

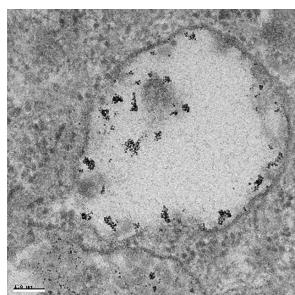
(Intra)Cellular Stability of Inorganic Nanoparticles: Effects on Cytotoxicity, Particle Functionality, and Biomedical Applications

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NP degradation:
-Release of toxic ions?
-Loss of NP function?
-Cancer-cell specific toxicity?

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1. INTRODUCTION

In recent years, the growth in the field of nanotechnology has stimulated many other areas, which eventually may involve everyday life uses as well as high-tech biomedical applications.^{1–3} Nanoparticles (NPs) constitute one of the most important “ingredients” in nanotechnology. Due to their small size, NPs display many unique chemical and/or physical properties that can differ quite extensively from the properties of the corresponding bulk material.⁴ A broad variety of materials that can be made on the nanosize scale, together with effective control over NP size, shape, and surface, has led to a huge library of NPs that offers a lot of potential for use in a plethora of future biomedical applications.⁵ Biomedical areas in which, despite many unsolved problems, the use of NPs holds promise include controlled drug release, targeted anticancer therapy, and development of contrast agents for noninvasive whole-body imaging.^{6,7} The strong interest in the exploitation of these NPs for biomedical purposes has also instigated concerns with respect to the possible drawbacks and potential dangers associated with their widespread distribution in the human body.^{8–12} As a result, the increasing interest is reflected by an increasing number of studies on “nanotoxicology”.

Given the relatively recent emergence of nanotoxicology as a research domain, this area is still in development and requires further optimization.¹³ The thriving industrial interest and onset of commercial use of NPs (e.g., in electronic devices, paint, etc.) call for urgent measures and appropriate legislation to be installed.¹⁴ This, in turn, should be based on scientific data that would allow for drawing conclusions concerning the safety of NPs. To date, the progress in the area of nanotoxicology does not keep up with the pace with which the field is advancing. In other words, more NP formulations exist than have been tested and comprehensive methods to measure in real time or to predict the behavior of a new type of NPs in a biological environment is still only in its developmental stage. Analyzing the interaction of NPs with living cells or organisms is hampered by a combination of factors including (i) the broad scale of different types of NPs, varying in chemical composition, size, shape, and surface coating,^{15,16} (ii) the large number of different cell types that can be used for testing that often interact with different NPs in a cell-type-dependent manner,¹⁷ (iii) the lack of standardized protocols for cell exposure (variations in incubation times, NP concentrations, ...),^{18,19} (iv) the lack of optimized methodologies for testing NP toxicity, where analysis is further complicated by undesired interactions of the NPs with the assay readout,^{20,21} (v) the need for unbiased methods to study NP behavior in the complex intracellular environment,¹³ (vi) the need for better analytical techniques to characterize NP properties and accurately define NP concentrations,²² and

(vii) the need for more appropriate and complex models to study NP toxicity in conditions that more adequately mimic the *in vivo* environment.²³

Several efforts in trying to optimize the methodology applied in the area of nanotoxicology have been made, including the use of high-throughput screening methodologies and theoretical modeling to keep up with the pace with which novel NPs are being developed.²⁴ In line with this approach, important common pathways involved in nanoinduced toxicity have been put forward as a key area to study since reliable and comparable data can be obtained by means of fast screening approaches.^{25,26} It has been suggested to focus on more subtle interactions, such as alterations in cell signaling,²⁷ genotoxicity,²⁸ or possible mutagenicity²⁹ of the materials as these effects would pose serious health hazards on any exposed individual. The use of multiparameter methodologies has also been put forward, where NPs are tested on multiple cell types for a variety of parameters under well-controlled conditions, allowing nontoxic concentrations to be defined based on a multitude of parameters and enabling a direct comparison of results obtained for different NPs tested in the same setup.^{18,30–32}

Nanotoxicological studies furthermore require a careful characterization of the NPs in order to generate reliable data, as the degree of agglomeration and sedimentation³³ or the presence of contaminants may affect the level of toxicity.^{34,35} Although many techniques are available to characterize NPs in a dry state or in simple liquids, there is an urgent need for appropriate techniques that will enable us to track NP properties in a subcellular environment as a function of time.^{13,22} As these NPs come into contact with cells, they are exposed to many changes in terms of ionic strength, pH, and protein concentrations and compositions, all of which will influence the NP properties and their interactions with the cells. Due to the low availability of validated technologies that allow studying NP behavior in an intracellular environment, this aspect has only recently received more attention. In this connection, it has been demonstrated that the local intracellular microenvironment to which the NPs are exposed can have significant effects on the colloidal, chemical, and biofunctional stability of the NPs,³⁶ resulting in NP degradation, which may lead to increased toxicity levels and loss of NP functioning and properties. A recent study has shown that when NPs were forced into the cell cytoplasm rather than being endocytosed, the toxic effect of all NPs tested could be largely overcome, indicating the endosomal presence of NPs to be a main cause of NP-mediated toxicity.³⁷ Given the clear problems that are associated with increased toxicity or loss of NP functionality, the present review aims at providing an overview of the current knowledge and understanding concerning intracellular NP stability and the effects thereof on cell viability and NP functionality demonstrated by four commonly used metal-based NPs: iron oxide NPs (IONPs), quantum dots (QDots), silver NPs, and ZnO NPs. Additionally, the degradation of NPs causing the gradual release of metal ions has also been described to elicit potent anticancer activity.³⁸ The present work will therefore also focus on data describing IONP, QDot, silver NP, and ZnO NP degradation and their potential use in anticancer therapy. The impact of NP degradation will be further discussed by comparing the effects of the four degrading inorganic NPs to Au NPs, which are chosen as a model system for nondegrading NPs.

2. BIOMEDICAL AND BIOTECHNOLOGICAL APPLICATIONS

The particular properties of these NPs have led to a series of applications, both in biomedical and in biotechnological areas. Please note that use of NPs in clinics does not necessarily involve NP administration to humans for therapeutic treatment or diagnostics. In particular, in the field of diagnostics NPs offer useful properties for a variety of assays (outside the human body), for example, for the detection of biomarkers. In the next sections, some applications of these NPs are highlighted.

2.1. Bioapplications of IONPs

IONPs possess useful properties for a variety of life science-related applications, comprising both basic as well as clinical research.^{39,40} For about 2 decades, IONPs have been used as FDA-approved contrast agents (dextran-coated Endorem (EU),⁴¹ Feridex (USA)⁴¹) in magnetic resonance imaging (MRI)⁴² for the detection of liver pathologies.⁴¹ Recently, a novel formulation of superparamagnetic IONPs coated with a carbohydrate shell (Ferumoxytol) has been FDA approved for the treatment of iron deficiency anemia in adults with chronic kidney disease.⁴³ A wide variety of IONPs are being tested for the *in vitro* labeling of cultured stem cells and their subsequent *in vivo* tracking by MRI after transplantation. Several of them are undergoing clinical trials.^{44,45} IONPs also have huge potential for functional MRI assays.^{46,47} In addition to their use as MRI contrast agents, the magnetic properties of IONPs can also be exploited in cancer treatment by targeting them to tumors by means of an extracorporeal magnet (Figure 1).^{48,49} By alternating magnetic fields local tissue can be destroyed by generation of heat (magnetic hyperthermia^{50–53}) or drugs can be locally trapped at the tumor site.^{54,55} While this method has been successfully used in veterinary medicine, clinical use in humans is still awaiting realization. IONPs can furthermore be used for magnetic separation and isolation of specific cell types or for enhanced gene or drug delivery.⁵⁶ IONPs are often also suggested as highly versatile theranostic tools where a single type of NP can serve both diagnostic and therapeutic purposes including follow-up of therapy.^{57–59}

2.2. Bioapplications of QDots

The brightness and high photostability of semiconductor QDots render them highly suitable for fluorescence-based imaging.⁶⁰ Their size-dependent, narrow emission bands allow efficient multiplexing, resulting in the separate detection of multiple-sized QDots by a single excitation source. Initially, QDots were employed as novel probes for fixed cells that enabled staining with reduced photobleaching compared to traditional organic fluorophores.^{61–63} Further development in this direction has led to the use of QDots for the simultaneous detection of multiple biomarkers in tissue sections, which was found to enhance the prediction of their clinical outcome (Figure 2).⁶⁴ Reduced photobleaching turned out to be particularly beneficial for live single-molecule tracking of membrane-bound receptors.^{65–67} It needs to be pointed out that, similar to other NPs, QDots are typically endocytosed by cells.^{68,69} This has been extensively used for long-term cellular tracking, cell fate mapping, and markers for identifying cells of different species.^{70,71} Concerning *in vitro* delivery recent advances in endosome escape strategies by employing disrupting peptides have facilitated their use for targeted intracellular applications,^{72,73} but this field still requires more extensive research as these polymers will facilitate endosomal

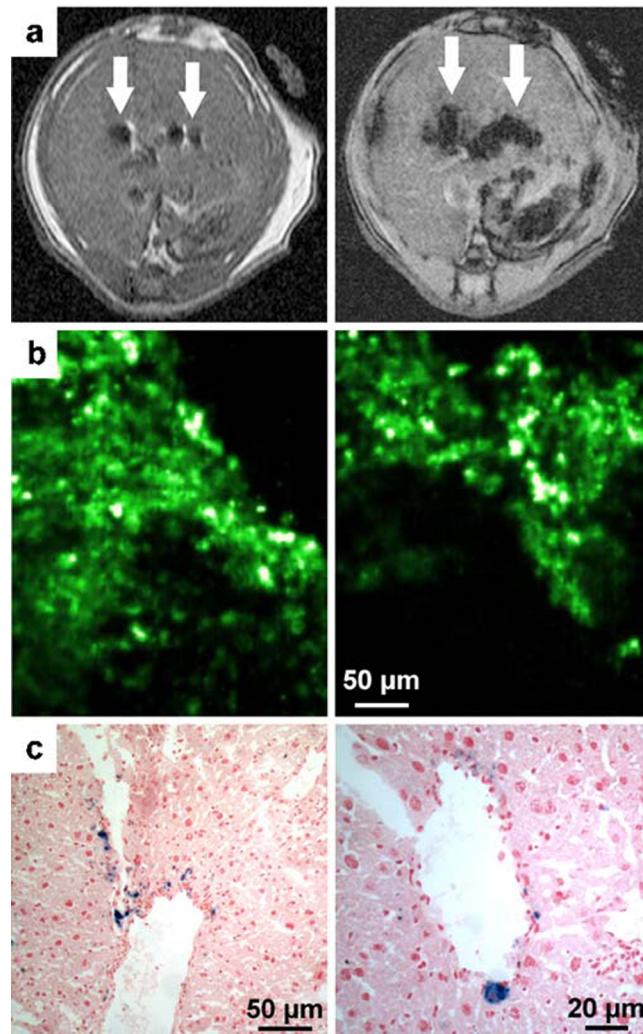


Figure 1. Mouse bearing an external magnet, imaged at day 10 following the intrasplenic injection of 2×10^6 Huh7 hepatoma cells labeled with anionic IONPs and fluorescently tagged with the membrane marker PKH67. Axial MRI pictures acquired using T_1 -weighted (left) and T_2 -weighted (right) GE sequences. Portal branches of the liver show a hypointense signal on the T_1 -weighted image, which is amplified on the T_2 -weighted image (white arrows). *In vivo* confocal fluorescence microscopy performed immediately after MR procedures. Fluorescent-labeled cells are observed in rows, consistent with the perivascular location of labeled cells. Histology of liver slices (Perl's staining) confirms the presence of Prussian blue-positive stained cells immediately in contact with main portal branches, consistent with perivascular topography. Reprinted with permission from ref 49. Copyright 2009 European Society of Radiology.

escape but will not determine intracellular targeting. QDots are also evaluated as *in vivo* probes for fluorescence imaging^{74,75} and hold appreciable promise for early cancer imaging and detection.^{76–78} In this connection, *in vivo* toxicity of the QDots needs to be considered as well as limited retention times and their specific biodistribution.⁷⁹ Recent studies, however, suggest that toxic effects can be tolerated under some circumstances as will be detailed later.⁸⁰ Apart from imaging, QDots have been also suggested for therapeutic applications, in combination with established drugs,⁸¹ or used directly for anticancer purposes by means of photodynamic UV or photothermal therapies.^{82,83}

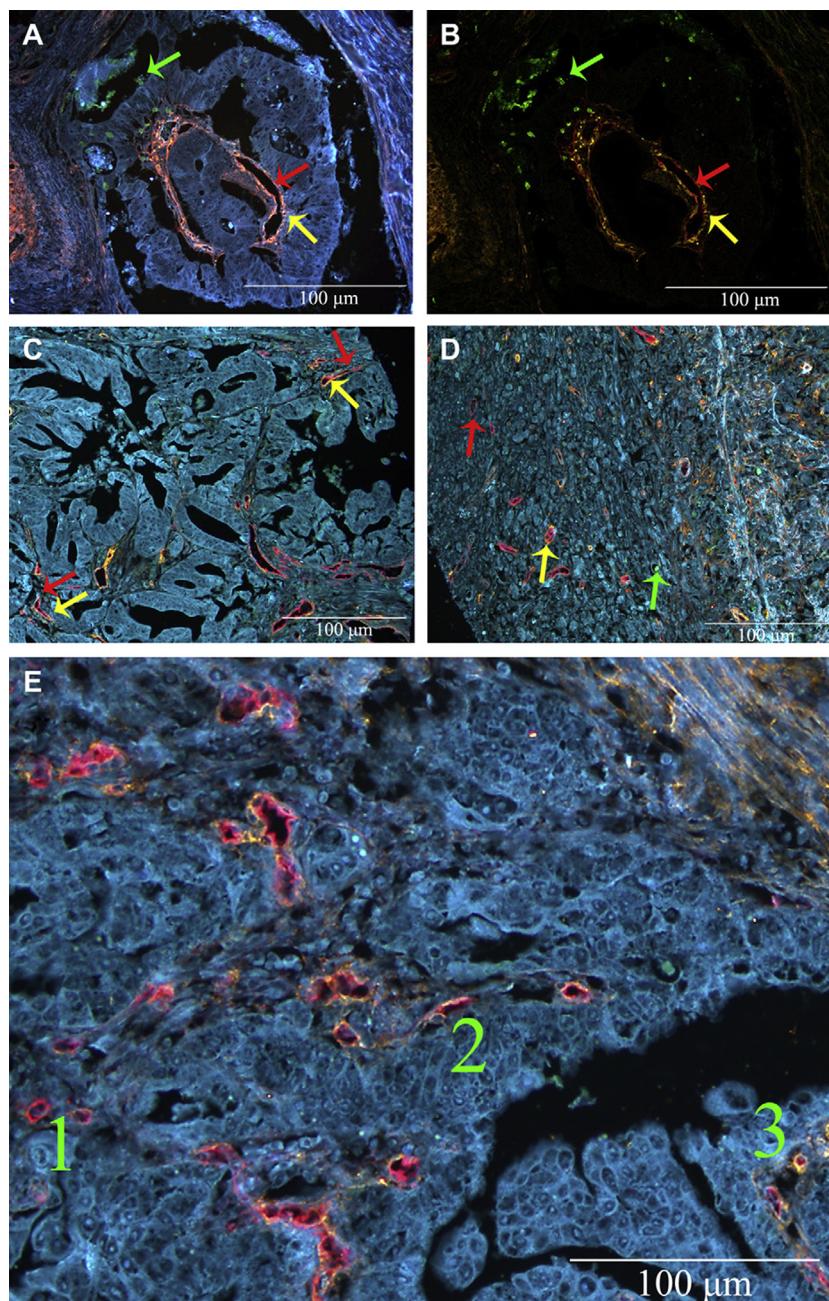


Figure 2. Multispectrum imaging indicates the maturity of neovessels. (A) Multiplexed imaging of neovessels (red), type IV collagen (yellow), and infiltrating macrophages (green) by targeted QDots of various sizes. (B) Corresponding unmixed image of A obtained by spectrum analysis with distinctive autofluorescence background that showed the spatial relationship between endothelial cells and type IV collagen. (C, D) Improved neovessels maturity was found in GC tissues with low macrophages density (C) compared with high macrophages density (D). (E) Immature neovessels (1), neovessels with intact type IV collagen (2), and mature neovessels (3) were showed in the same sample: (red arrow) neovessels; (green arrows) macrophages; (yellow arrows) type IV collagen. Scale bar: 100 μm . Reprinted with permission from ref 64. Copyright 2012 Elsevier.

The combined use of their fluorescent properties for diagnosis and therapy makes QDots promising theranostic tools.⁸⁴

2.3. Bioapplications of Silver NPs

Silver NPs are most frequently used because of their potent antimicrobial properties,^{85–87} which has led to their use in deodorants, clothing, and food packaging materials.⁸⁸ The antimicrobial property of silver NPs has further been exploited in various biomedical applications,⁸⁹ including dentistry, where silver NPs are used in treating dental plaque biofilms.⁹⁰ Silver NPs are also used for the treatment of infected wounds,⁹¹ where silver NPs have been found to promote wound healing

by accelerating re-epithelialization and improving collagen fibril alignment.⁹²

On the basis of the localized surface plasmon resonance (LSPR) of silver NPs, the NPs have also been used in various imaging applications, where the *in vivo* detection of silver NPs by photoacoustic imaging is depicted as a very promising and sensitive platform.⁹³ Silver NPs are also used for ultrasensitive label-free detection of biochemical markers, such as circulating RNA by surface-enhanced Raman-spectroscopy.⁹⁴ By combining silver NPs with drugs or using photothermal therapy, the NPs have been suggested as theranostic tools for both

diagnostic detection and therapy.⁹⁵ Silver NPs are furthermore investigated as potential anti-HIV agents given their high antiviral efficacy.⁹⁶ In this connection, it has been shown that silver NPs bind the viral envelope glycoprotein gp120 and thus inhibit the virus from binding to and entering host cells.⁹⁷

2.4. Bioapplications of ZnO NPs

ZnO NPs are currently among the most widely used NP types in daily life due to their high UV absorbance and near-transparent appearance. They are frequently used in many types of sunscreens for better protection against harmful UV-B radiation.^{98,99} ZnO NPs have been also reported to exert antibacterial properties,¹⁰⁰ although unlike silver NPs, ZnO NPs cannot be considered as a general antibiotic given the clear resistance of some commensal bacteria strains.¹⁰¹ ZnO is also used in ointments and creams for the treatment of diaper dermatitis¹⁰² and commonly used in combination with eugenol as dental cements for root canal fillings.¹⁰³

Furthermore, ZnO NPs are increasingly explored more for medical applications, where, for instance, fluorescent ZnO nanowires have been demonstrated to enable *in vivo* visualization of tumor vasculature.¹⁰⁴ Alternatively, ZnO nanorods targeted toward tumor cells can be detected by optical microscopy or even the naked eye by emission of purple light, facilitating detection of the tumor during tumor resection.¹⁰⁵ ZnO NPs can also be used in cancer therapy, either through the generation of reactive oxygen species after UV irradiation^{106,107} or by their inherent toxicity.¹⁰⁸

2.5. Bioapplications of Au NPs

Au NPs have a long history of clinical use in the treatment of rheumatoid arthritis.¹⁰⁹ Au NPs are also one of the most widely studied types of inorganic NPs with regard to new clinical applications.¹¹⁰ There are several main application routes of Au NPs. In a generic way Au NPs are used as “biocompatible” delivery vehicles, as demonstrated by current clinical trials such as the delivery of tumor necrosis factor alpha (TNF α) by means of PEGylated Au NPs (Aurimmune; CytImmune Sciences, Rockville, MD), which was found to significantly enhance specific tumor targeting efficacy compared to free TNF α .¹¹¹ More specifically, Au NPs are used because of their plasmonic properties, which can be harnessed as well in diagnostic as in therapeutic applications. Au NPs show a lot of diagnostic potential as contrast agents for optical imaging and spectroscopy using Au NPs as surface-enhanced Raman probes,^{112–114} where their therapeutic potential lies in photothermal therapy.^{112,115–117}

3. NANO(CYTO)TOXICITY

The widespread use of the NPs described brings along the risk of chronic exposure to increasing levels of these compounds, which calls for a thorough understanding of the possible side effects that may occur. Miniaturization of materials down to the nanoscale, which is in the order of the size of proteins, affects their physical properties but also alters their toxicological profile in comparison to their bulk counterparts.^{19,118} This can be largely attributed to their size only, as NPs display a high surface area over volume ratio, which increases exposure and the level of interaction of the material with their immediate surroundings.^{22,119} Their small size furthermore enables them to reach intracellular structures, such as, for instance, under special circumstances the nucleus, that are inaccessible for larger sized materials.^{120,121}

Despite the enormous diversity in NP types, the small size of metal- and metal-oxide-based NPs appears to result in several common effects on cell physiology,¹⁸ with the induction of reactive oxygen species (ROS) as the main contributor.^{25,122} Furthermore, NPs are known to interact with proteins and affect their conformation and functionality.^{123–130} Recently, several studies have also put forward the induction of autophagy as a common NP-induced effect.^{38,131–133} Another general observation is that toxicity is closely related to size, where nanosized NPs are generally found to be more toxic than micrometer-sized materials. The latter is, however, not true for all types of materials as other factors, such as differences in crystal structure between nano- and micrometer-sized materials, can also contribute to their degree of toxicity.¹³⁴ Even within a single type of material there is substantial variation between different types of NPs in terms of size, shape, and coating. These are all factors that contribute significantly to both the mechanism by which and the extent to which NPs interact with cells. This explains why a large amount of seemingly conflicting data have been published on this aspect.²² Another factor that has received only little attention thus far is the sterility of the NPs, especially for in-house-prepared novel NP types. In most studies, sterilization of the NPs is not even mentioned, although it is critical that for proper analysis the NPs need to be sterile and the method of sterilization must be optimized as it can influence the results obtained.¹³⁵ All these factors are standing in the way of a critical analysis of available literature data, while in order to draw any general conclusions regarding the toxicity of the NPs, it is important to take these factors into account. The following sections will, therefore, provide a brief overview of the most common and important findings relating to the (cyto)toxic effects of the various NPs.

3.1. IONP-Induced (Cyto)toxicity

IONPs have a long history in biomedical applications, where dextran-coated IONPs (Feridex in US; Endorem in EU) were FDA approved in 1996 and have been used in clinical settings for the noninvasive detection of liver lesions for nearly two decades.¹³⁶ IONPs were for a long time believed to be safe, given the high tolerance of cells to ferric iron, which is an essential element for cell growth and the initial studies by Weissleder et al.¹³⁷ These authors studied the pharmacokinetics and toxicity of dextran-coated IONPs in rats and Beagle dogs and found no adverse effects at doses that were 150 times higher than needed for MRI.¹¹⁹

3.1.1. Effect of the Coating on IONP Cytotoxicity. The recent surge in nanotechnology and the associated increased investigations on NP-associated adverse effects have refurbished the interest in toxic effects associated with IONPs, as evidenced by the large increase in publications on IONP-induced toxicity from the beginning of this century onward.^{119,138} One of the early studies addressing the *in vitro* toxicity of IONPs is the work by Berry et al.,¹³⁹ who compared the effects of dextran- or albumin-coated IONPs with bare IONP on human dermal fibroblasts. The authors found that NPs alter cell behavior and morphology depending on their surface coating. In subsequent studies coating with pullulan was found to reduce toxicity of IONPs on human dermal fibroblasts as compared to bare IONPs.¹⁴⁰ Furthermore, it was also demonstrated that altering the coating of the IONPs also influenced the way the NPs were internalized by the cells. This finding was further supported by a more recent study, where three types of IONPs coated with very similar carbohydrates (glucose, maltose, or lactose) were

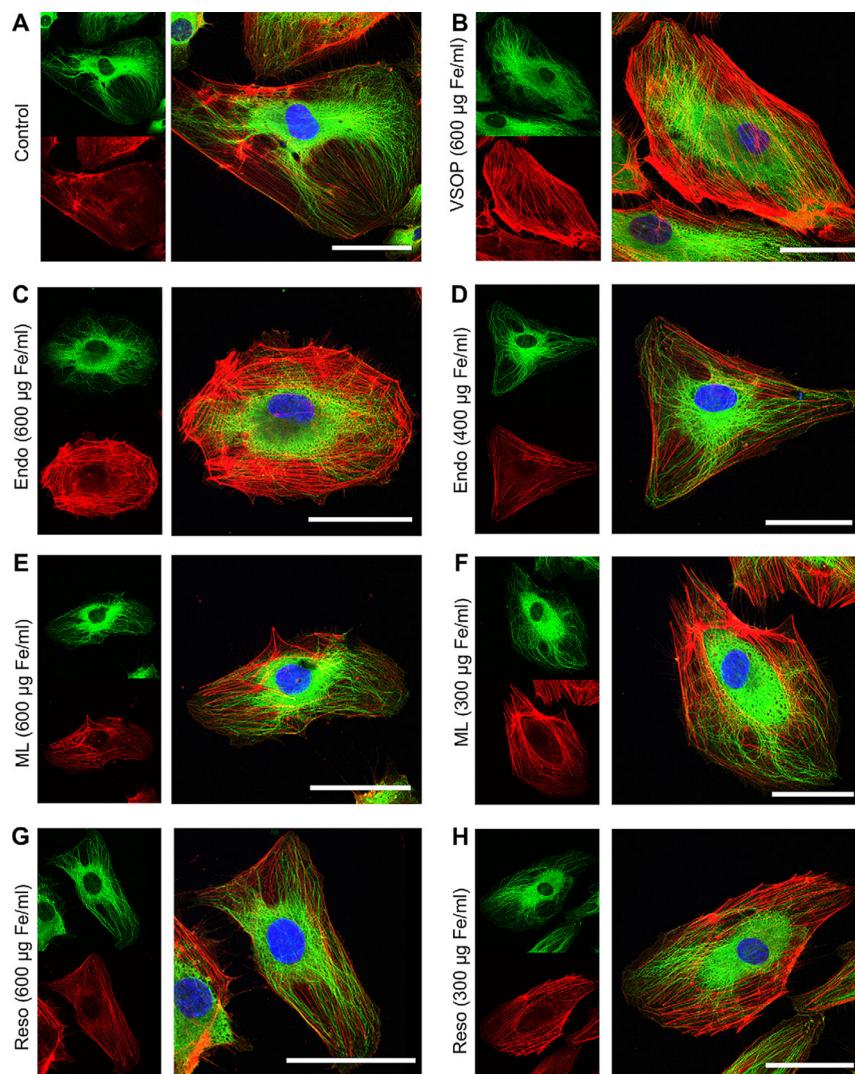


Figure 3. High intracellular NP concentrations affect cell spreading. Confocal micrographs of human blood outgrowth endothelial cells (hBOECs) either untreated (A) or incubated for 24 h with (B) VSOP, (C) Endorem, (E) MLs, or (G) Resovist at 600 μg Fe/mL for 24 h, (D) Endorem at 400 μg Fe/mL, and (F) MLs or (H) Resovist at 300 μg Fe/mL for 24 h. Cells were kept in culture for an additional 3 days. F-actin is colored red, the nucleus is colored blue by DAPI staining, and α -tubulin is indicated in green. Green and red channels are shown separately for every image. Scale bars: 50 μm . Reprinted with permission from ref 32. Copyright 2011 Elsevier.

found to affect human fibroblasts quite differently,¹⁴¹ revealing that even minimal differences in NP coating can have a substantial impact on the way the IONPs will interact with cells in culture.

The effect of coating on IONP toxicity is, similar to the effect of NP size, largely dependent on the degree to which they interact with cells and thus on the level and mechanism of cellular IONP internalization.¹⁴² The coating also determines the colloidal stability of the NPs in complex biological media and hereby affects NP agglomeration and the effect thereof on NP uptake and toxicity.¹⁴³ Obviously, differences in IONP uptake will influence the degree of toxicity of the NPs, which requires a careful determination of the intracellular localization of the IONPs as well as the average number of IONPs per cell.^{18,19} For *in vitro* studies, these data will allow us to compare the intrinsic differences in toxicity of various IONPs. Merely measuring the initial concentrations used for cell labeling is less meaningful considering, for example, that IONPs that are taken up to 5 times lower extent than control NPs are only two times

less toxic.¹⁴⁴ This obviously makes them less interesting for biomedical applications.

The coating can also influence IONP toxicity through various other mechanisms. Various coatings, such as cationic lipids, are intrinsically quite toxic and can affect cell viability regardless of the intrinsic effect of the iron oxide cores.¹⁴⁵ A careful design and composition of the coating is therefore necessary to generate IONPs that achieve sufficient cell uptake levels while not affecting cell homeostasis.^{146,147} Occasionally the coating does not completely cover the iron oxide core, leaving it partially exposed to the surrounding environment. The combination of the coating and the iron oxide core lead furthermore to a new entity with novel physicochemical parameters, which may result in different toxicological effects.¹⁴⁸

3.1.2. Key Effects of IONPs on Cytotoxicity. IONPs have been described to display various effects on the functional and/or morphological cell integrity. Typically NPs generate a strong but transient increase in ROS, reaching maximal levels after approximately 24–48 h.¹⁴⁹ This transient elevation of ROS can

subsequently result in a variety of effects, including cell death or effects on cell morphology.¹⁵⁰ The effect of IONPs on cell morphology has been reported in various studies. Morphological changes have been found to be connected to the intracellular number of NPs, with high intracellular concentrations of various types of IONPs resulting in cellular alterations, affected actin and tubulin cytoskeleton, and associated signaling processes (Figure 3).¹⁵¹ The extent of the effects depended on the intracellular level of the IONPs rather than on intrinsic differences between the IONPs and occurred at concentrations at which no acute cytotoxicity was observed.¹⁵² Another common effect observed for IONPs is inhibition of the differentiation of stem cells after labeling. Several studies have indicated a clear reduction in chondrogenic differentiation of mesenchymal stem cells upon labeling with dextran-coated IONPs,¹⁵³ where others described a diminishment of osteogenic differentiation of mesenchymal stem cells exposed to carboxydextrans-coated IONPs.¹⁵⁴ Additionally, IONPs have been found to reduce the capacity of human endothelial progenitor cells to form a vascular network¹⁵⁵ and to impede the outgrowth of neurites by PC12 cells stimulated with nerve growth factor.¹⁴⁸ Collectively, these data show that labeling stem cells with IONPs can affect the intracellular signal transduction and may as a result impede stem cell differentiation and functionality. Of course, these results must be translated in functional doses, for instance, their ability to efficiently generate MRI contrast. Several studies have indicated that at low NP concentrations, no effect on stem cell differentiation was observed, while transplanted cells could still be monitored through MRI.^{156,157} Together, the results indicate the importance of evaluating NP toxicity at functionally relevant concentrations.

As both Fe_3O_4 and Fe_2O_3 lead to a similar magnetic contrast and, in addition, have very similar crystal structures, only little attention has been paid to any potential effects on cellular well being. It has recently been shown, however, that for IONPs of identical size and identical surface chemistry the iron redox state may have a marked influence on the toxicity and DNA damaging ability of the NPs.¹⁵⁷ These findings suggest that it is important to carefully control the ratio of Fe^{2+} and Fe^{3+} ions in the IONPs as this affects the surface potential and surface coordination chemistry, which in turn may influence the mechanism of interaction with cultured cells. In this connection, we want to stress that it is not trivial to experimentally distinguish between Fe_3O_4 and Fe_2O_3 (and to be aware that oxidation can occur) and that thus any claim concerning differences between both types of NPs needs to be accompanied by a detailed chemical characterization of the NPs.¹⁵⁸

3.1.3. Positive Effects of IONPs on Cell Physiology. Some recent findings have also demonstrated the complexity of unraveling IONP-mediated toxicity. Whereas IONPs are generally considered to induce high but transient levels of ROS, others observed a peroxidase-like activity of the NPs, potentially enabling them to decrease cellular H_2O_2 levels.¹⁵⁹ The intrinsic peroxidase-like activity of the IONPs combined with the presence of free ferric iron has furthermore been found to promote the proliferation of mesenchymal stem cells.¹⁶⁰ Again, these data reveal that IONPs can affect intracellular signaling, which, due to the presence of important signal transductors such as nuclear factor kappa B (NF κ B), may result in a large variety of effects, that therefore may be hard to detect without rigorous testing.

3.2. QDot-Induced (Cyto)toxicity

3.2.1. Effect of QDot Composition on Cytotoxicity.

One of the main issues regarding QDot-toxicity is the wide variation in chemical composition of the QDots. Both core-only and core–shell QDots are used for biomedical applications and various materials can be used for either the core or the coating. CdTe QDots are most frequently used as core-only NPs, while CdSe/ZnS QDots are the most frequently used core–shell NPs. We want to point out that this is merely for historic reasons, as these “classical” NP materials were the easiest to synthesize at the beginning, whereas nowadays NPs can be made with a much larger variety of materials. The effect of QDot composition still requires more in-depth studies, considering that several factors such as shape of the QDots and thickness or composition of the shell layer have not been thoroughly investigated yet.

Most “classical” QDots are cadmium-based, which intrinsically brings along a potential danger, given the high toxicity of free cadmium.¹⁶¹ Recently, the use of so-called gradient alloy QDots,¹⁶² or cadmium-free NPs, such as ZnSe/InP/ZnS core/shell/shell NPs has been suggested as a less toxic alternative,¹⁶³ but their toxicity and functionality (e.g., quantum yield) remains to be adequately determined as initial studies have revealed that the benefit of the lower toxicity of such QDots is compromised by their lower brightness.¹⁶⁴ The presence of cadmium ions results in a higher toxicity than that of IONPs, the nontoxic level of QDots is often being less than 10 nM, whereas for IONPs this can be as high as several μM .^{31,32} These values must be carefully considered as the QDots are typically smaller than IONPs and therefore have a higher total surface area than an equal number of IONPs, which may result in inducing a much higher toxicity.

3.2.2. Effect of QDot Coating on Cytotoxicity. Similar to the IONPs, the nature of the QDot coating plays an important role in the toxicity of the QDots.³⁴ Various types of coating have been applied, including monothiolated or bidentate thiolated hydrocarbonic acids,^{165–167} silica,^{168,169} peptides and proteins,^{166,170} amphiphilic polymers,^{61,171} and lipid micelles.¹⁷² The surface coating, size, and charge largely determine the level of cellular QDot uptake as well as the mechanism by which the NPs are internalized.¹⁷³ The effect of the surface coating on cellular uptake and cytotoxicity is however quite complex, as demonstrated by evaluating the uptake and toxicity of two sizes of commercially available amphiphilic polymer-coated QDots functionalized with carboxylic acid, poly(ethylene glycol) (PEG), or PEG with amine groups.¹⁷⁴ In this particular case it was observed that the type of functionalization had no substantial effect on the internalization level by human epidermal keratinocytes. However, the coating did play an important role in the cytotoxicity and release of pro-inflammatory cytokines.

One possible issue concerning the effect of coating on QDot uptake levels is the lack of adequate methods to quantify their cellular levels. Often the number of QDot-positive spots (endosomes) per cell is determined based on fluorescence microscopy images of labeled cells.¹⁷⁵ However, due to their small size individual QDots cannot be laterally resolved with standard fluorescence microscopy.¹⁷⁶ Alternative methods consist of measuring the average fluorescence intensity of labeled cells with fluorescence microscopy or flow cytometry.¹⁷⁷ However, these methods suffer from various shortcomings that are standing in the way of a correct analysis: (i) QDots typically agglomerate when present in endosomes,

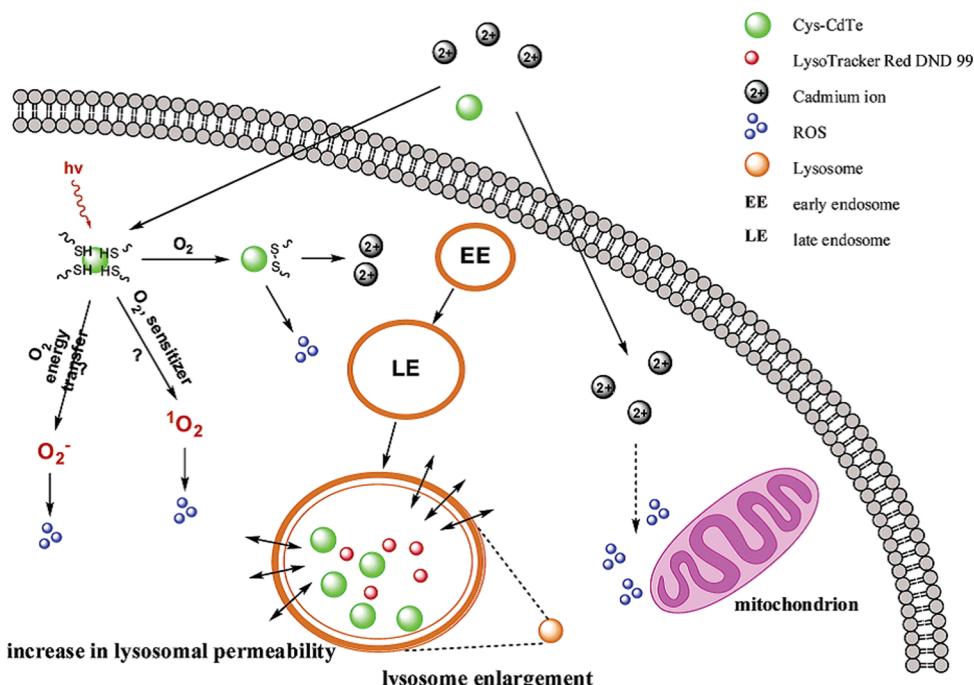


Figure 4. Schematic representation of the mechanistic pathways implicated in the cytotoxicity of CdTe QDs in live cells, highlighting the salient changes in cellular morphology, the chemical species involved, and the chemical reactions that can lead to ROS and free Cd²⁺ ion release. Reprinted with permission from ref 184. Copyright 2007 American Chemical Society.

which may affect the overall fluorescence of the QDots.³¹ (ii) The fluorescence intensity of QDots is quite sensitive to the local pH. As endosomes have lower pH values than the buffers at which the QDots are stored, the fluorescence intensity of intraendosomal QDots will not be comparable to that of free QDots.³¹ (iii) As QDots have been described to end up in various intracellular compartments depending on their size and coating, the microenvironment of the QDots in a cell can be quite variable, which again may seriously affect their fluorescence intensity.¹⁷⁸ Alternative methods to quantify QDots per cell involve lysis of cells in low osmolarity buffers of slightly alkaline pH followed by determining fluorescence intensity or quantifying Cd²⁺ ions by inductively coupled plasma-mass spectrometry (ICP-MS) or by using commercially available fluorescence assays such as the Measure-iT kit by Molecular Probes.^{31,178} The coating of QDots will furthermore influence the colloidal stability of the NPs. It has been reported that the level of agglomeration of QDots is an important factor contributing to their toxicity, as large QDots aggregates readily sediment and cover the cell surface, thus impeding their viability.³⁴

3.2.3. Key Effects in QDot Cytotoxicity. Next to coating, other parameters will also influence the toxic effects of QDots. The different intracellular distribution of QDots varied in size has been reported for both CdTe QDots¹⁷⁹ and CdSe/ZnS core–shell QDots.¹⁸⁰ This was also associated with a higher level of toxicity of the smallest QDots.¹⁷⁴

Similar to IONPs and nearly all types of NPs, QDots induce high (transient) levels of ROS, which can result in a variety of effects. CdSe-based QDots, for instance, were found to induce high levels of ROS which in turn led to activation of important signaling mediators (c-Jun N-terminal kinases; JNKs) and loss of mitochondrial membrane potential resulting in cell death.¹⁸¹ High levels of ROS are also known to potentially damage DNA,²⁹ possibly resulting in epigenetic as well as genomic

effects.¹⁸² Other than by the induction of ROS, some QDots have also been found to be able to penetrate into the nucleus, where they have been claimed to interact directly with the nuclear DNA.¹⁷⁴ The induction of ROS by QDots can also influence cytoplasmic calcium levels, which can have drastic effects on cell homeostasis, such as a high influx of extracellular calcium ultimately leading to cell death, for instance, by affecting the specificity of voltage-gated sodium channels.¹⁸³

The intracellular localization of QDots also affects cell homeostasis. Long-term intraendosomal localization of CdTe-based QDots has been described to result in a lysosomal enlargement the level of which depends on the presence or absence of a passivating shell layer (Figure 4).¹⁸⁴ The enlargement of the lysosomal compartment was found to be associated with an enhancement of intracellular glutathione concentrations and activation of transcription factor EB, a master regulator of lysosomal biogenesis.¹⁸⁵ These effects indicate an adaptation of the labeled cells to the presence of intraendosomally located QDots by altering signaling cascades. The adaptation of cells to the intracellular presence of QDots has been demonstrated to have a protective effect, inducing autophagy as a defense mechanism against QDot-induced stress.¹⁸⁶ The effect of free Cd²⁺ on cell viability is also substantial, as has been elegantly demonstrated by Gagné et al.,¹⁸⁷ who compared the effect of freshly prepared (2 months old) and aged (2 years old) CdTe QDots. The authors found that the aged QDots had stronger effects than the freshly prepared ones on cell viability, oxidative stress, and DNA damage as a result of free Cd²⁺ ions. Another area of concern which is actively being studied regards the effect of QDots and their heavy metal constituents on the environment.¹⁸⁸

3.2.4. Preliminary *In Vivo* Effects of QDots. The effect of QDots on animals or humans remains largely in the dark. Although an impressive amount of work has been performed on cultured cells, few *in vivo* studies have been reported. There

are also major discrepancies between data obtained in *in vitro* and *in vivo* studies, which probably stems largely from dosing effects, since QDot concentrations used for cell labeling remain fairly constant whereas QDot concentrations in the body will vary in time and space.¹⁸⁹ On the short term, QDots have been shown to affect hematopoiesis through oxidative stress-dependent mechanisms.¹⁹⁰ Clearance of small QDots from the body in relation to long-term toxicity of QDots retained within the body must be thoroughly analyzed, but presently, there are hardly any data available on long-term effects of QDots. Recently, a pilot study in nonhuman primates revealed no adverse effects after intravenous administration of QDots^{80,191} at doses sufficient for imaging. Additional research is needed, however, as some recent studies have indicated the ability of CdTe/CdS QDots to transfer across the placental barrier in mice.¹⁹² Additionally, CdSe core-only QDots were found to impede fetal development.¹⁹³ All these effects were again dependent on the size and surface coating of the QDots as well as on the presence of a passivating shell layer, suggesting the possibility that QDot composition can be finely tuned to minimize any adverse effects. The future, however, will clearly lie in Cd-free QDot materials, in which at least Cd²⁺-specific effects will no longer be present.

3.3. Silver NP-Induced (Cyto)toxicity

The antimicrobial, cytotoxic, and *in vivo* toxic effects of silver NPs have been widely studied, but as for nearly all types of NPs, numerous conflicting data have been published, preventing any attempt to draw general conclusions regarding their toxicity.^{86,194–197} Especially in recent years, a multitude of papers were published on the topic of silver NP toxicity, emphasizing the clear need for a deeper understanding of the interaction of these materials with living organisms.

3.3.1. Role of Oxidative Stress in Silver NP Cytotoxicity. The main cause of toxicity of silver NPs appears to be their strong induction of ROS, which has been described in many studies as the underlying mechanism of a variety of associated secondary effects.¹⁹⁸ Oxidative stress induced by silver NPs has been found to result in mitochondrial membrane destabilization,¹⁹⁹ activation of transcription factors,²⁰⁰ or diminished cell proliferation.²⁰¹

A matter of great concern regarding the toxicity of silver NPs is their potential mutagenicity. Due to their strong induction of ROS, exposure of cells to silver NPs has often been found to result in genotoxic effects,²⁰¹ such as chromosomal alterations resulting in a loss of heterozygosity.²⁰² Although the induction of DNA damage has been frequently reported, this issue remains a matter of debate as a significant number of studies have also indicated a lack of genotoxic effects. The apparent discrepancy between these findings likely stems from several factors, such as (i) the use of silver NPs of different sizes and coatings, (ii) the complication to distinguish between effects of released Ag⁺ ions and the actual Ag NP, as these two are in equilibrium,²⁰³ (iii) the complications related to the quantification of cellular uptake of silver NPs virtually prohibiting a direct comparison of results, and (iv) the use of different genotoxicity tests.^{204,205}

3.3.2. Key Aspects of Silver NP Cytotoxicity. The difficulty to quantify levels of silver NP uptake as well as to obtain information regarding their precise intracellular localization further hinders a straightforward analysis. Although several methods are available to assess cellular uptake, such as ICP-MS or atomic absorption spectrometry,²⁰⁶ these techniques

can generally determine only the total amount of Ag inside cells but cannot distinguish between ionic Ag and Ag bound to the NPs. This is a general problem for corrosive NPs, as also mentioned above for CdSe QDots. In addition, these studies do not provide information about the intracellular distribution of Ag. While it is safe to assume that most Ag NPs reside in endosomal/lysosomal compartments,²⁰⁷ released Ag⁺ ions as well as ultrasmall Ag clusters also can be located in other intracellular compartments. New techniques to study the ionic dissolution of silver NPs, such as tangential flow filtration, are being evaluated but must be further optimized prior to their full implementation in nanotoxicological research.²⁰⁸

Several studies have indicated a size-dependent toxicity of silver NPs, where the smallest NPs (10–20 nm diameter) were typically the most cytotoxic (compared to 50–100 nm diameter) (Figure 5).^{209,210} The shape of silver NPs also

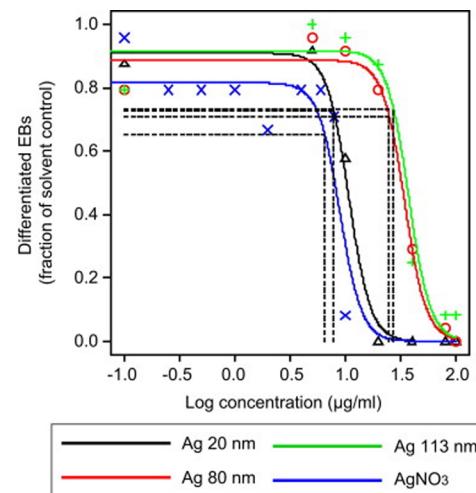


Figure 5. Differentiation of embryoid bodies (EBs) into spontaneously contracting cardiomyocytes as a function of the silver (NP or nitrate) concentration. Data was fitted with a log–logistic dose–response model: $y = a + (1 - a)/(1 + \exp(c \cdot \ln(b/x)))$. Dashed lines represent EC₂₀ values. Reprinted with permission from ref 210. Copyright 2011 Elsevier.

plays an important role. Silver nanowires (length 1.5–25 μm; diameter 100–160 nm) were found to be more toxic than spherical NPs (30 nm diameter) and resulted in stronger reductions in cell viability and induction of the NFκB activity.²¹¹ While most studies focus on silver NPs without any specific coating, applying a suitable coating has also been found to influence NP toxicity levels.^{203,206} Note that like with all other NPs there are no bare NPs under biological conditions, in particular, due to protein adsorption, which at any rate provides an organic coating. Without coating the NPs would immediately agglomerate,²² and agglomeration will strongly influence cellular NP uptake levels and hereby NP toxicity.²¹² This point often is not correctly addressed in the literature, where several reports speak about “bare” NPs, which however would not be stable in solution. Colloidal stability is one key parameter influencing the toxicity of NPs. Apart from differences between the NP properties, also major differences have been described between different cell types, giving rise to cell-type-dependent toxicity profiles.^{209,210,213,214} Given the strong induction of oxidative stress by the silver NPs, differences between cell types have been related to differences in their natural antioxidant levels.²¹⁵ However, these differences

or those obtained by means of applying an alternate coating or comparing differently sized silver NPs can also be explained by differences in uptake efficiency between cell types (whereby again colloidal stability is a key parameter). An important factor to take into account is the total surface area of cell-associated silver NPs which will determine the extent of interaction of the NPs with their environment. Therefore, all above-mentioned features (size, shape, coating, colloidal stability, cellular uptake efficiency) must be thoroughly assessed in order to be able to determine this value.

Silver NPs have further been described to affect the stability of the plasma membrane by inducing deformations of the lipid bilayer,²¹⁰ but these effects may be diminished upon applying an appropriate NP coating. Bowman et al.²¹⁶ further reported effects of silver NPs in a zebrafish model using differently sized NPs. These authors claimed that silver NPs exert a surface area-dependent but not size-dependent response, increased mortality, and heart anomalies. These results should not be overinterpreted, as different sizes of the Ag cores can be completely overshadowed by different degrees of agglomeration, which often is insufficiently taken into consideration. Silver NPs have been described to cause low levels of toxicity in stem cells. Moreover, the differentiation capacity of human adipose-derived stem cells was found to be unaffected.²¹⁷ Silver NPs can, however, affect intracellular signaling pathways, as demonstrated in a recent study by Comfort et al.,²⁷ who showed a significant effect of 10 nm diameter silver NPs on epidermal growth factor signal transduction in a human epithelial cell line.

Taking into account the strong antimicrobial potential of silver NPs, the widespread use of these NPs and their impact on the environment is being studied.⁸⁶ Several studies have indicated effects of silver NPs on, for instance, algae or rainbow trout, where it was found that both whole silver NPs and free Ag⁺ ions resulted in similar toxic effects.^{218,219} Note that this is in contradiction to other studies²⁰³ and relates again to the above-mentioned complication to distinguish between the effect of Ag⁺ released by Ag NPs and Ag NPs themselves, as these are in equilibrium with each other, whereas added Ag⁺ will have a different intracellular distribution than Ag⁺ released from incorporated Ag NPs. Important aspects to be considered are (i) the dissolution rate of the NPs and the release of Ag⁺ ions²²⁰ and (ii) other environmental transformations of the silver NPs, such as sulfidation of silver NPs, which results in poorly soluble silver sulfide and hereby decreases silver-induced toxicity.^{221,222} Recently, Xiu et al.¹⁹⁵ claimed that silver NPs themselves have negligible antibacterial and possibly cytotoxic effects by themselves (under certain conditions). This claim was based on their observation that upon exposure of bacteria to silver NPs under strict anaerobic conditions (precluding silver oxidation and release of Ag⁺) no toxic effects were apparent in contrast to exposure under normal aerobic conditions (and release of Ag⁺).

3.3.3. Preliminary *In Vivo* Effects of Silver NPs. The *in vivo* toxicity of silver NPs also remains rather unclear. Most studies indicate no or only minimal effects for the concentrations studied. Upon intravenous injection of silver NPs, the NPs were found to accumulate predominantly in liver and spleen, after which the NPs were excreted primarily through the biliary route.²²³ Higher levels of intravenously injected silver NPs have been shown to cause inflammatory reactions in liver and lung, but more remarkably, a clear gender-related clearance rate of silver NPs was noted.²²⁴ Van der

Zande et al.²²⁵ observed no effects after 28 days of oral exposure of rats to silver NPs. The NPs were found to accumulate predominantly in the liver and spleen, but significant amounts of silver NPs were found in all major organs. After 8 weeks of washing out, most organs were free of silver NPs, apart from the brain and the testis. Further research is necessary to evaluate the effects of long-term persistence of silver NPs in these organs and to evaluate the role of gender-related differences in NP biodistribution and toxicity.

3.4. ZnO NP-Induced (Cyto)toxicity

3.4.1. Role of Oxidative Stress in ZnO NP Cytotoxicity.

The toxicity of ZnO NPs has also been studied extensively, given their broad application in many daily life goods. Similar to the other NPs, ZnO NPs have been reported to induce high levels of ROS which can result in a variety of secondary effects, such as loss of mitochondrial membrane potential, upregulation of pro-apoptotic genes, and downregulation of antiapoptotic genes.^{226,227}

A key signaling transducer in ZnO-mediated toxicity is the tumor suppressor gene p53 which protects cells from developing cancer phenotypes through its control over several major pathways including apoptosis and cell cycle progression.^{228–230} The importance of studying the precise mechanism underlying NP toxicity was demonstrated in an elegant study by Ng et al.,²²⁸ who found activation of p53 in skin fibroblasts exposed to ZnO NPs. Since p53 is an important mediator involved in many different pathways and can be activated by DNA damage, it was suggested that ZnO NPs could have a strong carcinogenic effect. The authors demonstrated that exposure of the skin fibroblasts, in which p53 was knocked down, to ZnO NPs did not result in apoptosis but rather increased cell cycle progression. Together, these data show the importance of revealing key mediators involved in NP toxicity and express the relative impact of toxicity data dependent on the nature of the results obtained. While ZnO NPs can result in different degrees of cell death mediated by p53, depending on the concentrations of the NPs used, this is actually a protective effect which is favored over the induction of carcinogenesis. The induction of ROS also has been reported to result in concentration-dependent genotoxicity.^{28,231,232} Significant levels of DNA damage can then, among others, ultimately lead to activation of p53.^{227,228}

3.4.2. Key Aspects in ZnO NP Cytotoxicity. Like for the silver NPs, the levels of cell-associated ZnO NPs are not frequently evaluated, thus preventing an easy comparison of similar materials with regard to differences in cytotoxic profile due to alterations in their size, shape, or coating. In general, smaller NPs (e.g., ranging from 10 to 100 nm diameter) typically induce the strongest toxic effects.²³³ Contradictory results have been obtained for various coatings.²³⁴ These discrepancies are likely a result of differences in the level of cellular association/uptake of the various NPs and diversity of their total surface areas as well as possible intrinsic differences in their dissolution rate or their distinct uptake mechanism. For example, it has been reported that differently shaped ZnO NPs (spherical and sheet shaped) cause identical degrees of toxicity provided they had the same surface area.²³⁵

An important parameter of ZnO NPs-induced toxicity is the level of free Zn²⁺.²³⁶ Cellular uptake of ZnO NPs results in an elevation of both cytoplasmic and mitochondrial Zn²⁺ levels, which can ultimately cause mitochondrial damage and apoptosis (Figure 6).²³⁷ Dissolution of ZnO NPs is another

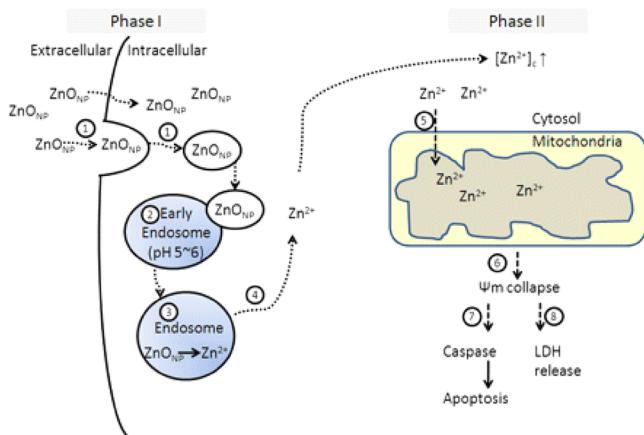


Figure 6. Mechanical toxicological pathway of ZnO-NPs. Phase I: The cytosolic entrance and dissolution of ZnO-NPs leads to an initial elevation in cytosolic Zn²⁺ concentrations ($[Zn^{2+}]_c$). (1). ZnO-NPs are engulfed via endocytosis. (2). Endocytic vesicles fuse with early endosomes. (3). Vesicular ZnO-NPs dissolve at low pH in endosomes. (4). Endosomal Zn²⁺ leaks into the cytosol to elevate $[Zn^{2+}]_c$. Phase II: (5). Mitochondria sequester excess cytosolic Zn²⁺ to result a rise in mitochondrial Zn²⁺ concentrations $[Zn^{2+}]_m$. (6). High $[Zn^{2+}]_m$ induces mitochondrial membrane potential collapse, which activates caspase-3 and leads to cell apoptosis (7) and LDH release (8). Reprinted with permission from ref 237. Copyright 2012 Oxford Journals.

serious concern for their environmental impact as dissolved Zn²⁺ ions either from nanosized ZnO or from bulk ZnO have been shown to result in acute toxicity in a zebrafish model.²³⁸ As mentioned earlier for CdSe and Ag, the equilibrium of free Zn²⁺ and ZnO NP prevents a clear distinction between effects caused by released Zn²⁺ and those induced by the intact ZnO NPs. However, there is some evidence in favor of a direct effect of ZnO NPs (apart from ZnO). The group of Nel demonstrated in an elegant study that toxicity of metaloxide NPs depends on their band gap.²³⁹ This directly correlated toxicity to a bulk material (the band gap), which is not connected to released ions. However, toxicity of ZnO is also correlated to the amount of released Zn²⁺ ions. This was demonstrated also by the Nel group, who doped ZnO NPs with iron. This was found to change the NP matrix, slow down the rate of dissolution, and thus release of Zn²⁺ and as such was shown to diminish ZnO NP toxicity in a zebrafish model.²⁴⁰

3.4.3. Preliminary *in Vivo* Effects of ZnO NPs. The widespread use of ZnO NPs in daily life goods has compelled the use of animal models to evaluate the effect of ZnO NPs. In most studies, ZnO NPs have been administered orally, demonstrating that within 72 h the NPs accumulate in the liver, lung, and kidneys (as most NPs), with small amounts of ZnO NPs being excreted with the urine and large amounts being excreted in the feces.²⁴¹ Transient histopathological lesions in the liver of the exposed animals have been observed,²⁴² as well as nephrotoxicity and alterations in kidney metabolism.²⁴³ Sharma et al.²⁴⁴ described the induction of oxidative stress associated with DNA damage and apoptosis in the liver of mice exposed to ZnO NPs at concentrations below which any acute toxicity was apparent. Taken together, these findings highlight the need for more data on the potential toxicity of ZnO NPs.

3.5. Au NP-Induced (Cyto)toxicity

3.5.1. Effect of Au NP Coating on Cytotoxicity. Au NPs were initially assumed to not pose any serious health hazards given their lack of chemical reactivity and the low toxicity of bulk gold. However, the miniaturization of Au into the nanoscale resulted in highly different toxicity profiles, suggesting that Au NPs may elicit clear toxic effects. One key aspect to Au NP toxicity is the coating which is applied onto the NPs in order to provide colloidal stability and to enable biomedical applications. The nature of the coating can influence Au NP toxicity through various mechanisms: First, the applied coating governs the colloidal stability of the NPs in complex media and will influence the agglomeration and sedimentation of the Au NPs. The formation of small agglomerates can either impede or enhance cellular NP internalization, depending on the nature of the cell types studied (i.e., capable of phagocytosis or not).²⁴⁵ Second, coatings that provide high colloidal stability, such as poly(ethylene glycol) chains, can also reduce interaction of NPs with cell membranes and hereby reduce their cellular uptake levels and thus reduce cell cytotoxicity.^{33,246} Alternatively, coatings can also be used to increase cellular uptake levels (e.g., positively charged polymers compared to negatively charged polymers), which is also accompanied by higher levels of toxicity.²⁴⁷ Third, the coating itself can also be toxic.²⁴⁸ During Au NP synthesis, cetyltrimethylammonium bromide (CTAB) is commonly used as a stabilizing surfactant. For CTAB-coated NPs, the release of CTAB molecules into surrounding cell media has been found to be a major cause of Au NP toxicity.^{249,250} Overcoating the CTAB-coated NPs with other agents could overcome these toxic effects.²⁴⁹ Small alterations in NP surface properties, such as changes in NP surface hydrophobicity, have also been shown to play a key role in NP toxicity, where the induction of autophagy and the level of cell membrane damage correlated with the degree of NP surface hydrophobicity.²⁶ In order to study the effect of NP surface chemistry on the intrinsic toxicity of the NPs, it is essential to determine cellular NP uptake levels. When comparing PEGylated and non-PEGylated Au NPs, the PEGylated NPs exerted lower toxicity levels, which were mainly due to their reduced uptake levels.²⁴⁶ When comparing their toxicity at similar intracellular NP numbers, the PEGylated NPs were found to be more toxic and result in higher levels of ROS induction.

3.5.2. Key Aspects in Au NP Cytotoxicity. Another parameter which is important in the toxicity of Au NPs is NP size. The size of Au NPs is negatively correlated with NP toxicity, where smaller NPs usually result in higher toxicity,²⁵¹ likely due to the higher total surface area of all the NPs combined. The impact of size can be very high, as demonstrated by Pan et al., who showed that spherical Au NPs of 1.2 nm diameter resulted in cellular apoptosis, whereas spherical Au NPs of 1.4 nm diameter resulted in necrosis, suggesting that the mechanism underlying Au NP toxicity is highly size dependent.²⁵² The effect of NP shape has thus far not been studied in depth. Although rod-shaped particles were found to result in lower cellular uptake levels than spherical NPs,²⁵³ initial studies revealed that Au nanorods were more toxic than Au nanospheres, but this finding was linked to the fact that the nanorods required CTAB as a stabilizing coating, which was not the case for the nanospheres.²⁵⁴ Overcoating the nanorods with polymers overcame the toxic effects and furthermore revealed that rods of different aspect ratios did

not result in any significant differences in toxicity. Given the wide variety in different shapes possible for Au NPs (spheres, rods, sheets, stars, cubes, ...), more detailed studies on the effect of NP shape must be undertaken to elucidate the full impact of this parameter.

3.5.3. Preliminary *in Vivo* Effects of Au NPs. The effect of Au NPs *in vivo* has also not been studied in depth and requires more data, especially regarding the impact of various parameters such as shape and coating. The effect of size has been studied more in depth.²⁵⁵ One key study was performed by Chen and colleagues, who studied the size-dependent toxicity of Au NPs.²⁵⁶ Their results displayed clear size-dependent effects, where NPs of intermediate size (between 8 and 37 nm diameter) induced prominent toxic effects in mice upon intravenous injection, which were not apparent for smaller and larger NPs. Interestingly, the *in vivo* effects observed were not seen when performing *in vitro* studies on HeLa cells, indicating the high discrepancy between *in vitro* and *in vivo* conditions. The main cause of toxicity may lie in their biopersistence and lack of clearance from the body.²⁵⁷

4. INTRACELLULAR NANOPARTICLE STABILITY

One issue that is receiving increasing attention recently is the chemical stability of the NPs exposed to the disintegrative endosomal environment. Typically, NPs are taken up via endocytosis, during which they will be exposed to highly varying conditions, such as pH changes from 7.4 (extracellular medium), via 5.5 (late endosomes), to 4.5 (lysosomes). In addition to their acidic pH, lysosomes also contain high levels of hydrolytic enzymes that can result in the degradation of the entire NPs or their coating. It has been demonstrated that upon endosomal uptake of NPs, any chemically conjugated or nonspecifically bound (attached during the NP presence in serum-containing cell media) proteins are rapidly degraded³⁶ by a low-specific protease called Cathepsin L, resulting in a major loss of function of bioconjugated NPs.²⁵⁸ In particular, for NPs containing intracellular targeting molecules or pharmaceutical drugs these effects can have major consequences. However, as most biologically relevant coatings are easily degraded, the NPs will be stripped from their coating, often resulting in altered physicochemical properties such as intraendosomal aggregation.²⁵⁹ Depending on the type of NP, the latter can have negative effects, such as diminishing QDot fluorescence intensity, or alternatively can have positive effects, such as enhancing magnetic moment for IONPs. For protein-coated Au NPs it has been demonstrated that as a result of the multifunctional molecular structure of proteins, these constructs exhibit pH-responsive reversible agglomeration/disaggregation behavior.³⁶ The exposure of the metal (oxide) surface to the disintegrative and acidic environment may also have grave effects on the NP itself, such as acid etching followed by release of metal ions. As deformations of the NP surface can have profound effects on their physical properties, the question remains how functional the NPs are after prolonged exposure to the endosomal microenvironment. Furthermore, as for many NPs (such as CdSe, Ag, ZnO) the release of ions is considered an important cause of toxicity, the intraendosomal degradation of NPs could also have significant effects on cellular integrity and/or function.²⁶⁰ The following sections will discuss these issues and provide an overview of the current state-of-the-art regarding intracellular NP stability.

4.1. Bioinduced IONP Degradation

4.1.1. Biological Effects of Free Fe Ions. The intracellular degradation of IONPs has been studied for quite some time.²⁶¹ The degradation of IONPs and the resulting release of ferrous (Fe^{2+}) ions was observed in several studies, where it was found that Fe^{3+} ions could bind low molecular weight molecules such as citrate in order to remain soluble. Free ions can then shuttle through the divalent metal transport channel present in late endosomes and lysosomes and as such reach the cytoplasm, where they will become part of the cell's labile iron pool (cytoplasmic iron levels).²⁵⁹ These increased cellular iron levels have been found to affect cellular iron metabolism. Upregulation of ferritin, the iron-storage protein, and transferrin receptor, the membrane receptor responsible for iron uptake, has been observed.^{262–264} Geppert et al.²⁶⁵ observed strong increases in cellular ferritin levels in primary astrocytes exposed to IONPs. This was associated with only negligible effects on cell homeostasis under the formation of transient ROS elevations. As astrocytes have potent antioxidant properties and naturally contain high levels of iron, the observed lack of any effects on cellular function may be attributed to these features and thus be cell-specific.

4.1.2. Cytotoxic Effects of IONP Degradation. The presence of free iron in an acidic environment typically results in high levels of oxidative stress by means of the well-known Fenton reaction,²⁶³ suggesting possible effects of IONP degradation on cell-type function. Exposure of cells to IONPs has been linked to an upregulation of Cathepsin L activity, alterations in ferritin levels, and secretion of pro- and anti-inflammatory cytokines.²⁶⁶ While free Fe ions can also potentially cause DNA damage as a result of oxidative stress,¹⁵⁷ these ions are also known to play a role in several cellular processes, such as cell cycle progression.²⁶⁴ Soenen et al.²⁶⁷ compared four different types of IONPs coated with a lipid bilayer, dextran, carboxydextrans, or citrate and optimized cell labeling conditions to reach similar intracellular iron loads for all NPs in either C17.2 neural progenitor cells or PC12 rat pheochromocytoma cells. All NPs were found to be susceptible to acid-induced degradation, the extent of which was dependent on the nature of the coating material where citrate-coated IONPs were found to be the least stable. Upon release of free Fe ions, high levels of oxidative stress were observed. This was correlated with loss of cell viability and loss of PC12 neurite outgrowth at subtoxic levels. Interestingly, these effects could be abolished by using desferrioxamine, an iron chelator. The extent of the observed effects correlated well with the respective differences in NP dissolution rates, where citrate-coated IONPs were found to be most toxic. The use of iron chelators such as desferrioxamine has also been found to suppress IONP-induced inhibition of stem cell differentiation.²⁶⁸

4.1.3. Advantageous Effects of IONP Degradation. Apart from the inherent danger of altering signaling pathways, several authors have also tried to exploit the release of free Fe ions into the cytoplasm and the associated signaling pathways to their advantage. For instance, Huang et al.¹⁶⁸ exposed human mesenchymal stem cells to carboxydextrans-coated IONPs and observed that whole NPs had a potent peroxidase-like activity, but upon degradation, oxidative stress levels increased as a result of the Fenton reaction. Using relatively low doses of intracellular IONPs the levels of induced ROS were not causing any harmful side effects. The free Fe ions that were generated did, however, alter the expression of several major cell cycle

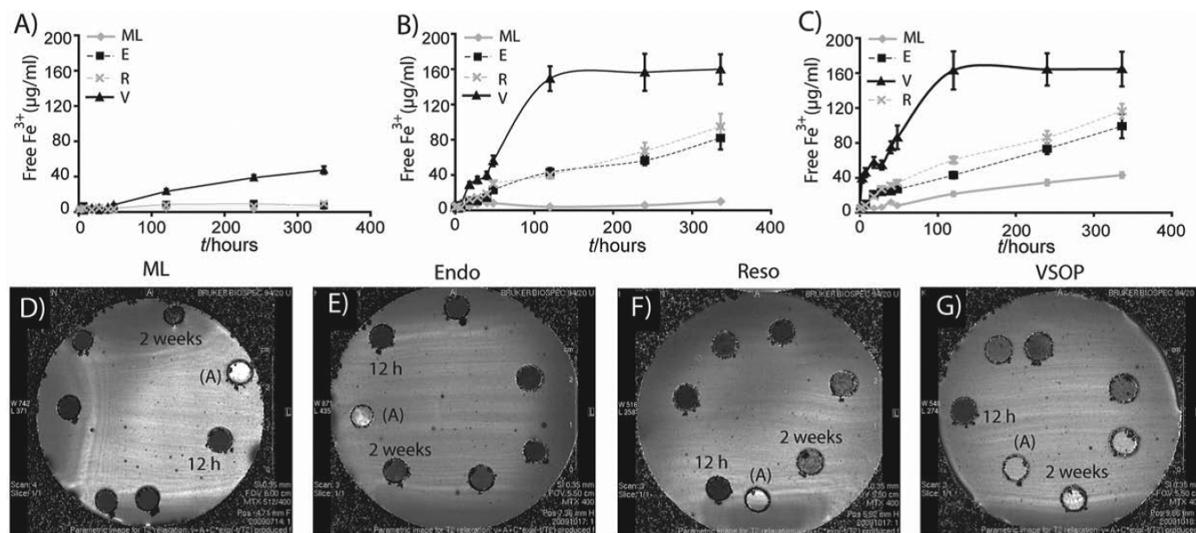


Figure 7. pH effect on NP degradation and MR signal intensities. The amount of free ferric iron measured as a function of time for 4 differently coated IONPs (lipid (MLs), dextran (Endorem), citrate (VSOP), and carboxydextrans (Resovist)) incubated at $200 \mu\text{g Fe mL}^{-1}$ in 20 mM sodium citrate containing cell culture medium at different pH values: (a) pH 7.0, (b) pH 5.5, and (c) pH 4.5; $n = 4$. Representative T_2^* maps measured for NPs in the above-described medium at pH 4.5: (d) ML, (e) Endorem, (f) VSOP, and (g) Resovist. Samples were collected at different time points after addition of the NPs to the acidic culture medium and are represented clockwise in terms of increasing incubation time; starting with the labeled position (A) for pure agar, the times were 12 h, 24 h, 48 h, 72 h, 1 week, and 2 weeks. Reprinted with permission from ref 267. Copyright 2010 John Wiley & Sons.

regulators such as cyclin B, cyclin D1, and cyclin-dependent kinase 4 (CDK4).

4.1.4. Effects of IONP Degradation on IONP Functionality.

Degradation of IONPs has also been studied in animal models. Several reports have described degradation of intravenously injected IONPs in liver and spleen macrophages.^{269–272} In these studies, the degradation of the NPs was associated with a loss of MRI contrast, which was explained by the compartmentalization of free iron in ferritin molecules.

The loss of MRI contrast has also been observed in several *in vitro* studies as a direct effect of degradation of the IONPs. The magnetic contrast of IONPs, which are typically used as T_2 contrast agents (darkening effect), is strongly dependent on the size of the iron oxide core. Degradation of the NPs results in a decrease in NP diameter and thus also in a reduction in T_2 magnetic contrast. Furthermore, the released Fe ions do not have a strong T_2 effect but rather interact with surrounding protons and as such qualify as a T_1 contrast agent. The elevated T_1 contrast subsequently suppresses the T_2 contrast of the remaining iron oxide core,²⁷³ resulting in a dual effect of both loss of IONP diameter and increase in T_1 contrast on the diminished T_2 contrast. In an *in vitro* endosomal model system, several types of IONPs have been found to undergo a pH-dependent degradation, resulting in loss of T_2 contrast.^{266,273} The extent of the degradation of the different IONPs was shown to be dependent on the nature of the coating (Figure 7). Furthermore, the extent of degradation was also correlated with the degree of loss of T_2 contrast. Citrate-coated IONPs were degraded most rapidly, which resulted in a significant loss of T_2 contrast within 1 week of exposure to lysosomal pH conditions (Figure 7). The degradation of differently coated IONPs has also been found to be pH dependent, with maximal degradation occurring at pH levels of approximately 4. Upon a further reduction of the pH, NP aggregation was favored over NP dissolution (Figure 8).²⁶¹ Besides degradation of the inorganic

IONP core, also degradation of the shell can play a decisive role.

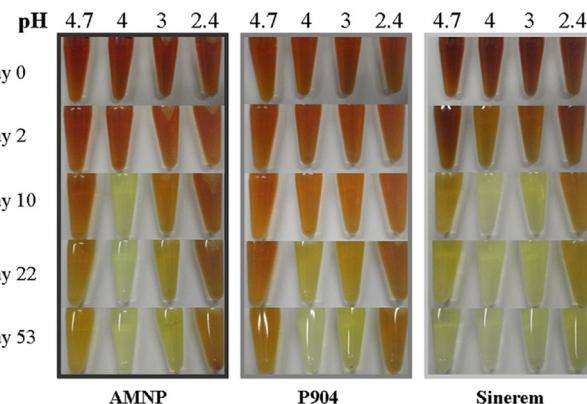


Figure 8. Visual follow-up of differently coated iron oxide NP (citrate (AMNP), aminoalcohol derivatives of glucose (P904), dextran (Sinerem)) degradation in the acidic medium with citrate chelate. Reprinted with permission from ref 261. Copyright 2010 IOP Publishing Ltd.

4.1.5. Possible Approaches To Impede IONP Degradation.

A more thorough investigation is necessary to unravel the extent of IONP degradation and to find possible ways of protecting the NPs against the destructive intraendosomal environment. Given the impact of the coating on the extent of IONP degradation, it is conceivable that the way in which IONPs are encapsulated and encapsulation degree will play a major role. Thus far, few studies have shown degradation-resistant IONPs, and results must be carefully analyzed in order to avoid misinterpretation. For instance, it has been reported that citrate- and poly(acrylic acid)-coated IONPs are resistant to degradation at pH values as low as 1.8.²⁷⁴ As in several studies degradation of citrate-coated IONPs was reported, the

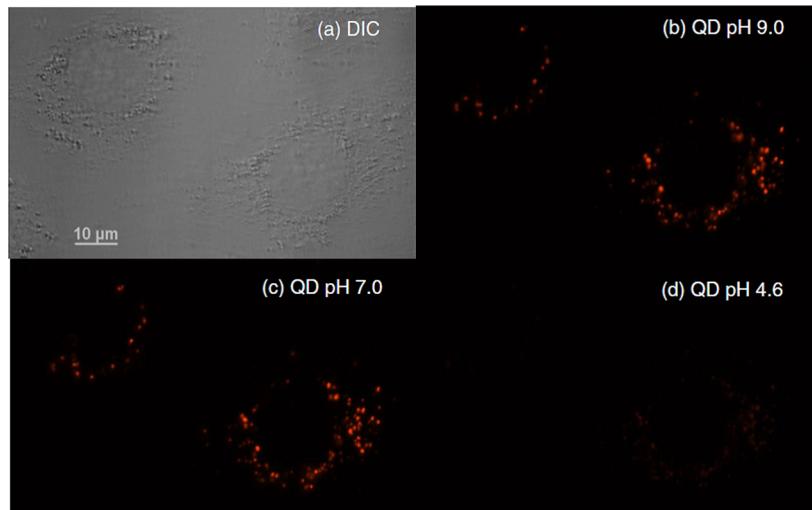


Figure 9. (a) DIC image of fixed SKOV-3 cells; (b, c, and d) fluorescence images of fixed QD-loaded SKOV-3 cells in pH 9.0, 7.0, and 4.6 buffers, respectively. Reprinted with permission from ref 284. Copyright 2006 IOP Publishing Ltd.

findings of Safi et al. are somewhat surprising. Possibly the lack of a buffered pH system (NP stability was measured *in situ* in the main IONP stock) and the lack of stabilizing low molecular weight agents or the use of a different characterization technique may all have contributed to these contradictory observations, as a proper mimicking of the endosomal microenvironment, including the presence of citrate, is essential for a proper analysis.²⁷⁵ The importance of surface engineering on improving IONP stability in a biological environment and hereby reduce degradation-dependent toxicity has been demonstrated.²⁷⁶ Using aberration-corrected high-resolution TEM, the degradation of iron oxide nanocubes could be evaluated *in situ*, showing that the degradation rate of the IONPs is governed by the availability and access rate of chelating agents to the nanocrystal surface, which in turn is determined by IONP surface reactivity (coating).²⁷⁷ Protection of the IONPs against degradation may potentially be achieved by creating core/shell-type NPs in which the IONPs form the magnetic core, surrounded by an inert shell, such as gold. These NPs have been frequently applied, and it has been demonstrated that the deposition of a gold shell has little effect on the magnetic properties of the iron oxide core.²⁷⁸

4.2. Bioinduced QDot Degradation

When QDots are exposed to a lysosomal microenvironment, the effect of proteases present in this compartment can have a substantial effect on the biologically-relevant coating of the QDots, as it has been demonstrated for other NPs. Recently, a novel methodology was described by which the stability of a monolayer of short surfactant molecules on the QDot surface in intracellular environments can be investigated.²⁷⁹ On the basis of this technology, the authors concluded that intracellular biothiols such as glutathione or cysteine can degrade QDot monolayers and thus drastically alter the colloidal stability and functionality of the QDots. The stability of the QDot monolayer was found to correlate with QDot size and ligand structure.

4.2.1. Effect of Low Endosomal pH on QDot Functionality. Exposure of QDots to an acidic microenvironment can have considerable effects on their physical properties, as holds true for the IONPs. For QDots, their fluorescence emission intensity depends on the surface of the NPs, where

oxidation or surface defects can lead to drastic reductions in NP photoluminescence quantum yield.²⁸⁰ Exposure of QDots to low pH values is also accompanied by a drop in fluorescence emission intensity.^{31,281} This effect depends on the QDot material as well as on the structure of the inorganic core. Inorganic core/shell structures which keep photogenerated charge carriers to the QDots core and away from the surface reduce effects of the environment on QDot fluorescence. Similar observations have been made in cells, where endosomal localization of QDots resulted in a gradual decrease in intensity of fluorescence emission. Although loss of cellular fluorescence with time will be affected by cell division and concurrent dilution of the QDots among daughter cells,²⁸² rapid effects within a short time span cannot be merely contributed to cell division effects. Several studies support this hypothesis, demonstrating that the loss of fluorescence intensity over time is steepest within the first few hours and then decreases gradually, likely indicating an initial pH-dependent effect followed by a slow dilution of the QDots upon cell division or QDot dissolution.²⁸³ This will be explained in more detail in the following section. It has been suggested that intracellular degradation of QDots depends on their location. Several groups reported that when QDots escape the endosomal pathway and thus avoid exposure to the degradative endosomal environment and low pH they remain stable and bright for long periods of time.^{284,285} Additionally, using fixed cells, the fluorescence emission intensity could be efficiently quenched upon exposing the cells to low-pH buffers (e.g., pH 4.6) (Figure 9).²⁸⁴

4.2.2. Impact of Low Endosomal pH on QDot Degradation. The rapid reduction in fluorescence emission intensities caused by exposure of QDots to low pH reveals that despite of the presence of organic shells on their surface the QDot cores may still be very susceptible to changes in their microenvironment. In other words, ligand shells around QDots in general are permeable to small molecules such as H⁺ or O₂. Exposure of CdSe/ZnS QDots coated with various types of polymers to UV light has been found to result in oxidative dissolution of the QDots, resulting in the release of Cd²⁺ ions.²⁸⁶ The rate of dissolution was dependent on the nature of the polymer coating, suggesting that the coating does provide some protection to the QDot cores. Cho et al.¹⁸⁴ described the effect of the endosomal microenvironment on the possible

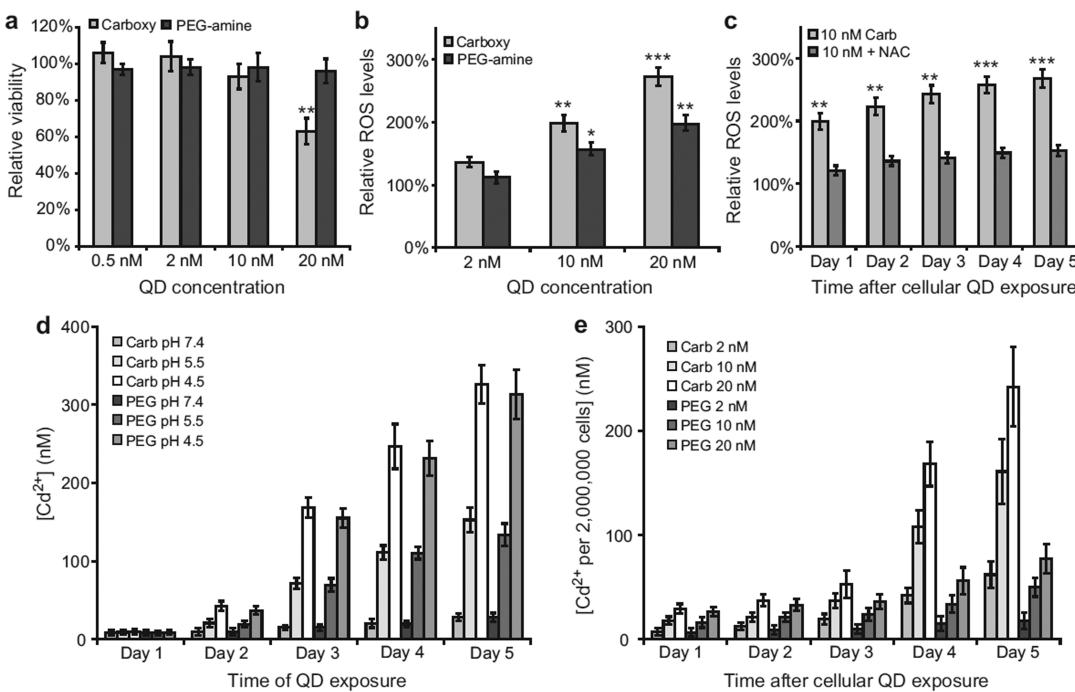


Figure 10. Evaluation of QDot toxicity and pH-dependent degradation. (a) Viability of C17.2 cells. (b) Levels of reactive oxygen species (ROS) in C17.2 cells as a function of QDot concentration for both carboxy- and PEG-amine QDs after 24 h incubation. Data are represented as mean \pm SEM ($n = 8$) and expressed as relative to untreated control cells, showing acute cytotoxic effects for carboxy-QDs at 20 nM and a clear concentration-dependent induction of ROS that is most pronounced for carboxy-QDs. (c) Levels of ROS in nonproliferating C17.2 cells exposed to 10 nM carboxy-QDs for 24 h and subsequently analyzed for ROS after 1, 2, 3, 4, or 5 days of additional culture showing a clear increase in cellular ROS levels. Cellular ROS levels could be reduced by coexposure of C17.2 cells to 5 mM N-acetyl-cysteine (NAC), a potent free radical scavenger. Data are expressed as mean \pm SEM ($n = 8$) against 60 nM Apigenin-treated control cells. (a–c) Degree of statistical significance of treated samples versus control samples is indicated when appropriate (**, $p < 0.01$; ***, $p < 0.001$). (d) Levels of free Cd²⁺ in suspensions of either carboxy- or PEG-amine QDs at various pH values (7.4, 5.5, 4.5) as a function of time showing a clear pH-mediated degradation of either type of QDot and similar associated Cd²⁺ release profiles. Data are expressed as mean \pm SEM ($n = 3$). (e) Levels of free Cd²⁺ in C17.2 cells exposed to either carboxy- or PEG-amine QDs for 24 h and subsequently kept in nonproliferating state after which the cellular Cd²⁺ levels are measured after 1, 2, 3, 4, and 5 days. Data are expressed as mean \pm SEM ($n = 3$) and show a clear time-dependent increase in cellular Cd²⁺ levels, being more pronounced for the carboxy-QDs, which is in line with their much higher cellular uptake efficiency. Reprinted with permission from ref 31. Copyright 2012 John Wiley & Sons.

degradation of CdTe and CdSe/ZnS QDs with three different coatings after internalization by human breast cancer cells. The authors found only a minor release of Cd²⁺ from the core/shell NPs while demonstrating a much higher (6–30-fold) release of Cd²⁺ for the core-only NPs, indicating the protective effect of the shell layer. In this study it was claimed that, in contrast to general belief, there is no dose-dependent correlation between CdTe dissolution and QDot toxicity, suggesting that factors other than free Cd²⁺ contributed to the QDot toxicity. Soenen et al.³¹ exposed two types of polymer-coated CdSe/ZnS QDs to an *in vitro* endosomal buffer system, similarly as described for the IONPs in section 4.1. Using this system, an immediate drop in QDot fluorescence emission intensity was observed, which was followed by a further gradual reduction of fluorescence intensity with time. These effects were correlated with a time-dependent release of Cd²⁺ for both types of QDs when exposed to the buffer systems. A time-dependent release of Cd²⁺ was observed in cell cycle-arrested cells, the extent of which depended on the level of cell-internalized QDs, carboxylated QDs being taken up much more efficiently than PEGylated ones (Figure 10). The time-dependent release of Cd²⁺ ions further augmented QDot toxicity as the viability of the cells gradually decreased with time in line with the augmented Cd²⁺ levels. These effects have been observed using various types of QDs, revealing that the

kinetics of QDot degradation are influenced by the nature of the QDot surface coating and chemical composition of the QDs.^{162,180}

4.2.3. Cytotoxic Effect of Intracellularly Released Cd²⁺ Compared to Free Cd²⁺. The contribution of Cd²⁺ to the overall toxicity of QDs has been found to be significant as “aged” QDs result in much higher toxicity levels than freshly prepared ones.¹⁸⁷ However, the results by both Cho et al.¹⁸⁴ and Soenen et al.³¹ indicate that although Cd²⁺ release significantly contributed to QDot toxicity, other factors such as NP-mediated ROS induction also play an important role. It is likely that direct QDot effects (such as ROS induction) result in acute toxicity, whereas Cd²⁺ release is a gradual process which will rather contribute to long-term toxicity, especially in nonproliferating cells. This hypothesis is confirmed by other studies, in which QDs were found to be more cytotoxic than free Cd²⁺ ions, when given at similar doses of unbound Cd²⁺.²⁸⁷ Likely, the effect of QDot compartmentalization in small delineated structures plays an important role. It is conceivable that upon degradation of the QDs much higher local Cd²⁺ concentrations within the endosomes and at the perinuclear region will be attained than can be achieved by external administration of Cd²⁺.^{31,288} Next to the degradation of QDs in cultured cells, the degradation of QDs in mice has also been described, where CdTe QDs were found to slowly

dissolve, releasing their metal ions in the bloodstream. Interestingly, Te^{2+} ions were mainly found in the kidneys, whereas Cd^{2+} ions mostly accumulated in the liver.²⁸⁹

4.2.4. Environmental Effects of QDot Degradation.

The widespread use of QDots also raises questions concerning their long-term effects on the environment as a result of their degradation. In the environment, the QDots can be subjected to widely varying pH conditions, low pH values as a rule resulting in a rapid release of Cd^{2+} ions (within 1 min) due to loss of the organic coating.²⁹⁰ The released ions can augment microbial toxicity. Similar to the situation in eukaryotic cells, QDots and their gradual release of Cd^{2+} have been found to be more toxic than free Cd^{2+} , suggesting the importance of NP-related effects.²⁹¹ The rate of dissolution also plays an important role, toxicity increasing with the rate of dissolution, which in turn is governed by the chemical composition and coating of the QDots.²⁹² Additionally, the interaction of QDots with other compounds, such as chelating agents, can result in chemical transformations, dictating their mobility and degradation.²⁹³ Studies have revealed that QDots that end up in the soil are slowly degrading, and any animals present in the soil, such as earthworms, will accumulate Cd^{2+} ions over time with the main source of the Cd^{2+} being the free ions rather than the whole QDots.²⁹⁴

4.2.5. Possible Protection of QDots against Intracellular Degradation. Protection of QDots against acid-induced degradation is not easily achieved. One possibility lies in the use of alternative materials for the synthesis of Cd^{2+} -free QDots, but these materials are less characterized and often contain heavy metals that are likely to induce toxicity.²⁹⁵ Alternatively, the coating of the QDots can be altered. However, there are certain limitations. Covering the QDots with a protective shell of a layer of gold or other metals or metal alloys, as used in the case of IONPs, would ruin the fluorescent properties of the QDots.^{296,297} One interesting approach that has shown some success is the use of dual-layered polymer/silica coating,^{168,298,299} which resulted in highly stable QDots with fluorescence intensity remaining nearly constant across the entire pH range (Figure 11).^{34,169,300} Such QDots were also found to be stable in the low gastric pH and could be used to visualize the gastrointestinal tract of mice.³⁰¹

4.3. Bioinduced Silver NP Degradation

4.3.1. Parameters Influencing Silver NP Degradation.

The dissolution of silver NPs is well known, and the effects of various parameters have been thoroughly investigated.²²⁰ Although separate detection of released Ag^+ and Ag NPs is challenging, it is technically possible.³⁰² Liu and Hurt³⁰³ found an increased dissolution rate of silver NPs with temperatures, rising from 0 to 37 °C and with increased acidity of the medium. The authors further showed that silver NPs can absorb Ag^+ on their surfaces, indicating that colloidal suspensions of silver NPs consist of three forms of silver: Ag(0) solids, free Ag^+ or its complexes, and surface-adsorbed Ag^+ . They further concluded that silver NPs cannot persist in realistic environments in the presence of dissolved oxygen and that under such conditions degradation will occur. Clear correlations between the dissolution rates of silver NPs at pH 7.0 and at 25 °C with increasing ionic strength have been described,³⁰⁴ suggesting that the dissolution rate of silver NPs in cell culture media will be much higher than in water.³⁰⁵ The dissolution rate of silver NPs can be influenced by the nature of

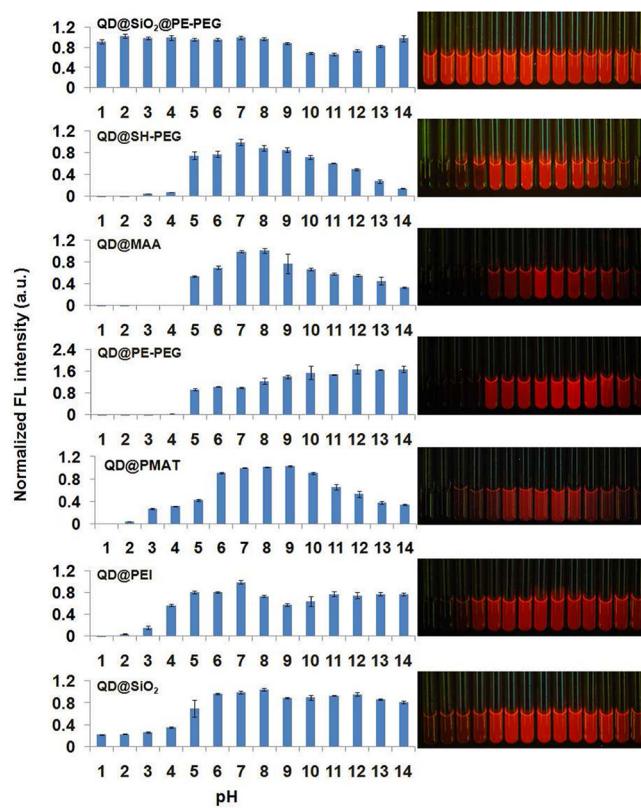


Figure 11. pH stability comparison of QD@ SiO_2 @PE-PEG with QDs with traditional surface coatings, QD@SH-PEG, QD@MAA, QD@PE-PEG, QD@PMAT, QD@PEI, and QD@ SiO_2 . Panels on the right show the corresponding fluorescence images of QDs dispersed in pH 1–14 solutions (illuminated with a 365 nm handheld UV lamp). Reprinted with permission from ref 300. Copyright 2010 American Chemical Society.

the coating applied,³⁰⁶ though this effect is not always as strong as one may expect.²⁰³

4.3.2. Toxic Effects of Silver NP Degradation. The effect of silver NP dissolution on their toxicity has been an issue of debate for a long time. Several studies compared the effect of silver NPs with the effect of free silver ions, where some reported comparable levels of toxicity for silver NPs and AgNO_3 .³⁰⁷ However, the mechanisms of toxicity were different: exposing cells to silver NPs did not affect the expression of metal-responsive metallothioneins, whereas exposure of cells to AgNO_3 did. To give an example, as metallothioneins are considered to present essential biomarkers in metal-induced toxicity,³⁰⁸ it can be concluded that the toxicity induced by the silver NPs was not a direct effect of free Ag^+ but rather caused by silver NP-induced oxidative stress.^{308,309} Thus, toxicity of Ag NPs is clearly not exclusively caused by release of Ag^+ ions. On the other hand, the observation that silver NPs and silver ions result in similar levels of effects, both in terms of cell viability and induction of oxidative stress, supports the notion that silver ions play a major role in the toxic effects induced by silver NPs.³¹⁰ Again, we emphasize that this apparent “contradiction” in fact is due to different experimental conditions. Ag administered in the form of free Ag^+ ions from a salt such as AgNO_3 , in general, will have a different intracellular distribution than incorporated Ag NPs and Ag^+ released from those. Interesting results in this connection were obtained by Eom and Choi,³¹¹ who found that the time of exposure was critical in

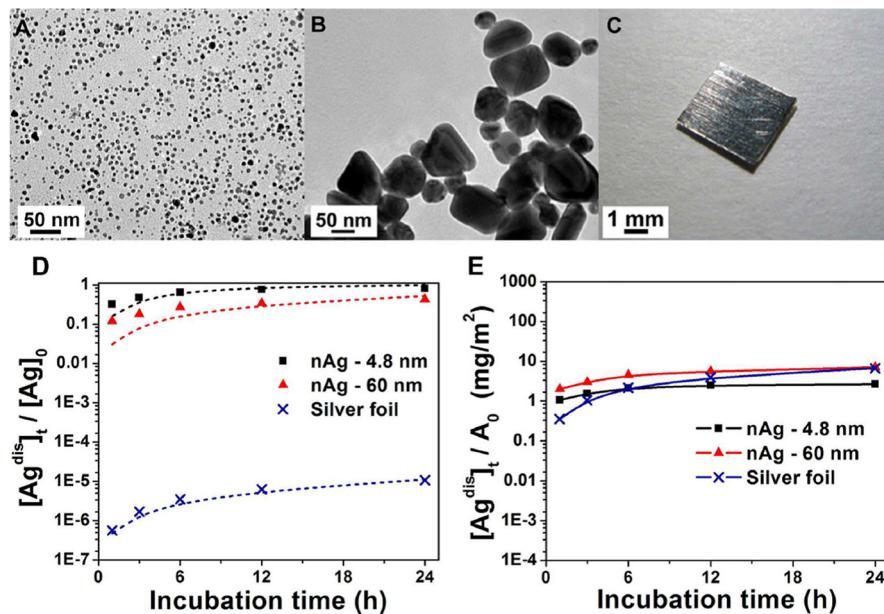


Figure 12. Effect of NP size on silver release rates. (A) Typical TEM image of nAg-4.8 nm synthesized by reduction of AgClO_4 with NaBH_4 in the presence of trisodium citrate, showing monodispersed spherical silver NPs with an average diameter of 4.8 ± 1.6 nm. (B) Typical TEM image of nAg-60 nm synthesized by reducing AgNO_3 with trisodium citrate while boiling, showing pseudospherical and truncated triangular NPs in a size range of 40–80 nm. (C) Image of a macroscopic square silver foil ($4 \text{ mm} \times 4 \text{ mm} \times 0.127 \text{ mm}$). (D) Time-resolved mass-based soluble silver release measurements in air-saturated acetate buffer (pH 4). Initial concentration of nAg-4.8 nm and nAg-60 nm is 0.05 mg/L, while initial concentration of silver foil is 10 700 mg/L (a $4 \text{ mm} \times 4 \text{ mm} \times 0.127 \text{ mm}$ piece in 2 mL buffer). Release can be described by first-order kinetics: $-(dm/dt) = k \cdot m$, shown by dashed lines. (E) Surface area-based soluble silver release renormalized from (D). Reprinted with permission from ref 318. Copyright 2012 American Chemical Society.

this aspect. Upon short incubation times toxic effects demonstrating themselves in oxidative stress were similar for silver NPs and free Ag^+ . However, after 24 h, the effects of the NPs had increased and were much higher than those caused by Ag^+ ions. Other studies also reported this higher toxicity of silver NPs than of free Ag^+ ions.³¹² This can be explained in a similar manner as the relatively high toxicity of dissolved IONPs or QDots by different intracellular distribution, leading for NPs to locally very high concentrations of Ag^+ ions which will not be reached using free Ag^+ . In addition, the presence of silver NPs provides a long-term source for continued release of Ag^+ ions to their environment, thus conceivably further augmenting toxic effects at later time points.³¹³ As higher ionic strengths enhance silver NP dissolution, the rate of intracellular dissolution of silver NPs proceeds approximately 50 times faster than their dissolution in water,¹⁹⁹ which will further increase NP toxicity. Using two sets of silver nanoclusters, either rich and poor in Ag^+ ions, it has been demonstrated that cytotoxic effects were mainly driven by the release of Ag from the nanoclusters and their subsequent oxidation to Ag^+ in the lysosomal environment, resulting in high levels of oxidative stress.³¹⁴

The high oxidative stress induced by the NPs or by free silver ions potentially causes DNA damage, as reflected by high levels of chromosomal damage in human hepatocarcinoma cells (HepG2).³¹² As these effects could be partially overcome by treating the cells with cysteine, a strong ionic Ag^+ ligand, this suggests an important role of free Ag^+ ions. In inhalation studies, the slow but persistent degradation of Ag NPs has also been shown to result in mild pulmonary fibrosis at later time points (21 days) in contrast to acute toxic effects of more rapidly degrading NPs, suggesting that biodegradation of NPs can have serious delayed effects.³¹⁵

4.3.3. Environmental Effects of Silver NP Degradation.

The effect of silver NPs on the environment is an area of intense research. Although silver NPs are frequently used because of their potent antimicrobial effects, their widespread use and distribution into the environment may present a serious (health) hazard and therefore calls for due caution in their use. Similar to the cytotoxic effects of silver NPs, their antimicrobial effects have been ascribed to either the whole NPs³¹³ or the presence of free Ag^+ ions.³¹⁶ Recently, Xiu et al.¹⁹⁵ presented an elegant strategy to distinguish between the effects caused by the two. Using various silver NPs under anaerobic conditions that preclude oxidation of the NPs and thus release of Ag^+ ions the antibacterial activity of the silver NPs was completely abolished, demonstrating the essential importance of free Ag^+ ions for the antibacterial effect. The authors suggested that modulating the release rate of Ag^+ , the antibacterial activity could be controlled and the environmental impact mitigated.

4.3.4. Possible Control over Silver NP Dissolution.

Control of silver NP dissolution rates might be achieved by modulation of a variety of factors, such as the nature of the coating applied. For example, similar to the other NPs, polymer coatings such as polyvinylpyrrole (PVP) seem to offer a better protection than short ligands such as citrate,³¹⁷ although other studies present less optimistic data in this respect.²⁰³ An interesting overview of possible strategies has been presented by Liu et al.,³¹⁸ who performed a systematic study of chemical concepts to control the dissolution of silver NPs. The authors claim that by carefully optimizing NP design the release rate of Ag^+ ions could be modulated by 4 orders of magnitude. Reduced dissolution rates could be obtained by thiol ligand binding, formation of sulfidic coatings, or scavenging of peroxy intermediates. Release of Ag^+ ions could be enhanced by

preoxidizing the NPs or by reducing NP size. In their quantification release rates expressed per units of surface area factors such as the size of the silver NPs did not greatly influence the release rate of Ag^+ , although, obviously, increased surface area over volume ratios resulted in a larger number of released ions per mass unit of silver compared to larger NPs (Figure 12).

4.3.5. Therapeutic Benefits of Silver NP Degradation.

For silver NPs, their optimal design remains an issue of debate. As both their antibacterial activity and their toxicity are dependent on the release of Ag^+ ions, the dissolution rates of the NPs will have to be carefully controlled in order to find conditions that enable the NPs to exert their function without running a potential risk. Once the fine control over their dissolution rates is achieved, it may also become possible to finely tune NPs that have a high potential for biomedical purposes by exploiting their toxic effects for specific targets. Recently, several interesting findings have been published, including the proposition that the toxic effects of silver NPs are strongly dependent on the type of cell used and that at specific conditions silver NPs did not cause cell death but rather resulted in a block of cell proliferation.³⁰⁸ More interestingly, several groups showed that under these conditions silver NPs specifically act at highly proliferative cancer cells, whereas normal, healthy cells either remained completely unaffected or displayed only minor, transient effects.^{308,319} Ag NPs have, for instance, been shown to affect the leukemic cells from patients with chronic myeloid leukemia, suggesting an important role of Ag NPs in future treatment of the disease.³²⁰ Liu et al.³²¹ demonstrated the potential of silver NPs as anticancer agents by conjugating them with TAT-peptide. These constructs were compared with the classical chemical anticancer agent doxorubicin, which is known to cause many side effects. The TAT-conjugated silver NPs were found to efficiently inhibit tumor growth in mice bearing malignant melanoma (Figure 13) without any obvious side effects on the animals. Interestingly, the TAT-conjugated silver NPs were also found to be very effective against multidrug-resistant (MDR) cancer cells in comparison to doxorubicin. The authors postulated that the

size of the silver NPs was too large for it to be pumped out of the cells by *p*-glycoproteins, one of the major efflux pumps for chemical therapeutics. Accordingly, cells labeled with silver NPs then contained a reservoir of Ag^+ ions which upon their release would result in cell death.

4.4. Bioinduced ZnO NP Degradation

4.4.1. Parameters Influencing ZnO NP Degradation.

Similar to the silver NPs, dissolution of ZnO NPs has also been studied intensively, and many parameters have been carefully assessed. ZnO NPs can easily be dissolved in aqueous solutions, whereby the rate of dissolution increases by lowering pH and NP size is an important parameter in determining the total number of released Zn^{2+} ions, with an increased release rate for smaller sized ZnO NPs.³²² This effect is again due to the increased surface area over mass of the ZnO NPs, keeping in mind that, at equal total surface areas, the total number of released ions may not significantly differ. In terms of cell exposure, the type of medium used is very important as clear differences in both aggregation state and dissolution rates have been observed for ZnO NPs dispersed in various types of cell media.³²³ These findings should be taken as a warning for caveat in comparative studies of ZnO toxicity in multiple cell types using different media compositions since either sedimentation or dissolution may substantially influence their effects on cell viability.

4.4.2. Toxic Effects of ZnO NP Degradation.

While the importance of ZnO NP dissolution with regard to their toxicological effect became widely accepted³²⁴ and at the same time ZnO NP dissolution was found to be greatly affected by the nature of the cell medium, other groups set out to elaborate on the precise mechanisms underlying ZnO toxicity. Gilbert et al.³²⁵ recently reported the fate of ZnO NPs in human bronchial epithelial cells and found that upon internalization the ZnO NPs completely dissolved (Figure 14). This led to high levels of intracellular Zn^{2+} ions complexed to naturally occurring molecular ligands. Obviously, the massive dissolution of ZnO NPs in a cellular environment will also influence the toxicity of the NPs. It has been shown that intracellular ZnO NP dissolution interferes with cellular Zn^{2+} ion homeostasis. Likely, ZnO NPs were taken up by the cells and directed into the endo/lysosomal compartment where they dissolve. This then elevates the cytoplasmic Zn^{2+} levels, resulting in sequestration of Zn^{2+} to the mitochondria leading to mitochondrial dysfunction, which in turn activates caspases and induces apoptosis.³²⁷ In an interesting study, Shi et al.³²⁶ observed that in most cases the viability of MCF-7 breast cancer cells exposed to various types of NPs could be restored by overexpressing microsomal glutathione transferase 1 (MGST1), an antioxidant enzyme. Where the NP-mediated effects were mostly related to induction of oxidative stress, as in the case of SiO_2 , the dissolution of ZnO NP and release of Zn^{2+} ions affected cell viability through other mechanisms that could not be overcome by MGST1 activity. The authors also postulated that Zn^{2+} ions could directly interfere with the activity of MGST1 and thereby abrogate its ability to restore cell viability. ZnO NPs have also been shown to readily dissolve in macrophages, where their dissolution resulted in minimal oxidative damage, but instead generated strong responses in cellular metabolism and proteasomal protein degradation efficacy, which are both processes known to be sensitive to cellular Zn^{2+} levels.³²⁷

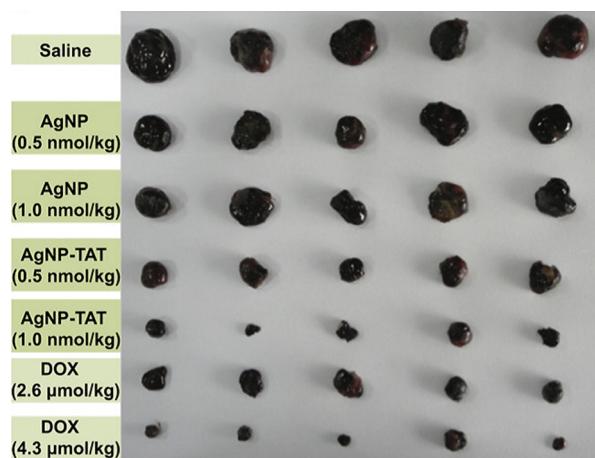


Figure 13. In vivo anticancer activity of nanosilver. Photomicrographs of representative tumors excised from animals treated with the conditions indicated (saline; silver NP (0.5 nmol/kg; 1.0 nmol/kg); TAT-conjugated silver NPs (0.5 nmol/kg; 1.0 nmol/kg); doxorubicin (2.6 $\mu\text{mol}/\text{kg}$; 4.3 $\mu\text{mol}/\text{kg}$)) showing their individual sizes. Reprinted with permission from ref 321. Copyright 2012 Elsevier.

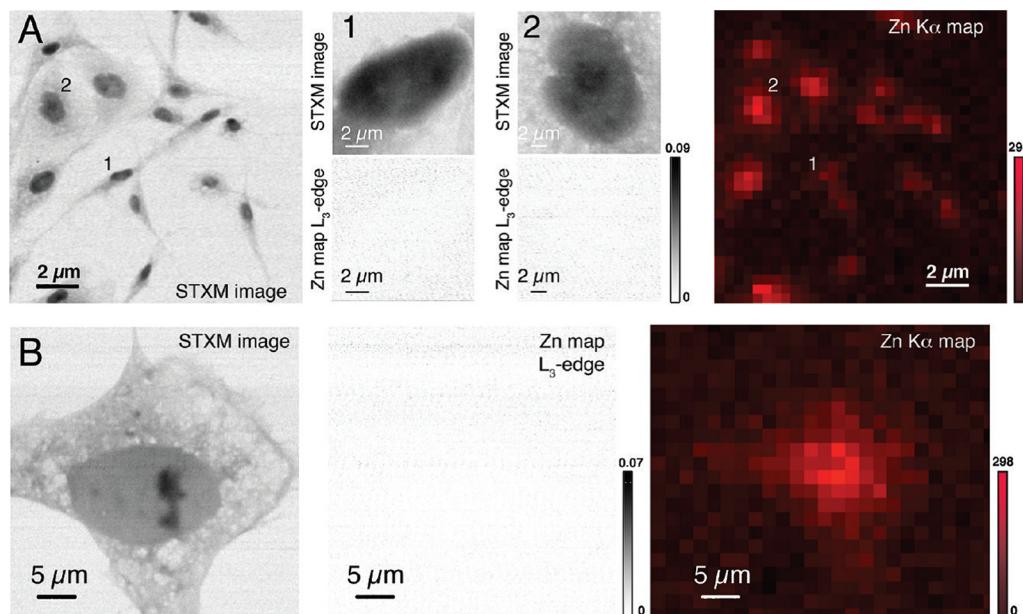


Figure 14. Scanning transmission X-ray microscopy (STXM; gray images) and micro-X-ray fluorescence (μ XRF; red images) mapping of intracellular zinc within BEAS-2B cells exposed to (A) 600 μ M ZnSO₄ or (B) BSA-coated ZnO NPs. Individual cells are clearly imaged by STXM at 30 nm resolution, but intracellular zinc is undetectable at the Zn L₃-edge. By contrast, μ XRF elemental mapping performed on the same cells show zinc-rich areas. XRF maps were recorded at 11 keV using a 6 μ m \times 6 μ m beam spot size and 3 μ m \times 3 μ m pixel size. Reprinted with permission from ref 325. Copyright 2010 American Chemical Society.

4.4.3. In Vivo and Environmental Effects of ZnO NP Degradation.

ZnO NP dissolution has also been found to be the main cause of lung injury in animal models. For instance, similar pathologies have been described in rats instilled with either ZnO NPs or free Zn²⁺ as both resulted in, among others, eosinophilia and pulmonary fibrosis. The toxic effects were explained by the presence of high levels of free Zn²⁺ instead of bound Zn²⁺. Zn²⁺ is an essential element in cell homeostasis and typically will remain bound inside cells as free ions are highly toxic. The endosomal dissolution of the NPs results in a rapid release of Zn²⁺, which was found to damage lysosomal membranes (Figure 15), resulting in its translocation to the cell cytoplasm where it can lead to cell death.³²⁸ The dissolution of ZnO NPs is also thought to have effects on the environment, as ZnO NP toxicity on zebrafish was reduced by sedimentation of the NPs (which results in a lower exposure of the zebrafish, as the NPs are on the bottom only) and augmented by ZnO NP dissolution.²³⁸ Again, these results confirm the importance of ZnO NP dissolution and the total available surface area in their reactivity toward the environment.

4.4.4. Possible Control over ZnO NP Degradation. As discussed in section 4.4, the dissolution rate of ZnO NPs can be modulated by incorporation of iron into the NP which alters the NP's matrix and slows down dissolution.²⁴⁰ The use of iron doping in ZnO NPs has been found to reduce the toxicity of the NPs toward zebrafish²⁴⁰ and to diminish their antibacterial activity.³²⁹ The same authors also demonstrated the reduced toxicity of iron-doped ZnO NPs in bronchial epithelial and macrophage cell lines³³⁰ as well as in a rodent lung model.²⁴⁰ Another possible solution is the application of alternative coatings, such as a TiO₂ shell, modulating the toxicity of the ZnO/TiO₂ core/shell structures by the thickness of the shell layer impeding Zn²⁺ release.³³¹

4.4.5. Therapeutic Benefits of ZnO Degradation. Like for the silver NPs, the avid dissolution of ZnO NPs has been suggested to be a potentially useful feature for the development

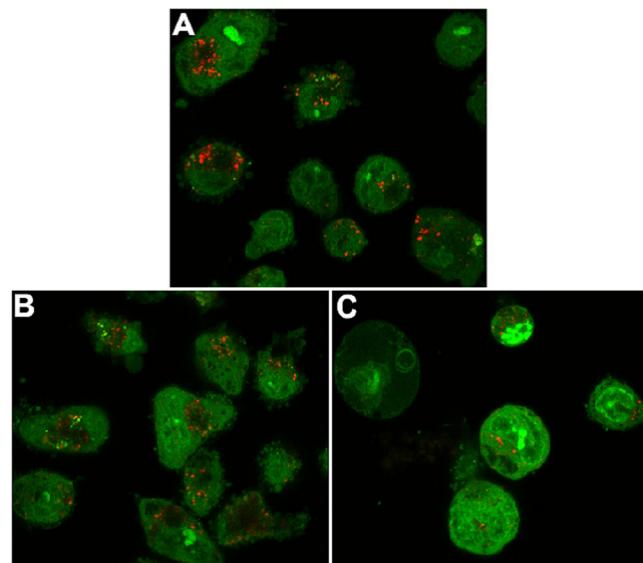


Figure 15. Lysosomal destabilization by ZnO NPs in THP-1 macrophage cells. Differentiated THP-1 cells by PMA (10 ng/mL) were stained with acridine orange for 15 min, and cells were treated with NP for 24 h: (A) vehicle control; (B) TiO₂ NP at 10 cm²/mL (36.4 μ g/mL); (C) ZnO NP at 10 cm²/mL (20.6 μ g/mL). Acridine orange dye aggregates inside of lysosomes which show red fluorescence. ZnO NP-treated cells showed less signals compared to vehicle control or TiO₂ NP-treated cells. Reprinted with permission from ref 328. Copyright 2011 BioMed Central Ltd.

of a novel anticancer therapy. Several authors have indeed reported a selective killing of cancer cells by the ZnO NPs under conditions where normal healthy cells remained unaffected.^{332–334} Given the rapid dissolution and high toxicity of ZnO NPs, their use in animals or humans is approached with more caution than for the silver NPs. Several elegant strategies

have however been proposed to exploit the potential anticancer effect of the ZnO NPs. As cancer cells are metabolically very active, they generate lactic acid, resulting in a decrease in the pH surrounding the tumor cells. However, several questions remain to be answered, such as the nature of selectivity between cancer cells and other metabolically active cells such as stem cells. It has been shown that ZnO NPs display strong pH-dependent dissolution kinetics. Irrespective of the size or nature of their polymeric surface coating, ZnO NPs undergo rapid dissolution in the region near cancer cells, which typically has a low pH (5–6), resulting in selective killing of the cancer cells.³³⁵ An interesting study by Muhammad et al.³³⁶ further demonstrated a synergistic effect of Zn²⁺ ions and chemical agents on the destruction of cancer cells. In this study, the authors employed ZnO NPs as plugs to seal the pores of mesoporous silica NPs containing doxorubicin. These ZnO NPs prevented premature release of the drug, but upon internalization by cervical cancer cells (HeLa), the ZnO NPs rapidly dissolved, releasing the drug and killing the cancer cells. Interestingly, the authors observed that the ZnO NPs not only functioned as a plug but actually exerted a synergistic effect on killing the cancer cells.³³⁶

4.5. Stability of Au NPs against Bioinduced Degradation

4.5.1. Toxic Effects of Nondegrading Au NPs. The biopersistence of Au NPs and their resistance against biodegradation in the intracellular environment may impart specific toxic effects. Au NPs, for instance, have been described to induce autophagy,^{337–339} a cellular defense mechanism, which at higher levels can result in cell death.¹³³ The precise mechanisms underlying the induction of autophagy remain unclear but are likely related to the high payload of nondegradable material occupying the cellular degradative compartment (endosomes). This will result in a loss of the total cellular degradative capacity, which can activate transcription factor EB (TFEB), which is a main regulator of both the lysosomal expression and the regulation (CLEAR) network, controlling the degradative capacity of the cells as well as the autophagy pathway.^{340,341}

4.5.2. In Vivo and Environmental Effects of Nondegrading Au NPs. The long biopersistence of Au NPs may have profound long-term effects on the toxicity of the NPs. As mentioned in section 3.5.3, the persistence of medium-sized NPs resulted in clear toxic effects *in vivo* compared to smaller and larger sized NPs that were cleared more efficiently.³⁴² For any therapeutic application, it is therefore important that the size and shape of the NPs is well controlled in order to allow a proper clearance of the NPs.

4.5.3. Therapeutic Benefits of Nondegrading Au NPs. The biopersistence of Au NPs may also offer some benefits in terms of biomedical applications. For any photothermal cancer therapies, the NPs could be used for repeated treatments at similar conditions over long time periods. For IONPs, which are prone to degradation, the application of repeated treatments would be quite challenging, as the degradation of the NPs would affect the efficacy of the thermal heating process. Additionally, when NPs are to be used in bioapplications, one of the main concerns are the possible long-term effects. In the case of nondegrading Au NPs, these effects can be studied and data can be extrapolated to later time points more easily than degrading NPs where not only the NPs but also their metabolites must be considered and their kinetics over time.

5. CONCLUSION AND FUTURE PERSPECTIVES

The use of NPs in daily life and biomedical research is rapidly increasing. The great versatility in different types of materials, coatings, sizes, and shapes offers great potential for both technological and medical applications, but at the same time, they are a cause of concern with regard to their impact on living organisms and the environment. Considerable progress has been made in our understanding of how NPs interact with living matter such as cells and tissues, but several aspects have only recently been addressed and need to be further unraveled. One such topic is the effect of the microenvironment on the chemical stability of metal (oxide) NPs.

Here, we provided an overview of the current understanding of the behavior of IONPs, QDots, silver and ZnO NPs, and Au NPs as a result of highly varying conditions to which they will be exposed when used in biomedical research or when distributed in the environment. It was shown that depending on the nature of the core material degradation of the NPs can occur, and its extent depends on the microenvironment to which they are exposed (pH, ionic strength). In this connection, distinction should be made between degradation of the inorganic core, which highly depends on the material of the core, and degradation of the organic surface coating, which in particular affects colloidal stability and thus biodistribution of the NPs. For nondegrading NPs such as Au NPs, the biopersistence of these NPs may cause long-term effects; however, by appropriate control over various physicochemical parameters, any toxicological impact of nondegrading materials can be better controlled and more easily studied. For degrading NPs, the process of degradation brings along substantial difficulties in understanding their toxicological profile as not only the NP but also its released ions and the combination of the two can all have different biodistributions and toxic effects, which must be studied in detail. This degradation may have significant effects on the functionality of the NPs and thus hinder their use in various conditions because the NPs themselves become less effective or because of augmented NP toxicity. The underlying mechanisms are multifold and depend on the chemical composition of the inorganic core. In essence, leaching of metal ions from the core NPs will result in high local concentrations of the metal ion. Some metal ions, e.g., Cd²⁺ or Ag⁺, are intrinsically toxic and thus pose serious hazards at low concentrations. Other metal ions such as Fe²⁺ or Zn²⁺ are biologically useful and control various biological processes such as cell cycle progression, but when present at high levels, cellular signaling cascades can be perturbed, resulting in significant toxicity. Alternatively, these ions would normally be present in specific cellular compartments (e.g., ferritin for Fe²⁺), whereas the release of the ions from the NPs occurs in the lysosomal compartment which has a much lower pH value than the cell cytoplasm. The presence of high levels of these metal ions in a different cellular compartment with its unique acidic and degradative microenvironment can also result in toxicity by the induction of high levels of ROS. For NP functionality, acidic etching of the NP surface will affect the structure of the inorganic core and hereby alter its physical parameters (e.g., loss of fluorescence intensity for QDots or MRI contrast for IONPs). To overcome these problems, several strategies have been proposed by which the respective NPs could be protected more effectively against environment-induced dissolution. The use of protective coatings that are more resilient against pH-induced degradation (e.g., thick

polymer shells, silica layers, or gold shells instead of short ligands) can improve NP stability. Other focus points are NP shape and size, where the ratio of surface area over volume should be kept as low as possible as degradation will occur at the surface of the NPs. Furthermore, the composition of the chemical core can be adjusted by the addition of other metal ions and hereby altering the matrix of the inorganic cores, which can result in enhanced chemical stability against pH-dependent degradation. Depending on the envisaged application, all these parameters should be optimized, where highly stable NPs should be used for most imaging purposes. Alternatively, unstable NPs could offer additional therapeutic benefits in cancer research if targeting of the NPs can be made highly efficient to allow local dissolution of the NPs at the desired target site. As the control over many synthesis-related parameters is increasing and the dissolution rate of the NPs can be tailored to demand more precisely, this opens up numerous possibilities to exploit the intrinsic toxicity of optimally designed NPs as a novel anticancer therapeutic.

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Notes

The authors declare no competing financial interest.

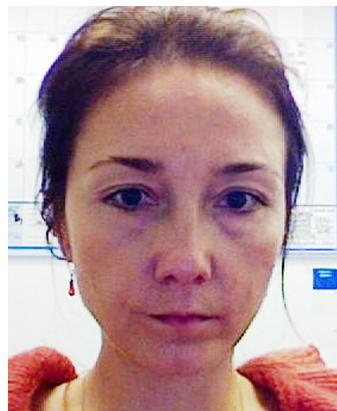
Biographies



Stefaan J. Soenen (1983) graduated with his M.S. degree in Industrial Sciences (Biochemistry) in 2005 and a second M.S. degree in Molecular Medical Biotechnology at Ghent University (Belgium) in 2006. He then pursued Ph.D. studies until 2010 at the lab of Marcel De Cuyper (KULeuven, Belgium) focusing on cell–nanoparticle interactions. After this he became a postdoctoral fellow at Ghent University under the guidance of Kevin Braeckmans and Stefaan De Smedt and is currently also working at the KULeuven, Biomedical MRU Unit, under the supervision of Uwe Himmelreich, focusing on biomaterials use in live cells. His main research interests lie in studying cell–nanoparticle interactions, unraveling the mechanisms involved, and investigating the possible health effects and medical uses of nanomaterials.



Wolfgang J. Parak obtained his Ph.D. degree at the LMU Munchen, Germany (1999), in the group of Hermann Gaub. After a postdoctoral stay at the University of California, Berkeley, CA, in the group of Paul Alivisatos he returned to Munich as an Assistant Professor in 2002 with a temporary position as Associate Professor in 2005. Since 2007 he has been a Full Professor at the Physics Department of the Philipps Universitat Marburg, Marburg, Germany. In addition, he was appointed as Head of the Biofunctional Nanomaterials Unit at CIC biomaGUNE, San Sebastian, Spain, in 2013. He is also Associate Editor of *ACS Nano*.



Joanna Rejman (1971) graduated at the University of Wroclaw. She was granted a Marie Curie Individual Fellowship to perform a postdoctoral research project on lipid-mediated cell transfection at the University of Groningen (The Netherlands) in the group of Prof. Hoekstra. Two years later she joined the group of Prof. Conese in the research institute of San Raffaele Hospital in Milan (Italy). She continued her research in the field of gene and cell therapy at the Faculty of Pharmaceutical Sciences in Gent (Belgium). There she was also involved in projects on the development of noninvasive methods to image migration and survival of stem cells *in vivo*. She is currently employed in the Biophotonic group of Philipps University of Marburg. Her main scientific interests include noninvasive cell labeling, (transient) modification of cell functions, and understanding the processes involved in internalization, intracellular trafficking, processing, and potential toxic effects of nanoparticles.



Bella B. Manshian completed her Ph.D. degree in Genetic Toxicology studying genotoxic effects of ultraviolet radiation and chemical mixtures. During her Ph.D. studies she worked part time as a research assistant in newborn immunity and allergy studies following child developmental medicine and stem cell research in collaboration with Celgene Cellular Therapeutics, world leaders in stem cell therapy, and the U.K. National Health System. Following this she was a research officer in the Swansea Nano medicine department, where she investigates the genotoxicity of nanoparticles. Currently, she works at KULeuven, Biomedical MRI Unit, focusing on the possible health effects and medical uses of nanomaterials.

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