a first step in increasing our understanding in this field.

Especially in the case of *in vivo* applications, a lot of research still needs to be done to generate sufficient data. Another important field is the ecological impact of inorganic NPs. Whereas, most studies focus on the biomedical

applications of these particles and expose cells directly to these NPs, the main use of inorganic NPs lies in technological applications. A great number of NPs are then widely distributed into the air or soil and can be ingested by bacteria or enter our food chain [146]. A recent study by Werlin et al. [147] has furthermore shown that engineered nanomaterials such as QDs can be transferred from prey to predator in the typical food chain. Furthermore, the QDs were shown to be biomagnified, resulting in higher concentrations in organisms higher up the food chain.

The increasing use of NPs in present industry and medicine therefore warrants a careful assessment of the possible negative effects associated with these particles. The use of these NPs should be dealt with great care and time and effort should first be spent on investigating the possible impact of these nanomaterials in order to allow them to be used in a safe and well-controlled manner for the benefit of mankind.

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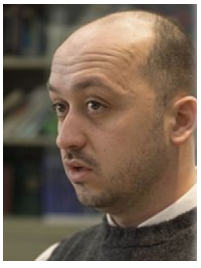

Stefaan De Smedt (1967) studied pharmacy at Ghent University (Belgium) and graduated in 1995. He joined the pharmaceutical development group of Janssen Research Foundation. In 1999 he became Professor at Ghent University where he is chairing the Ghent Research Group on Nanomedicines. Since 2004 he serves as European Associate Editor of the Journal of Controlled Release. His research is at the interface between drug delivery, biophysics, material sciences and advanced optical imaging. He received the 2006 CRS Young Investigator Award and the 2010 APV Research Award for Outstanding Achievements in Pharmaceutical Sciences. He is scientific founder of spin-off Memobead Technologies.

Kevin Braeckmans obtained an MS in Physics at Ghent University (Belgium) in 1999, and received his Ph.D. from Ghent University (Belgium) on advanced optical microscopy methods for which he received first price (FBBF) in 2005. He stayed at the group of Prof. Braüchle at the Ludwig-Maximilians Universität München developing algorithms for single particle tracking. Later, he returned to Ghent University as a FWO postdoctoral researcher. In 2008 he became a professor at the Faculty of Pharmaceutical Sciences of Ghent University where he is leading the Biophotonic Imaging Group, focusing on microscopy techniques for measuring molecular dynamics in gene therapy.