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The Biological Effects and Possible Modes of Action of Nanosilver

Carolin Völker, Matthias Oetken, and Jörg Oehlmann

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1 Introduction

Engineered nanomaterials are increasingly employed in a variety of applications. The size of nanoparticles, by definition, ranges between 1 and 100 nm in at least one dimension (The Royal Society and The Royal Academy of Engineering 2004). Such dimensions result in a high surface area to volume ratio. The subsequent chemical, physical, and biological properties of nanomaterials are unique, and lead to diverse technical applications and prospectively to widespread use in commercial products. In 2004, the production volume of nanomaterials was estimated to be 2,000 t worldwide, and is expected to rise to 58,000 t within the next decade (The Royal Society and The Royal Academy of Engineering 2004).

Currently, the majority of nanotechnology-enabled consumer products are based on nanoscale silver (Woodrow Wilson International Center for Scholars 2011).

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Because of its antimicrobial properties that were initially used to dress wounds, sanitize medical equipment, and treat water (Gaiser et al. 2009), nanosilver use has been extended to a variety of products, including textiles, cosmetics, food packaging materials, and electronics (Wijnhoven et al. 2009). Silver compounds have long been used for their bactericidal properties (Kim et al. 2002; Silver et al. 2006). The number of applications to which nanosilver is increasingly being put suggests that it has higher activity than its bulk counterpart (Choi et al. 2008). The majority of nanosilver applications require a nanoparticle size range of 1–10 nm; nanosilver in this size range is synthesized by reducing dissolved silver salts (bottom-up technique), usually silver nitrate (Tolaymat et al. 2010). Capping agents are used to prevent silver nanoparticles from aggregating, and the dominant ones are polyvinylpyrrolidone (PVP) and citrate (Tolaymat et al. 2010). These capping agents predominantly lead to a negative particle charge under standard environmental pH conditions (Tolaymat et al. 2010).

As the number and diversity of silver nanoparticle applications increase, concerns about the potential environmental impact of silver nanoparticles are growing (Wijnhoven et al. 2009; Woodrow Wilson International Center for Scholars 2011). Benn and Westerhoff (2008) have demonstrated that silver particles are released from commercially available sock fabrics during the laundering process. Farkas et al. (2011) demonstrated the release of nanosilver from a silver nanowashing machine. Other sources of silver derive from the use and degradation of various consumer products that are disposed of and enter wastewaters (Bradford et al. 2009). Mueller and Nowack (2008) modeled the environmental exposure to engineered nanomaterials. Because estimates for the production and application of nanomaterials vary considerably, the authors' modeling predicted environmental concentrations (PEC) under two exposure scenarios: a realistic one and a high emission scenario. The PEC results for nanosilver were estimated to be in the range from 1.7×10^{-3} to 4.4×10^{-3} μg/m^3 in air, 0.03 to 0.08 μg/L in water, and 0.02 to 0.1 μg/kg in soil.

The environmental behavior and toxicity of silver are known to be influenced by several physicochemical factors of the media in which it exists, including pH, organic carbon content, and cation exchange capacity (Ratte 1999). Toxicity depends on and varies with the type of silver species involved, particularly for free silver ions, which show the highest potential toxicity (Ratte 1999). The ionic form of silver is known to be highly toxic to aquatic organisms (Eisler 1996), and has well-documented antibacterial properties (Bragg and Rainnie 1974; Ghandour et al. 1988; Schreurs and Rosenberg 1982). In 1954, silver was registered as a pesticide in the USA for the first time (U.S. EPA 1993). Recently, an antibacterial product for textile preservation based on nanosilver was conditionally registered. However, the manufacturer was urged to develop further product chemistry, toxicology, exposure, and environmental data as a condition of this registration (U.S. EPA 2010).

The majority of toxicity data available on nanosilver are on bacteria species (Choi et al. 2008; Fabrega et al. 2009; Lok et al. 2006; Morones et al. 2005; Sondi and Salopek-Sondi 2004) and cell lines (Arora et al. 2009; AshaRani et al. 2009a; Bouwmeester et al. 2011; Carlson et al. 2008; Foldbjerg et al. 2011). One proposed mechanism by which nanosilver produces toxicity is by enhancing intracellular levels of reactive oxygen species (ROS). ROS, when formed, produce subsequent cellular damage such as disrupting membrane integrity and damaging proteins and

DNA (Arora et al. 2009; AshaRani et al. 2009a; Bouwmeester et al. 2011; Carlson et al. 2008; Foldbjerg et al. 2011; Gogoi et al. 2006; Lok et al. 2006; Morones et al. 2005; Sondi and Salopek-Sondi 2004).

Aquatic organism toxicity studies have focused primarily on the adverse effects of nanosilver on embryonic development and altered stress enzyme levels in freshwater fish species (Laban et al. 2010; Yeo and Kang 2008). Among freshwater species, *Daphnia* had the highest acute susceptibility to nanosilver, probably because particles are effectively taken up due to their filter-feeding strategy (Griffitt et al. 2008). Unfortunately, limited chronic exposure data are currently available.

In this review, we focus on the biological effects of nanosilver and its possible modes of toxic action. In addition, we identify current knowledge gaps and future research needs. The review is based on peer-reviewed papers found in ISI Web of Science until the end of 2011.

In addition to nanosilver, we also address ionic silver in this review, because of its potential role (silver ions released from nanosilver) in causing toxicity. Furthermore, size dimensions of the nanoparticles used in the cited studies are indicated, because there is evidence that effects of nanoparticles may be size dependent. The size dimensions refer to the actual particle sizes in the test media and size scales are provided as described in the studies. However, when there was no characterization of particle sizes in the test media, primary particle sizes are provided.

2 The In Vitro Toxicity of Silver

2.1 Silver as Disruptor of Basal Cell Functions

In vitro test systems allow for specific cellular processes to be directly studied with high reproducibility, and have been used to evaluate the mechanisms by which silver nanoparticles are toxic (Braydich-Stolle et al. 2005; Carlson et al. 2008; Foldbjerg et al. 2011; Hussain et al. 2005). Existing studies have primarily been focused on mammalian cell lines. Because cell types, culture conditions, and types of silver nanoparticles vary, effects noted among studies are not directly comparable. Nevertheless, some conclusions can be drawn.

In vitro study results show that silver nanoparticles are able to enter cells, probably by phagocytosis (AshaRani et al. 2009a; Carlson et al. 2008; Wei et al. 2010) or passive diffusion through the cell membrane (Carlson et al. 2008). Once inside the cell cytoplasm, silver nanoparticles appear in intracellular vesicles and are able to enter organelles like mitochondria and nuclei (Arora et al. 2009; AshaRani et al. 2009a; Carlson et al. 2008; Wei et al. 2010). The entry of silver nanoparticles into cells and their toxic potential is size dependent. Wei et al. (2010) showed that silver microparticles (diameters of 2–20 μm) did not enter mouse fibroblast cells, whereas silver nanoparticles with diameters of 50–100 nm were detected inside the same cells. Studies that have compared different-sized silver nanoparticles have shown smaller particles to be more cytotoxic, probably from the increased reactive surface area of smaller nanoparticles (Carlson et al. 2008; Wei et al. 2010).

Size-dependent toxicity occurred not only for nanosilver but also for nanoparticles of different chemical composition (Griffitt et al. 2008; Xia et al. 2006). However, a particular particle size is not the pivotal factor that determines toxicity, because particles of a similar size that have different chemical composition show different toxicities (Buzea et al. 2007; Griffitt et al. 2008; Liu et al. 2009). Among nanometals, nanosilver is most toxic to bacteria and freshwater organisms, followed by nanoforms of copper and zinc oxide (Griffitt et al. 2008; Kahru and Dubourguier 2010). Titanium dioxide, for example, is less toxic than other nanometals of a similar size. Therefore, the chemical composition and resulting intrinsic properties of nanoparticles greatly influence toxicity (Griffitt et al. 2008).

Silver nanoparticles primarily seem to cause their toxicity by enhancing intracellular levels of reactive oxygen species (ROS). ROS include radicals containing oxygen, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl ($^{\bullet}OH$), or nitroxyl ($NO^{\bullet}$) radicals, and by-products, e.g., alkoxyl radicals ($RO^{\bullet}$) (Lesser 2006; Simon et al. 2000). Radical oxygen intermediates are produced during oxidative metabolism by the reduction of oxygen to water in the mitochondrial respiratory chain (Fridovich 1978). Under normal conditions, nonenzymatic and enzymatic detoxification mechanisms (i.e., reduced glutathione (GSH), superoxide dismutase (SOD), and peroxidases-like catalase) prevent free radical reactions from occurring in cells (Apel and Hirt 2004; Lesser 2006; Simon et al. 2000; Yu 1994). Although free radical reactions may lead to oxidative damage of lipids, proteins, and DNA, ROS may also retain a role in cellular function, and can act as chemical messengers in cellular pathways (Lesser 2006; Simon et al. 2000). ROS are not exclusively generated as metabolic by-products; they also may result from the environmental impact caused by UV radiation or chemicals (Bindhumol et al. 2003; Robertson and Orrenius 2000; Sies 1997). If the amount of ROS exceeds the level of cellular antioxidants, cells will be exposed to oxidative stress.

Several in vitro studies have demonstrated that nanosilver exposure increased intracellular ROS levels (AshaRani et al. 2009b; Carlson et al. 2008; Eom and Choi 2010). Elevated levels of hydrogen peroxide and superoxide, for example, were detected in human fibroblasts after treatment with silver nanoparticles (diameters of 6–20 nm) at concentrations of 25 and 50 μg/mL (AshaRani et al. 2009b). The detection of silver nanoparticles in mitochondria and the resultant effects on mitochondrial function (viz., membrane potential or respiratory chain effects; Arora et al. 2009; Carlson et al. 2008; Foldbjerg et al. 2011; Wei et al. 2010) may also alter ROS production, and thereby cause oxidative stress. Table 1 summarizes studies in which an involvement of ROS in the toxic mode of action of nanosilver was demonstrated.

Other indicators of oxidative stress are increased activities of GSH, GSH-related enzymes, and other enzymes that are involved in cellular antioxidant defense mechanisms. Various mammalian cell-line studies have shown that nanosilver treatment altered the activities of antioxidant enzymes and lipid peroxidation, have increased secretion of inflammatory cytokines/chemokines, and have increased the expression of stress response genes (Arora et al. 2009; AshaRani et al. 2009a; Bouwmeester et al. 2011; Carlson et al. 2008; Foldbjerg et al. 2011). Increased levels of GSH and SOD, for example, were detected in primary mouse fibroblasts and primary mouse

Table 1 Evidence for involvement of ROS (reactive oxygen species) in the toxic mode of action of nanosilver

Cell line	Silver nanoparticles (size)	Evidence for involvement of ROS	References
Human lung fibroblast cells (IMR-90) Human glioblastoma cells (U251)	Starch-capped AgNPs (TEM: 6–20 nm)	Permeability changes of mitochondria leading to disruption of calcium homeostasis probably ROS mediated	AshaRani et al. (2009a)
Human lung fibroblast cells (IMR-90) Human glioblastoma cells (U251)	Starch-capped AgNPs (TEM: 6–20 nm)	Increase in hydrogen peroxide and superoxide production; probably ROS mediated: mitochondrial dysfunction, DNA damage resulting in cell cycle arrest	AshaRani et al. (2009b)
Human lung carcinoma epithelial-like cell line (A549)	PVP-coated AgNPs (stock solution (MilliQ), TEM: 69 ± 3 nm, DLS: 121 ± 6 nm; RPMI 1,640 media, DLS: 149 ± 37 nm)	Increased levels of ROS; cytotoxicity, bulky DNA adducts and ROS levels reduced by pretreatment with antioxidant NAC	Foldbjerg et al. (2011)
Jurkat T cells	AgNPs (DLS: 28–35 nm)	Increased levels of ROS leading to increased levels of NF-κB, Nrf-2 which activate MAPK causing apoptosis	Eom and Choi (2010)
Primary mouse fibroblasts Primary mouse liver cells	AgNPs (DLS: 6.5–43.8 nm, average size of 16.6 nm)	Increased levels of GSH, SOD	Arora et al. (2009)
Mouse fibroblast cells (L929)	AgNPs (TEM: 50–100 nm)	DNA damage (possibly ROS mediated) leading to cell cycle arrest and apoptosis	Wei et al. (2010)
Mouse lymphoma cell line (L5178Y thymidine kinase $(tk)^{+/-}$-3.7.2C cells) Human bronchial epithelial cells (BEAS-2B)	AgNPs (manufacturer: <100 nm)	DNA damage (possibly ROS mediated) and cytotoxicity	Kim et al. (2010)
Rat alveolar macrophages	Hydrocarbon-coated AgNPs (SEM: primary sizes 15, 30, 55 nm, larger agglomerates in suspension)	Increased levels of ROS and decreased levels of GSH in cells exposed to AgNPs (15 nm)	Carlson et al. (2008)
Baby hamster kidney cells (BHK21) Human colon adenocarcinoma cells (HT29)	AgNPs (TEM: 10–15 nm)	Apoptosis with involvement of caspases could be ROS mediated	Gopinath et al. (2008)
Danio rerio embryos (whole organism)	AgNPs, supporter material TiO_2 (TEM: 10–20 nm)	Increased catalase activity indicates oxidative stress-related toxicity	Yeo and Kang (2008)
Caenorhabditis elegans (whole organism)	AgNPs (uncoated, DLS: 14~20 nm)	Increased *sod-3* gene expression indicates oxidative stress-related toxicity	Roh et al. (2009)

liver cells, respectively, after spherical silver nanoparticle (6.5–43.8 nm, average size 16.6 nm) treatment (Arora et al. 2009). In contrast, Carlson et al. (2008) correlated the increased ROS levels in rat alveolar macrophages with depleted GSH levels, after treatment with hydrocarbon-coated spherical-silver nanoparticles with a primary size of 15 nm. As the authors suggested, this could be the result of silver nanoparticles reacting with GSH-maintenance enzymes, for example, direct binding to GSH-reductases. In conclusion, increased ROS levels that are accompanied by depleted GSH levels produce oxidative stress.

Foldbjerg et al. (2011) observed minor cytotoxic effects of nanosilver (149 ± 37 nm) on the human lung carcinoma epithelial cell line (A549) after adding *N*-acetylcysteine (NAC), a precursor for GSH; this suggests that the observed cytotoxicity was ROS mediated. The authors suggested direct binding of silver to thiol groups of cysteine as another possible reason for the appearance of minor toxic effects after NAC treatment. Exposure to NAC also inhibited bulky DNA adducts that emerged after nanosilver treatment of A549 cells, suggesting that this effect was also ROS mediated.

In several in vitro studies performed with nanosilver, a strong correlation between enhanced ROS levels and apoptosis was detected (Carlson et al. 2008; Foldbjerg et al. 2011; Hsin et al. 2008; Sanpui et al. 2011). Enhanced levels of nuclear factor-kappaB (NF-κB) and nuclear factor-E2-related factor-2 (Nrf-2) were detected in Jurkat T cells that showed increased ROS levels after nanosilver (28–35 nm) treatment. These transcriptional factors are known to activate mitogen-activated protein kinase (MAPK), which is relevant to the induction of apoptosis (Ashkenazi and Dixit 1998; Eom and Choi 2010; Janssen-Heininger et al. 2000). Eom and Choi (2010) revealed an elevated level of p38 MAPK in nanosilver-treated cells that showed apoptosis. Additionally, ROS-induced DNA damage may have produced cell cycle arrest, and may have contributed to the observed apoptosis in nanosilver-treated cells.

Apoptosis induced by DNA damage, with resulting cell cycle arrest, also occurred in mouse fibroblasts (L929) treated with 100 μg/mL nanosilver (diameters of 50–100 nm) (Wei et al. 2010). Nanosilver-induced DNA damage and cytotoxicity also occurred in mouse lymphoma cells (L5178Y) and in human bronchial epithelial cells (BEAS-2B) (Kim et al. 2010). Furthermore, nanosilver-treated cells displayed decreased mitochondrial function (Arora et al. 2009). Disruption of the mitochondrial respiratory chain by silver nanoparticles was suggested by Wei et al. (2010) and Carlson et al. (2008). Such disruption may enhance ROS levels and produce cellular damage, but could also be the cause of a mitochondrial-driven apoptosis from cytochrome C release and caspase cascade activation. The role of mitochondria in nanosilver-mediated apoptosis was also evaluated by AshaRani et al. (2009a). Nanosilver treatment of human lung fibroblasts (IMR-90) and human glioblastoma cells (U251) disrupted calcium homeostasis, probably from mitochondrial permeability changes caused by oxidative stress. Calcium transients in mitochondria could impair mitochondrial function, leading to higher ROS levels and inhibiting ATP synthesis (Orrenius et al. 1992). Furthermore, mitochondrial membrane permeability from calcium overload led to the release of apoptogenic factors like cytochrome C, which initiates the activation of caspases (Belizário et al. 2007).

The general role of ROS in causing apoptosis has been evaluated in several previous studies (Buttke and Sandstrom 1994; Fadeel et al. 1998; Sakon et al. 2003; Simon et al. 2000). ROS are responsible for activating caspase cascades (Fadeel et al. 1998; Simon et al. 2000), which have a critical role in causing programmed cell death (Cohen 1997). Apoptosis is known to result from moderate oxidative stress, whereas severe oxidative stress produces necrosis (Bonfoco et al. 1995; Curtin et al. 2002). Necrosis is associated with inflammation and is characterized by cell swelling and lysis, whereas apoptosis is an active cellular process that leads to morphological cell changes (viz., cell shrinkage, membrane blebbing, nuclear condensation, DNA fragmentation), and to the formation of apoptotic bodies that are engulfed by phagocytic cells (Robertson and Orrenius 2000).

The type (apoptosis vs. necrosis) of nanosilver-induced cell death appears to be dependent on the particle concentration with which cell lines are treated. Nanosilver-treated (spherical, 6.5–43.8 nm, average size 16.6 nm) primary mouse fibroblasts and primary mouse liver cells showed a dose-dependent form of cell death (Arora et al. 2009). Concentrations of 3.12 μg/mL (primary fibroblasts) and 12.5 μg/mL (primary liver cells) produced an apoptotic cell population; the necrotic concentration, at which total lack of caspase-3 activity occurred, was much higher (100 μg/mL in primary fibroblasts, 500 μg/mL in primary liver cells). Apoptotic bodies, cell membrane blebbing, and condensed chromatin occurred in baby hamster kidney (BHK21) and human colon adenocarcinoma (HT29) cell lines after treatment with 11.0 μg/mL silver nanoparticles (10–15 nm) (Gopinath et al. 2008). Furthermore, caspase gene expression increased in nanosilver-treated cells. Necrotic cells resulted from treatment with higher concentrations (>44.0 μg/mL).

In summary, the results of in vitro cytotoxicity studies indicate that one toxic mechanism of nanosilver may be a dose-dependent programmed cell death driven by several apoptotic pathways that are potentially induced by ROS as summarized in Table 1 and Fig. 1. Similar results were obtained for nickel ferrite nanoparticles and zinc oxide nanoparticles in in vitro studies (Ahamed et al. 2010; Xia et al. 2008). As observed for nanosilver, zinc oxide nanoparticles and zinc ions accumulated in cell organelles and produced oxidative stress, mitochondrial damage, and enhanced calcium release (Xia et al. 2008). Titanium dioxide nanoparticles are redox active, capable of generating ROS (Farré et al. 2009), and also become internalized into cells and organelles (Xia et al. 2008). However, the in vitro and in vivo adverse effects elicited by titanium dioxide are relatively minor, and are only observed at high concentrations (Heinlaan et al. 2008; Ivask et al. 2010; Xia et al. 2008).

In contrast to other nanometals that cause increased ROS levels, cerium oxide nanoparticles appear to protect cells from oxidative stress by suppressing ROS generation (Xia et al. 2008).

The antioxidant activity of cerium oxide is explained by its mixed valence state (trivalent (3+) and tetravalent (4+)), and its ability to change its oxidation state (Tarnuzzer et al. 2005). Additionally, cerium oxide with a higher Ce^{3+}/Ce^{4+} ratio shows superoxide dismutase mimetic capabilities, and higher efficiency than the authentic enzyme (Korsvik et al. 2007). Although the adverse effects caused by increased ROS levels were observed for all nanometals (except cerium oxide), the

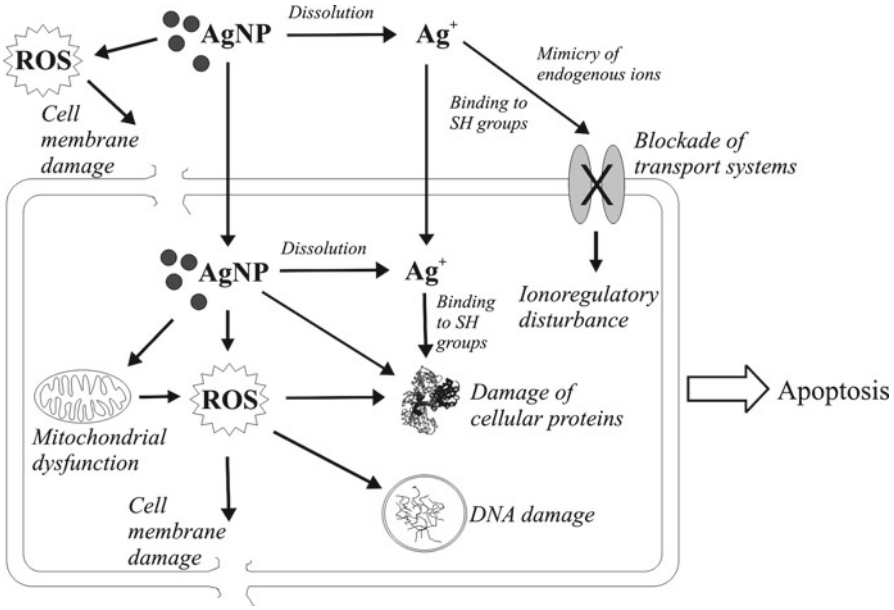

Fig. 1 Possible modes of action of nanosilver toxicity

toxicities differed significantly and were dependent on the particles' chemical composition.

The release of metal ions from nanoparticle surfaces is considered to be a further cause that influences toxicity. Indeed, higher acute toxicities are observed for nanomaterials of higher solubility (viz., zinc oxide, copper oxide), not excluding a possible involvement of both, released ions and the particulate form (Auffan et al. 2009; Brunner et al. 2006).

In general, ionic as well as nanoparticulate forms of heavy metals are capable of producing ROS and subsequent oxidative damage to cellular structures, and of inducing stress proteins and activating protein kinases (Stohs and Bagchi 1995). Another important mechanism of metal toxicity at the cellular level is the replacement or mimicry of essential ions (e.g., lead's well-known mimicry of calcium (Clarkson 1993)). We also note that metals unfold their toxic potential by an incomplete mimicry of endogenous ions in some metabolic steps that leads to, for example, a blockade of a particular metabolic process (Clarkson 1993). Hussain et al. (1994) considered ionic silver mimicry, and the replacement of endogenous ions such as K^+ and Na^+, as one reason for the inhibition of isolated Na^+/K^+-ATPase, after they treated the enzyme with silver nitrate. Because binding of the silver to the enzyme is reversible by adding cysteine, silver ions may block the transport system by binding to the enzyme's thiol groups (Hussain et al. 1994). It is well documented that silver ions show a strong affinity to free thiol groups, since these compounds neutralize the biological effects of silver ions (Hussain et al. 1992; Liau et al. 1997).

Moutin et al. (1989) also hypothesized that ionic silver has the property of structurally mimicking endogenous ions. These authors investigated the effects of silver

ions on sarcoplasmic reticulum vesicles that were prepared from rabbit skeletal muscle. They found a concentration-dependent ability of silver ions to trigger calcium release, suggesting that silver ions act on the same site as do calcium ions. This conclusion was confirmed by inhibition of calcium release at higher silver concentrations, which led to a result similar to that shown for calcium-induced calcium release. However, disruption of calcium homeostasis could also result from impairment of calcium translocases (located in membranes) by ROS, as reported for other heavy metals (Viarengo and Nicotera 1991). ROS are capable of oxidizing the SH groups of Ca^{2+}/Mg^{2+}-ATPases, leading to dysfunction and altered calcium levels. In addition, silver ions could bind to the thiol groups of the enzyme, and thereby disrupt its function.

Moreover, it has been documented that silver ions form complexes with isolated DNA (Jensen and Davidson 1966; Yamane and Davidson 1962). These complexes are chemically and biologically reversible (Jensen and Davidson 1966), and there is an indication that the helical structure of the DNA is not disrupted (Yamane and Davidson 1962). In contrast to other cations, Ag-DNA complexes are not formed by an interaction with silver and phosphate groups. Rather, silver binds specifically to purine and pyrimidine bases, probably by replacing the hydrogen bond between complementary base pairs (Jensen and Davidson 1966; Luk et al. 1975; Yamane and Davidson 1962).

It is not yet clear to what extent the toxic mode of action of nanosilver is comparable to that of ionic silver, or whether it is related to the release of silver ions. Studies that compared the effects of silver in its ionic forms and nanoforms show contradictory results, and this will be addressed later in this chapter. However, results of in vitro studies reveal that nanosilver and silver ions, respectively, affect cellular components and functions. Whether these in vitro results can be extrapolated to live animal studies is not yet clear.

To date, only two in vivo studies have been performed that provide evidence for oxidative stress as an important mechanism for nanosilver toxicity: Yeo and Kang (2008) exposed zebrafish embryos (*Danio rerio*) to nanosilver (10–20 nm) and observed an increase of catalase activity, indicating an involvement of ROS in the toxicity of nanosilver. Comparable results are reported by Roh et al. (2009), using the soil nematode *Caenorhabditis elegans* as model organism. After exposure to nanosilver (0.1 and 0.5 mg/L, 14–20 nm) *C. elegans* showed an increased expression of the superoxide dismutases-3 (sod-3) gene. Increased sod-3 gene expression was linked to the involvement of oxidative stress since superoxide dismutases are known to scavenge ROS (Lesser 2006).

2.2 The Antibacterial Properties of Silver Compounds

The antibacterial properties of silver compounds are well documented: silver has long been used to treat wounds and disinfect water (Kim et al. 2002; Silver et al. 2006). The antibacterial action of silver probably derives from silver ions attaching to the negatively charged bacterial cell wall, where they can disrupt its permeability

(Ratte 1999). In addition, silver ions are presumed to enter bacterial cells by an essential copper transport system (Ghandour et al. 1988). The toxic mechanism in bacteria is by inhibition of the respiratory chain, collapse of the proton motive force, and interference with phosphate uptake (Bragg and Rainnie 1974; Ghandour et al. 1988; Schreurs and Rosenberg 1982). Because of the high affinity that silver ions have for thiol groups (Liau et al. 1997), the interference with respiratory enzymes could be mediated by silver ions binding to thiol sites on these enzymes (Holt and Bard 2005; Kim et al. 2008b). This was confirmed by a study with *Escherichia coli*, wherein effects of the silver ions on phosphate uptake and exchange were reversed by the addition of thiols (Schreurs and Rosenberg 1982). However, study results indicate that silver does not exclusively act with thiols (Schreurs and Rosenberg 1982).

Feng et al. (2000) documented effects on DNA molecules of *Staphylococcus aureus* and *E. coli* after treatment with silver ions, and showed that the affected DNA molecules condensed and lost their replicating ability. In addition, proteins that surrounded the nuclear region were expressed, perhaps to protect the DNA from the silver ions. In general, effects of the silver ions were more profound on Gram-negative *E. coli* cells, indicating a better protection of Gram-positive *S. aureus* against silver penetration (Feng et al. 2000). This conclusion is in agreement with the results of a study from Jung et al. (2008). Gram-negative cells only possess a thin peptidoglycan layer between their inner and outer membranes, whereas Gram-positive cells possess a thick peptidoglycan layer and lack an outer membrane (Li et al. 2010b; Thiel et al. 2007). In Gram-negative bacteria the outer membrane serves to protect from agents that would normally damage the peptidoglycan layer (Li et al. 2010b). However, in the case of silver ions, the size of the peptidoglycan layer seems to be of particular importance in protecting against the penetration of silver (Feng et al. 2000).

Considering the well-documented bactericidal effects of silver ions, the numerous studies that have dealt with silver nanoparticles have been focused on bacteria. Such studies have either had the goal of evaluating nanoparticle effectiveness for antibacterial applications (Jain and Pradeep 2005; Lee et al. 2007), or of elucidating the environmental impact of the particles, e.g., effects on nitrifying organisms in wastewater treatment plants (Choi et al. 2008). Additional information on the antibacterial effects of silver nanoparticles is presented below. Moreover, a comprehensive look at this topic is provided by Marambio-Jones and Hoek (2010), who reviewed the antibacterial effects of silver nanomaterials in detail.

The interaction of silver nanoparticles with bacterial cell walls has been documented in several studies. Silver particles are known to have attached to the cell wall of Gram-negative bacteria (*E. coli*) (Choi et al. 2008; Gogoi et al. 2006), which resulted in the formation of pits and cell death (Choi et al. 2008; Gogoi et al. 2006; Sondi and Salopek-Sondi 2004). Such adhesions and interactions with bacterial cell surfaces are possibly due to electrostatic forces that exist between the negatively charged cell surface of the bacteria and the silver nanoparticles (Thiel et al. 2007). Furthermore, silver nanoparticles were found to bind to and accumulate in bacterial cell membranes (Morones et al. 2005; Sondi and Salopek-Sondi 2004). When this occurred, effects were produced on membrane structure, permeability, and leakage

of cellular components like reducing sugars and proteins (Li et al. 2010b; Morones et al. 2005; Sondi and Salopek-Sondi 2004). Nanosilver may inactivate respiratory chain dehydrogenase (Li et al. 2010b), which results in inhibition of respiration and growth. In addition, nanosilver could affect phosphate lipids of the membrane and deactivate membrane enzymes, finally leading to cell death (Li et al. 2010b).

According to the mechanism hypothesized for silver ions, the interaction of nanosilver with components of bacterial membranes is suggested to be mediated by binding to thiol groups. Bacterial membranes are rich in sulfur-containing proteins, and may be preferential sites for silver nanoparticles (Morones et al. 2005). In general, thiol groups show metal complexing properties (Deratani and Sebille 1981; Jiménez et al. 1997), and may therefore contribute to the antibacterial effects observed to occur for other nanometals. Both zinc and copper oxide nanoparticles show high toxicities to bacteria, followed by aluminum and nickel oxide (Baek and An 2011; Heinlaan et al. 2008; Jiang et al. 2009). Zinc oxide particles were found to attach to bacterial surfaces, although at a lower intensity than aluminum oxide nanoparticles (Baek and An 2011). The attachment of nanoparticles to bacterial surfaces may have toxic manifestations, but is only one aspect of their toxic potential. As evidence for this, the more soluble zinc oxide particles showed lower attachment, but higher toxicity than did aluminum oxide particles (Baek and An 2011).

Studying the effects of nanosilver (diameter of 9.3 ± 2.8 nm) on bacterial membranes (*E. coli*), Lok et al. (2006) found that nanosilver induced the collapse of proton motive force, which decreased cellular potassium and ATP levels. Intact *E. coli* cells normally maintain their membrane potential by a high content of intracellular potassium. Membrane destabilization and the absence of ATP observed after nanosilver treatment resulted in the accumulation of envelope protein precursors in the cytoplasm. Normally, after conversion to mature forms, newly synthesized envelope proteins are incorporated into the outer membrane of bacteria. The conversion to mature forms and the translocation across the cytoplasm membrane requires a membrane potential and energy in form of ATP (Zimmermann and Wickner 1983). Nanosilver treatment disrupted this process, and produced an accumulation of protein precursors (Lok et al. 2006). The detrimental effects of nanosilver on cell membranes may also occur in nitrifying bacteria (Choi and Hu 2008). Interference with bacterial membranes may also apply for nanoparticles of different chemical composition; altered membrane permeability of bacterial membranes was reported for zinc oxide (Huang et al. 2008; Liu et al. 2009).

In addition to interacting with bacterial cell walls and membranes, silver nanoparticles are able to enter bacterial cells (Lok et al. 2006; Morones et al. 2005; Shrivastava et al. 2007; Sondi and Salopek-Sondi 2004). In mammalian cell lines, the uptake rate of silver nanoparticles is size dependent. Morones et al. (2005) revealed that silver particles having an average size of 1–10 nm bound to the membranes of *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Salmonella typhus*, and these particles were incorporated into the cells. As nanoparticle size decreased, the toxicity increased; this relationship was linked to the greater reactive surface area of the smaller particles (Lok et al. 2007; Morones et al. 2005). Pal et al. (2007) estimated a 109-fold increased surface area as the size of a spherical particle was reduced from

10 μm to 10 nm, which was concomitantly followed by enhanced antibacterial activity. Size-dependent results were also obtained in a study in which *E. coli* and *S. aureus* were treated with silver nanoparticles of three diameters, viz., 7, 29, and 89 nm (Martínez-Castañón et al. 2008). Silver nanoparticles having a size of 7 nm showed the lowest minimum inhibition concentrations (MIC) (viz., 6.25 and 7.5 μg/mL) for *E. coli* and *S. aureus*, respectively. MIC increased with particle size.

As documented for ionic silver, Gram-negative bacteria show higher susceptibilities to nanosilver than do Gram-positive bacteria. When treated with 89-nm-sized particles, *S. aureus* (Gram-positive) showed a MIC of 33.7 μg/mL; values that were approximately threefold those of Gram-negative *E. coli* cells (11.8 μg/mL) (Martínez-Castañón et al. 2008). Shrivastava et al. (2007) demonstrated strong inhibition of Gram-negative *E. coli* cells at 25 μg/mL of nanosilver, whereas Gram-positive *S. aureus* only showed partial inhibition at a concentration of 100 μg/mL.

The mechanisms by which silver nanoparticles unfold their toxic potential inside bacterial cells may also involve interference with sulfur-containing proteins, as has been observed to occur for ionic silver (Morones et al. 2005). This suggests DNA damage, and possible consequences could be disturbance of cell division and cell death (Morones et al. 2005). In addition, research suggests that ROS may be involved in the toxicity of silver nanoparticles to nitrifying bacteria cultures (Choi and Hu 2008), *E. coli* (Hwang et al. 2008), and *P. aeruginosa* (Kora and Arunachalam 2011). Similar observations were made for *E. coli* cells exposed to copper and zinc oxide nanoparticles (Ivask et al. 2010).

In general, size, specific surface area, and shape are important factors that influence the silver nanoparticle toxicity to bacteria (Fabrega et al. 2009; Pal et al. 2007). Differently shaped silver nanoparticles (e.g., spherical, rod shaped, and truncated triangular) inhibited *E. coli* differentially (Pal et al. 2007). Truncated triangular particles showed the strongest antibacterial activity, with complete inhibition of *E. coli* at a concentration of 10 μg/mL. In contrast, spherical particles only reduced growth at a level of 125 μg/mL. Rod-shaped particles did not fully inhibit *E. coli* cells, even at concentrations of 1,000 μg/mL. These differences in the activity of differently shaped particles may be explained by the different atomic structure that is characteristic of different shaped particles.

In summary, the toxic effects produced by nanosilver are primarily from the interference nanosilver has with bacterial cell membranes, and susceptibilities of the bacteria differ as different membrane structures (Gram-negative vs. Gram-positive) are encountered.

3 The In Vivo Toxicity of Silver

Ionic silver is one of the most toxic metals to aquatic organisms (Eisler 1996). Studies with silver nitrate show acute effective concentrations in the low microgram-per-liter range (Bury et al. 1999; Davies et al. 1978; Morgan et al. 1997; Nebeker et al. 1983; Zhao and Wang 2011). The most acutely sensitive freshwater

organisms are cladocerans and amphipods (Bianchini et al. 2002; Ratte 1999). The severity of the toxic effects depends on the amount of free silver ions present, which is influenced by the physicochemical parameters of the surrounding medium (Brauner and Wood 2002; Bury et al. 1999; Eisler 1996). When testing the toxicity of silver nitrate, adding food reduces the toxic effects in the test systems (Bianchini and Wood 2002; Hook and Fisher 2001). The reason for the reduction is thought to derive from the presence of organic material that alters silver bioavailability, and enhances the complexation of silver ions by algae.

Mechanisms by which toxic effects are mediated were evaluated in freshwater fish, and one important mechanism by which silver causes toxicity appears to be inhibition of branchial enzymes that are involved in ion transport (Brauner and Wood 2002; Morgan et al. 1997). Normally, sodium and chloride ions are mainly transported across the gills by means of branchial sodium-potassium pump (Na^+/K^+-ATPase), which is directly related to the uptake of these ions (Bianchini and Wood 2003). Silver ions at concentrations of 2 and 10 μg/L, respectively, inhibited gill enzymes like Na^+/K^+-ATPase and carbonic anhydrase in rainbow trout (*Oncorhynchus mykiss*), leading to inhibition of active Na^+ and Cl^- uptake (Morgan et al. 1997). The silver-mediated inhibition of Na^+/K^+-ATPase was also demonstrated by Hussain et al. (1994), who studied the isolated enzyme in vitro. When gill system enzymes were disrupted, ion uptake from water was inhibited in vivo, and resulted in a net loss of ions and death of the organisms (Bianchini and Wood 2003).

An ionoregulatory disturbance induced by silver occurred not only in fish, but in daphnids as well; the daphnids showed a decrease in whole-body sodium concentration after treatment with 5 μg silver nitrate/L (Bianchini and Wood 2002). As the authors suggested, silver and sodium ions may share the same mechanism of transport across the gills, resulting in a quick accumulation of silver in *Daphnia magna* during the experiment. The activity of whole body Na^+/K^+-ATPase increased by 60% from the loss of the sodium ions, probably as a compensatory response to the reduced sodium level. The ionoregulatory disturbance in daphnids is probably mediated by silver mimicking sodium in Na^+ channels that inhibits the function of these channels.

As observed for silver ions, nanoparticulate forms of silver also show high toxic effects on freshwater species. The high toxicity to such aquatic species is in contrast to what occurs in mammals, where the in vivo toxicity of nanosilver is relatively low. For example, a 28-day inhalation study performed with Sprague-Dawley rats that were exposed to concentrations of nanosilver (average size 60 nm) up to 1,000 mg/kg did not show any changes in body weight or hematology and blood biochemical values (Kim et al. 2008a). The only observation was a gender-related difference in accumulation of silver in the kidneys. Female rats showed a twofold higher accumulation compared to male rats, but it was not elucidated as to whether this accumulation was animal-size dependent or if hormones were involved.

The concentrations that are effective in producing silver nanoparticle effects on aquatic organisms vary and depend on the physicochemical properties of the particular nanoparticles used in the tests (Allen et al. 2010; Gaiser et al. 2009). How silver nanoparticles are prepared and how the animals are dosed also affects the

intensity of the observed effects (Roh et al. 2009). Effect values range from the low microgram-per-liter range (toxicity comparable to silver nitrate; Allen et al. 2010; Li et al. 2010a) to the high microgram-per-liter range (Gaiser et al. 2011; Zhao and Wang 2011). The effective concentrations obtained for different types of silver nanoparticle dispersions on aquatic organisms are summarized in Table 2.

Allen et al. (2010) studied the effects of different nanosilver dispersions either prepared from uncoated particles or those with different surface coatings. The authors found that the aggregation state of the particles that was affected by particle size produced different LC_{50} values for *D. magna*. Smaller particles had a higher reactive surface and were probably more bioavailable. Laban et al. (2010) also showed differential LC_{50} values for fathead minnow (*Pimephales promelas*) embryos that were affected by how test solutions were prepared. Sonicated nanosilver dispersions led to tenfold higher toxicity (LC_{50} 1.25–1.36 mg/L) than did stirred solutions (LC_{50} 9.40–10.6 mg/L). Stirred solutions showed higher aggregation rates of the nanoparticles. Since aggregation of nanoparticles is positively correlated with their tendency to settle out of the water in which they are dispersed (Chen and Elimelech 2006; Keller et al. 2010), the organisms may have been exposed to lower nanosilver concentrations. Sonicated samples produced to a more stable colloidal suspension and, therefore, a higher particle concentration was delivered to the organisms (Laban et al. 2010). Gaiser et al. (2011) showed size-dependent effects for both nanosilver particles that had a primary size of 35 nm (agglomerated in the test medium to 588 nm) and for microsilver-sized particles of 811 nm. The smaller agglomerates showed approximately a tenfold higher toxicity and increased mortality levels at concentrations of 0.1 mg/L.

Li et al. (2010a) did not find a size-dependent effect on *D. magna* exposed to particles having primary sizes of 36, 52, and 66 nm, but they obtained a relatively low median LC_{50} value (~3 µg/L). The absence of size-dependent effects is explained by aggregation of the particles to diameters of 438, 378, and 553 nm, after 24 h. The low LC_{50} value may have resulted from an extremely fast silver nanoparticle uptake by *D. magna*, as has been shown to occur with silver ions (in <1 h) in previous studies (Glover and Wood 2005). The filter-feeding strategy of daphnids renders particle uptake from the water phase very effective, and, therefore, may explain why daphnids are the most susceptible of organisms to nanosilver exposure (Griffitt et al. 2008).

In most studies, LC_{50} values for daphnids were in the low microgram-per-liter-range (Table 2) (Allen et al. 2010; Griffitt et al. 2008; Li et al. 2010a). Bivalve molluscs are also a filter-feeding taxonomic group, and similarly show a high susceptibility to nanosilver exposure as a result of their feeding strategy. Ringwood et al. (2010) demonstrated adverse effects from nanosilver exposure (diameter of 25 nm, stabilized with sodium citrate). The effects noted were on embryonic development and lysosomal integrity of adult hepatopancreas tissues in oysters (*Crassostrea virginica*) at concentrations of 1.6 µg/L and 0.16 µg/L, respectively. In contrast, fish (viz., zebrafish (*D. rerio*) and fathead minnow (*P. promelas*)) showed tenfold to 100-fold higher lethal concentrations (Griffitt et al. 2008; Laban et al. 2010).

Sublethal effects, derived from silver nanoparticles, and their possible mechanisms were studied in zebrafish. *D. rerio* embryos showed lower hatch rates and

Table 2 Effective concentrations of nanosilver observed in vivo

Test organism	Silver nanoparticles (size)	Effective concentration	References
Caenorhabditis elegans	AgNPs (uncoated, DLS: 14~20 nm)	Significant decrease in reproduction at 0.05; 0.1; 0.5 mg/L	Roh et al. (2009)
Crassostrea virginica (embryos and adults)	AgNPs (stabilized with sodium citrate, DLS: 25 nm)	Significant effects on embryonic development at 1.6 µg/L Lysosomal destabilization (adult oysters, hepatopancreas cells) at 0.16 µg/L	Ringwood et al. (2010)
Daphnia magna neonates	Citrate-coated AgNPs (DLS: 5.94–39.75 nm) Coffee-coated AgNPs 1:100 dilution (DLS: 101.5–773.6 nm) Uncoated AgNPs (Sigma-Aldrich, DLS: 681.4–5,412) Coated AgNPs (Sigma-Aldrich, coating unknown, DLS: 39.39–249.8 nm)	LC_{50} (48 h) 1.1 µg/L LC_{50} (48 h) 1.0 µg/L LC_{50} (48 h) 1.4 µg/L (filtered 100 nm); 16.7 µg/L (unfiltered) LC_{50} (48 h) 4.4 µg/L (filtered 100 nm); 31.5 µg/L (unfiltered)	Allen et al. (2010)
Daphnia magna neonates *Cyprius carpio*	AgNPs (uncoated, nominal size 35 nm), micro-Ag (nominal size 0.6–1.6 µm)	*Daphnia magna* (96 h): 60% mortality at 0.1 mg/L (AgNPs); 80% mortality 1 mg/L (micro Ag) *Cyprius carpio* showed Ag (both forms) in liver, intestine, gills, and gall bladder	Gaiser et al. (2009)
Daphnia magna neonates	AgNPs (uncoated, nominal size 35 nm, DLS: 588 nm in reconstituted hard water), micro-Ag (nominal size 0.6–1.6 µm, DLS: 811 nm in reconstituted hard water)	96 h acute tests AgNPs: 10 and 1 mg/L 100% mortality, 0.1 mg/L 56.7% mortality 96 h acute tests micro Ag: 10 mg/L 100% mortality, 1 mg/L 80% mortality, 0.1 mg/L no significant toxicity	Gaiser et al. (2011)
Daphnia magna (7-day adults)	Carbonate-coated AgNPs (TEM: 20 nm)	Uptake rate constant (k_u): 0.060 L/g/h at 2, 10, and 40 µg/L; 2.2 L/g/h at 160 and 500 µg/L >70% of AgNP in daphnids was accumulated through the dietary route	Zhao and Wang (2010)

(continued)

Table 2 (continued)

Test organism	Silver nanoparticles (size)	Effective concentration	References
Daphnia magna (neonates)	Carbonate-coated AgNPs (TEM: 20 nm, DLS: 40–50 nm)	Significant decrease in body length and reproduction at 50 µg/L	Zhao and Wang (2011)
Daphnia pulex (adults) *Ceriodaphnia dubia* (neonates) *Danio rerio* (adult and juvenile <24 h)	AgNPs (coated with metal oxide, primary size 20–30 nm, major particle diameters observed in suspension (SEM) 44.5, 216, 94.5 nm)	*Daphnia pulex*: LC_{50} (48 h) 0.040 mg/L *Ceriodaphnia dubia*: LC_{50} (48 h) 0.067 mg/L *Danio rerio* (48 h): LC_{50} (adult) 7.07 mg/L, LC_{50} (juvenile) 7.20 mg/L	Griffitt et al. (2008)
Danio rerio (embryos)	Starch-coated AgNPs, BSA-coated AgNPs (TEM: both 5–20 nm)	LC_{50} (dependent on growth stage): 25–50 mg/L	AshaRani et al. (2008)
Danio rerio (embryos)	AgNPs, supporter material TiO_2 (TEM: 10–20 nm)	Reduced hatch rates and larval abnormalities at 10 ng/L	Yeo and Kang (2008)
Pimephales promelas (embryos)	AgNPs (uncoated, TEM: 29–100 nm, majority 31–50 nm) AgNPs (uncoated, TEM: 21–280 nm, majority 21–60 nm)	LC_{50} (96 h): 9.4 (stirred), 1.25 (sonicated) mg/L LC_{50} (96 h): 10.6 (stirred), 1.36 (sonicated) mg/L	Laban et al. (2010)

several larval abnormalities after exposure to nanosilver (10–20 nm) (Yeo and Kang 2008). Roh et al. (2009) used the soil nematode *C. elegans* as a model organism to study nanosilver toxicity. After exposure to nanosilver (0.1 and 0.5 mg/L, diameter 14–20 nm), *C. elegans* showed decreased reproductive ability, accompanied by increased expression of the superoxide dismutases-3 (sod-3) gene. Laban et al. (2010) demonstrated a concentration-dependent increase of larval abnormalities in nanosilver-exposed embryos of *P. promelas*. In addition, uptake of particles ranging in size from 29 to 100 nm to 21 to 280 nm was observed. Possible uptake mechanisms suggested by the authors were diffusion across membrane pores, or active uptake by endocytosis. Entry of silver nanoparticles by diffusion into cells, or by endocytosis, was proposed by AshaRani et al. (2008), after they evaluated nanosilver-treated *D. rerio* embryos. The nanosilver-treated embryos displayed phenotypic defects like abnormal body axes, degeneration of body parts, pericardial edema, and cardiac arrhythmias. In addition, an increase in apoptosis and necrosis in the body parts that accumulated blood was detected. The particles having an average size of 5–20 nm were detected in the brain, heart, yolk, and blood of embryos, and a high particle accumulation occurred in the nucleus. The authors opined that DNA damage and chromosomal aberrations, derived from the nanoparticles located in the nucleus, may have accounted for the observed toxic effects.

Uptake of silver nanoparticles by *D. magna* was studied by Zhao and Wang (2010), and these authors used their results to develop hypotheses on possible mechanisms.

Low concentrations of nanosilver (2–40 μg/L) in the water phase led to uptake rates in proportion to the nanosilver concentration, whereas uptake rates at higher concentrations (160 and 500 μg/L) increased disproportionately. Besides endocytosis as possible route of nanosilver uptake the authors proposed direct ingestion into the gut as explanation for the higher uptake rates at higher concentrations. The authors also investigated the dietary ingestion of nanosilver via algal food. Because nanosilver showed a strong accumulation in or on algae in the experiments, the authors concluded that nanosilver incorporated into the daphnids mainly via the diet. Ingested silver nanoparticles could not be completely depurated and led to sublethal effects like decreased reproduction and growth in *D. magna* treated with 5 and 50 μg/L (Zhao and Wang 2011). In addition, low depuration of nanosilver from the daphnids may be important in the potential transport of nanosilver along the aquatic food chain (Zhao and Wang 2010, 2011).

In summary, there are only a few studies in which the toxic mode of action of nanosilver was evaluated as an in vivo exposure. Of those studies that were completed results indicated that ROS are involved in producing the observed toxicity in the test systems.

4 Are Effects Caused by Nanoparticles or Released Silver Ions?

Release of silver ions from nanoparticle surfaces is considered to be important to nanosilver toxicity. In several studies that have dealt with silver nanoparticles, the degree to which silver ions were released was regarded to play a role in toxicity (AshaRani et al. 2008; Bouwmeester et al. 2011; Carlson et al. 2008; Eom and Choi 2010; Foldbjerg et al. 2011; Griffitt et al. 2008; Laban et al. 2010; Morones et al. 2005; Roh et al. 2009). Treating mammalian cell lines in vitro with silver nitrate often revealed effects that were comparable to those obtained for nanosilver (Bouwmeester et al. 2011; Carlson et al. 2008; Eom and Choi 2010). Notwithstanding the similarity of action, there also appears to be a unique nanoparticle effect. Eom and Choi (2010) and Foldbjerg et al. (2011) demonstrated that silver nanoparticles induced higher titers of ROS than did silver ions. In addition, increased expressions of transcriptional factors like Nrf-2 and NF-κB, and accumulating DNA damage that produced apoptosis, were observed for nanosilver, but were not evident effects of silver ions (Eom and Choi 2010).

Results with bacteria also suggest that toxic effects may not exclusively result from the release of silver ions from nanoparticle surfaces (Choi and Hu 2008; Fabrega et al. 2009; Lok et al. 2007; Morones et al. 2005). Choi and Hu (2008) revealed higher toxic effects from silver nanoparticles than silver ions on nitrifying bacteria. The authors hypothesized that the small size (<5 nm) of the uncharged nanoparticles potentially enhanced effective uptake, whereas charged ions are not easily transported across the cell membrane. In contrast, Hwang et al. (2008) found an equal induction of ROS, when they compared the effects of silver nanoparticles

with those of silver ions released from silver nitrate. This result was confirmed by Pal et al. (2007), who proposed that silver ions constitute the cause of the detrimental effects.

The results of in vivo studies indicate that silver nanoparticles and silver ions have different mechanisms of toxicity. AshaRani et al. (2008) did not find that silver ions were involved in the toxicity of nanosilver to *D. rerio*, because none of the larval abnormalities found for nanosilver could be shown to occur for silver nitrate. Reproductive effects on *C. elegans* were slightly more prominent, when the organism was treated with silver nanoparticles than with silver ions provided as silver nitrate (Roh et al. 2009). Stress-related gene expression also differed, suggesting different toxicity mechanisms. Different uptake mechanisms for silver nanoparticles and silver ions were proposed by Zhao and Wang (2010). They showed that nanoparticle uptake was about 4.3-fold lower than silver ion uptake. Griffitt et al. (2008) suggested that the toxicity of silver nanoparticles did not result from silver ion release in several aquatic species. The ion concentrations released from silver nanoparticles during the test were lower than those of silver nitrate that produced lethal effects. Laban et al. (2010) hypothesized that ions from silver nitrate caused greater toxicity than did ions released from silver nanoparticles. The toxicity of silver ions from silver nitrate was three times higher to *P. promelas* as compared to the silver ions from nanoparticles. This effect is explained by the more rapid dissociation of silver nitrate than silver ions from silver nanoparticles.

From the foregoing, we conclude that the observed toxic effects of silver nanoparticles are caused by both silver ions and the particulate form. The release of free silver ions may contribute to some, but not to all, toxic effects observed for nanosilver (Gaiser et al. 2011; Laban et al. 2010). This conclusion may also apply to other nanometals, as similar observations were made for zinc oxide nanoparticles, nanonickel, and nanocopper (Franklin et al. 2007; Griffitt et al. 2008).

5 Conclusions and Future Research

Based on the results of a comprehensive literature study on toxic effects of nanosilver and silver ions, we offer the following conclusions as to what the several mechanisms of action are for silver nanoparticles (Fig. 1); some may also apply for other nanometals:

1. *ROS generation*. Increased ROS levels caused by nanosilver may account for observed cases of cellular damage and apoptosis. ROS generation and oxidative stress may either result from the catalytic properties of silver nanoparticles, an effect of mitochondrial dysfunction caused by nanosilver, or constitute a combination of both mechanisms.
2. *Interaction with cellular enzymes*. The evidence suggests that silver nanoparticles may interact with cellular enzymes. Since silver ions show strong affinities to free thiol groups, nanosilver may show similar effects. Binding to thiol groups can lead to damage and inactivation of proteins and enzymes and, therefore, to

cellular damage. In addition, silver ions bind to DNA molecules, specifically to purine and pyrimidine bases.

3. *Mimicry of endogenous ions*. Researchers have also demonstrated that silver is probably capable of structurally mimicking endogenous ions, like calcium, sodium, or potassium ions. Such action can block transport systems and induce ionoregulatory disturbances.
4. *Release of silver ions*. When silver ions are released from nanosilver forms, organisms may be affected by the released ions. Silver ions may affect organisms by similar modes of action as reported for nanosilver. However, some evidence indicates that the effects produced may not totally derive from the released silver ions.

Finally, the results reported in this review are primarily from in vitro test systems. Such systems have limitations and do not allow one to confidently predict probable effects on whole organisms in vivo. Based on the reviewed studies, we believe there is compelling evidence that nanosilver is active via an ROS-mediated toxic mechanism, in both in vivo and in vitro systems. Of course, other possible mechanisms may be operating and may be involved either separately or in tandem.

To evaluate whether an increased production and application of silver nanoparticles pose an environmental risk, additional in vitro data are needed. Most importantly, observations are needed in in vivo test systems to better predict and prevent future adverse effects on communities and ecosystems as nanosilver use increases. Special emphasis should be given to chronic in vivo studies, ideally covering the entire life cycle of test organisms. Furthermore, systematic studies investigating dietary uptake of silver nanoparticles and the potential biomagnification risk in the food web should be undertaken.

6 Summary

Novel physicochemical and biological properties have led to a versatile spectrum of applications for nanosized silver particles. Silver nanoparticles are applied primarily for their antimicrobial effects, and a variety of commercially available products have emerged. To better predict and prevent possible environmental impacts from silver nanoparticles that are derived from increasing production volumes and environmental release, more data on the biological effects are needed on appropriate model organisms.

We examined the literature that addressed the adverse effects of silver nanoparticles on different levels of biological integration, including in vitro and in vivo test systems. Results of in vitro studies indicate a dose-dependent programmed cell death induced by oxidative stress as main possible pathway of toxicity. Furthermore, silver nanoparticles may affect cellular enzymes by interference with free thiol groups and mimicry of endogenous ions.

Similar mechanisms may apply for antibacterial effects produced by nanosilver. These effects are primarily from the interference nanosilver has with bacterial cell membranes.

Few in vivo studies have been performed to evaluate the toxic mode of action of nanosilver or to provide evidence for oxidative stress as an important mechanism of nanosilver toxicity. Organisms that are most acutely sensitive to nanosilver toxicity are the freshwater filter-feeding organisms.

Both in vitro and in vivo studies have demonstrated that silver ions released from nanoparticle surfaces contribute to the toxicity of nanosilver. Contradictory results exist on the extent to which silver ions contribute to toxicity, and, indeed, some findings indicate a unique nanoparticle effect.

For an adequate evaluation of the environmental impact of nanosilver, greater emphasis should be placed on combining mechanistic investigations that are performed in vitro, with results obtained in in vivo test systems. Future in vivo test system studies should emphasize long-term exposure scenarios. Moreover, the dietary uptake of silver nanoparticles and the potential to bioaccumulate through the food web should be examined in detail.

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