Toxicology Research

REVIEW

Silver nanoparticles – wolves in sheep's clothing?

Rasmus Foldbjerg,[a,b] Xiumei Jiang,[a,c] Teodora Miclăuş,[d] Chunying Chen,[c] Herman Autrup[a] and Christiane Beer*[a]

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Silver nanoparticles (Ag NPs) are one of the most widely utilized engineered nanomaterials (ENMs) in commercial products due to their effective antibacterial activity, high electrical conductivity, and optical properties. Therefore, they have been one of the most intensively investigated nanomaterials in terms of their toxic effects on humans and the environment. It has become clear during recent years that nanomaterials can behave unexpectedly due to new and unique characteristics when their particle size reaches the nanoscale (1–100 nm). Consequently, their effect on human health and the environment has been hard to predict. Widespread applications increase the chances of public and environmental exposure to Ag NPs and have thereby increased concerns regarding the potential adverse effects of Ag NPs on human health and environmental safety. To fully understand and predict possible health effects following exposure to Ag NPs, information about the mechanisms for their cytotoxicity and genotoxicity is necessary. The present paper attempts to review the cellular and molecular mechanisms behind Ag NP toxicity. In addition, the role of silver ions in the toxicity of Ag NPs is discussed.

1 Introduction

Nanotechnology is one of the most rapidly growing fields holding great promise for scientific advancements in many sectors such as medicine and consumer products. Nanotechnology exploits the unique properties that arise at a size smaller than 100 nm affecting the physical, chemical, and biological behavior of engineered nanomaterials (ENMs). However, the same characteristics which make ENMs attractive for their use in new products have led to concerns that they

[a]*Department of Public Health, Aarhus University, Bartholins Alle 2, 8000 Aarhus C, Denmark. E-mail: cbee@ph.au.dk; Tel: +45 8716 8027*
[b]*The Faculty of Medicine, Department of Health Science and Technology, Fredrik Bajers Vej 3, Building B, 9220, Aalborg Ø, Denmark*
[c]*The National Center for Nanoscience and Technology, Chinese Academy of Science, No. 11, Beiyitiao Zhongguancun, Beijing 100190, China*
[d]*iNANO Center, Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark*

Rasmus Foldbjerg

Rasmus Foldbjerg is an Assistant Professor in the Laboratory of Immunology at Aalborg University. In 2003 and 2007, respectively, he earned his B.S and M.S degrees in Molecular Biology at Aarhus University. Later, he joined the group of Professor Herman Autrup at the Department of Environmental and Occupational Medicine, Aarhus University and here he received his Ph.D. in Medicine in 2013. His research interests are nanotoxicology, immunology and the development of vaccines.

Xiumei Jiang

Xiumei Jiang is a PhD student at the CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China and has been an exchange PhD student at the Department of Public Health, Aarhus University in Denmark during 2012–2013. She received her Bachelor's Degree in Biology from Northeast Normal University, China (2010). Her research interests include the biomedical applications and potential toxicity of noble metal nanoparticles.

may pose a risk to humans and the environment. As a consequence of their small size, nanoparticles (NPs) have a very high surface to volume ratio, rendering them potentially more reactive than larger particles. From studies on the ultrafine nano-sized particle fraction from air pollution it is known that exposure to these particles increases the risk of developing airway and cardiovascular diseases. The increased use and exposure of consumers and workers to ENMs and their potentially higher reactivity has resulted in concerns about potential adverse health effects of ENMs and the development of a new toxicological field, nanotoxicology. Based on the definition of toxicology by the Society of Toxicology (SOT),[1] nanotoxicology has been described by Oberdorster as the study of the adverse effects of ENMs on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects.[2]

Ag NPs are one of the most widely used ENMs, especially due to their effective antibacterial activity. They are used in medical devices and supplies as well as in consumer products such as surface cleaners, room sprays, toys, antimicrobial paints, home appliances, food storage containers, and textiles.[3,4] Therefore, they have been in the focus of a number of toxicological investigations during recent years. However, silver and its antibacterial capacity have been used for centuries. Already in ancient Italy and Greece silver was utilized, *e.g.*, for storage vessels to keep the water fresh. The antibacterial effect of silver was scientifically described in the late 19th century[5] and has subsequently been exploited in a wide range of medical applications like medical equipment, implants and prostheses, catheters, wound therapy and surgical textiles.[6] Today, silver is still used for wound treatment, especially in burn victims to prevent infections.[6] The absorption of silver is most likely to occur in the gastro-intestinal tract, the lungs or through damaged skin.[7,8] Soluble silver compounds are considered more likely to cause adverse health effects compared to metallic or insoluble silver.[8] Prolonged exposure to silver compounds has been shown to be associated with the development of a characteristic, irreversible pigmentation of the skin (argyria) and/or the eyes (argyrosis)[7] that is due to the precipitation of silver in dermis and mucosal membranes.[9] However, conventional silver compounds are not thought to be carcinogenic or toxic to the immune, cardiovascular, nervous or reproductive systems.[7,8] However, based on the fact that ENMs may behave physically, chemically and toxicologically in a different manner than the bulk material, there are concerns that exposure to Ag NPs might lead to health effects that cannot be predicted by studying the parent material. For example, due to the higher reactivity of ENMs it is reasonable to assume that ENMs could, at least theoretically, react with biological systems in new unpredicted ways and might be quantitatively and qualitatively more toxic.

We will here review the present knowledge on the cellular and molecular mechanisms behind Ag NP toxicity. In addition, the role of silver ions in the toxicity of Ag NPs is discussed.

2 Physicochemical properties that may influence the toxicity of Ag NPs

A number of toxicological investigations have shown that the physicochemical properties of ENMs are of great importance for the toxic potential of these materials, as they may influence the interaction of ENMs with the organism. The most important physicochemical properties in this regard are: size including surface area, agglomeration and aggregation state; surface chemistry including surface charge and coating; shape; and chemical composition.

2.1 Size

The physical behavior of particles changes dramatically when they reach sizes lower than 100 nm. Below this size, the smaller the particles are, the more the rules of quantum

Teodora Miclăuş

Teodora Miclăuş is currently a PhD student at the Interdisciplinary Nanoscience Center, Aarhus University. Her project, under the supervision of Assoc. Prof. Duncan Sutherland, involves the study of protein-mediated surface phenomena at silver nanoparticles in the context of toxicology. She received her B.Sc. in 2009 and her M.Sc. in 2011 from the University of Bucharest.

Prof. Chunying Chen

Prof. Chunying Chen is a principal investigator at CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China. She received her PhD degree in Biomedical Engineering (1996) from Huazhong University of Science and Technology of China. Her research interests include the potential toxicity of nanoparticles, therapies for malignant tumors and vaccine nanoadjuvants using theranostic nanomedicine systems. She serves as an editorial member in Nanotoxicology, Particle and Fibre Toxicology and Toxicology Research.

physics apply, resulting in new chemical, mechanical, electrical, optical and/or superparamagnetic characteristics of the particles.[10,11] Considering the possibility of novel and unique properties of materials on the nanoscale, it is nearly impossible to extrapolate the biological reactivity and toxicity of ENMs from their larger-sized counterparts. Therefore, the size is undoubtedly an extremely important property of ENMs, also from a toxicological point of view, as it influences a number of particle characteristics which themselves have a strong influence on the toxicity of ENMs. The characteristics are high surface to volume ratio, high surface reactivity, adsorption of compounds, the ability to cross cellular membranes and strong interparticle forces. Indeed, studies which have compared Ag NPs of different sizes have shown that smaller NPs are more cytotoxic than their larger counterparts.[12–15] The increased specific surface area of smaller particles leading to higher reactivity or an enhanced release of toxic silver ions from the particle surface may explain the size-dependent toxicity of Ag NPs. However, it should be noted that the studies mentioned above do not demonstrate a clear association between nanoscale phenomena such as quantum effects or surface plasmon resonances and toxicity. It has also recently been pointed out that size-dependent toxicities of NPs often are scalable effects, meaning that small particles show greater or lower tendencies to behave in a certain manner compared to large particles, but that their behavior is predictable from that of larger particles.[16]

2.2 Surface chemistry

Surface chemistry, including surface charge and coating, also has an influence on the toxicity of Ag NPs. In general, the coating of ENMs has several purposes: to avoid agglomeration/aggregation, to change the surface charge, to target ENMs for uptake by specific cell types, or to change the bioavailability and degradation of the particles. For example, Ag NPs coated with the polysaccharide gum arabic were able to penetrate cell organelles whereas uncoated Ag NPs with a hydrocarbon surface layer aggregated and did not penetrate cell organelles. Not surprisingly, the polysaccharide gum arabic coated Ag NPs that are distributed throughout the cells caused the highest toxicity.[17] This difference in their toxicity might be caused by a higher degree of agglomeration of the uncoated Ag NPs and differences in their uptake and dissolution. Noticeably, despite presenting different surface chemistries, the two types of Ag NPs had comparable negative surface charges. However, another study from the same group showed that Ag NPs with a hydrocarbon surface layer were more toxic than polysaccharide-coated Ag NPs, suggesting that other factors such as cell type, media and binding of serum proteins may influence the toxicity.[18] Another recent study focused on four types of Ag NPs with various surface coatings that covered high negativity to high positivity.[19] In this study, poly(diallyldimethylammonium)-coated Ag NPs were found to be the most toxic, followed by biogenic-Ag and oleate-Ag NPs, whereas uncoated Ag NPs were found to be the least toxic in both mouse macrophage and lung epithelial cells. In other words, the more positively charged NPs were found to be the most toxic.[19] This corresponds to results from a study on gold NPs which concluded that positively charged particles have greater efficiency in cell membrane penetration and cellular internalization.[20] In contrast to the findings by Suresh and coworkers,[19] Yang *et al.* compared Ag NPs with similar sizes but different coatings (citrate, PVP and gum Arabic) and found the toxicity of Ag NPs to be independent of surface charge when using the model organism *C. elegans*. They concluded that toxicity was mainly due to NP dissolution, which depended on the surface coating.[21]

The complexity of NP surface chemistry is further complicated by the fact that the surfaces of NPs are immediately

Herman Autrup

Herman Autrup, PhD, is a professor in environmental medicine, Department of Public Health, University of Aarhus, Denmark. He is a member of the Danish Academy of Technical Sciences and of the Academy of Toxicological Sciences. He has received the Frits Sobel award from the European Environmental Mutagen Society, the EUROTOX Merit Award (2013), and the SOT Education award (2014). He is the president of IUTOX and the chair of ILSI Health Environment Science Institute. Herman Autrup has published more than 200 papers in international peer-reviewed journals. He is currently an associate editor of Toxicological Sciences and an Advisory Board member of Toxicological Research.

Christiane Beer

Christiane Beer is a researcher at Aarhus University Hospital, Department of Occupational Medicine and an external Assoc. Prof. at the Department of Public Health, University of Aarhus, Denmark. She received her PhD in natural sciences from the University of Braunschweig, Germany. In 2003 she received a postdoctoral fellowship from the German National Academy of Sciences, Leopoldina. She is a board member of the Danish Society of Pharmacology, Toxicology and Medicinal Chemistry. Her present research interests are nanotoxicology and health effects of exposure to quartz and coal dust.

covered by proteins when they come in contact with a biological medium where they can interact, in theory, with any protein of the plasma proteome that consists of approximately 3700 different proteins.[22] This interaction between particles and proteins results in a protein corona covering the surface of the particles. It is notable that the formation of the corona is a dynamic, competitive process. Over time, the most abundant proteins that bind first are displaced by proteins with higher affinity. The resulting protein corona consists of a "hard corona" with only a few proteins in a relatively immobile layer, and a "soft corona" with a more loosely bound protein layer that is less well-understood.[23] Which proteins bind to the particles is, however, dependent on their chemical composition and surface charge. For carbon black, silica, titanium dioxide and acrylamide nanoparticles several of the proteins of the protein corona have been identified.[24–27] A number of these proteins are ligands to receptors at the cell surface. Through the interaction with an appropriate receptor, these proteins are affecting the uptake of the ENMs they are bound to. Ashkarran *et al.* found that depending on the NP shape and the NP/protein ratio the protein corona can evolve quite differently, thereby affecting the composition and thickness of the corona.[28] This could have serious implications for *in vitro* to *in vivo* extrapolations since the NP/protein ratio is often very different in these two situations. In another recent study, Hayashi *et al.* showed that the presence of a specific protein secreted by earthworm *Eisenia fetida* in the corona of Ag NPs leads to increased cell uptake.[29] These examples, while not exhaustive, suggest that the formation of a protein corona at the surface of NPs leads to a new "biological identity" in the biological milieu, which has an effect on the subsequent cellular/tissue responses. Therefore, the NPs that cells of tissues and organs actually come in contact with are completely different from the original surface of the NP.[22] In addition, the coating and the protein corona of ENMs also have an effect on the surface reactivity. If the surface atoms of the ENMs are covered by proteins, their surface reactivity is affected and the biological responses might be reduced. However, the dynamics of the Ag NP corona in response to FBS concentrations, incubation time, NP size, NP surface coating *etc.* are still unresolved.

3 Mechanisms of Ag NP toxicity

3.1 Toxicity of Ag NPs – silver ion or particle?

Compared to micro-sized particles, NPs have a remarkably high surface-to-volume ratio. In a 10 nm NP the fraction of surface atoms accounts for more than 10% of all the atoms composing the crystallite, and for a 2 nm particle it increases to approximately 60%.[30] This high availability of surface atoms increases the potential for releasing, in the case of Ag NPs, silver ions as both solubility and dissolution kinetics may vary as a function of size.[31,32]

As it is known that silver ions are toxic, there is broad agreement that they strongly contribute to the biological activity of Ag NPs and several studies have reported the influence of size, coating, concentration, temperature, pH, ionic strength, and time on the dissolution behavior of Ag NPs.[33–36] Although the high surface area of metal-based NPs increases the possibility that metal ions are released from these NPs,[31,32] it is unclear to what degree the toxicity of Ag NPs results from released silver ions and how much toxicity is related to the Ag NPs *per se*. Therefore, understanding the ion release kinetics for Ag NPs is critical for understanding the mechanisms of Ag NP toxicity.

Particles, such as Ag NPs, that are composed of elemental silver (Ag^0) are generally not considered to be soluble or reactive in pure water.[37] However, they can dissolve under oxidizing conditions involving two coupled processes: (1) oxidation with a release of reactive oxygen species and (2) a proton-mediated release of dissolved silver.[35] In agreement with this mechanism, silver ion release could be controlled through the manipulation of the oxidation pathways by changing the surface area (size), ligand binding, polymeric coatings, scavenging of peroxy-intermediates, and pre-oxidation treatments.[35] The surface oxidation of Ag NPs results in the formation of highly reactive silver ions. These silver ions are adsorbed on the surface of the NP but are also released to the surrounding environment and a colloidal suspension of Ag NPs will therefore contain at least three forms of silver: Ag NPs, dissolved silver (both ionic silver and soluble silver complexes), and ionic silver adsorbed on the surface of NPs.[34] Oxidative dissolution is a complex chemical reaction influenced by pH, coatings, temperature and ligands in the surrounding fluid.[36] PVP-stabilized Ag NPs dissolved faster than citrate-stabilized Ag NPs and an increase in temperature led to increased dissolution.[33] But also the presence of cysteine or BSA can enhance the dissolution of Ag NPs.[36,38]

Dissolution of Ag NPs *in vivo* might be completely different, and to simulate the biodissolution of Ag NPs, experiments have been conducted in artificial body fluids like artificial interstitial fluid (Gamble's solution, pH 7.4) and artificial lysosomal fluid (ALF, pH 4.5). These were used to simulate dissolution in the airway surface liquid or in the macrophage phagolysosome, respectively. However, no dissolution of Ag NPs into silver ions in either of the simulated biological fluids was detected.[39] This is in agreement with our own findings where we investigated the silver ion release from Ag NPs in cell culture medium at pH 4.5 and pH 7.0. We found that Ag^+ release in the medium was lower than expected, with only 7.5% at pH 4.5 and 5% at pH 7.0 after 24 h incubation.[40] However, the fluids used by Stebounova *et al.* and us contained significant amounts of sodium chloride leading to precipitation of silver chloride complexes. In synthetic gastric fluid (pH 1.12), Ag NPs (5 nm) dissolved relatively rapidly whereas the dissolution was very slow in wound fluid (pH 7.52). Interestingly, the addition of BSA greatly increased the dissolution.[36] Rogers *et al.* found that citrate-stabilized Ag NPs (1–10 nm and 40 nm) agglomerate, release silver ions, and partially react to form silver chloride complexes in synthetic stomach fluid (SSF, pH 1.5).[41] Thus, many recent studies have

investigated the chemical transformations of Ag NPs in biological environments, but exact knowledge about the importance of silver speciation inside cells is needed to establish the mechanism of action. Using the Triton X-114-based cloud point extraction method described by Guibin Jiang's group[42] that allows the separation of silver ions released from intracellular Ag NPs to investigate the intracellular fate of Ag NPs, we have shown that more than half (55%) of the internalized Ag NPs dissolved in silver ions after 1 h incubation and dissolution increased over time.[43]

It has been suggested that the toxicity of Ag NPs is mainly due to oxidative stress and is independent of silver ions.[44] Other studies reported that the measured silver ion content of the Ag NP suspension could not fully explain the observed toxicity of the Ag NP suspension and that both silver ions and Ag NPs contribute to the toxicity.[45,46] Although these reports are to some degree conflicting, most of the evidence suggests that silver ions at least account for a part of the toxicity of Ag NPs. It is, however, difficult to determine to what extent the Ag NPs in a solution contribute to cellular toxicity because the Ag ions in the solution are relatively toxic to cells and thus tend to overshadow the toxicity of the Ag NPs themselves. For example, for an Ag NP suspension containing 5.5% silver ions we could not detect any difference in toxicity between the Ag NP suspension and its supernatant.[47] Therefore, it seems that at low metal ion concentrations the uptake of NPs leads to an additional toxicity, whereas at higher metal ion concentrations the presence of NPs does not add further measurable toxicity. This is in agreement with the findings of Navarro *et al.* (2008) and Kim *et al.* (2009).[44,46] In both these studies the Ag NP suspension contained low amounts of silver ions and in both cases the ionic fraction of the Ag NP suspension could not fully explain the measured toxicity. This was also the case in a recent study on *C. elegans* where the authors found a linear correlation between Ag NP toxicity and NP dissolution. Noticeably, none of the Ag NPs used in this study exhibited greater toxicity than would be predicted by complete dissolution of the same mass of silver as silver ions.[21] This is in agreement with results from a study which investigated the effect of Ag NPs and silver nitrate on gene expression in CaCo-2 intestinal cells. Both give rise to very similar responses, leading to the conclusion that the toxic effects observed from Ag NPs are likely due to silver ions that are released from the NPs.[48] In addition, the toxicity of 20–80 nm Ag NPs could fully be explained by released silver ions whereas 10 nm Ag NPs proved more toxic than predicted.[49] Although the cytotoxicity of Ag NPs may largely be explained by silver ions, gene expression data indicate that Ag NPs may affect cells in a more complex way than silver ions.[50] In conclusion, it is still uncertain by which mechanism and to what degree silver ions play a role in Ag NP-mediated toxicity.

3.2 Uptake of Ag NPs

Several authors have reported that different pathways, such as clathrin-mediated and caveloe-mediated endocytosis, phagocytosis and pinocytosis,[51–53] are involved in the uptake process of nanomaterials into cells. Which of these cellular uptake mechanisms apply is greatly dependent on the size of the particles and on their surface coating. If particles reach a size larger than approximately 500 nm they are mainly taken up *via* phagocytosis by so-called professional phagocytes like neutrophils, monocytes, macrophages, dendritic and mast cells; smaller particles are primarily processed by endocytic pathways. An alternative for the uptake of larger aggregates (0.5–5 μm) might be micropinocytosis.[53]

Size also plays a role in the uptake of Ag NPs. Liu *et al.* reported that higher levels of silver were present in cells exposed to small Ag NPs than in cells exposed to larger Ag NPs (20 nm and 50 nm), indicating a size-dependent cellular uptake.[15] However, the assessment of which pathways are involved in the uptake of Ag NPs has not been investigated in much detail. We have previously used transmission electron microscopy to study the uptake of Ag NPs by the Chinese hamster ovary cell line CHO-K1.[43] We observed that the uptake of Ag NPs into cells is a time dependent process, with increased amount of nanoparticles in cells over time. To investigate the cellular uptake pathway for BSA-coated Ag NPs' internalization into CHO-K1 cells, we used several chemical uptake inhibitors and low temperature.[40] Cells incubated at 4 °C or treated with methyl-β-cyclodextrin or dynasore exhibit a significant decrease in uptake of Ag NPs, indicating that Ag NPs are mainly taken up *via* energy-dependent and lipid-raft mediated endocytosis pathways, such as caveolae-mediated endocytosis. However, at 4 °C cells are still able to take up Ag NPs, suggesting that energy-independent uptake pathways may also be involved in the internalization of Ag NPs.[40] Parameters such as nanoparticle composition, size, and surface chemistry (especially coating) may play a significant role for the preferred uptake pathways for a specific nanomaterial.[53] In addition, the cell line used to investigate the uptake is of importance too. PVP-coated Ag NPs were mainly taken up by clathrin-dependent endocytosis and macropinocytosis into human mesenchymal stem cells.[54] Uptake of Ag NPs into macrophages seems to involve clathrin-dependent endocytosis mechanisms.[55] In the bronchial epithelial cell line BEAS-2B uptake mechanisms included nearly all known active uptake pathways: clathrin- and caveolin-dependent endocytosis, micropinocytosis, and phagocytosis.[56] Several studies showed that Ag NPs are intracellularly localized in endosomal/lysosomal structures after uptake.[43,54]

3.3 Intracellular fate of Ag NPs

Limbach *et al.* noted that NPs, in general, could be carriers for heavy metal uptake into human lung epithelial cells, accentuating the toxicity of the NP. They termed this a "Trojan horse-type mechanism".[57] It has been suggested that Ag NPs may also act as a "Trojan horse", bypassing typical barriers and then releasing silver ions that damage the cell machinery.[58] This theory is supported by our investigations concerning the intracellular fate of Ag NPs using X-ray Absorption Near Edge Structure (XANES). This technique has been used to study the degradation and bio-interaction of nanoparticles in biological

systems.[59,60] We used XANES to study the intracellular fate of Ag NPs in CHO-K1 cells over time.[40] Our findings suggest that 14.2% of the internalized silver was oxidized to Ag–O– after 12 h incubation. After 24 h, 61.5% of the silver was present as Ag–S–, suggesting that binding of Ag to sulfide groups of amino acids and proteins may be involved in the dissolution of Ag NPs. This intracellular Ag–S– binding can be toxic to cells as the binding of silver ions to proteins may disrupt the protein structure and function, thereby resulting in toxic effects. In addition, the presence of Ag in the form of Ag–S– might be an indication that the increased formation of ROS observed after cellular exposure to Ag NPs might be due to a depletion of sulfide group containing antioxidants like glutathione (GSH). The intracellular ROS scavenger GSH is an important redox balancer in cells and was shown to be an efficient silver ion chelator.[61] Adsorption of silver ions to GSH could reduce the availability of GSH in cells, which will, in turn, induce an imbalance of intracellular redox levels, thereby leading to oxidative stress.

3.4 Necrosis and apoptosis

Cell death is often attributed to either necrosis or apoptosis. However, in a very recent review from the Nomenclature Committee on Cell Death, at least 13 different types of regulated cell death were enumerated, thereby describing the complexity of cell death.[62] Nevertheless, necrosis and apoptosis are probably the best known types of cell death, where the former is characterized as accidental and pathological and the latter is considered to be a controlled, programmed and physiological mechanism that is regulated by specific genes and an activation of specific molecular pathways. Previously, programmed cell death was considered to occur either by the extrinsic, receptor-mediated pathway or the intrinsic, mitochondria-mediated pathway. But autophagic cell death and necroptosis (regulated necrosis) have also been identified.[62]

ENMs may activate several pathways of programmed cell death.[63] However, mitochondria-dependent (intrinsic) apoptosis seems to be the dominating mechanism of programmed cell death caused by Ag NPs. The intrinsic apoptosis pathway is activated in response to numerous types of cellular stress including DNA damage, oxidative stress, cytosolic calcium overload, and endoplasmic reticulum (ER) stress as a function of the accumulation of unfolded proteins.[63] This pathway is characterized by insertion of Bax and/or Bak into the mitochondrial outer membrane and an activation of procaspase-9, which triggers the caspase cascade by activation of procaspase-3 leading to apoptotic cell death and phagocytosis by macrophages.[64]

Exposure of human and animal cells to Ag NPs led to down-regulation of the pro-survival protein Bcl-2, and enhanced expression of pro-apoptotic gene products such as Bax and Bad (Bcl-2-associated death promoter).[65,66] In addition, it triggered the release of cytochrome *c* into the cytosol and translocation of Bax into the mitochondria in NIH3T3 cells, indicating activation of the intrinsic apoptotic pathway.[67] In human liver cells increased protein levels of active caspase-9 (activated by cytochrome *c* released from mitochondria) were observed.[66] In agreement with this, Ag NP exposure led to activation of procaspase-3 in various human and animal cell lines.[65,66,68,69] Although oxidative stress is generally considered to be a key factor in Ag NP-mediated apoptosis, the mechanism by which Ag NPs trigger apoptosis has not been completely resolved. For example, a recent study reported that Ag NPs may exert cytotoxic effects through the modulation of ER stress pathways.[70]

3.5 Oxidative stress

The toxicological effects of ENMs on cells include cytotoxicity and genotoxicity. One, if not the most important, underlying mechanism for these effects is the induction of oxidative stress in the cells. Oxidative stress, which is caused by an imbalance between the production of reactive species in an organism and its antioxidant capacity, has been described as an important mechanism in nanotoxicology.[71,72] Reactive oxygen species are chemically reactive molecules and, as the name suggests, do contain oxygen. Examples of reactive oxygen species are oxygen itself, superoxide anion, peroxide, hydroxyl radicals and ions, and hydrogen peroxide. These molecules are always present in cells as they are natural byproducts of the oxygen metabolism but, *e.g.*, cellular stress, infection or other environmental factors can lead to an excessive formation of reactive oxygen species. In addition to reactive oxygen species, reactive nitrogen species containing nitric oxide can also be involved in the induction of oxidative stress.[73]

There are different mechanisms of how exposure to ENMs might lead to an increased formation of reactive oxygen species. The generation of free radicals by ENMs themselves is one possibility. Another possibility is an increased production of ROS in mitochondria. In addition, for Ag NPs the depletion of antioxidants and the subsequent impairment of the antioxidant capacity have been discussed as possible mechanisms. As ROS can induce a number of cellular damages, eukaryotic organisms have evolved a comprehensive range of proteins to detoxify ROS and repair oxidative damage to DNA, lipids and proteins. These antioxidants include enzymatic scavengers such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione S-transferase (GST) and the peroxiredoxins, as well as non-enzymatic factors such as GSH and vitamins.[74]

Several *in vitro* studies have demonstrated cellular responses related to oxidative stress after Ag NP exposure. Reactive oxygen intermediates are formed when oxidative dissolution of Ag NPs occurs, suggesting that a direct NP-mediated mechanism is possible.[34] When cells were pretreated with cyanide, an inhibitor of the mitochondrial electron-transferring activity of cytochrome *c* oxidase, ROS production that is otherwise induced by Ag NP exposure was inhibited.[67] These results suggest that mitochondria are involved in Ag NP-mediated ROS production. In another study, the antioxidant capacity of human serum was lowered by *ex vivo* Ag NP treatment, indicating that the Ag NPs induced depletion of antioxidants.[75] GSH is a major endogenous antioxidant scavenger

able to bind to and reduce ROS, thereby protecting cells against oxidative stress. Whereas some studies reported increased levels of GSH in response to Ag NP treatment[69,76] others found decreased levels of GSH to correlate with ROS markers,[13,68] suggesting an inhibition of GSH-synthesizing enzymes or depletion of GSH. Noticeably, as silver ions bind strongly to thiol groups present in GSH[36] this binding may play a role in GSH depletion. Other indications of oxidative stress found in response to Ag NP exposure include the increase of transcription of stress-related genes,[48,50] lipid peroxidation[66,68] and protein carbonylation.[14,66] Noticeably, although both Ag NPs and silver ions increased the intracellular ROS level, silver ions induced more ROS than Ag NPs at the same silver concentration.[43]

In summary, several lines of evidence suggest that oxidative stress may derive from depletion of antioxidants, oxidative dissolution of Ag NPs or following perturbation of mitochondria.

3.6 Genotoxicity

Genotoxicology is the study of genetic aberrations following exposure to test agents and is considered an important area in risk assessment, as DNA damage may initiate carcinogenesis.[77] Previous studies showed that Ag NPs could enter mitochondria and the nucleus[78] but localization of Ag NPs in endoplasmic reticulum and mitochondria[79] or endosomes/lysosomes[43,54] has also been reported. Silver ions are released from endocytosed Ag NPs and can thereby induce the formation of ROS. Increased formation of ROS has been suggested as a common pathway for nanomaterial-induced toxicity and is associated with, *e.g.*, membrane damage, DNA and protein damage, apoptosis or necrosis.[67,80,81] In fact, ROS has been considered the major source of spontaneous damage to DNA and several *in vitro* studies with Ag NPs have indicated genotoxic effects in different types of human and mammalian cells.[17,43,45,80,82–87] However, it has to be noted that not all studies found statistically significant genotoxic effects.[88,89] The most common outcomes considered in these studies include: DNA strand breaks, micronuclei and chromosomal aberrations. The chemical reactions that result in such DNA damages are based on the formation of highly reactive and short-lived hydroxyl radicals ($OH^{\bullet}$) in close proximity to DNA.[90]

It is generally believed that DNA damages like the formation of DNA adducts are an essential first step in the multistage process of carcinogenesis. One marker for oxidative DNA damage is 8-oxodG that has been studied both *in vivo* and *in vitro.*[91,92] The formation of 8-oxodG can lead to chromosomal aberrations and the induction of mutations, which mainly involve GC to TA transversions. We have previously reported that Ag NPs induced bulky DNA adducts in A549 cells which could be inhibited by antioxidants.[84] Importantly, an epidemiological study linked bulky DNA adducts to an increased risk of cancer.[93] Using the comet assay Nymark *et al.* showed dose-dependent DNA damage after the exposure of BEAS-2B cells to PVP-coated Ag NPs for 4 h and 24 h. However, the particles did not induce chromosomal aberrations; nor did they cause formation of micronuclei.[87] In contrast, Kim *et al.* found that Ag NPs stimulated DNA breakage and micronuclei formation in a dose-dependent manner in BEAS-2B cells. Noticeably, ROS scavengers, especially superoxide dismutase, could reduce the genotoxic effects in both assays, thereby suggesting oxidative stress as a mechanism for Ag NP induced DNA damage.[80] In another study, both the comet assay and the chromosomal aberration test showed DNA damage in human mesenchymal stem cells after 1, 3, and 24 h at Ag NP concentrations of 0.1–10 μg ml^{-1}.[85] A number of other *in vitro* studies have also reported that Ag NPs (in the size range 1–50 nm) are able to induce DNA and chromosomal damage in different cell types.[17,45,82,86] In contrast, in a human testicular embryonic carcinoma cell line, in primary testicular cells from C57BL6 mice and in MEF-LacZ cells no significant genotoxic effects were detected.[88,89] Further indications for Ag NP-mediated genotoxicity come from a study on DNA damage repair proteins in mouse embryonic and fibroblast cells.[17] In particular, protein expression was up-regulated for the cell cycle checkpoint protein, p53, and the DNA damage repair proteins, Rad51, and phosphorylated-H2AX.[17] In line with these studies we demonstrated that both Ag NP and silver ion exposure increased the bulky DNA adduct and the 8-oxodG levels as well as the micronucleus formation in CHO-K1 cells in a concentration-dependent manner.[43] However, there are differences in the genotoxicity of Ag NPs and silver ions. While silver ions induced the formation of bulky DNA adducts and micronuclei approximately 2-fold more than Ag NPs, the amount of 8-oxodG was 44% higher in cells exposed to Ag NPs than that in cells exposed to silver ions. In general, both exposure to Ag NPs and silver ions induced cyto- and genotoxicity but silver ions appeared to be more (geno)-toxic than Ag NPs to CHO-K1 at the same silver concentration.[43]

In summary, genotoxic effects following Ag NP exposure may occur within mammalian cells and different modes of action could be speculated. For example, it is possible that ROS, produced by exposure to Ag NPs, interact with and damage proteins or DNA. However, it is also possible that Ag NPs or liberated silver ions interact directly with proteins or DNA and thereby cause genotoxic effects.

3.7 Toxicogenomics

To date, only very few studies have been performed to investigate the effect of Ag NP exposure on the gene expression or on epigenetic changes. One of the first studies used microarrays to investigate the gene expression in Caco-2 cells (derived from a human colorectal adenocarcinoma) after treatment with Ag NPs and silver ions. The authors showed that exposure to Ag NPs induced changes in gene expression in a range of stress responses including oxidative stress, endoplasmic reticulum stress response, and apoptosis. Interestingly, the gene expression response to Ag NPs was reported to be very similar to that of silver ions.[48] This was in contrast to a microarray study we performed where a higher number of genes were regulated in the presence of the Ag NPs compared to silver ions. This was in agreement with Eom *et al.* who also found a more complex regulation of genes upon exposure to Ag NPs.[94]

Noticeably, especially genes from the metallothionein superfamily that are involved in metal binding and response to metal exposure were up-regulated in many microarray studies.[48,50,94,95] This is expected, as metals/metal ions are known to induce the expression of these genes.[96] However, metallothioneins are not only induced as a response to metal exposure but also by oxidative stress. The cysteines of metallothioneins have been shown to bind oxidant radicals like superoxide and hydroxyl radicals.[97,98] The up-regulation of metallothioneins is therefore consistent with the induction of ROS by silver ions and Ag NPs. The induction of ROS by silver ions and Ag NPs has been shown earlier.[13,47,50,66] Furthermore, Ag NP exposure induced the up-regulation of stress response genes including genes encoding heat shock proteins (HSPs).[48,50,95] Like metallothioneins, HSPs have been classified as stress response proteins due to their induction by several kinds of cellular stress like infection and inflammation.[99] We reported an up-regulation of HSP genes after Ag NP and Ag ion exposure (small HSPs, HSP40, HSP70, HSP90 and HSP110 family)[50] which is in agreement with previous studies showing the induction of HSPs by several stress conditions that include exposure to heavy metals.[100,101] Under these conditions HSPs play a role in maintaining the correct folding of nascent and stress-induced misfolded proteins by preventing protein aggregation or facilitating selective degradation of misfolded or denatured proteins.[102–104] The induc-

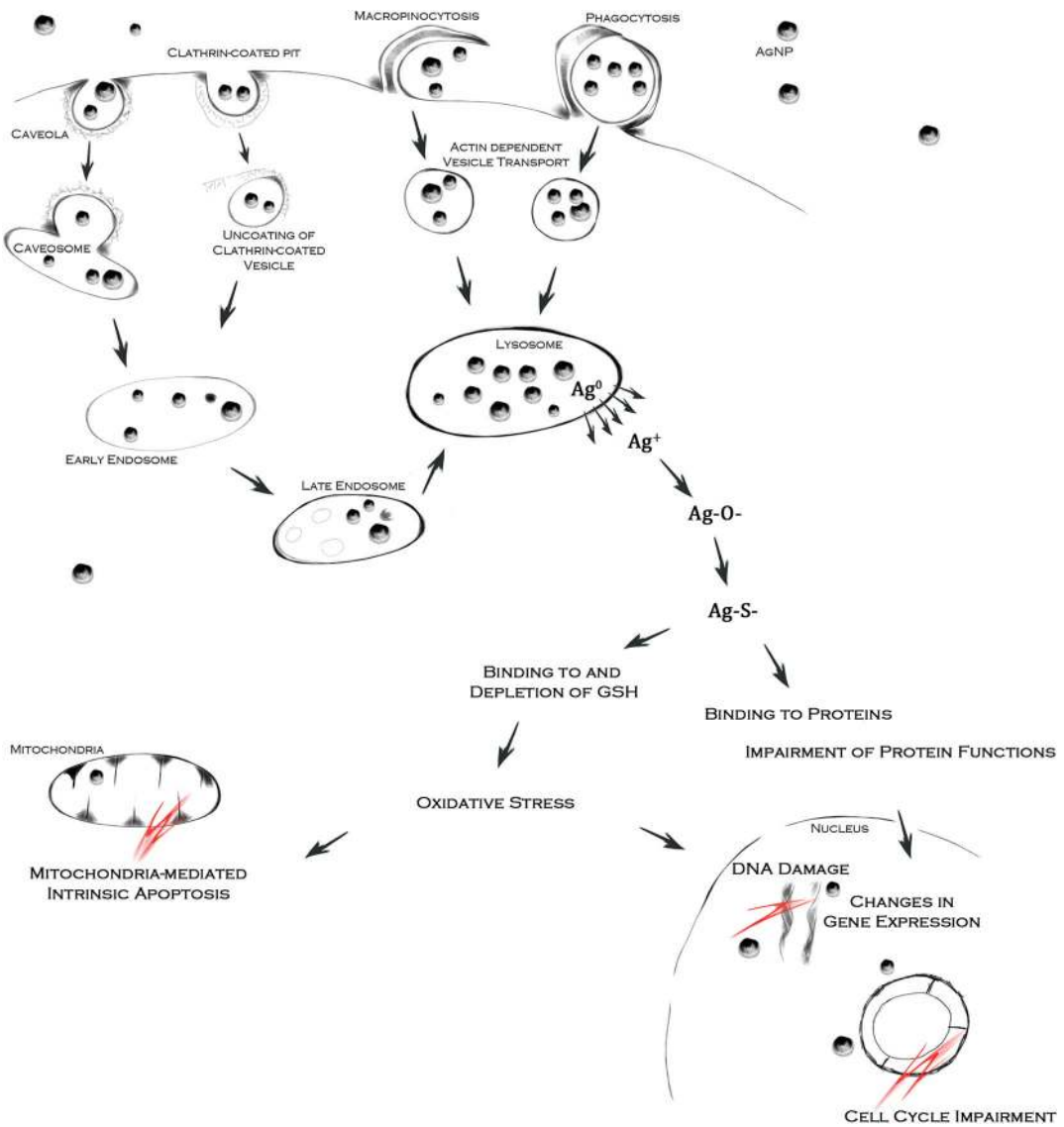

Fig. 1 Uptake and toxicity of Ag NPs. Ag NPs can be taken up by caveola- or clathrin-mediated endocytosis, macropinocytosis and phagocytosis, and a non-identified energy-independent uptake process. The particles are subsequently found in endosomes and lysosomes and dissolve quite fast in silver ions. The intracellular silver is then oxidized to Ag–O– and finally stabilizes as Ag–S– most likely binds to proteins. This binding to proteins may disturb the protein functions leading to changes in gene expression. The high affinity of Ag to –SH groups will also affect the antioxidant defense of the cells as Ag will most likely also bind to GSH, thereby leading to oxidative stress and mitochondria-mediated intrinsic apoptosis, DNA damage and impairment of the cell cycle.

tion of HSPs has previously been associated with oxidative stress[105] and recently a correlation between ROS and induction of HSP70 was found in *Drosophila melanogaster* after exposure to Ag NPs.[17]

In our study,[50] Ag NP treatment also had an extensive effect on the expression of genes coding for proteins involved in the regulation and maintenance of the cell cycle. Noticeably, the vast majority of these regulated genes were down-regulated after 24 hours of exposure to Ag NPs whereas treatment with silver ions had no effect on the cell cycle. It cannot be excluded that Ag NPs could directly interact with proteins involved in the cell cycle *via* binding to thiol groups. Strikingly, the few genes of the cell cycle regulation pathway that were up-regulated encoded proteins with inhibitory functions, like GADD45, which has been shown to be involved in cell cycle arrest at the G2/M checkpoint by overexpression of GADD45a in primary human fibroblasts[106] and DNA repair.[107] Furthermore, GADD45 has been shown to interact with and inhibit the kinase activity of the Cdk1/cyclinB1 complex, which itself plays a key role in the G2/M transition.[108] In addition to the up-regulation of GADD45, Cdk1 and cyclinB1 were down-regulated in response to the Ag NP treatment and our data suggest that 24 hours exposure to Ag NP results in cell cycle arrest at the G2/M boundary.[50] This cell cycle arrest was specific for Ag NPs under the experimental conditions (concentration, initial silver ion fraction, exposure time) used by us. The arrest of cells in the G2/M phase of the cell cycle has previously been reported for Ag NPs.[109–111]

In conclusion, although the transcriptional response to exposure with silver ions is highly related to the responses caused by Ag NPs, our and others' data suggest that Ag NPs, due to their particulate form, affect cells in a more complex way. Even when the studies did not use the same array platform, cell line, and Ag NP and silver ion concentrations, genes from the metallothionein and heat shock protein family were consistently up-regulated in response to Ag NPs.[48,50,94,95]

4 Conclusion

Based on the discussed literature and on our own results, we present an overview of Ag NP toxicity (Fig. 1). Depending on the cell line, Ag NPs can be taken up by caveola- or clathrin-mediated endocytosis, macropinocytosis and phagocytosis, and a non-identified energy-independent uptake process. The particles are subsequently found in endosomes and lysosomes. The internalized Ag NPs dissolve quite fast into silver ions. The intracellular silver is then oxidized to Ag–O– and finally stabilizes as Ag–S– most likely binds to proteins. This binding to proteins may disturb the protein functions leading to changes in gene expression. The high affinity of Ag to –SH groups will also affect the antioxidant defense of the cells as Ag will most likely also bind to GSH, thereby leading to oxidative stress and mitochondria-mediated intrinsic apoptosis, DNA damage and impairment of the cell cycle. In this way, silver ions represent the real hazard (wolves) that are disguised as NPs with a protein corona (sheep's clothing) that enables the Ag NPs to enter cells and release silver ions leading to toxic responses.

Competing interests

The authors declare that they have no competing interests.

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Notes and references

1 Society of Toxicology SOT, http://www.toxicology.org/ai/pub/si05/SI05_Define.asp. 2005. Ref Type: Online Source.
2 G. Oberdorster, Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology, *J. Intern. Med.*, 2010, **267**, 89–105.
3 T. M. Tolaymat, A. M. EI Badawy, A. Genaidy, K. G. Scheckel, T. P. Luxton and M. Suidan, An evidence-based environmental perspective of manufactured silver nanoparticle in syntheses and applications: a systematic review and critical appraisal of peer-reviewed scientific papers, *Sci. Total Environ.*, 2010, **408**, 999–1006.
4 S. W. Wijnhoven, W. J. Peijnenburg, C. A. Herberts, W. I. Hagens, A. G. Oomen, E. H. Heugens, *et al.*, Nano-silver - a review of available data and knowledge gaps in human and environmental risk assessment, *Nanotoxicology*, 2009, **3**, 109–U78.
5 A. D. Russell and W. B. Hugo, Antimicrobial activity and action of silver, *Prog. Med. Chem.*, 1994, **31**, 351–370.
6 A. B. Lansdown, Silver in health care: antimicrobial effects and safety in use, *Curr. Probl. Dermatol.*, 2006, **33**, 17–34.
7 ATSDR: Agency for Toxic Substances and Disease Registry, *Toxicological Profile for Silver. Prepared by Clement International Corporation, under Contract 205–88-0608*. U.S. Public Health Service, ATSDR/TP-90-24, 1990.
8 P. L. Drake and K. J. Hazelwood, Exposure-related health effects of silver and silver compounds: A review, *Ann. Occup. Hyg.*, 2005, **49**, 575–585.
9 L. Jonas, C. Bloch, R. Zimmermann, V. Stadie, G. E. Gross and S. G. Schad, Detection of silver sulfide deposits in the skin of patients with argyria after long-term use of silver-containing drugs, *Ultrastruct. Pathol.*, 2007, **31**, 379–384.
10 T.-D. Nguyen and T.-O. Do, Size- and Shape-Controlled Synthesis of Monodisperse Metal Oxide and Mixed Oxide Nanocrystals, in *Nanocrystal*, ed. Y. Masuda, InTech, 2011, ISBN: 978-953-307-199-2, DOI: 10.5772/17054. Available from: http://www.intechopen.com/books/nanocrystal/size-and-shape-controlled-synthesis-of-monodisperse-metal-oxide-and-mixed-oxide-nanocrystals.

11 A. Baeza-Squiban, S. Boland, S. Hussain and F. Marano, *Health Effects of Nanoparticles, In General, Applied and Systems Toxicology*, John Wiley & Sons, Ltd, 2009.

12 L. K. Braydich-Stolle, B. Lucas, A. Schrand, R. C. Murdock, T. Lee, J. J. Schlager, *et al.*, Silver nanoparticles disrupt GDNF/Fyn kinase signaling in spermatogonial stem cells, *Toxicol. Sci.*, 2010, **116**, 577–589.

13 C. Carlson, S. M. Hussain, A. M. Schrand, L. K. Braydich-Stolle, K. L. Hess, R. L. Jones, *et al.*, Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species, *J. Phys. Chem. B*, 2008, **112**, 13608–13619.

14 A. Haase, H. F. Arlinghaus, J. Tentschert, H. Jungnickel, P. Graf, A. Mantion, *et al.*, Application of laser postionization secondary neutral mass spectrometry/time-of-flight secondary ion mass spectrometry in nanotoxicology: visualization of nanosilver in human macrophages and cellular responses, *ACS Nano*, 2011, **5**, 3059–3068.

15 W. Liu, Y. Wu, C. Wang, H. C. Li, T. Wang, C. Y. Liao, *et al.*, Impact of silver nanoparticles on human cells: effect of particle size, *Nanotoxicology*, 2010, **4**, 319–330.

16 A. D. Maynard, D. B. Warheit and M. A. Philbert, The new toxicology of sophisticated materials: nanotoxicology and beyond, *Toxicol. Sci.*, 2011, **120**(Suppl 1), S109–S129.

17 M. Ahamed, M. Karns, M. Goodson, J. Rowe, S. M. Hussain, J. J. Schlager, *et al.*, DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells, *Toxicol. Appl. Pharmacol.*, 2008, **233**, 404–410.

18 L. K. Braydich-Stolle, B. Lucas, A. Schrand, R. C. Murdock, T. Lee, J. J. Schlager, *et al.*, Silver nanoparticles disrupt GDNF/Fyn kinase signaling in spermatogonial stem cells, *Toxicol. Sci.*, 2010, **116**, 577–589.

19 A. K. Suresh, D. A. Pelletier, W. Wang, J. L. Morrell-Falvey, B. Gu and M. J. Doktycz, Cytotoxicity induced by engineered silver nanocrystallites is dependent on surface coatings and cell types, *Langmuir*, 2012, **28**, 2727–2735.

20 E. C. Cho, J. Xie, P. A. Wurm and Y. Xia, Understanding the role of surface charges in cellular adsorption versus internalization by selectively removing gold nanoparticles on the cell surface with a I2/KI etchant, *Nano Lett.*, 2009, **9**, 1080–1084.

21 X. Yang, A. P. Gondikas, S. M. Marinakos, M. Auffan, J. Liu, H. Hsu-Kim, *et al.*, Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*, *Environ. Sci. Technol.*, 2012, **46**, 1119–1127.

22 M. P. Monopoli, C. Aberg, A. Salvati and K. A. Dawson, Biomolecular coronas provide the biological identity of nanosized materials, *Nat. Nanotechnol.*, 2012, **7**, 779–786.

23 M. P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. B. Bombelli, *et al.*, Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles, *J. Am. Chem. Soc.*, 2011, **133**, 2525–2534.

24 T. Cedervall, I. F. Lynch, M. F. Foy, T. F. Berggard, S. C. Donnelly, G. Cagney, G. F. Cagney, *et al.*, Detailed identification of plasma proteins adsorbed on copolymer nanoparticles, *Angew. Chem., Int. Ed. Engl.*, 2007, **46**(30), 5754–5756.

25 Z. J. Deng, G. F. Mortimer, T. F. Schiller, A. F. Musumeci, D. F. Martin and R. F. Minchin, Differential plasma protein binding to metal oxide nanoparticles, *Nanotechnology*, 2009, **20**(45), 455101.

26 S. Boland, S. Hussain and A. Baeza-Squiban, Carbon black and titanium dioxide nanoparticles induce distinct molecular mechanisms of toxicity, *WIREs Nanomed. Nanobiotechnol.*, 2014, **6**, 641–652.

27 A. A. Vertegel, R. W. Siegel, J. Dordick and J. S. Dordick, Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme, *Langmuir*, 2004, **20**(16), 6800–6807.

28 A. A. Ashkarran, M. Ghavami, H. Aghaverdi, P. Stroeve and M. Mahmoudi, Bacterial effects and protein corona evaluations: crucial ignored factors in the prediction of bioefficacy of various forms of silver nanoparticles, *Chem. Res. Toxicol.*, 2012, **25**, 1231–1242.

29 Y. Hayashi, T. Miclaus, C. Scavenius, K. Kwiatkowska, A. Sobota, P. Engelmann, *et al.*, Species differences take shape at nanoparticles: protein corona made of the native repertoire assists cellular interaction, *Environ. Sci. Technol.*, 2013, **47**, 14367–14375.

30 C. Nützenadel, A. Züttel, D. Chartouni, G. Schmid and L. Schlapbach, Critical size and the surface effect of the hydrogen interaction of palladium clusters, *Eur. Phys. J. D*, 2000, **8**, 245–250.

31 I. A. Mudunkotuwa and V. H. Grassian, The devil is in the details (or the surface): impact of surface structure and surface energetics on understanding the behavior of nanomaterials in the environment, *J. Environ. Monit.*, 2011, **13**, 1135–1144.

32 S. W. Bian, I. A. Mudunkotuwa, T. Rupasinghe and V. H. Grassian, Aggregation and dissolution of 4 nm ZnO nanoparticles in aqueous environments: influence of pH, ionic strength, size, and adsorption of humic acid, *Langmuir*, 2011, **27**, 6059–6068.

33 S. Kittler, C. Greulich, J. Diendorf, M. Koller and M. Epple, Toxicity of Silver Nanoparticles increases during storage because of slow dissolution under release of Silver Ions, *Chem. Mater.*, 2010, **22**, 4548–4554.

34 J. Liu and R. H. Hurt, Ion release kinetics and particle persistence in aqueous nano-silver colloids, *Environ. Sci. Technol.*, 2010, **44**, 2169–2175.

35 J. Liu, D. A. Sonshine, S. Shervani and R. H. Hurt, Controlled release of biologically active silver from nanosilver surfaces, *ACS Nano*, 2010, **4**, 6903–6913.

36 J. Liu, Z. Wang, F. D. Liu, A. B. Kane and R. H. Hurt, Chemical transformations of nanosilver in biological environments, *ACS Nano*, 2012, **6**, 9887–9899.

37 E. Wiberg, N. Wiberg and A. F. Holleman, *Holleman-Wiberg's Inorganic Chemistry*, in ed. N. Wiberg, Academic Press, San Diego, CA, 2001.

38 A. P. Gondikas, A. Morris, B. C. Reinsch, S. M. Marinakos, G. V. Lowry and H. Hsu-Kim, Cysteine-induced modifi-

cations of zero-valent silver nanomaterials: implications for particle surface chemistry, aggregation, dissolution, and silver speciation, *Environ. Sci. Technol.*, 2012, **46**, 7037–7045.
39 L. V. Stebounova, E. Guio and V. H. Grassian, Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution, *J. Nanopart. Res.*, 2011, **13**, 233–244.
40 X. Jiang, T. Miclaus, L. Wang, R. Foldbjerg, D. S. Sutherland, H. Autrup, *et al.*, Fast intracellular dissolution and persistent cellular uptake of silver nanoparticles in CHO-K1 cells: implication for cytotoxicity, *Nanotoxicology*, 2014, DOI: 10.3109/17435390.2014.907457.
41 K. R. Rogers, K. Bradham, T. Tolaymat, D. J. Thomas, T. Hartmann, L. Ma, *et al.*, Alterations in physical state of silver nanoparticles exposed to synthetic human stomach fluid, *Sci. Total Environ.*, 2012, **420**, 334–339.
42 S. J. Yu, J. B. Chao, J. Sun, Y. G. Yin, J. F. Liu and G. B. Jiang, Quantification of the uptake of silver nanoparticles and ions to HepG2 cells, *Environ. Sci. Technol.*, 2013, **47**, 3268–3274.
43 X. Jiang, R. Foldbjerg, T. Miclaus, L. Wang, R. Singh, Y. Hayashi, *et al.*, Multi-platform genotoxicity analysis of silver nanoparticles in the model cell line CHO-K1, *Toxicol. Lett.*, 2013, **222**, 55–63.
44 S. Kim, J. E. Choi, J. Choi, K. H. Chung, K. Park and J. Yi, *et al.*, Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells, *Toxicol. In Vitro*, 2009, 23, 1076–1084.
45 K. Kawata, M. Osawa and S. Okabe, In vitro toxicity of silver nanoparticles at non-cytotoxic doses to HepG2 human hepatoma cells, *Environ. Sci. Technol.*, 2009, **43**, 6046–6051.
46 E. Navarro, F. Piccapietra, B. Wagner, F. Marconi, R. Kaegi, N. Odzak, *et al.*, Toxicity of Silver Nanoparticles to *Chlamydomonas reinhardtii*, *Environ. Sci. Technol.*, 2008, **42**, 8959–8964.
47 C. Beer, R. Foldbjerg, Y. Hayashi, D. S. Sutherland and H. Autrup, Toxicity of silver nanoparticles - nanoparticle or silver ion?, *Toxicol. Lett.*, 2012, **208**, 286–292.
48 H. Bouwmeester, J. Poortman, R. J. Peters, E. Wijma, E. Kramer, S. Makama, *et al.*, Characterization of translocation of silver nanoparticles and effects on whole-genome gene expression using an in vitro intestinal epithelium co-culture model, *ACS Nano*, 2011, **5**, 4091–4103.
49 A. Ivask, I. Kurvet, K. Kasemets, I. Blinova, V. Aruoja, S. Suppi, *et al.*, A size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells in vitro, *PLoS One*, 2014, **9**, e102108.
50 R. Foldbjerg, E. S. Irving, Y. Hayashi, D. S. Sutherland, K. Thorsen, H. Autrup, *et al.*, Global gene expression profiling of human lung epithelial cells after exposure to nanosilver, *Toxicol. Sci.*, 2012, **130**, 145–157.
51 A. E. Nel, L. Madler, D. Velegol, T. Xia, E. M. Hoek, P. Somasundaran, *et al.*, Understanding biophysicochemical interactions at the nano-bio interface, *Nat. Mater.*, 2009, **8**, 543–557.
52 A. Verma and F. Stellacci, The effect of surface properties on nanoparticle-cell interactions, *Small*, 2010, **6**, 12–21.
53 F. Zhao, Y. Zhao, Y. Liu, X. Chang, C. Chen and Y. Zhao, Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials, *Small*, 2011, **7**, 1322–1337.
54 C. Greulich, J. Diendorf, T. Simon, G. Eggeler, M. Epple and M. Koller, Uptake and intracellular distribution of silver nanoparticles in human mesenchymal stem cells, *Acta Biomater.*, 2011, **7**, 347–354.
55 H. Wang, L. Wu and B. M. Reinhard, Scavenger receptor mediated endocytosis of silver nanoparticles into J774A.1 macrophages is heterogeneous, *ACS Nano*, 2012, **6**, 7122–7132.
56 A. R. Gliga, S. Skoglund, I. O. Wallinder, B. Fadeel and H. L. Karlsson, Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release, *Part. Fibre Toxicol.*, 2014, **11**, 11.
57 L. K. Limbach, P. Wick, P. Manser, R. N. Grass, A. Bruinink and W. J. Stark, Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress, *Environ. Sci. Technol.*, 2007, **41**, 4158–4163.
58 E. J. Park, J. Yi, Y. Kim, K. Choi and K. Park, Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism, *Toxicol. In Vitro*, 2010, **24**, 872–878.
59 L. Wang, J. Li, J. Pan, X. Jiang, Y. Ji, Y. Li, *et al.*, Revealing the binding structure of the protein corona on gold nanorods using synchrotron radiation-based techniques: understanding the reduced damage in cell membranes, *J. Am. Chem. Soc.*, 2013, **135**, 17359–17368.
60 Y. Qu, W. Li, Y. Zhou, X. Liu, L. Zhang, L. Wang, *et al.*, Full assessment of fate and physiological behavior of quantum dots utilizing *Caenorhabditis elegans* as a model organism, *Nano Lett.*, 2011, **11**, 3174–3183.
61 C. N. Lok, C. M. Ho, R. Chen, Q. Y. He, W. Y. Yu, H. Sun, *et al.*, Silver nanoparticles: partial oxidation and antibacterial activities, *J. Biol. Inorg. Chem.*, 2007, **12**, 527–534.
62 L. Galluzzi, I. Vitale, J. M. Abrams, E. S. Alnemri, E. H. Baehrecke, M. V. Blagosklonny, *et al.*, Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012, *Cell Death Differ.*, 2012, **19**, 107–120.
63 F. T. Andon and B. Fadeel, Programmed Cell Death: Molecular Mechanisms and Implications for Safety Assessment of Nanomaterials, *Acc. Chem. Res.*, 2013, **46**(3), 733–742.
64 M. Ott, V. Gogvadze, S. Orrenius and B. Zhivotovsky, Mitochondria, oxidative stress and cell death, *Apoptosis*, 2007, **12**, 913–922.
65 P. Gopinath, S. K. Gogoi, P. Sanpui, A. Paul, A. Chattopadhyay and S. S. Ghosh, Signaling gene cascade in silver nanoparticle induced apoptosis, *Colloids Surf., B*, 2010, **77**, 240–245.
66 M. J. Piao, K. A. Kang, I. K. Lee, H. S. Kim, S. Kim, J. Y. Choi, *et al.*, Silver nanoparticles induce oxidative cell

damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis, *Toxicol. Lett.*, 2011, **201**, 92–100.
67 Y. H. Hsin, C. F. Chena, S. Huang, T. S. Shih, P. S. Lai and P. J. Chueh, The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells, *Toxicol. Lett.*, 2008, **179**, 130–139.
68 S. Arora, J. Jain, J. Rajwade and K. Paknikar, Cellular responses induced by silver nanoparticles: In vitro studies, *Toxicol. Lett.*, 2008, **179**, 93–100.
69 S. Arora, J. Jain, J. Rajwade and K. Paknikar, Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells, *Toxicol. Appl. Pharmacol.*, 2009, **236**, 310–318.
70 R. Zhang, M. J. Piao, K. C. Kim, A. D. Kim, J. Y. Choi, J. Choi, *et al.*, Endoplasmic reticulum stress signaling is involved in silver nanoparticles-induced apoptosis, *Int. J. Biochem. Cell Biol.*, 2012, **44**, 224–232.
71 A. Nel, T. Xia, L. Madler and N. Li, Toxic potential of materials at the nanolevel, *Science*, 2006, **311**, 622–627.
72 G. Oberdorster, J. Ferin, G. Finkelstein, P. Wade and N. Corson, Increased pulmonary toxicity of ultrafine particles? II. Lung lavage studies, *J. Aerosol Sci.*, 1990, **21**, 384–387.
73 C. C. Winterbourn, Reconciling the chemistry and biology of reactive oxygen species, *Nat. Chem. Biol.*, 2008, **4**, 278–286.
74 R. Franco, R. Sanchez-Olea, E. M. Reyes-Reyes and M. I. Panayiotidis, Environmental toxicity, oxidative stress and apoptosis: menage a trois, *Mutat. Res.*, 2009, **674**, 3–22.
75 E. J. Rogers, S. F. Hsieh, N. Organti, D. Schmidt and D. Bello, A high throughput in vitro analytical approach to screen for oxidative stress potential exerted by nanomaterials using a biologically relevant matrix: human blood serum, *Toxicol. In Vitro*, 2008, **22**, 1639–1647.
76 S. J. Kang, Y. J. Lee, E. K. Lee and M. K. Kwak, Silver nanoparticles-mediated G2/M cycle arrest of renal epithelial cells is associated with NRF2-GSH signaling, *Toxicol. Lett.*, 2012, **211**, 334–341.
77 N. Singh, B. Manshian, G. J. Jenkins, S. M. Griffiths, P. M. Williams, T. G. Maffeis, *et al.*, NanoGenotoxicology: The DNA damaging potential of engineered nanomaterials, *Biomaterials*, 2009, **30**, 3891–3914.
78 P. V. AshaRani, M. G. Low Kah, M. P. Hande and S. Valiyaveettil, Cytotoxicity and genotoxicity of silver nanoparticles in human cells, *ACS Nano*, 2009, **3**, 279–290.
79 L. Wei, J. Tang, Z. Zhang, Y. Chen, G. Zhou and T. Xi, Investigation of the cytotoxicity mechanism of silver nanoparticles in vitro, *Biomed. Mater.*, 2010, **5**, 044103.
80 H. R. Kim, M. J. Kim, S. Y. Lee, S. M. Oh and K. H. Chung, Genotoxic effects of silver nanoparticles stimulated by oxidative stress in human normal bronchial epithelial (BEAS-2B) cells, *Mutat. Res.*, 2011, **726**, 129–135.
81 L. Wang, Y. Liu, W. Li, X. Jiang, Y. Ji, X. Wu, *et al.*, Selective targeting of gold nanorods at the mitochondria of cancer cells: implications for cancer therapy, *Nano Lett.*, 2011, **11**, 772–780.
82 P. V. AshaRani, G. L. K. Mun, M. P. Hande and S. Valiyaveettil, Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells, *Acs Nano*, 2009, **3**, 279–290.
83 N. A. Flower, B. Brabu, M. Revathy, C. Gopalakrishnan, S. V. Raja, S. S. Murugan, *et al.*, Characterization of synthesized silver nanoparticles and assessment of its genotoxicity potentials using the alkaline comet assay, *Mutat. Res.*, 2012, **742**, 61–65.
84 R. Foldbjerg, D. A. Dang and H. Autrup, Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549, *Arch. Toxicol.*, 2011, **85**, 743–750.
85 S. Hackenberg, A. Scherzed, M. Kessler, S. Hummel, A. Technau, K. Froelich, *et al.*, Silver nanoparticles: evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells, *Toxicol. Lett.*, 2011, **201**, 27–33.
86 Y. Li, D. H. Chen, J. Yan, Y. Chen, R. A. Mittelstaedt, Y. Zhang, *et al.*, Genotoxicity of silver nanoparticles evaluated using the Ames test and in vitro micronucleus assay, *Mutat. Res.*, 2012, **745**, 4–10.
87 P. Nymark, J. Catalan, S. Suhonen, H. Jarventaus, R. Birkedal, P. A. Clausen, *et al.*, Genotoxicity of polyvinylpyrrolidone-coated silver nanoparticles in BEAS 2B cells, *Toxicology*, 2013, **313**(1), 38–48.
88 N. Asare, C. Instanes, W. J. Sandberg, M. Refsnes, P. Schwarze, M. Kruszewski, *et al.*, Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells, *Toxicology*, 2012, **291**, 65–72.
89 M. V. Park, A. M. Neigh, J. P. Vermeulen, L. J. de la Fonteyne, H. W. Verharen, J. J. Briede, *et al.*, The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles, *Biomaterials*, 2011, **32**, 9810–9817.
90 J. Cadet, T. Delatour, T. Douki, D. Gasparutto, J. P. Pouget, J. L. Ravanat, *et al.*, Hydroxyl radicals and DNA base damage, *Mutat. Res.*, 1999, **424**, 9–21.
91 C. Chen, L. Qu, B. Li, L. Xing, G. Jia, T. Wang, *et al.*, Increased oxidative DNA damage, as assessed by urinary 8-hydroxy-2′-deoxyguanosine concentrations, and serum redox status in persons exposed to mercury, *Clin. Chem.*, 2005, **51**, 759–767.
92 S. Dwivedi, Q. Saquib, A. A. Al-Khedhairy and J. Musarrat, Butachlor induced dissipation of mitochondrial membrane potential, oxidative DNA damage and necrosis in human peripheral blood mononuclear cells, *Toxicology*, 2012, **302**, 77–87.
93 H. Bak, H. Autrup, B. L. Thomsen, A. Tjonneland, K. Overvad, U. Vogel, *et al.*, Bulky DNA adducts as risk indicator of lung cancer in a Danish case-cohort study, *Int. J. Cancer*, 2006, **118**, 1618–1622.
94 H. J. Eom, N. Chatterjee, J. Lee and J. Choi, Integrated mRNA and micro RNA profiling reveals epigenetic mech-

anism of differential sensitivity of Jurkat T cells to AgNPs and Ag ions, *Toxicol. Lett.*, 2014, **229**, 311–318.
95 D. H. Lim, J. Jang, S. Kim, T. Kang, K. Lee and I. H. Choi, The effects of sub-lethal concentrations of silver nanoparticles on inflammatory and stress genes in human macrophages using cDNA microarray analysis, *Biomaterials*, 2012, **33**, 4690–4699.
96 C. D. Klaassen, J. Liu and B. A. Diwan, Metallothionein protection of cadmium toxicity, *Toxicol. Appl. Pharmacol.*, 2009, **238**, 215–220.
97 M. V. Kumari, M. Hiramatsu and M. Ebadi, Free radical scavenging actions of metallothionein isoforms I and II, *Free Radical Res.*, 1998, **29**, 93–101.
98 A. Formigari, P. Irato and A. Santon, Zinc, antioxidant systems and metallothionein in metal mediated-apoptosis: biochemical and cytochemical aspects, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.*, 2007, **146**, 443–459.
99 M. G. Santoro, Heat shock factors and the control of the stress response, *Biochem. Pharmacol.*, 2000, **59**, 55–63.
100 M. H. Mutwakil, J. P. Reader, D. M. Holdich, P. R. Smithurst, E. P. M. Candido, D. Jones, *et al.*, Use of stress-inducible transgenic nematodes as biomarkers of heavy metal pollution in water samples from an english river system, *Arch. Environ. Contam. Toxicol.*, 1997, **32**, 146–153.
101 K. Guven and D. I. De Pomerai, Differential expression of HSP70 proteins in response to heat and cadmium in *Caenorhabditis elegans*, *J. Therm. Biol.*, 1995, **20**, 355–363.
102 M. J. Schlesinger, Heat shock proteins, *J. Biol. Chem.*, 1990, **265**, 12111–12114.
103 R. I. Morimoto, Cells in stress: transcriptional activation of heat shock genes, *Science*, 1993, **259**, 1409–1410.
104 S. C. Gupta, A. Sharma, M. Mishra, R. K. Mishra and D. K. Chowdhuri, Heat shock proteins in toxicology: how close and how far?, *Life Sci.*, 2010, **86**, 377–384.
105 A. M. Gorman, B. Heavey, E. Creagh, T. G. Cotter and A. Samali, Antioxidant-mediated inhibition of the heat shock response leads to apoptosis, *FEBS Lett.*, 1999, **445**, 98–102.
106 X. W. Wang, Q. Zhan, J. D. Coursen, M. A. Khan, H. U. Kontny, L. Yu, *et al.*, GADD45 induction of a G2/M cell cycle checkpoint, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 3706–3711.
107 M. L. Smith, J. M. Ford, M. C. Hollander, R. A. Bortnick, S. A. Amundson, Y. R. Seo, *et al.*, p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes, *Mol. Cell Biol.*, 2000, **20**, 3705–3714.
108 M. Vairapandi, A. G. Balliet, B. Hoffman and D. A. Liebermann, GADD45b and GADD45 g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress, *J. Cell Physiol.*, 2002, **192**, 327–338.
109 Y. S. Lee, D. W. Kim, Y. H. Lee, J. H. Oh, S. Yoon, M. S. Choi, *et al.*, Silver nanoparticles induce apoptosis and G2/M arrest via PKCzeta-dependent signaling in A549 lung cells, *Arch. Toxicol.*, 2011, **85**, 1529–1540.
110 L. Wei, J. Tang, Z. Zhang, Y. Chen, G. Zhou and T. Xi, Investigation of the cytotoxicity mechanism of silver nanoparticles in vitro, *Biomed. Mater.*, 2010, **5**, 044103.
111 L. A. Austin, B. Kang, C. W. Yen and M. A. El-Sayed, Nuclear targeted silver nanospheres perturb the cancer cell cycle differently than those of nanogold, *Bioconjugate Chem.*, 2011, **22**, 2324–2331.