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Review Article

Mechanisms of Silver Nanoparticle Toxicity

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Abstract
The rapidly growing field of nanotechnology holds great promise for scientific advancement in many sectors such as medicine, consumer products, energy, and materials. In general terms, nanotechnology covers engineered structures, devices, and systems that have a length scale between 1 and 100 nanometers. At this size, materials begin to exhibit unique properties that affect physical, chemical, and biological behavior. However, the same characteristics which make nanomaterials attractive for exploitation in new products have led to concerns that nanomaterials may pose a risk for humans and the environment. Specific concerns have been raised about the possible toxicity of engineered nanoparticles (NPs) supported by studies which indicated that NPs are more toxic than larger particles on a mass for mass basis. As a consequence of their small size, NPs have a very high surface to volume ratio rendering them potentially more reactive than larger particles. Accordingly, there are strong indications that particle surface area and surface chemistry are responsible for the observed responses in cell cultures and animals. Silver nanoparticles (Ag NPs) are among the most commonly utilized nanomaterials due to their anti-microbial properties, high electrical conductivity, and optical properties. Information about the mechanisms involved in the cytotoxicity of Ag NPs is important in order to evaluate the potential hazards posed by these particles. Several studies have suggested oxidative stress plays a major role in NP toxicity. But, to what degree and by which mechanism Ag NPs cause oxidative stress in cells is unresolved. The present paper attempts to critically review the present knowledge about the underlying factors and mechanisms which may influence Ag NP toxicity.

Keywords: Toxicology; Nanoparticles; Silver; Reactive oxygen species; Apoptosis; Genotoxicity

The concept of nanotoxicology

The rapid development, increased production and use of nanomaterials have raised concerns that such materials may introduce new hazards during occupational, consumer, and environmental exposures. Nanotoxicology, a term coined by Donaldson and colleagues in 2004 (Donaldson *et al.* 2004), refers to the study of the potential toxic impacts of nanoparticles (NPs) on biological and ecological systems. The basis for this new subcategory within toxicology was the idea that particles at the nanoscale behave differently from their larger counterparts. In the following year, the concept of nanotoxicology was consolidated with a highly cited review paper by Oberdörster et al. titled "Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles" (Oberdorster *et al.* 2005) and as the title suggests, early nanotoxicity studies arose from aerosol experiments examining size-dependent toxicological effects of particles. While conventional particle toxicology deals with both natural and anthropogenic particles of a broader size range, nanotoxicology deals specifically with engineered NPs. Widely accepted definitions of nanotechnology refer to a size range of 1-100 nm "where unique phenomena enable novel applications" (NSET 2010). However, it is still uncertain whether materials which were engineered to utilize unique properties at the nanoscale also exhibit nanoscale-specific toxicity and likewise the major question whether conventional risk assessment methods can be applied to nanomaterials is not completely resolved.

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has concluded that there is no scientific evidence supporting the appropriateness of a 100 nm upper size limit and that engineered NPs do not necessarily exhibit novel physico-chemical properties. However, a decreasing size will always result in a corresponding change in biodistribution and distribution kinetics in an organism or in an ecosystem (SCENIHR 2010).

New types of NPs are constantly being developed. The categories which are commonly studied within toxicology include carbon-based materials (fullerenes and carbon nanotubes), inorganic NPs (metal oxides and metals) and quantum dots (Ju-Nam and Lead 2008). Each structure exhibits its own characteristics, such as tubes, wires, fibers, capsules and dots. Apart from chemical composition and shape, the properties of NPs also depend on various factors such as size, shape, purity, crystallinity, electronic properties, type of surface function, solubility and stability (Nel *et al.* 2006). Currently, silver (Ag) is by far the most common material explicitly referenced in nanotechnology consumer products according to the Woodrow-Wilson database on nano-products (PEN 2012) (figure 1).

The perception of nanotechnology as something novel, unknown and somewhat exotic combined with the memory of previous particle-related hazards has fueled concerns about

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the safety of nanomaterials. For example, since the early 20th century, asbestos exposure has been associated with increased risk for mesothelioma (Tetley 2007). *In vivo* studies have demonstrated that long, straight multi-walled carbon nanotubes (MWCNT) also have the potential to induce mesothelioma following intraperitoneal or intrascrotal injection in mice and rats and thus may behave in a manner similar to asbestos (Aschberger *et al.* 2010). The possibility that engineered nanomaterials could pose hazards similar to asbestos or air pollution particles has been a powerful driving force in raising concerns about nanotechnology.

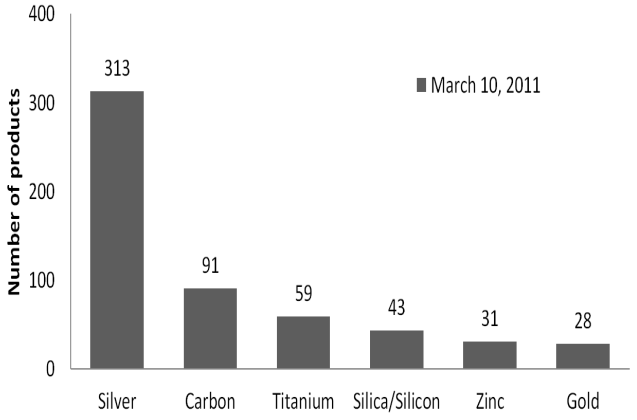

Figure 1
Nanomaterials in consumer products. The graph depicts the most common materials mentioned in product descriptions of nanotechnology consumer products. The graph was modified from (PEN 2012).

Lessons from history

For many years, ambient particulate matter (PM) has been regarded as a serious health problem. PM is divided into different size fractions, PM10 (PM ≤ 10 μm), PM2.5 (PM ≤ 2.5 μm) and PM0.1 (PM ≤ 0.1 μm), based on the aerodynamic diameters, and can originate from natural (e.g., forest fires) or anthropogenic (e.g., diesel exhaust) sources. In the 1990s, studies comparing ultrafine (PM0.1) and fine (PM2.5) particles found significantly greater pulmonary inflammation and interstitial translocation from ultrafine than fine particles although the same particle mass was intratracheally instilled in rats (Ferin *et al.* 1990; Oberdorster *et al.* 1990). Numerous studies have subsequently reported that the increased pulmonary toxicity of ultrafine particles is related to their larger surface area and increased potential for translocation (Oberdorster *et al.* 2007). As research advanced, particles were found to act by a variety of pathways to induce inflammation and oxidative stress in the lungs thereby creating a link between particle exposure and disease (Donaldson and Seaton 2012).

These results were followed by epidemiological studies, describing an association between exposure to ultrafine particles and respiratory diseases, cardiovascular diseases, cancer and possibly allergy in humans (Schwarze *et al.* 2006). Taken together, the results from studies on ultrafine particles indicate that a small size, a large surface area, and a propensity to generate reactive oxygen species (ROS) play a role in the ability of particles to induce cellular injury. In a highly cited review, Nel and coworkers suggested a hierarchical oxidative stress model as a central paradigm for explaining toxic effects of nanosized particles. This model suggests that the cellular response to oxidative stress is a tiered process ranging from induction of antioxidant defenses to inflammatory responses and cell death depending on the level of stress (Nel *et al.* 2006). Since 2004, when the field of nanotoxicology was proposed, the number of publications on the subject has increased exponentially and the involvement of oxidative stress has been a major hypothesis in most of the studies.

Silver

Since the earliest times, silver has been used in daily life as well as in medicine. In ancient Italy and Greece silver was used for storage vessels to keep water fresh. However, the antibacterial effect of silver was not scientifically described until the late 19th century (Russell and Hugo 1994). Subsequently, silver has been used in a wide range of medical devices including bone prostheses, surgical sutures and needles, cardiac implants, catheters, dental devices, wound therapy and surgical textiles (Lansdown 2006). In 2003, the largest consumers of silver were industrial sectors; particularly jewelry, silverware and the photographic industries (Wijnhoven *et al.* 2009). Soluble silver compounds such as silver salts have been used to treat a variety of diseases (e.g. ophthalmia- neonatorium) even today, silver is still used for wound treatment especially in burn victims to prevent infections (Lansdown 2006).

Silver can be absorbed orally, by inhalation and through damaged skin (ATSDR 1990; Drake and Hazelwood 2005). Soluble silver compounds are more readily absorbed than metallic or insoluble silver and are thus more likely to cause adverse health effects (Drake and Hazelwood 2005). The most common adverse health effect associated with prolonged exposure to silver compounds is the development of a characteristic, irreversible pigmentation of the skin (argyria) and/or the eyes (argyrosis) (ATSDR 1990). This discoloration is due to precipitation of silver in dermis and ophthalmic mucosal membranes respectively (Jonas *et al.* 2007). Human case studies have shown that long term ingestion of colloidal silver solutions could lead to argyria (Kim *et al.* 2009b; Mayr *et al.* 2009). But, silver is not thought to be carcinogenic or toxic to the immune, cardiovascular, nervous or reproductive systems (ATSDR 1990; Drake and Hazelwood 2005).

Silver nanoparticles

Silver nanoparticles (Ag NPs) are synthesized using various techniques resulting in different shapes and sizes for use in numerous applications. The most common technique involves the dissolution of silver salt into a solvent and the subsequent addition of a reducing agent e.g., with the supplemental use of stabilizing agents to prevent agglomeration of the NPs. Some of the most commonly used stabilizing agents are sodium citrate and polyvinylpyrrolidone (PVP) which yield particles with a negative surface charge at physiological pH (Tolaymat *et al.* 2010). The solvents and reducing agents used in the synthesis process affect the physical and morphological characteristics of the resulting Ag NPs.

Most applications of Ag NPs are based on their antibiotic effects. It is well established that Ag NPs have strong antibacterial, antiviral and antifungal effects (Wijnhoven *et al.* 2009). Therefore, Ag NPs are widely used in medical devices and supplies such as wound dressings, medical catheters, contraceptive devices, and surgical instruments. Furthermore, products containing Ag NPs have been used for decades as algaecides in e.g. swimming pools (Nowack *et al.* 2011). Ag NPs have also been used in consumer products such as surface cleaners, room sprays, toys, antimicrobial paints, home appliances (e.g., washing machines, air and water filters), food storage containers, and textiles (Tolaymat *et al.* 2010; Wijnhoven *et al.* 2009).

Toxicokinetics of nanosilver

Ag NPs may be absorbed into the human body via inhalation, ingestion, and dermal exposure. Absorption represents the process by which unchanged compounds proceed from the site of administration and enter the central blood circulation. After entering the blood stream, the toxicant is distributed to tissues throughout the body.

- *Inhalation*

The respiratory system represents a major portal of entrance. Sprays containing nanosilver are available on the market and aerosol exposure during production is likely in occupational settings (EPA 2012). Several inhalation studies in mice and rats demonstrated that inhalation of Ag NPs resulted in absorption of silver into the systemic circulation and distribution to organs (Hyun *et al.* 2008; Ji *et al.* 2007; Kim *et al.* 2011b; Song *et al.* 2012; Stebounova *et al.* 2011a; Sung *et al.* 2011; Sung *et al.* 2008; Sung *et al.* 2009; Takenaka *et al.* 2001).

- *Gastrointestinal absorption*

In general, application of NPs in products related to food is still rare (Chaudhry *et al.* 2008). But several food contact materials containing Ag NPs have been developed and aqueous dispersions of colloidal silver are being increasingly marketed as health supplements (Chaudhry *et al.* 2008). Thus, ingestion of Ag NPs is a relevant exposure route. Several recent reports described absorption of silver into the systemic circulation after oral administration of Ag NPs or silver ions to rats (Kim *et al.* 2010; Loeschner *et al.* 2011; Hadrup *et al.* 2012a; van der Zande *et al.* 2012), and two of these studies indicated that ionic silver is the main bioavailable form of silver following oral ingestion (Loeschner *et al.* 2011; van der Zande *et al.* 2012).

- *Dermal absorption*

Antibacterial textiles and lotions containing Ag NPs are marketed worldwide, and within the health care system, dressings containing Ag NPs are used to treat burns and other types of damaged skin (Vlachou *et al.* 2007; Wijnhoven *et al.* 2009). Increased serum silver levels in burn victims treated with a wound dressing (Acticoat) containing Ag NPs (15 nm) provides evidence of dermal absorption of silver in to the bloodstream (Vlachou *et al.* 2007). Ag NPs (25 nm) have also been shown to penetrate the upper layers of the epidermis in excised human skin in static diffusion cells (Larese *et al.* 2009). In accordance with these data, Samberg et al found Ag NPs in the superficial layers of the stratum corneum in skin from dermally exposed pigs (Samberg *et al.* 2010). Though, serum silver levels in the exposed pigs were not reported the possibility exists that silver ions from the Ag NPs were absorbed to the bloodstream.

- *Distribution to organs and tissues*

Distribution of Ag NPs may be affected by protein binding and is also likely to depend on dose, particle size and exposure time. Differences in distribution patterns could lead to differences in toxicity. The liver and kidneys have been described as the primary organs for silver deposition, whether the exposure was orally (Kim *et al.* 2010; Kim *et al.* 2008; Loeschner *et al.* 2011), intravenously (Lankveld *et al.* 2010; Park *et al.* 2011a), subcutaneously (Tang *et al.* 2009), or through inhalation (Sung *et al.* 2009; Takenaka *et al.* 2001). The spleen was also found to be a major target organ in mice and rats exposed to Ag NPs by intravenous injection (Chrastina and Schnitzer 2010; Lankveld *et al.* 2010), intraperitoneal injection (Garza-Ocanas *et al.* 2010), and oral gavage (van der Zande *et al.* 2012). Some studies reported silver distribution to the testis and to the brain (Kim *et al.* 2010; Kim *et al.* 2008; van der Zande *et al.* 2012) However, it was uncertain whether silver was present in the brain endothelial cells or in the brain tissue. Elimination of silver occurred at an extremely slow rate in brain and testis, which still contained high concentrations of silver two months after the final exposure (van der Zande *et al.* 2012). Importantly, two oral studies in rats indicated that the biodistribution patterns were similar for silver ions and Ag NPs (Loeschner *et al.* 2011; van der Zande *et al.* 2012).

- *Metabolism/ Biotransformation*

Biotransformation describes the process where the physicochemical properties of an absorbed foreign compound are enzymatically altered to favor elimination from the body. However, no evidence exists for metabolism of Ag NPs by enzymes in the liver and the rest of the body. It is plausible to assume that Ag NPs or released silver ions are able to interact with metallothioneins (Wijnhoven *et al.* 2009). Metallothionein are proteins which are present in all living cells have a unique structure, depending on their ability to bind metals like zinc and silver. They regulate the cellular metal homeostasis and play a cytoprotective role (Coyle *et al.* 2002). Another mechanism for detoxification of silver is by sequestering it in the tissues as non-toxic silver-protein complexes or silver sulfide (ATSDR 1990; Drake and Hazelwood 2005). The end product of internalized silver ions is most likely silver sulfide or silver selenide molecules that accumulate intracellularly as nanocrystals in lysosomes (Kristiansen *et al.* 2008). Importantly, Loeschner et al reported that silver granules containing selenium and sulfur were found in lysosomes of various tissues after oral administration of silver acetate and Ag NPs which indicates that silver from both silver sources can be sequestered in tissues as secondary Ag NPs (Loeschner *et al.* 2011). A proposed mechanism for this process was recently reported (Liu *et al.* 2012).

- *Elimination*

It is known that following oral or inhalation exposure to bulk silver or silver compounds, large amounts of silver are excreted via feces and to some extent urine (ATSDR 1990; Drake and Hazelwood 2005). The limited data on Ag NP elimination suggest excretion through feces as a main route. In rats injected with Ag NPs, silver was detected in feces at

24 h after treatment, which suggests biliary excretion (Park *et al.* 2011a). After intravenous or oral exposure to Ag NPs, the concentration of silver in the urine of rats was extremely low compared with the levels in the feces (Loeschner *et al.* 2011; Park *et al.* 2011a) . A recent rat study reported comparable levels of silver in urine and feces after intravenous injection of Ag NPs (Dziendzikowska *et al.* 2012). Remarkably, the level of silver in urine and feces peaked at days 1 and 2 post exposure, respectively, and was much higher for Ag NPs (20 nm) than the larger Ag particles (200 nm) (Dziendzikowska *et al.* 2012). This appears to allude to the more readily absorbed nature of NPs A human case report described elevated levels of silver ions in the urine following dermal exposure to the silver coated wound dressing, Acticoat (Trop *et al.* 2006).

Factors that may affect Ag NP toxicity

Several factors influence the ability of a metal to produce toxic effects. For nano-sized particles, size, solubility, coating, morphology, surface charge and surface area are recognized as important determinants for toxicity (Maynard *et al.* 2011).

- *Size*

Studies which have compared Ag NPs of different sizes have shown that smaller NPs are more cytotoxic than their larger counterparts (Braydich-Stolle *et al.* 2010; Carlson *et al.* 2008; Haase *et al.* 2011; Liu *et al.* 2010b). Liu et al reported that higher levels of silver were present in cells exposed to small Ag NPs than to larger Ag NPs (20 nm and 50 nm), indicating a size-dependent cellular uptake of the NPs (Liu *et al.* 2010b).

The size-dependent toxicity of Ag NPs may be explained by the increased specific surface area of smaller particles leading to high reactivity or by an enhanced release of toxic silver ions from the particle surface. 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. In contrast, it was recently pointed out that observations on size-dependent toxicity of NPs often are scalable effects meaning that small particles show greater or less tendency to behave in a certain way compared to large particles, but their behavior is predictable from that of larger particles (Maynard *et al.* 2011).

- *Surface chemistry*

Ag NPs are often coated for the purpose of promoting stability and dispensability (Tolaymat *et al.* 2010). Ahamed et al. (2008) compared the uptake by mouse embryonic fibroblasts of uncoated Ag NPs with a hydrocarbon surface layer and Ag NPs coated with the polysaccharide gum Arabic. After 24 hours, most of the uncoated Ag NPs had formed clusters and had not penetrated cell organelles, while the polysaccharide-coated Ag NPs were distributed throughout the cells. The investigators reported that the polysaccharide-coated Ag NPs caused the highest levels of toxicity (Ahamed *et al.* 2008). Despite different surface chemistry, the two types of Ag NPs had comparable negative surface charges. The differential toxicity in the specific study may be caused by a higher degree of agglomeration for the uncoated Ag NPs and differences in bioavailability (e.g., uptake and dissolution). However, conclusions should be drawn with caution considering another study from the same group investigating identical Ag NPs in spermatogonial cells (Braydich-Stolle *et al.* 2010). In this study, Ag NPs with a hydrocarbon surface layer were more toxic than polysaccharide-coated Ag NPs suggesting that other factors such as cell type and media may influence the toxicity.

A recent study focused on four types of Ag NPs with nearly uniform size and shape distribution but with different surface coatings, imparting overall high negativity to high positivity (Suresh *et al.* 2012). 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 (Suresh *et al.* 2012). 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 (Cho *et al.* 2009). In contrast to the findings by Suresh and coworkers, Yang et al. found the toxicity of Ag NPs to be independent of surface charge using the model organism C. elegans (Yang *et al.* 2012). These investigators compared Ag NPs with similar sizes but different coatings (citrate, PVP and gum Arabic) and found differential toxicity towards C. elegans. Gum Arabic-coated NPs were ~9-fold more toxic than PVP-coated NPs, which in turn was ~3-fold more toxic than citrate-coated NPs. The authors concluded that toxicity was mainly due to NP dissolution which depended on the surface coating (Yang *et al.* 2012).

Clearly, the surface charge and surface coatings could have an effect on the bioavailability or interaction of Ag NPs with cellular systems. However, the complexity of NP surface chemistry is further complicated by the fact that the surfaces of NPs are immediately covered by proteins when they come in contact with a biological medium (Monopoli *et al.* 2012). The formation of a protein corona at the surface of NPs confers a new "biological identity" in the biological milieu, which determines the subsequent cellular/tissue responses. Therefore, what biological entities, such as cells, tissues, and organs, actually "see" when interacting with NPs is completely different from the original pristine surface of the NPs (Monopoli *et al.* 2012). It is note worthy that the formation of the corona is a dynamic, competitive process. Over time, the most abundant protein (having bound first) is displaced by those with higher affinity, and the resulting biomolecules, "hard corona" contains only a few proteins in a relatively immobile layer, with a more loosely bound layer ("soft corona") that is less well-understood (Monopoli *et al.* 2011). Ashkarran et al. found that for Ag NPs with different shapes the composition and thickness of the protein corona can evolve quite significantly depending on the NP shape and the NP/protein ratio (Ashkarran *et al.* 2012). This could have serious implications for *in vitro* to *in vivo* extrapolations since the NP/protein ratio is often very different. In the case of spherical Ag NPs (16 nm) five proteins (serotransferrin, serum albumin, alpha-fetoprotein, kininogen-1 and fibrinogen alpha chain) were found to be associated with the hard corona after one hour incubation in FBS (Ashkarran *et al.* 2012). However, the dynamics of the Ag NP corona in response to FBS concentrations, incubation time, NP size and NP surface coating etc. are still unresolved.

- *Ion release*

There is a broad agreement that silver ions strongly contribute to the biological activity of Ag NPs and several studies have reported influences of size, coating, concentration, temperature, pH, ionic strength, and time on the dissolution behavior of Ag NPs (Kittler *et al.* 2010; Liu *et al.* 2010a; Liu *et al.* 2012; Liu and Hurt 2010). Understanding the ion release kinetics for Ag NPs is critical for risk assessment; if Ag NP toxicity is driven by the release of silver ions, our current knowledge about dissolved silver speciation and toxicity may be highly informative for risk assessment. If on the other hand Ag NPs have unique toxicities deriving from their nanoparticulate form, additional studies will be required.

Ag NPs are composed of elemental silver (Ag^0), which is not soluble or reactive in pure water (Wiberg *et al.* 2001), but is soluble in acidic solutions (i.e., nitric acid). However, Ag NPs are soluble in aqueous solutions under oxidizing conditions. The dissolution of Ag NPs in aqueous solutions involves two coupled processes: (1) oxidation with release of reactive oxygen species and (2) proton-mediated release of dissolved silver (Liu *et al.* 2010a). In accordance, ion release could be controlled through manipulation of the oxidation pathways, involving surface area (size), ligand binding, polymeric coatings, scavenging of peroxy-intermediates, and pre-oxidation treatments (Liu *et al.* 2010a). The surface oxidation of Ag NPs results in the formation of highly reactive ionic silver both adsorbed to the surface of the NP and released to the surrounding milieu. Colloidal suspensions 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 to the surface of NPs (Liu and Hurt 2010).

Based on surface area, smaller NPs should dissolve faster than larger NPs. Liu et al. (2010a) examined the release rate of soluble silver from Ag NPs of different sizes (4.8 and 60 nm) and from macroscopic silver foil. As expected, the mass-based release rates were inversely proportional to particle size, and the first-order release rate constant for the 4.8-nm particle was five orders of magnitude higher than that for macroscopic silver. When the data were renormalized by surface area, however, the variation in rate constants decreased from five orders of magnitude to one order of magnitude, demonstrating that in this scenario, surface area drives silver ion release rates (Liu *et al.* 2010a).

Particle concentration is also relevant when considering dissolution at the nanoscale. In general, the dissolution rate is higher for lower concentrations of Ag NPs. At higher concentrations, key factors that influence dissolution, like available oxygen and presence of protons (i.e., pH), may be depleted, and high concentrations of dissolved silver and ligands, which can inhibit surface reactions, might further inhibit dissolution (Liu and Hurt 2010). Oxidative dissolution is a complex chemical reaction influenced by pH, coatings, temperature and ligands in the surrounding fluid (Liu *et al.* 2012). The rate of dissolution and the final degree of dissolution were shown to be higher for PVP-stabilized Ag NPs than for citrate-stabilized Ag NPs and an increase in temperature led to increased dissolution (Kittler *et al.* 2010). Other studies have shown that the presence of cysteine or BSA enhanced the dissolution of Ag NPs (Gondikas *et al.* 2012; Liu *et al.* 2012).

To simulate the bio dissolution of Ag NPs, experiments have been conducted in several artificial body fluids, e.g., representing the stomach, lysosomes, wounds and blood. Artificial interstitial fluid (Gamble's solution, pH 7.4) and artificial lysosomal fluid (ALF, pH 4.5) were used to simulate dissolution in the airway surface liquid or in the macrophage phagolysosome, respectively (Stebounova *et al.* 2011b). No dissolution of Ag NPs into silver ions in either of the simulated biological fluids was detected (Stebounova *et al.* 2011b). However, the fluids used by Stebounova et al. contained significant amounts of sodium chloride and thus dissolution may have occurred followed by precipitation of silver-chloride complexes (a silver ion control was not included). Furthermore, the measurements of silver ions included a filtration step and some filters have been reported to bind high amounts of silver (Kennedy *et al.* 2010). Another study demonstrated that Ag NP (5 nm) dissolution in synthetic gastric fluid (pH 1.12) was relatively rapid whereas it was very slow in wound fluid (pH 7.52), but, addition of BSA greatly increased the dissolution (Liu *et al.* 2012). Rogers et al. conducted studies in synthetic stomach fluid (SSF, pH 1.5) with citrate-stabilized Ag NPs (1-10 nm and 40 nm) and found that they agglomerate, release silver ions, and partially react to form silver-chloride complexes (Rogers *et al.* 2012). 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 still needed to establish the mechanism of action.

Results from studies investigating the toxicological role of Ag NP dissolution appear equivocal. Some studies proposed that Ag NP cytotoxicity was independent of Ag+ concentration and resulted primarily from oxidative stress (Kim *et al.* 2009a; Eom and Choi 2010). However, several studies suggest that the mechanism of Ag NP toxicity is largely explained by Ag ions (Navarro *et al.* 2008; Bouwmeester *et al.* 2011; Beer *et al.* 2012; Yang *et al.* 2012). Limbach et al. (Limbach *et al.* 2007) have observed 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". It has also been suggested that Ag NPs may act as a "Trojan horse", bypassing typical barriers and then releasing silver ions that damage the cell machinery (Park *et al.* 2010). This view was partly supported by results from the studies of Navarro et al. who examined the rate of photosynthesis in algae exposed to Ag NPs or silver ions in the presence and absence of cysteine (a chelator of free silver ions). Their results suggested that interactions between algae and NPs may enhance the release of silver ions, i.e., NPs acted as an effective delivery vehicle for silver ions (Navarro *et al.* 2008). A recent study used Ag NPs with various sizes and coatings to examine the mechanism of toxicity in C. elegans and found a linear correlation between Ag NP toxicity and NP dissolution (Yang *et al.* 2012). Remarkably, 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 (Yang *et al.* 2012). This appears to corroborate results from a study which investigated the effect of Ag NPs and silver nitrate on gene expression in CaCo-2 intestinal cells and found very similar responses, leading to the conclusion that the effects observed from Ag NPs are likely exerted by silver ions that are released from the NPs

(Bouwmeester *et al.* 2011). Although, the cytotoxicity of Ag NPs may largely be explained by silver ions (Beer *et al.* 2012), gene expression data indicate that Ag NPs, may affect cells in a more complex way than silver ions alone (Foldbjerg *et al.* 2012). In conclusion, it is still uncertain by which mechanism(s) and to what degree silver ions play a role in Ag NP-mediated toxicity.

Mechanisms of Ag NP toxicity in vitro

In vitro test systems allow for specific cellular processes to be directly studied with high reproducibility, and have been used in numerous studies to evaluate the mechanisms by which Ag NPs are toxic. Existing studies have primarily been focused on mammalian cell lines and several studies have demonstrated cellular uptake of Ag NPs and their subsequent effects as reviewed by several authors (Wijnhoven *et al.* 2009; EPA 2010; Johnston *et al.* 2010). However, because cell types, culture conditions, and types of Ag NPs vary, the results of these studies are not directly comparable. Nevertheless, the following sections will review the current knowledge of the mechanisms behind Ag NP-induced toxicity including oxidative stress, cell death and genotoxicity.

- *Oxidative stress*

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 (Oberdorster *et al.* 2005; Nel *et al.* 2006). A main determinant for oxidative stress is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The term ROS encompasses the initial species generated by oxygen reduction (superoxide or hydrogen peroxide) as well as their secondary reactive products whereas RNS is used to describe reactive species derived from nitric oxide (Winterbourn 2008). Reactive species may also be produced by neutrophils and macrophages during inflammation. 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 glutathione (GSH) and vitamins (Franco *et al.* 2009).

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 (Liu and Hurt 2010). Pretreatment of cells with cyanide, an inhibitor of the mitochondrial electron-transferring activity of cytochrome c oxidase, leads to inhibition of ROS production induced by Ag NPs (Hsin *et al.* 2008). This suggests 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 (Rogers *et al.* 2008). GSH is a major endogenous antioxidant scavenger that protects cells against oxidative stress through its ability to bind to and reduce ROS. Some studies reported increased levels of GSH in response to Ag NP treatment (Arora *et al.* 2009; Kang *et al.* 2012) which could be cellular responses (upregulation) for coping with Ag NP-mediated oxidative damage. In contrast, other studies found decreased levels of GSH to correlate with ROS markers (Arora *et al.* 2008; Carlson *et al.* 2008), suggesting either an inhibition of GSH-synthesizing enzymes or depletion of GSH. Remarkably, silver ions bind strongly to GSH and other molecules with thiol groups (Liu *et al.* 2012) and may thus play a role in GSH depletion. Other examples of oxidative stress indications found in response to Ag NP exposure include stress-related gene transcription (Bouwmeester *et al.* 2011; Foldbjerg *et al.* 2012), lipid peroxidation (Arora *et al.* 2008; Piao *et al.* 2011) and protein carbonylation (Haase *et al.* 2011; Piao *et al.* 2011).

It should be noted that the genetic background of human cell lines is very diverse and when assessing the *in vitro* response of a given toxicant, this could be important. For example, the Glutathione S-transferase Mu 1 (GSTM1) and Glutathione S-transferase theta-1 (GSTT1) isoforms are both involved in cellular antioxidant defense mechanisms (Autrup 2000). Our unpublished results demonstrated that the human lung cell line, A549, is negative for both genotypes whereas the monocytic cell line, THP-1, is positive for both (data not shown). These cell lines may thus exhibit differential sensitivity towards Ag NP-induced oxidative stress (Foldbjerg *et al.* 2009; Foldbjerg *et al.* 2011).

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. A recent *in vivo* study investigated the effect of silver dissolution and oxidative stress on toxicity in C. elegans. Using mutant sensitivity analysis and pharmacological rescue experiments with trolox and N-acetylcysteine, the study concluded that the determining factor for Ag NP toxicity was NP dissolution but that some of the Ag NPs (three out of five) also acted via oxidative stress (Yang *et al.* 2012). This finding may provide an explanation for the differing results between studies.

Cell death

Cell death is often attributed to either necrosis or apoptosis, where the former is characterized as accidental and pathological, and the latter is considered to be a controlled, programmed and physiological mechanism. The term "programmed" in this context implies that the dismantling of the cell is regulated by specific genes and involves the 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. Today, other modalities such as autophagic cell death and necroptosis (regulated necrosis) have also been identified. In a very recent review from the Nomenclature Committee on Cell Death (NCCD), at least 13 different types of regulated cell death were enumerated thereby alluding to the complexity of cell death (Galluzzi *et al.* 2012).

Different nanomaterials may activate several pathways of programmed cell death (Andon and Fadeel, 2012). However, the pre dominat mechanism of programmed cell death caused by Ag NPs appears to be mitochondria-dependent (intrinsic) apoptosis. The intrinsic apoptosis pathway is activated in response to numerous types of cellular stress including DNA damage, oxidative stress, cytosolic calcium overload, endoplasmic reticulum (ER) stress as a function of the accumulation of unfolded proteins, etc. (Andon and Fadeel,

2012). The intrinsic pathway (figure 2) is characterized by insertion of Bax and/or Bak into the mitochondrial outer membrane. This is followed by the release of several proteins from the mitochondrial intermembrane space, including cytochrome c, which forms a cytosolic apoptosome complex with Apaf-1 (apoptosis activating factor-1) and procaspase-9 in the presence of dATP. This results in the activation of procaspase-9, which triggers the caspase cascade by activation of procaspase-3 leading to apoptotic cell death and phagocytosis by macrophages (Ott *et al.* 2007).

Studies in human and animal cells treated with Ag NPs demonstrated 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) (Gopinath *et al.* 2010; Piao *et al.* 2011). In addition, Ag NP exposure triggered release of cytochrome c into the cytosol and translocation of Bax into the mitochondria in NIH3T3 cells, indicating activation of the intrinsic apoptotic pathway (Hsin *et al.* 2008). Increased protein levels of active caspase-9 (activated by cytochrome c released from mitochondria), were observed in human liver cells (Piao *et al.* 2011). In accordance with these findings, activation of procaspase-3 was detected in various Ag NP-treated human and animal cell lines (Arora *et al.* 2008; Arora *et al.* 2009; Gopinath *et al.* 2010; Piao *et al.* 2011).

Although oxidative stress is generally considered to be a key factor in Ag NP-mediated apoptosis, the mechanism by which Ag NPs trigger apoptosis is not completely resolved. For example, a recent study reported that Ag NPs may exert cytotoxic effects through modulation of ER stress pathways (Zhang *et al.* 2012).

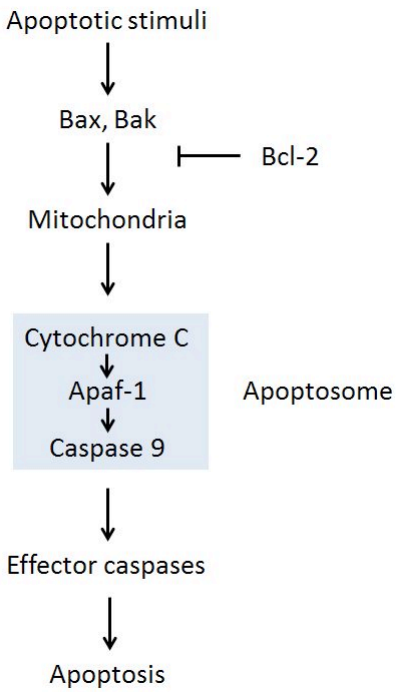

Figure 2
Schematic overview of main events in intrinsic apoptosis. Refer to the text for details.

- *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 (Singh *et al.* 2009). Several *in vitro* studies with Ag NPs have indicated genotoxic effects in different types of human and mammalian cells (Ahamed *et al.* 2008; AshaRani, V *et al.* 2009; Kawata *et al.* 2009; Foldbjerg *et al.* 2011; Hackenberg *et al.* 2011; Kim *et al.* 2011a; Flower *et al.* 2012;; Li *et al.* 2012; Nymark *et al.* 2012). However, some investigators did not find statistically significant genotoxic effects (Asare *et al.* 2012; Park *et al.* 2011b). The most common effects considered in these studies include: DNA strand breaks, micronuclei induction and chromosomal aberrations.

In a study by Nymark et al., the genotoxic effects of Ag NPs (42.5 nm) coated with PVP were investigated in a human bronchial epithelial cell line (BEAS2B) (Nymark *et al.* 2012). The particles did not induce chromosomal aberrations after 24 h or 48 h of exposure, nor did they cause formation of micronuclei after 48 h. However, DNA damage detected by the comet assay was seen after both 4 h and 24 h exposures, and the induction was dose-dependent (Nymark *et al.* 2012). In corroboration, Kim et al. (2011a) found that Ag NPs stimulated DNA breakage and micronuclei formation in a dose-dependent manner in BEAS-2B cells. Remarkably, ROS scavengers, especially superoxide dismutase (SOD), could reduce the genotoxic effects in both assays thereby implicating oxidative stress as a mechanism (Kim *et al.* 2011a). In another study, both the comet assay and chromosomal aberration test showed DNA damage in human mesenchymal stem cells after 1, 3, and 24 h at Ag NP concentrations from 0.1-10 µg/ml (Hackenberg *et al.* 2011). A number of other *in vitro* studies have also shown that Ag NPs (in the size range 1–50 nm) are able to induce DNA and chromosomal damage in different cell types (Ahamed *et al.* 2008; AshaRani, V *et al.* 2009; Kawata *et al.* 2009; Li *et al.* 2012). In contrast, Park et al detected no significant genotoxic effects based on gene mutations in MEF-LacZ cells (Park *et al.* 2011b). Furthermore, no statistically significant DNA damage was found in a human testicular embryonic carcinoma cell line and in primary testicular cells from C57BL6 mice after Ag NP exposure (Asare *et al.* 2012). Interestingly, 5 nm Ag NPs did not induce mutations in five different S. typhimurium strains when the Ames test was performed according to OECD guidelines (Li *et al.* 2012). In contrast, the same study found concentration-dependent genotoxicity in the human lymphoblast TK6 cell micronucleus assay (Li *et al.* 2012). It has been suggested that negative results in the Ames test may be due to the inability of the nanomaterials to penetrate the bacterial cell wall. Another explanation is low sensitivity of the Ames test strains towards oxidative DNA damage (Landsiedel *et al.* 2009; Li *et al.* 2012). Further indications of Ag NP-mediated genotoxicity were observed when investigating DNA damage repair proteins in mouse embryonic and fibroblast cells (Ahamed *et al.* 2008). In particular, protein expression was upregulated for the cell cycle checkpoint protein, p53, and the DNA damage repair proteins, Rad51, and phosphorylated-H2AX (Ahamed *et al.* 2008).

In summary, genotoxic effects following Ag NP exposure may occur within mammalian cells and different modes of action could be speculated. E.g., 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.

Toxic effects of Ag NPs *in vivo*

Several *in vivo* studies on Ag NP toxicity have been conducted (Wijnhoven *et al.* 2009; Christensen *et al.* 2010; EPA 2012). No severe effects after acute exposure to Ag NPs by inhalation (Sung *et al.* 2011) or ingestion (Kim *et al.* 2012) were observed in rats. Chronic inhalation studies (28-day and 90-day) in rats demonstrated decreased lung function in male rats and lung inflammation (Song *et al.* 2012) and minimal levels of bile-duct hyperplasia in the liver (Sung *et al.* 2009). When Ag NPs were administered orally to rats, the observed adverse effects included: slight liver damage in part reflected by increased serum levels of alkaline phosphatase and cholesterol (Kim *et al.* 2010; Kim *et al.* 2008). In regards to genotoxicity, a 90-day inhalation study and a 28-day oral study which measured erythrocyte micronuclei found no differences between treated and control animals (Kim *et al.* 2011b; Kim *et al.* 2008). As part of a 28-day oral rat study it was reported that antibody levels (IgG and IgM) in blood, *ex vivo* lymphocyte proliferation and cytokine release (upon stimulation), and NK-cell activity did not indicate immunotoxicity (van der Zande *et al.* 2012). Another recent 28-day oral rat study demonstrated Ag NP-related effects on brain neurotransmitter concentrations (Hadrup *et al.* 2012b). Additionally, brains from mice exposed to Ag NP for 24 h by intraperitoneal injection exhibited gene expression changes indicating that Ag NPs may produce neurotoxicity by generating free radical-induced oxidative stress and altered gene expression (Rahman *et al.* 2009). It should be kept in mind that the effects mentioned above depend on the Ag NP dose and may sometimes only appear at very high doses.

Perspectives

The magnitude of engineered nanomaterials is enormous and thus efficient screening methods for toxicity are desirable to, provide quick answers, lower the expenses of testing, and to fulfill the strategy to reduce the cost, refine and replace animal testing. Only animal experiments (*in vivo*) can provide sufficient answers to the complex issues of absorption, distribution, metabolism, and excretion (ADME). *In vitro* studies may provide additional evidence to explain the mechanisms behind the potential toxic effects of NPs. Potentially, knowledge of the biological processes involved in the pathogenesis of NP may be useful in developing biomarkers o f adverse effects. A major question is whether *in vitro* investigations can be used to predict *in vivo* effects. A recent study compared the effects caused by Ag NPs in a human hepatocyte cell line and rat livers and found similarities between the *in vitro* and *in vivo* responses, illustrating that this cell line may be suitable to predict acute *in vivo* responses to Ag NP toxicants (Gaiser *et al.* 2012). High-throughput *in vitro* screening procedures were also reported to be useful for predicting mechanisms of NP injury *in vivo* (Nel *et al.* 2012). Thus, ongoing research indicates that predictions from *in vitro* data to *in vivo* effects, to some extent, may be possible. Recently, we initiated toxicogenomic studies with different types of NPs (including silver) to compare the transcriptional regulation in human cells to that in similar murine cells. It is expected that this type of transcriptional profiling will reveal fundamental differences and similarities dependent on NP type, organ and species. Based on these results, it is our hope that the potential adverse effect biomarkers may be selected for validation in rodents to assess the significance of *in vitro* NP toxicity data.

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