BACTERIAL RESISTANCE TO SILVER NANOPARTICLES

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Abstract

Silver nanoparticles (NPs) exhibit significant antimicrobial activity against a broad range of bacteria and fungi at concentrations ranging from a few ppm to tens of ppm that are not cytotoxic to human cells. Silver NPs also strongly enhance antibacterial activity against multiresistant, β-lactamase and carbapenemase-producing *Enterobacteriaceae* when combined with antibiotics. As a result, silver NPs have already been applied in various biomedical and antimicrobial technologies and products of every-day life as an alternative to conventional antibiotics. While antibiotic resistance has been discussed extensively in the literature, the possible development of resistance to silver NPs after repeated long-term exposure has not been fully explored. We report that bacteria can develop resistance to silver NPs after prolonged exposure. The observed resistance consists in aggregation of silver NPs which consequently eliminates their antibacterial effects. The resistance mechanism can be overcome by preventing the aggregation of silver NPs via their deposition on suitable inorganic substrate such as graphene oxide.

Keywords: Silver nanoparticle, graphene oxide, bacteria, antibacterial, antibiotic, resistance

1. INTRODUCTION

Bacterial resistance to antibiotics currently represents a very serious and complex problem transcending the borders of healthcare. The mounting resistance of pathogenic bacteria increasingly limits antibiotic effectiveness, significantly increasing the likelihood of failure of antibiotic therapy and the related morbidity and mortality of patients [1]. The development of entirely new antimicrobial drugs and their introduction into clinical practice is a lengthy and costly process. An option to overcome bacterial resistance is restoring antibacterial effect of antibiotics by their combination with other antibacterial substances. A promising way to overcome bacterial resistance is a combination of conventional antibiotics with nanomaterilas showing antibacterial effect such as silver based nanoparticles and nanocomposites. Silver-based nanostructured materials are considered to be promising nanomaterials in biomedical applications or biotechnology for their high antimicrobial activity [2]. Silver nanoparticles (NPs) exhibit high bactericidal activity at concentrations showing no cytotoxicity to mammalian cells and also strongly enhance the antibacterial activity of conventional antibiotics even against multiresistant bacteria through their synergistic effect [3-8]. Unlike the specific antibiotic's mode of action, the effects of silver NPs are not specific to a single cellular level, but influence many bacterial structures and metabolic processes concurrently [4,9-11]. The question whether silver NPs could be used in medicine to enhance the effectiveness of antibiotics or fully replace them in the treatment of local and systemic infections in the future is rather premature. However, it is no doubt too early now to deal with potential development of bacterial resistance to silver NPs. Silver in various forms has been used as an effective antibacterial agent for many centuries and its antibacterial effect is still maintained. Bacterial resistance to silver is mainly associated with the ionic form of silver [12,13]. Only few studies demonstrating possible resistance or decreased susceptibility to silver NPs have appeared recently, nevertheless resistance to silver NPs was driven by mechanisms eliminating ionic form of silver [14,15]. On the other hand, unlike the intensive, long-term and repeated administration of classical antibiotics or ionic forms of silver (silver sulfadiazine), silver NPs have not been commonly used in medical practice. It remains unclear whether

bacteria may develop resistance to silver NPs, as is the case with long term and repeated exposure to antibiotics. Here we show that bacteria originally susceptible to silver NPs are able to develop a mechanism by which they can resist the antibacterial effect of silver NPs after long-term and repeated exposure.

2. MATERIALS AND METHODS

2.1. Chemicals and biological material

Silver nitrate (p.a., Fagron), ammonia (28-30 % [w/w], p.a., Sigma-Aldrich), sodium hydroxide (p.a., Lach-Ner), D-maltose (p.a., Sigma-Aldrich), and graphene oxide (powder, Sigma-Aldrich) were used for silver NPs synthesis and silver decorated graphene oxide nanocomposite, respectively. The bacterial strains used in this work were *E. coli* CCM 3954 obtained from the Czech Collection of Microorganisms (Masaryk University, Brno) and *S. epidermidis* 008 obtained from the culture collection of the Department of Microbiology, Faculty of Medicine and Dentistry, Palacký University Olomouc.

2.2. Synthesis and characterization of silver NPs and silver decorated graphene oxide (Ag@GO)

The modified Tollens process involving reduction of the complex cation $[Ag(NH_3)_2]^+$ by D-maltose was used to synthesize dispersions of silver NPs with a diameter of 28 nm and silver concentrations of 108 $mg \cdot L^{-1}$ or 432 $mg \cdot L^{-1}$. The concentrations of all the reaction components were as follows: silver nitrate 1×10^{-3} $mol \cdot dm^{-3}$; ammonia 5×10^{-3} $mol \cdot dm^{-3}$; sodium hydroxide 9.6×10^{-3} $mol \cdot dm^{-3}$, and D-maltose (as a reducing agent) 1×10^{-2} $mol \cdot dm^{-3}$. To prepare silver NP dispersions at a concentration of 432 mg L^{-1}, the concentrations of all the reaction components were increased fourfold except that of sodium hydroxide. The same procedure was applied for deposition of silver NPs on the surface of graphene oxide sheets. For this purpose, 10 mg of graphene oxide was dispersed in 10 ml of $AgNO_3$, NH_3 and NaOH solution followed by 30 min sonication. After that, maltose solution was added under vigorous stirring to reduce silver NPs on the surface of graphene oxide sheets. Silver NPs decorated graphene oxide was three times separated by centrifugation at 150 x g for 15 minutes and wash by distilled water. Silver amount in Ag@GO nanocomposite was determined using atomic absorption spectrophotometer (AAS) (ContrAA 300, Analytik Jena, Germany) using method of flame atomization. Before analysis, vacuum dried Ag@GO nanocomposites were dispersed in 2 % HNO_3 solution under 30 min of sonication to dissolve silver NPs followed by graphene oxide separation using syringe filter (Polyethersulfone membrane, 45 µm). The average size of the synthesized silver NPs were determined by the dynamic light scattering method using a Zetasizer Nano ZS instrument (Malvern, UK). Transmission electron microscopy measurements were conducted using a JEM 2010 instrument (Jeol, Japan).

2.3. Culture of bacteria in the presence of silver NPs

Bacteria were repeatedly exposed to subinhibitory concentrations of silver NPs by twenty repeated cultures in microplates. Dispersion of silver NPs was diluted in geometric progression with Mueller-Hinton Broth (Becton, Dickinson and Company) and inoculated with a bacterial strain at a concentration of 10^6 CFU/mL. Incubation was carried out at 37°C for 24 hours. After the incubation, the MICs of silver were read after each culture step as the lowest silver concentrations inhibiting the visible growth of microorganisms. Immediately after the 24-hour cultivation, 10 µL of Mueller-Hinton broth containing the surviving bacteria were taken from wells with subinhibitory concentrations of silver (concentrations below MIC) and consequently sub-cultured on blood agar (TRIOS) at 37°C for 24 hours. Bacteria grown on blood agar were used for inoculum preparation at a density of 10^6 CFU/mL and for the next culture step.

2.4. Proteins identification and detection bacterial biofilm formation

Silver aggregates were separated from 500 ml (10 × 50 ml) of bacterial suspension containing 27 mg of silver by centrifugation at 100 × g for 2 minutes. The pelleted aliquots with silver concentration 4.5 $mg \cdot mL^{-1}$ were

each individually incubated with 1 mL of 2.5 % trifluoroacetic acid (TFA). Extracts obtained after overnight incubation at 37 °C with shaking at 800 rpm and the subsequent pelleting of the solid particles by a centrifugation at 20,000 g for 20 min, were vacuum-dried and the recovered proteins were dissolved in 50 µL of 50 mM NH_4HCO_3 (facilitated by a 5-min sonication). Then, 1.5 µL of 200 µM modified trypsin was added for in-solution protein digestion and the mixture was again incubated at 37 °C overnight. After vacuum drying, peptides from the digests were purified using ZipTip-C18 pipette tips (Merck-Millipore, Tullagreen-Carrigtwohill, Ireland) according to the manufacturer's instructions. Vacuum-dried peptide samples were dissolved in 10 µL of 0.1 % TFA and separated using a Dionex UltiMate3000 RSLCnano liquid chromatograph (Thermo Fisher Scientific, Germering, Germany), coupled to an amaZon speed ETD ion trap equipped with a CaptiveSpray ion source (Bruker, Bremen, Germany). The scan speed was 8,100 u s^{-1} for both mass spectrometry (MS) and tandem mass spectrometry (MS/MS); acquisition was performed by collision-induced fragmentation using helium as the collision gas.

Christensen method was applied for semi-quantitative detection of bacterial biofilm formation. *S. epidermidis* 008 biofilm was formed on sterilized 96-well microplates. A 10 µl of cell suspension was inoculated in 190 µl Mueller-Hinton Broth medium in each well to get 10^6 CFU/mL. Then microplate was incubated for 24 h at 37°C. After aspiration of planktonic cells biofilms were fixed with 99 % methanol. Plates were washed twice with phosphate buffer saline or sterile saline water and air-dried. Then, 200 µl of crystal violet solution (1 %) was added to all wells. After 10 min, the excess crystal violet was removed and plates were washed twice and air dried. Finally, the cell bound crystal violet was dissolved in 33 % acetic acid. Biofilm growth was monitored in terms of $O.D._{570}$ nm using micro plate reader (DYNEX MRX).

3. RESULTS AND DISCUSSION

The well-known and reliable modified Tollens process [5] was used for synthesis of silver NPs with a diameter of 28 nm with a very narrow size distribution. The silver NPs synthesized in this way exhibit good antibacterial activity as documented by their low minimum inhibitory concentrations (MICs) equal to 3.38 mg/L after the first culture step (**Table 1**). Nevertheless, it can be presumed that bacteria may resist the antibacterial effect of silver NPs after long-term exposure at subinhibitory silver concentrations thanks to their genetic material and ability to adapt to the effects of antimicrobials. This presumption has been confirmed by the gradually increasing MICs of silver NPs following repeated culture, as documented by **Table 1** summarizing MICs of silver NPs after each second cultivation step against tested bacterial strains. **Table 1** also includes MICs of silver NPs against reference (non-induced) strains after the 1st culture step in order to compare MICs of silver NPs against induced and non-induced bacteria. It can be clearly seen from the increasing MICs in consecutive culture steps that bacteria gradually developed resistance to silver NPs. In the case of *E. coli* CCM 3954 significantly higher MICs equal to 13.5 mg/L as compared to MIC of the reference strain equal to 3.38 mg/L was determined already in the sixth culture step and from the eighth step onwards, the MICs increased to as much as 54 mg/L. *S. epidermidis* 008 became gradually more resistant from the 14th to the 18th culture steps; in the 18th step, the MIC of silver NPs reached 54 mg/L. Dispersion of silver NPs with a silver concentration as high as 432 mg/L was used to determine the final MICs of silver NPs higher than 54 mg/L against all induced resistant strains. The final MICs of silver NPs against resistant strains increased to 108 mg/L after twenty culture steps (**Table 1**). Gradual development of bacterial resistance to silver NPs was also accompanied by precipitation of silver NPs at the bottom of microplate wells (**Figure 1a**). By contrast, in the presence of susceptible bacterial strains, the dispersion of silver NPs diluted in Mueller-Hinton broth retained the yellow color (**Figure 1b**). Because no precipitates were formed and the yellow color of the dispersion of silver NPs remained unchanged during culture with reference strains, aggregation stability of silver NPs as well as their antibacterial activity were unaffected. The good aggregation stability is also evidenced by transmission electron microscopy (TEM) images of silver NPs after culture with susceptible bacteria (**Figure 2a**). In the presence of resistant strains, however, the dispersion of silver NPs changed its

color from yellow to grey- black due to the formation of a precipitate at the bottom of the well. The precipitate consists of large aggregates of silver NPs with sizes on the order of hundreds of nanometers as seen from TEM measurements (**Figure 2b**). Such large aggregates do not show antibacterial activity as the originally small silver NPs. It is evident that bacteria developed the ability to induce aggregation instability of silver NPs during repeated culture in order to minimize or even totally eliminate their antibacterial effects.

Table 1 Minimum inhibitory concentrations (MICs) of silver NPs after each second culture step against bacteria compared with MICs of silver NPs against reference strains after the first culture step.

bacteria	MIC (mg/L) after appropriate culture step										
	1st	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
***E.coli* CCM 3954**	3.38	6.75	6.75	13.5	54	>54	>54	>54	>54	>54	108
***S. epidermidis* 008**	3.38	6.75	3.38	1.69	1.69	6.75	6.75	13.5	27	54	108

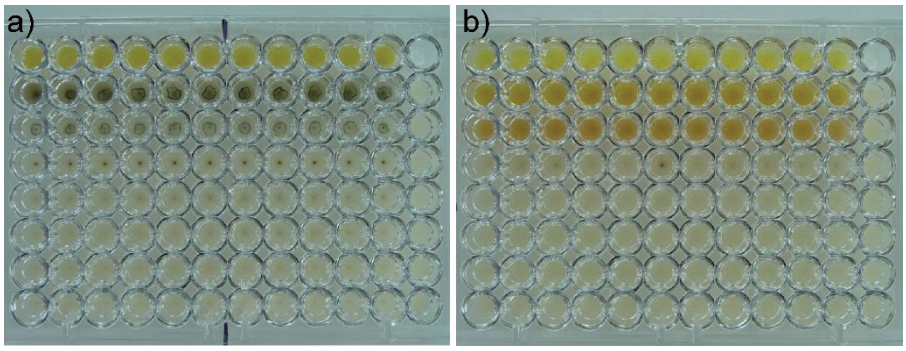

Figure 1 Microplates after culture of resistant (a) and sensitive (b) *E. coli* CCM 3954 with silver NPs diluted in Mueller-Hinton broth from the second to eighth rows to concentrations ranging from 54 mg/L to 0.84 mg/L. The upper row contains dispersion of silver NPs only.

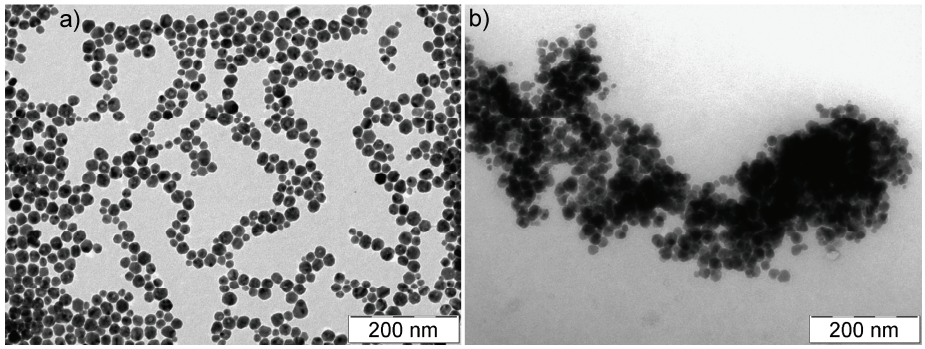

Figure 2 TEM images of stable non-aggregated silver NPs (a) and aggregated (b) silver NPs after culturing for 24 hours with “Ag-sensitive” and “Ag-resistant” *E. coli* CCM 3954, respectively.

It can be suggested that “Ag-resistant” bacteria induce aggregation of silver NPs by extracellular secretion of an agent that remains adsorbed on the aggregates’ surfaces. This agent is likely to be a high molecular weight substance such as a protein. Therefore, proteins extracted from silver NPs precipitated by Ag-resistant bacteria were subjected to proteomic mass spectrometry analysis to identify proteins that might be responsible for the observed NP aggregation. In the case of *E. coli* CCM 3954, the most abundant proteins in the extract prepared with 2.5 % TFA were flagellin followed by type-1 fimbrial protein. To investigate the proposed role of flagellin in triggering the aggregation process, we conducted a simple experiment with a commercially

available flagellin solution (1 mg/l), which was added to a dispersion of silver NPs in MH broth under conditions similar to those used in the culture experiments. The initially yellow dispersion of silver NPs immediately became grey upon adding flagellin, and a black silver precipitate was observed at the bottom of the Eppendorf tube after 24 hours at 37 °C. In the case of *S. epidermidis* 008, any proteins have not been analyzed on the surface of precipitated silver NPs. On the other hand, Ag-resistant *S. epidermidis* 008 produced bacterial biofilm much more than Ag-sensitive *S. epidermidis* 008 as it was confirmed by Christensen detection method. We therefore suppose that bacterial biofilm formation was responsible for aggregation of silver NPs and consequently for resistance of *S. epidermidis* 008 to silver NPs.

The mechanism of bacterial resistance based on aggregation of silver NPs can be overcome by preventing the particle aggregation using a strong deposition of silver NPs on the surface of appropriate nanostructured substrate. For example, graphene oxide can be considered as a suitable substrate for deposition of silver NPs. In order to overcome particle aggregation and bacterial resistance to silver NPs we synthesized silver NPs decorated graphene oxide (**Figure 3**). Diameter of silver NPs reduced on the GO sheets ranged from 15 to 30 nm, which is similar to diameter of silver NPs in the used water dispersion. Concentration of silver (determined using AAS) per 1 g of Ag@GO composite was 273 mg/g. Ag@GO nanocomposite showed a high antibacterial activity against both Ag-sensitive and Ag-resistant bacterial strains. Determined MIC of Ag@GO recalculated to silver content were as low as 2.5 mg/l for both Ag-sensitive *E. coli* CCM 3954 and *S. epidermidis* 008 and 5 mg/l for both Ag-resistant *E. coli* CCM 3954 and *S. epidermidis* 008. MICs of silver NPs equal to 5 mg/l against Ag-resistant *E. coli* CCM 3954 and Ag-resistant *S. epidermidis* 008 represent a very low concentration compared to MIC of water dispersion of silver NPs against sensitive bacterial strains. Bacterial resistance to uncoated silver NPs was overcome by preventing the particle aggregation through the deposition of silver NPs on the graphene oxide sheets.

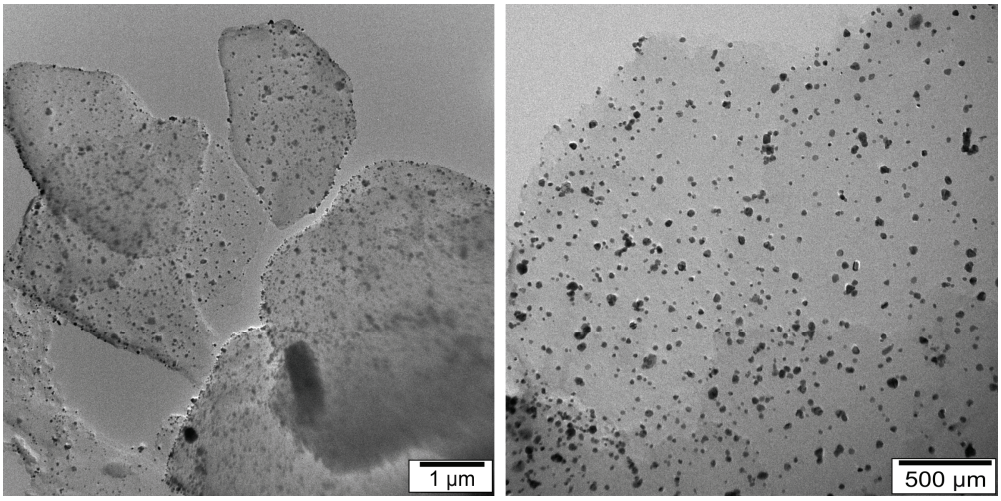

Figure 3 TEM images of Ag@GO nanocomposite at different magnifications

4. CONCLUSION

In summary, we have demonstrated that bacteria repeatedly exposed to subinhibitory silver concentrations are able to develop a resistance to silver NPs in relatively short time. The mechanism based on a physical effect by which bacteria can resist the antibacterial effect of silver NPs involves disruption of the colloidal stability of silver NPs through the flagellin (*E. coli* CCM 3954) or biofilm production (*S. epidermidis* 008). Bacterial resistance to silver NPs can be overcome by deposition of silver NPs of graphene oxide sheets preventing particle aggregation. These findings will certainly help to better understand the mechanisms that drive bacterial resistance to antibacterial agents and will be useful in prevention of bacterial drug resistance and fight against infectious bacteria.

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