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Antibacterial Mechanisms of Metallic Nanoparticles: A Review

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ABSTRACT

Given the slow approval rate for new antibiotics and the inability of current antibiotics to fully control bacterial infection, it is obvious that there is a great demand for unconventional biocides. Metallic nanoparticles, another possible route for fighting bacteria, should be considered. Metallic bactericidals have been in use for several years as external sanitizers and disinfectants and have shown biocidal effectiveness against both Gram-positive and Gram-negative bacteria, as well as against fungi. The mechanism of interaction of these metallic biocides includes protein membrane damage, production of superoxide radicals, and ions release that interact with the cellular granules and form condensed molecules. This article presents a review of the metallic nanoparticles antimicrobial mode of interaction against bacteria.

Keywords: antibacterial action, scanning electron microscope, silver ions, target site
Abbreviations: CFU, colony-forming unit; **LB medium**, Luria-Bertani medium; **MRSA**, methicillin-resistant *Staphylococcus aureus*; **NP**, nanoparticle; **SDS**, sodium dodecyl sulfate; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **SEM**, scanning electron microscope; **TEM**, transmission electron microscope

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INTRODUCTION

The last few years have seen an enormous increase of a host of antibiotic-resistant bacteria. In 2002, the US Center for Disease Control and Prevention (CDC) estimated that at least 90,000 deaths a year in the US could be attributed to bacterial infection, more than half caused by bacteria resistant to at least one commonly used antibiotic. In October, 2008 the CDC reported that the number of serious methicillin-resistant *Staphylococcus aureus* (MRSA) infections was close to 100,000 a year with almost 19,000 related fatalities (Taubes 2008). The inability of current antibiotics to fully control bacterial infection coupled with the slow approval rate for new antibiotics necessitates the research for unconventional biocidals.

The use of nanoparticles (NPs) as antibacterial agents have been the subject of many studies (Sondi and Salopek-Sondi 2004; Lok *et al*. 2006; Song *et al*. 2006; Jung *et al*. 2008) and since Ag possesses natural antibacterial properties that are strengthened at the nanoscale, the most NPs produced are made from Ag or combination of Ag and other compounds (Furno *et al*. 2004; Sondi and Salopek-Sondi 2004; Morones *et al*. 2005; Lok *et al*. 2006; Song *et al.* 2006; Shrivastava *et al*. 2007; Fernández *et al*. 2008; Krutyakov *et al*. 2008; Raffi *et al.* 2008).

Antibacterial NPs are being incorporated into different commercial products such as paints (Kumar *et al*. 2008), antibacterial medical devices (Furno *et al*. 2004), dental fillings (Beyth *et al*. 2006) and household items like refrigerators (SAMSUNG, SRS583HDP 585L pewter side by side refrigerator) (Bi *et al*. 2008).

The incorporation of NPs into different consumer products resulted mainly in health protection; however, the mechanisms of the antibacterial NPs are not well understood and still an expanding field of research. Antibacterial action of NPs are studied by using different assays to detect the damage occur to bacterial cells. The antibacterial impact of NPs is induced commonly on two macromolecules, which are the genetic material DNA and the proteins found either throughout the cell membrane or inside the cells.

MECHANISMS OF ANTIBACTERIAL SILVER AGENTS

The most used antibacterial NPs are the Ag NPs which are produced extensively and used as antibacterial agents in various fields; to reduce infections in burn treatment (Klaus *et al*. 1999) and to eliminate microorganisms on textile fabrics (Perelshtein *et al.* 2008a). Moreover, Ag NPs showed effective antiviral results against HIV infected cells (Elechi-

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guerra *et al.* 2005).

The antibacterial activities of Ag NPs are often related to the Ag content where the antibacterial property of Ag is well known. After the isolation of bacteria resistance to the commercial antibiotics (Finland 1979), the Ag gained an extensive interest as being the alternative for other antibiotics. Since more studies had proven the antibacterial activity of Ag against a wide spectrum of microorganisms, it is being used extensively in the medical applications. It was thought that one of the important advantages of using Ag as antibacterial agent is that bacteria cannot generate resistance to Ag, however this was nullified after the isolation of bacteria resistant to Ag such as; *Salmonella* and *Escherichia coli* (McHugh *et al*. 1975; Hendry and Stewart 1979). Gupta *et al*. (1999) was the first study to detect the development of Ag resistant *Salmonella* based on genetic and molecular basis.

Since Ag NPs were considered in some aspects to have the same mode of antibacterial action as Ag ions, they target the same molecules and structures in bacterial cells. The target sites for Ag ions that were identified in literature are represented in the following section.

Silver ion interaction with proteins

The mechanism of the antimicrobial action of Ag ions is closely related to their interaction with thiol groups in the side groups of cysteine residues in the protein chain as shown in **Fig. 1** (Bragg and Rainnie 1974; Spadaro *et al*. 1974; Liau *et al*. 1997; Gupta *et al*. 1998). Since there is no much difference in the electronegativity between Ag ions and sulfur a covalent bond is formed.

The disulfide bond is formed, as a result of oxidation reaction, between two thiol groups of cysteine residues at protein chain and contributes to the tertiary structure of a protein, the formed bond between Ag ions and sulfur may result in altering the protein structure and the folding of the protein, this affects the shape of the active site of the enzyme resulting in the inhibition of enzyme. Liau *et al.* (1997) proved the interaction of Ag ions with thiol groups found in enzymes and proteins; this was suggested to play an essential role in bacterial inactivation; however they indicated the involvement of other compounds in the interaction with Ag ions. Since Ag ions displace other heavy essential metals ions like Ca^{2+} or Zn^{+} (Schierholz *et al*. 1998), it can be assumed that other cations do not have the same mechanisms of interaction with Ag target sites. Another target site for the Ag ions is an enzyme of the respiratory chain of bacteria, Na^{+}-NQR (Na^{+}-translocating NADH ubiquinone oxidoreductase (NQR), which considered as the entry point for electrons into the respiratory chain of a number of marine and pathogenic bacteria. The interaction of Ag ions with this enzyme results in the inhibition of the NADH dehydrogenase (Schreurs and Rosenberg 1982; Holt and Bard 2005).

Other studies demonstrated that the antibacterial activity of Ag ions is not the result of interaction with specific target site, Dibrov *et al*. (2002) found that the antibacterial Ag ions attack several sites of *Vibrio cholera* rather than targeting specific target site. In addition to the interaction of Ag ions with the NQR found in the membrane, Ag ions may interact with any of the membrane proteins and lead to the leakage of the protons from the cell membrane. In Yakabe *et al*. (1980) study it was shown that Ag ion interacts with nucleic acids, where it interacts preferentially with the bases in DNA rather than with the phosphate group.

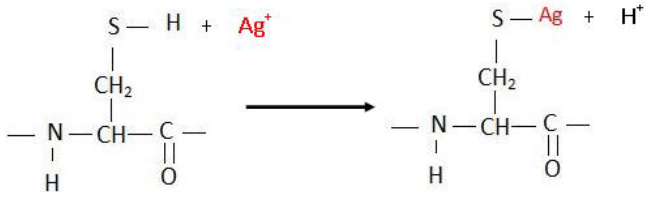

Fig. 1 The reaction of the cysteine residue in protein chain with silver ion.

Yamanaka *et al*. (2005) applied an antibacterial Ag solution with concentration of 900 ppb (part per billion) against *E. coli* and studied the treated bacteria using energy-filtering transmission electron microscopy (EFTEM). The results revealed that Ag ions penetrated inside *E. coli* cells and various phases of the cell death process were detected. Plasmolysis and partial disappearance of the cytoplasmic membrane were observed and indicated an intermediate stage of cell disruption. It was concluded that the Ag ions penetrated through ion channels without causing damage to the cell membranes. The penetrated Ag ions denature the ribosome and suppress the expression of enzymes and proteins essential to ATP production, these processes result in the cell disruption. This is in contrast to other studies that demonstrated that the Ag ions primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain, through binding to thiol groups (Bragg and Rainnie 1974; McDonnell and Russell 1999).

Feng *et al*. (2000) used TEM to visualize the morphological changes occurred in *E. coli* and *S. aureus* cells after Ag ions treatment. Ag treated cells showed shrunk and detached cytoplasm membrane from the cell wall; the appearance of an electron-light region in the center of the cells, with condensed DNA molecules located in the center of it; and electron-dense granules were observed around the cell wall and sometimes inside the cells. These electron dense granules were observed in the study of Jung *et al*. (2008) that revealed the presence of these granules after Ag ions treatment. However, in Feng *et al*. (2000) study the treated cells with the electron light region had no electron-dense granules inside the cells. The presence of Ag and sulfur in the observed dense granules and inside the cytoplasm of *E. coli* can be explained in relation to what was reported in previous studies that Ag reacts with phosphorus and sulfur compounds (Ahrland *et al.* 1958; Vitanov and Popov 1983; Hatchett and White 1996; Morones *et al*. 2005). Based on this observation, Feng *et al.* (2000) suggested that Ag ions entered the cells and combined with cell components containing sulfur, and the electron light region prevented the electron-dense granules from entering into it. In the same study *S. aureus* had a large amount of phosphorus detected in the condensed region in the middle of the cells, which was assumed to be the condensed form of DNA molecules, since phosphorus is a primary component of DNA molecules. It was assumed that the electron light region provided protection to DNA molecules. Although the formation and the components of this electron light region were unknown except for DNA molecules. Moreover, as Ag is considered as a heavy metal, it can cause the deposition of proteins *in vitro*. Therefore, the entrance of Ag into bacterial cells may lead to the deposition of proteins in cells. Thus, the small electron-dense granules outside the electron-light region can be a combination of Ag and the deposition of proteins.

Some of the morphological appearance revealed after Ag treatment can be explained by the work of Nover *et al*. (1983), where it was found that as a response to an outer environmental stimuli, some proteins are produced and activated inside the cells. Based on this result Feng *et al.* (2000) made the conclusion concerning the formation of light region with condensed form that appeared under TEM inside Ag treated *E. coli*. Although the stimuli were different, the response was suggested to be the same. It can be concluded that after the subjection of bacterial cells to Ag treatment, some proteins may surround the DNA in order to provide protection. This was appeared as an electron- light region under transmission electron microscope (TEM) and scanning electron microscope (SEM). However, if the risk is so harmful that the response cannot work enough for protection, the electron- light region, or even the cell wall, may be impaired and the electron-dense granules invaded the cell.

Yamanaka *et al.* (2005) found that the expression of 30S ribosomal subunit protein S2 was inhibited due to the effect

of the Ag ions. Explanation was made as following; the decreased expression of the S2 protein, after interaction with Ag ions, led to the denaturation of the ribosome, and as a result the protein- and enzyme-synthesizing function is impaired and the function of enzymes of the respiratory process would be inhibited. The inhibition in the expression of the enzymes and proteins pertaining to the respiration process will result in the reduction of ATPs; this may threat the cell life.

Experimental studies demonstrated that the reaction of bacterial cells and Ag depends on the type of the bacterial cells, some differences were observed between *S. aureus* and *E. coli* after treatment with Ag solution, in comparison to *E. coli*, *S. aureus* had less amount of electron dense granules, remained integral, and the electron-light region was comparatively darker than that of *E. coli*. All these phenomena suggested that *S. aureus* may have a stronger defense system against Ag due to the presence of thicker cell wall. Furthermore, it was reported that the cell surface of the Gram negative has higher negative charge than the Gram positive (Chung *et al*. 2004) and if the NPs have more positive charge than the negative it will interact with Gram negative bacteria and exert their antibacterial activity in higher extent compared to the Gram positive bacteria.

Jung *et al*. (2008) did not notice any difference in the morphologies of *E. coli* and *S. aureus* after the treatment with 0.2 ppm (part per million) of Ag ions for 2 hrs. TEM analysis showed shrunk and separated cytoplasm membrane observed in both cells, also cellular contents were found released from the cell wall and the cell wall was degraded. It was common to find electron-dense particles or precipitates around damaged bacterial cells.

On the other hand, the time required for the inhibition of the growth of Gram positive and Gram negative was found to be different in the study of Jung *et al*. (2008). A concentration of 0.2 ppm of Ag inhibited the growth of *E. coli* after 30 min while *S. aureus* was inhibited after 90 min. This indicates that the cell wall component of Gram-positive bacteria provides more resistance protection against antibacterial agents. However, Jung *et al*. (2008) recommended further analytical electron microscopy to be done to identify the elemental composition of the electron-dense particles or precipitates that appeared around damaged bacterial cells. The results of Feng *et al*. (2000) indicated that these electron dense particles are Ag and sulfur, whereas Yamanaka *et al*. (2005) revealed that the electron dense particles appeared in the cytoplasm of *E. coli* cells after treatment with Ag solutions was found to be formed due to condensation of osmium tetroxide used for cell fixation, and not Ag aggregates as mentioned in Feng *et al*. (2000) study.

THE ANTIBACTERIAL SILVER NANOPARTICLES

The observed NPs located on the cell membrane or inside the bacterial cells lead to many suggestions. Different methods were used in literature to detect the changes occur to the bacterial cells after the treatment with Ag NPs; the most used detection method is the electron microscopy (SEM and TEM). The major analysis pattern is to detect the damage occurred to bacterial cell after the treatment and to suggest the possible ways of the antibacterial mechanism as shown in **Table 1**.

Table 1 Common nanoparticles used in the literature.

NPs[a]	Size (nm)	Organism	Time[b]	Con.[c]	Target site	Reference
Ag	12	10^5 CFU/ml *E. coli*	24 hrs	10, 50, 100 µg/ml	Cell membrane	Sondi and Salopek-Sondi 2004
Ag	10	5 ×10^7 CFU/ml *E. coli*, *P. aeruginosa*, *V. cholera*, *S. typhus*	30 min	25, 50, 75 µg/ml	Cell membrane	Morones *et al*. 2005
Ag	10	*E. coli*, *S. aureus*, *Mycobacterium*, *tuberculosis*	60 min	1-10 ppm	-	Song *et al*. 2006
Ag	10	10^7 CFU/ml *E. coli*	24 hrs	1-100 µg/ml	Sulfur and phosphorus containing molecules	Pal *et al*. 2007
Ag	9.8	*E. coli*, *S. aureus*, *L. monocytogenes*	24 hrs	12.5, 25 µg/ml	Cell membrane and sulfur and phosphorus containing compounds	Fernández *et al*. 2008
Ag	10	Recombinant bioluminescent *E. coli*	-	0.1-1.0 µg/ml	Cell membrane	Hwang *et a*l. 2008
Ag	9.3	*E. coli*	10 min	-	Cell membrane	Lok *et al*. 2007
Ag	40	10^2 CFU/ml *B. subtilis*, *E. coli*	24 hrs	>70 µg/ml	-	Yoon *et al*. 2007
Ag	7, 29, 89	*E. coli*, *S. aureus*	24 hrs	6.25, 7.5 µg/ml	Cell membrane	Castanon *et al*. 2008
Ag	25	10^5-10^6 CFU/ml *S. aureus*, *E. coli*	24 hrs	6.75, 3.38 µg/ml	phosphorous and sulfur containing compounds	Panácek *et al*. 2006
Ag	16	10^4 CFU/ml *E. coli*	24 hrs	0, 20, 40, 60, 80, 100 µg/ml	Cell membrane and phosphorus and sulfur-containing compounds	Raffi *et al*. 2008
Fe_3O_4 Ag	60	10^8-10^9 CFU/ml *E. coli*, *S. epidermidis*, *Bacillus subtilis*	24 hrs	60-70 µg/ml	Cell membrane	Gong *et al*. 2007
Styrene-acrylic acid/Ag NPs	10-25	10^8 CFU/ml *E. coli*, *S. aureus*	24 hrs	0.61-1.22 ppm	Cell membrane	Paula *et al*. 2009
Ag-nHA /nTiO_2	-	10^7-10^8 CFU/ml *E. coli*, *S. aureus*, *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. sanguis*, *A. viscosus*, *A. naeslundii*, *P. anaerobius*	1 hr	291 mg/ml	Cell membrane	Liao *et al*. 2007
MgO	4	10^6 CFU/ml of *E. coli*, *B. megaterium*	20-60 min	-	Cell membrane	Stoimenov *et al*. 2002
ZnO	230-2417	10^6–10^7 CFU/ml of *E. coli*	-	0.1-0.25 g/l	Cell membrane	Zhang *et al*. 2007
Cu	100	10^2 CFU/ml of *B. subtilis*, *E. coli*	24 hrs	60 µg/ml	-	Yoon *et al*. 2007
Chitosan/ copper ions	53.99	10^7 CFU/ml of *E. coli*	24 hrs	9 µg/ml	Cell membrane	Du *et al*. 2008
CNP-CU chitosan copper	257	*E. coli*, *S. choleraesuis*, *S. typhimurium*, *S. aureus*	30, 60, 180 min	64 µg/ml	Cell membranes	Qi *et al*. 2004
TiO_2/Ag^+	70	*E. coli*, *Staphylococci*	-	200-400 ppm	-	Cheng *et al*. 2006
Cu-SiO_2	88.48	*S. aureus*, *E. coli*, *E. cloacae*, *C. albicans*, *P. citrinum*	-	-	Interaction with proteins	Kim *et al*. 2006

[a]: Nanoparticles, [b]: Time measured by hour (h) and minutes (min), [c]: concentration

The target sites of silver nanoparticles

Raffi *et al*. (2008) and Sondi and Salopek-Sondi (2004) studied the changes to bacterial cells after treatment with Ag NPs and considered these changes as the cause of cell death. They observed the presence of coagulated Ag NPs in the membrane of *E. coli* cells and NPs were found penetrated inside bacterial cells. The morphological changes recorded in Sondi and Salopek-Sondi (2004) study involve; pits formation in the cell wall of bacteria and leakage of the cellular content as an indication of cell lysis. These changes were suggested to significantly increase the membrane permeability that causes improper transport through the plasma membrane.

Amro and co-workers (2000) detected pits formation that was related to the metal depletion from the cell membrane, which is the consequence of progressive release of lipopolysaccharide (LP) molecules and membrane proteins. A bacterial membrane with this morphology exhibits a significant increase in its permeability, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, as result the cell dies. A similar mechanism would cause the degradation of the bacterial membrane structure during treatment with Ag NPs Raffi *et al.* (2008). In spite of the detailed research about Ag NPs mechanisms, the induction of Ag NPs to pits formation and the reaction that cause the release of LPs are still unknown.

In a study of Panácek *et al.* (2006) the mechanisms of Ag particles were suggested to involve the attachment of the particles to the surface of cell membrane causing disturbance to the permeability and the respiration. Morones *et al*. (2005) stated that NPs are bactericidal since they located on the membrane and may bind to the carbamate group of the amino acids as shown in **Fig. 2**.

Moreover, the accumulated NPs found inside the cell indicates their interaction with phosphorous and sulfur containing compounds. Panácek *et al*. (2006) study strongly recommended the use of Ag NPs as antibacterial substitute to the antibiotics. However, the target site was not specified and multiple mechanisms were suggested for the antibacterial mode of the Ag NPs; it could be the interaction with thiol and phosphorous compounds. Until now researchers could not find the way the NPs introduce their antibacterial action, it could be through the interaction of the particles with the cells or through the released ions or both ways.

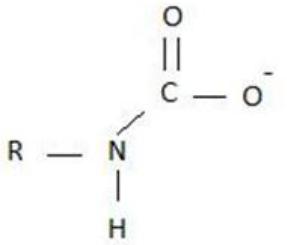

Fig. 2 Carbamate formed at the N-terminus of amino acid.

Detection of variations in the antibacterial activity of Ag NPs

The study of Song *et al.* (2006) revealed the differences in the morphologies of different types of treated bacterial cells; SEM and TEM showed the separation of the cytoplasm of the *E. coli* from the bacterial cell wall, plasmolysis, while for the *S. aureus* the synthesis of cell wall was inhibited and for other Gram positive *Mycobacterium tuberculosis* the only observable change was the presence of NPs inside the cells. In agreement with the above results of the differences in the effect of Ag ions on the morphology of different bacterial types, Panácek *et al.* (2006) found that Ag NPs with concentration of 6.75 µg/ml was toxic to *Staphylococcus aureus* (MRSA) compared to 3.38 µg/ml concentration used against *E. coli*.

Furthermore, it was found that the morphological differences exist between the bacteria of the same type; although *M. tuberculosis* and *S. aureus* are Gram-positive bacteria and it is expected to have similar morphological changes, it was not demonstrated in Song *et al.* (2006) study. Moreover, the antibacterial activity was observed against the Gram negative bacteria; *E. coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* by using concentration around 1 ppm, while against *S. aureus* and *M. tuberculosis* the used concentration was around 10 ppm. Therefore, it was concluded that the antibacterial activity of Ag NPs depends on the type of the bacteria; higher concentration of NPs was needed against the Gram positive due to the presence of thicker peptidoglycan layer in their cell walls, which provides more resistance to the passage of the NPs.

Ag-hydroxyapatite/titania NPs were investigated against oral bacteria in study of Liao *et al.* (2007), and were found to have an efficient antibacterial activity which varied according to the bacterial type, where the Gram negative bacteria were more sensitive to the NPs than the Gram positive, it was supposed that the negatively charged and the thick cell wall of the Gram positive bacteria bind more Ag ions and allow less amount to interact with the plasma membrane. The mechanism of the antibacterial action was suggested to be related to the active oxygen species that may destroy the outer membrane.

The same results were shown in a study of Morones *et al*. (2005); where higher Ag NPs concentration was required to inhibit the growth of the Gram positive bacteria; *P. aeruginosa* and *V. cholera* compared to the concentration used to inhibit the growth of Gram negative bacteria; *E. coli* and *S. typhus*. However, the highest concentration used, above 75 µg/ml, completely inhibited the growth for all types of bacteria. However they did not indicate any difference in the morphologies of different bacteria after the treatment unlike Song *et al.* (2006) study, the morphological differences of bacteria reflected the different in the antibacterial mechanism of NPs exerted on the different bacterial type. It was suggested that the attachment of NPs to the cell membrane as well as the accumulation of NPs inside the cell indicates cell death.

In contrast to the previous results, *E. coli* was more resistant than *S. aureus* to the antibacterial cellulose incorporated with Ag NPs; this was demonstrated in the study of Maneerung *et al*. (2008). The ability of *E. coli* to develop resistance against Ag NPs was related to the presence of the LP content in the cell wall of the Gram negative bacteria. Also the study of Yoon *et al*. (2007) found that the Gram positive bacteria *B. subtilis* was more sensitive than Gram negative bacteria *E. coli* after the application of Ag NPs and Cu NPs against 10^2 CFU/ml of each bacterial type.

Moreover the antibacterial activity of Ag NPs were found to be different according to the microbiology media used for the antibacterial test, the work of Sondi and Salopek-Sondi (2004) applied Ag NPs against *E. coli* on agar plates and in liquid medium. Differences in the antibacterial activity were recorded between the two methods, where NPs applied on agar plates resulted in a complete inhibition in bacterial growth, even though the bacterial number CFU that was subjected to treatment was high and rarely found in real life system, so it was suggested that these particles have an excellent antibacterial effect in reducing bacterial growth. In contrast, Ag NPs in liquid medium, even at high concentrations, caused only delay in the growth of *E. coli*. SEM microscopy showed aggregates composed of nanosized Ag particles and dead bacterial cells. This was related to the process of the interaction of these particles with the intracellular substances of the destroyed cells, that caused them to coagulate and removed from the liquid media. Therefore, it was concluded that these particles have a limited use as antibacterial substances in liquid media because of their low colloidal stability.

In the work of Pal *et al*. (2007) the antibacterial properties of differently shaped Ag NPs were investigated against *E. coli*, both in liquid systems and on agar plates. Energy-filtering transmission electron microscopy (EFTEM) images

revealed damage in the cell membranes after treatment and NPs were found accumulated inside cells, resulting in cell death. Truncated triangular Ag NPs had the strongest biocidal action, compared to the spherical and rod-shaped NPs, so the shape of Ag NPs affects the interaction of Ag NPs with bacteria. Although agar plate -growth experiments allow the detection of distinguishable antimicrobial properties of Ag NPs with different shapes, liquid-growth experiments showed basically similar results in the previous study (Pal *et. al*. 2007). However, the study (Sondi and Salopek-Sondi 2004) pointed out a distinct difference between these two methods.

Small size NPs have highest biocides effect compared to the large NPs, this is due to their large surface area that allows efficient binding with bacterial surface, therefore small NPs demonstrate effective cytotoxicity to the microorganisms (Tokumaru *et al.* 1974; Morones *et al*. 2005; Panácek *et al.* 2006; Castañón *et al*. 2008; Raffi *et al.* 2008).

In Morones *et al*. (2005) study the scanning transmission electron microscopy (STEM) was used to evaluate the effect of Ag NPs diameter on its antibacterial activity against several types of bacteria. A high-angle annular dark field (HAADF) was used as a detector in STEM to allow high spatial resolution and compositional imaging. NPs smaller than 10 nm showed the highest antibacterial activity and interacted with the bacteria through adhesion and penetration inside the cells, while the smallest NPs size that exhibited the highest antibacterial activity in the Panácek *et al.* 2006 study was 25 nm. It can be concluded that although the antibacterial activity of Ag NPs is size dependent, the biocidal size of NPs differs from one NPs system to other.

Based on the experimental studies (Feng *et al.* 2000; Morones *et al.* 2005; Song *et al.* 2006; Raffi *et al.* 2008) the overall effect of Ag NPs is less fairly coincides with the effect of Ag ions. The difference is the formation of a low molecular weight region in the center of bacteria, as reported in Feng *et al.* (2000), after the treatment with Ag ions solution. This low-density region supposed to be a kind of defense against Ag ions while Morones *et al.* (2005) study did not reveal the formation of a low density region in *E. coli* after Ag NPs treatment as reported by Feng *et al.* (2000); instead, a large number of small Ag NPs found inside the bacteria. Also this light region did not appear in bacteria treated with Ag NPs in Song *et al.* (2006) and Raffi *et al.* (2008) studies. This indicates the difference in the mode of action between Ag NPs and the Ag solution.

Other studies showed that the antibacterial activity of Ag NPs is related to the adsorption of Ag ions on the surface of particles (Henglein 1998). This was approved in the study of Lok *et al.* (2007) where the partially (surface) oxidized Ag NPs have antibacterial activities. The antibacterial activities were correlated with the levels of chemisorbed Ag ions formed on the particle's surface.

The mechanism by which the NPs are able to penetrate the bacteria is not totally understood, but the work by Sondi and Salopek-Sondi (2004) suggested that in the case of *E. coli* treated with Ag NPs the changes created in the membrane morphology might produce a significant increase in its permeability and affect proper transport through the plasma membrane. The interaction of Ag NPs can be related to Ag ions interaction in some aspects, the released Ag ions from the NPs interact with sulfur-containing proteins found in the membrane, resulting in changes in bacterial membrane. Moreover, many studies suggested that the opposite charges of bacteria and NPs attributed to their adhesion due to the electrostatic forces. It has been reported in the literature that, at biological pH values, the overall surface of the bacteria is negatively charged due to the dissociation of an excess number of carboxylic and other groups in the membrane (Stoimenov *et al.* 2002). However, the study of Lia *et al.* (2006) showed that the overall inhibitory effect of the Ag nitrate and titanium dioxide NPs against bacterial species may be due to damage to the bacterial enzymes or plasma membrane. Death of bacterial cells results from impaired metabolic pathways and leakage of the cytoplasmic content to the surroundings.

In spite of the negative charge surface of Ag particles used in Pal *et al.* (2007) study, they interacted with "building elements" of the bacterial membrane, causing structural changes, degradation and finally cell death. On the other hand, NPs found inside cells tend to react with other sulfur-containing proteins, as well as with phosphorus-containing compounds such as DNA (Hatchett and white 1996; Feng *et al*. 2000).

A recent study on the antibacterial effect of Ag NPs was conducted by Hwang *et al*. (2008). In this study different bioluminescent bacteria responsive to different toxic modes of actions were subjected to Ag NPs. The results showed that Ag NPs induce their antibacterial activities via the production of Ag ions. Other antibacterial mechanism was found to be through oxidative damages, mainly caused by the formation of superoxide radicals. Ag NPs were found to generate superoxide radicals that target many sites and molecules; also they attack the fatty acids in membranes and initiate lipid peroxidation. As a result, the membrane properties are changed because of the insufficient membrane fluidity through the significant disruption of the membrane-bound proteins Cabiscol *et al.* (2000). DNA is also a main target; reactive oxygen species attack both the nitrogen base and the sugar resulting in single and double-strand breaks in the backbone (Sies and Menck 1992; Sies 1993). Proteins are other targets; Reactive oxygen species (ROS) manifest their toxicity by the oxidation of sulfhydryl groups, reduction of disulfides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalyzed oxidation, reaction with aldehydes, modification of prosthetic groups or metal clusters, protein-protein cross-linking and peptide fragmentation. All these reactions affect the function of membranes and proteins, and block DNA replication or cause mutations (Stadtman 1990).

While there are several methods reported for the production of Ag NPs; few of them used biological system for the production of Ag NPs. The biological methods have been used in some studies in order to prevent using toxic substances in the production protocol. The green method for the preparation of Ag NPs involves the addition of $AgNO_3$ into filtrated biomass of fungi. Maliszewska and Sadowski (2009) produced Ag NPs by the addition of $AgNO_3$ into filtrated biomass of *Penicillium*, the prepared Ag NPs were 10-100 nm in size and appeared surrounded by a thin layer of material that were suggested to be an organic material from the fungi that contributed to the stability of the NPs. The produced NPs showed interesting antibacterial results against wide spectrum of bacteria. Potato plant fungus, *Phytophthora infestans* was used in the study of Thirumurugan *et al*. (2009) to synthesize Ag NPs, which displayed a broad biocidals effect at 5 µg/ml against bacteria. Moreover, Eby *et al*. (2009) have introduced a unique method for the preparation of Ag NPs that involves using the enzyme Lysozyme to reduce the Ag precursor, silver nitrate, in methanol. This stable Ag NPs combined the antibacterial properties of Ag and the lysozyme and were effective biocides against a fungus and broad spectrum of bacteria including highly silver-resistant bacteria such as *Proteus mirabilis*.

The toxicological test is necessary to be conducted to ensure the safety of the applied concentration of Ag NPs, although many studies showed promising results in using Ag NPs as antibacterial agent, they did not include the toxicological assay to improve the safety of their materials. Eby *et al*. (2009) study is one of the few studies in literature that included *in-vitro* tissue culture experiment as a test to determine the toxicological effects of Ag NPs against mammalian cells. The antibacterial concentration required to kill the bacterial strains was 25 µg/ml, this concentration is below the concentration that was toxic to the mammalian cells.

However the toxicity of Ag NPs was reported at higher concentration compared to the concentration used with other metallic NPs, the toxicological study of Hussain *et al*. (2005) showed a decrease in the mitochondrial function

after the treatment with 5-50 µg/ml of Ag NPs, whereas other metallic NPs like Fe_3O_4 and TiO_2 NPs induced their toxicity at higher levels (100-250 µg /ml). Therefore, it was concluded that Ag NPs is highly toxic material.

A complex of Fe_3O_4@Ag NPs was produced in the study of Gong *et al*. (2007). The complex was first synthesized by the production of Fe_3O_4 NPs, then $AgNO_3$ was added to the mixture and reduced using sodium bromide, $NaBH_4$. The produced non-aggregative NPs were spherical, with an average diameter of about 60 ± 20 nm. This morphology of the NPs was related to the reduction of Ag ions onto the surface of Fe_3O_4 NPs. This material complex was used as antibacterial agent against a wide spectrum of bacteria, the results indicated that Fe_3O_4@Ag NPs have broad antibacterial capability for different bacterial types including; *S. epidermidis*, *B. subtilis* and *E. coli*.

It is uncommon to find in the literature the application of different antibacterial Ag NPs against bacteria using the same biocidal concentration. The antibacterial concentration depends on the design of NPs system that involves the method of production and the reagents supplied and whether if the NPs were incorporated into polymer matrix or used as frees NPs. Therefore, many studies prefer to use a range of NPs concentration against bacteria rather than using one concentration in order to detect the minimum inhibitory concentration of NPs.

Although the free Ag NPs show efficient antimicrobial activity that depends on critical factors such as the size, the shape and the concentration applied, they sometimes form aggregate that render their antibacterial activity and this makes them unsuitable for some applications. Therefore, there is a growing interest in the combination of polymer with Ag NPs.

The natural fibers are considered as an organic polymer and usually they are biocompatible, they become biocides after metallic NPs incorporation, so metallic NPs loaded fibers is an example of the inclusion of NPs into an organic matrix. Chen and Chiang (2008) claimed that the cellulosic cotton integrated with Ag NPs could be used in different applications such as wound dressings, personal care products and clothing. In addition to the incorporation into organic polymer, the metallic elements are incorporated into inorganic matrix such as the ceramics, Bakumov *et al*. (2007) used the polysilazanes as the precursor system to generate silicon carbon nitride ceramic matrix integrated with Ag NPs. This material revealed an efficient antibacterial activity against *S. aureus* and *E. coli*. Antibacterial ceramic can be used in different industries such as food and medicine. The generation of the antibacterial products incorporated with metallic NPs can increase the price and the biocompatibility purpose of the product.

Enhancement of the ion release as one antibacterial mechanism of Ag NPs

Ag NPs have to release Ag ions to attack the pathogenic bacteria in order to be effective, therefore the steady and prolonged release of Ag ions are critical in the production of Ag NPs and considered as a challenge to the industry of antimicrobial Ag NPs. The elemental Ag must be oxidized to release Ag ions according to the following equation

$$Ag^0 \longrightarrow Ag^+ + e^-$$

The agglomerated Ag NPs affects the efficiently of the antimicrobial activity by reducing the ions release, indeed particles having low specific surface area show higher release of Ag ions (Kumar and Münstedt 2005).

Since the antibacterial activity of Ag NPs depends on the release of Ag ions, the incorporation of Ag NPs into organic polymer matrix is one critical approach to enhance the release of Ag ions. The organic polymer acts as suitable medium for the stabilizing of Ag NPs and preventing the aggregation of the NPs. In the study of Maneerung *et al*. (2008) the high oxygen content of bacterial cellulose anchors Ag ions to the cellulose fibers through ion dipole interaction, this design exhibited strong antibacterial activity against different bacteria and it was suggested to protect the wound infections via using it as wound dressing. Kumar and Münstedt (2005) incorporated Ag NPs into the molten polyamide polymer and used it as antimicrobial agent against *E. coli* and *S. aureus*, where the antibacterial activity increased as a function of time. This increase was related to the increase of Ag ions release. A styrene-acrylic acid polymer mixture incorporated with Ag NPs had good antimicrobial activity against *E. coli* and *S. aureus*, the biocidals property was related proportionally to the increase of Ag content (Paula *et al*. 2009). Another study incorporated the polyamide polymer with Ag NPs by thermal reduction of Ag acetate during melting processing of the polyamide polymers. 1.5 % Ag incorporated in polyamide was tested against 10^6 CFU/ml of *E. coli* and after 24 hours no bacteria were detected (Damm and Münstedt 2008).

Well-dispersed NPs were synthesized in the study of Thiel *et al*. (2007) using the sol-gel synthesis technique. Ag doped titania particles were synthesized with a concentration up to 1.01% Ag and sizes of 26 and 56 nm. Ag doped particles were applied with concentration of 25 µg/cm^2 against *E. coli* and found to be an affective antibacterial agents using liquid medium and agar plates. Xu *et al*. (2009) produced well dispersed Ag NPs coated with silica, Ag core and silica shell with size of 14-26 and 15-28 nm, respectively showed excellent antibacterial activity, where 0.39 mg/l concentration of Ag-SiO_2 core shell NPs was effective against *E. coli* and *S. aureus*.

The properties of the filler particles as well as those of the polymer govern the Ag ions release. It was found that interaction of Ag particles with the amide groups is stronger than the interaction between the Ag particles (Damm and Münstedt 2008); therefore, the Ag particles were surrounded by the polyamide chains that prevented their aggregation. It was confirmed that the interaction of water with the metallic Ag particles forms Ag ions. Damm and Münstedt (2008) found that the strong hydrophilic polyamides incorporated with Ag NPs showed increased Ag ions release in comparison to the less hydrophilic polyamides. They found that the polyamide provides a hygroscopic media where the water diffuses inside and controls the release of Ag ions through oxidation to the metallic Ag. Therefore, Ag ions release increases with growing water content of a polymer, as a result the antibacterial activity increased (Kumar and Münstedt 2005).

The molar ratio of reactants, ratio of the reducing agent to the metal precursor, is a critical factor in NPs production, the higher the molar ratio the smaller the size of NPs produced and therefore the less aggregated they are. In a kinetic study of Maneerung *et al*. (2008) the total Ag ions release was higher for the high molar ratio compared to the low ratio, because Ag ions spread increased gradually with time.

Moreover, Ag ions release can be enhanced by the addition of trace elements into Ag NPs. Dowling *et al*. (2003) added platinum to Ag NPs incorporated with polyurethane and silicon polymers to enhance the release of Ag ions through galvanic action and as a result the antibacterial activity increased.

Au was added as filler with Ag and together were incorporated into polytetrafluorethylene (PTFE) polymer and showed more efficient antibacterial affect against *S. aureus* compared to the polymer incorporated with Ag NPs only (Zaporojtchenko *et al*. 2006). A volume filling of 1% Au and 15% Ag integrated into the polymer showed the largest inhibition zone in the disk diffusion test compared to the incorporated polymer having no Au. This was correlated to the highest release of Ag ions recorded with Au-Ag NPs/ polymer. However increased filler content reduced the antibacterial activity of Ag NPs/polymer complexes where the increasing of Ag volume was found to negatively affect the antibacterial activity of the Au-Ag NPs/polymer, this was explained that the higher Ag concentration reduced the Ag

ions release due to the decreasing total surface area of the percolated particles (Zaporojtchenko *et al*. 2006). While the Ag polyamide nanocomposites with large surface area were produced by Damm *et al*. (2008), are efficient biocides, Ag NPs/polymer incorporated with weight percent of Ag content from 0.06% to 0.19% was tested against 10^6 CFU/ml of *E. coli* and resulted in complete inhibition after 24 hours of incubation. This was correlated with the Ag ions release.

The reducing agents play an important role in the production of NPs, gallic acid, sodium borohydride and ascorbic acid are examples of reducing agents used in literature (Castañón *et al*. 2008; Maneerung *et al*. 2008; Xu *et al*. 2009). In addition to acting as a reducing agent, sodium borohydride ($NaBH_4$) was found to act as a stabilizer for the Ag NPs, where the excess BH^{+4} form thick layer and prevent NPs aggregation (Song *et al*. 2009). The degree of the dispersion of NPs can be improved by using SDS as stabilizer to prevent NPs aggregation, where the higher the concentration the better the protection is against growth and aggregation (Song *et al*. 2009).

OTHER ANTIBACTERIAL METALLIC NPs

Other types of NPs are being used in antibacterial experiment rather than using the Ag NPs, a study conducted by Stoimenov *et al*. (2002) treated *E. coli* and *Bacillus megaterium* with magnesium oxide (NP-MgO) NPs and halogennated magnesium oxide NPs (AP-MgO/Cl_2 and AP-MgO/Br_2). The results showed that *E. coli* cell membrane was damaged after 60 min of treatment, the content was leaked out and the particles were observed inside all of the cells, the same results were obtained after treatment of *E. coli* with Ag NPs in Sondi *et al*. (2004) study, although all the NPs used resulted in the same morphological changes, the halogenated forms (AP-MgO/Cl_2 and AP-MgO/Br_2) resulted in an extensively more damaging effect. Moreover, the obvious morphological changes observed in *Bacillus megaterium* treated with the halogen particles was the presence of a large number of cell membrane pieces, this represented a different morphological changes after the treatment compared to *E. coli* morphological changes and it can be concluded that the different cell types treated with NPs resulted in different morphological changes. Based on TEM scanning results, it was concluded that the target site of NPs was the cell membrane of the bacteria and since the halogenated NPs charge is positive and opposite to that of cell membrane the NPs interacted with bacteria.

The antibacterial activity of the MgO NPs and the halogenated one that were used in Stoimenov *et al*. (2002) study was related to the chemical property of MgO that acted as a desiccant compound, this is was supported in Papenguth *et al.* (2000) study, and the oxidizing action of the halogen substance. Moreover, it was found that the NPs of AP-MgO were abrasive and caused mechanical damage to the cell membrane. Stoimenov *et al*. (2002) also shared the same suggestion of the study of Song *et al*. (2006) that the presence of NPs in the cytoplasm of bacterial cells induce metabolic disturbance. The direct contact between the particles and the cell membrane is essential to induce the antibacterial activity.

Du *et al*. (2008) loaded a polymer matrix with metal rather than Ag, they synthesized chitosan NPs loaded copper ions and used the complex against *E. coli*. The bacterial cells were killed through damage to the cell membrane. The damage to the cells was represented by the visualization of morphological changes involving the disappeared flagellum, irregularities in the morphology, mainly at both ends, changes in the basic shape of bacterial cell, where the *E coli* changed from the original rod shape to a nearly spherical shape. The integrity of the bacterial cell disappeared and holes of various sizes appeared on the cell surface, which revealed signs of membrane disruption These morphological changes were similar to what were observed in the study of Qi *et al.* (2004), Who investigated the antibacterial activity of chitosan NPs and copper-loaded NPs on the morphological appearance of *S. choleraesuis* by using the Atomic Force Microscopy (AFM), which is used to observe the morphological changes of bacteria treated with the NPs since it has a high-resolution imaging technique with high resolution properties and can resolute the small molecules found in liquid. The morphologies were observed after 30, 60 and 180 min treatment with NPs. The cells were transferred from a spherical shape to irregularly condensed masses after 60 min of treatment. After 3 hrs the cell membrane was broken into fragments and the cytoplasm content was leaked out. The results showed that the exposure of bacterial cells to the chitosan NPs led to the disruption of cell membranes. Morphological changes of bacterial cells were similar when Ag NPs used or other type of NPs; these represented by changes in the basic shape, membrane disruption, and cytoplasm leakage.

The antibacterial activity of the chitosan NPs used in Du *et al.* (2008) and Qi *et al*. (2004) studies can be explained according to Muzzarelli *et al.* (1990) who assumed that the positively charged amino groups in chitosan could interact with negatively charged anionic groups on the cell surface and form polyelectrolyte complexes. This leads to the formation of an impermeable layer around the cell that prevents transport of essential solutes into the cells this result in cell death (Choi *et al*. 2001; Halender *et al*. 2001).

Different assays were used in the study of (Xing *et al*. 2009) to determine the target sites of antibacterial chitosan. It was found that the content of the bacterial cells increased via destroying the integrity of the membrane and resulted in an efficient permeabilization of the membrane of the bacterial cells. SDS-PAGE assay indicated that the protein content decreased in the treated cells.

Du *et al*. (2009) evaluated the antibacterial activity of chitosan NPs loaded with different metallic ions against Gram-negative and Gram-positive, the highest antibacterial activity was recorded for chitosan NPs loaded with Ag ions that exhibited the most stable colloidal particles, the stability of the colloids were represented by zeta potential values, where the highest value obtained was for chitosan NPs loaded with Ag.

Antibacterial copper NPs loaded into modified cotton fiber (Mary *et al*. 2009) were used against *E. coli*. The material showed excellent antibacterial activity. It was recommended to use this complex in wound dressing and in fabrication of antibacterial dressing.

Zhang *et al.* (2007) used SEM to scan *E. coli* treated with zinc oxide NPs, ZnO NPs, with concentration of 0.2 g/l. After 5 hrs of treatment the damage occurred to bacterial cells was very clear and resulted in breakdown of the membrane of the bacteria. One of the possible reasons for the damage was suggested to be the direct interaction between ZnO NPs and the external membrane surface. The study of Perelshtein *et al*. (2008b) is one of the few studies in literature that loaded the cotton fabrics with ZnO NPs. The complex was used as antibacterial agent against *S. aureus* and *E. coli* and resulted in killing both bacteria after 3 hrs. The concentration of ZnO incorporated into the fiber was less than 1% and it was claimed that this concentration had never been reported in literature with using the antibacterial ZnO NPs.

Metallic NPs, Fe_3O_4@Ag NPs, were produced in Gong *et al*. (2007) study and characterized as superparamagnetism, this property allows them to be recollected and reused for several times. Therefore, the best potential application of these NPs is to be used as disinfectants for water, where they can be removed easily from the water.

What can be concluded regarding the antibacterial activity of metallic NPs is the attachment of the particles with the cell membrane as a necessary primary step, where the particles themselves manifest their bactericidal activity through the oxidizing agent on their surfaces, the electrostatic force results from the two opposite charges of the particles and the cell membrane, all these factors contribute to disturbance of the cell membrane leading to increased permeability that results from pit formation, as a result the

particles pass inside the bacterial cell, where the penetrated NPs accumulated inside the cells; and induce the biocides affect through the released ions that interact with the different sulfur and phosphorus containing molecules inside the cells such as the proteins and DNA, all these lead to cell death. The variations in the design of the NPs contribute to the manifestation of different physical and chemical properties that need to be characterized before the application.

CAN BACTERIA DEVELOP RESISTANCE TO SILVER NANOPARTICLES?

Many studies succeeded in the isolation of resistance bacteria against the Ag such as *E. coli* (Hendry and Stewart 1979), *Salmonella typhimurium* (McHugh *et al.* 1975) and many others species (Haefeli *et al.* 1984; Deshpande *et al.* 1994). A well-studied defense mechanism against antibacterial Ag ions is the developing of efflux system against the Ag ions (Li *et al.* 1997). Since it is well understood that in some aspects Ag NPs share similar mode of antibacterial actions with Ag ions, bacteria may generate the same resistance against Ag NPs. A recent study claims that the excess use of the Ag NPs can end up with the evolution of bacteria resistance to these particles (Anderson 2008).

On the other hand, there is another point of view that considers Ag resistant bacteria not a serious problem, Ag has multi target sites inside the bacteria and furthermore it is difficult for the bacteria to develop resistance to the broad and unspecific antibacterial activity of Ag (Woodbury 2008).

CONCLUSIONS

Metallic bactericidals have been in use for several years as external sanitizers and disinfectants and have shown antibacterial activity against both Gram-positive and -negative bacteria, as well as against fungi. The mechanism of interaction of these metallic biocides includes protein membrane damage, production of superoxide radicals, and release of ions that interact with the cellular granules and form condensed molecules. Since Ag ions have non selective toxicity and are bactericidals at very low concentrations, which is due to the ability of bacterial cells to absorb and concentrate Ag from very dilute solutions, Ag is the most nanoparticulate material and was reported to act as highly efficient biocides.

It can be concluded that the overall interaction of NPs with the bacterial cell depends on the characteristics of the bacterial cell surfaces and the NPs material combination. The ions release that interacts with the cellular components is one suggested mode of action of the antibacterial Ag NPs. Increasing the ion release enhances the antibacterial properties of the NPs. The combination of metal NPs with polymers enhances their distribution and makes them suitable for a variety of potential antimicrobial applications. Concerning the parameters that affect their biocidals property, the antibacterial activity of metallic NPs is being described as size dependent or shape dependent, however the most proper description for metallic NPs, especially Ag NPs, would be the highly efficient biocides. For future work the cooperation of many scientific fields may lead to complete and deep understanding of the antibacterial activity of metallic NPs. Toxicological study are needed in combination with bacterial assay in order to prove that the antibacterial activity of the designed NPs is safe for human cells.

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