

## Full Paper

# In vitro assessment of Ag<sub>2</sub>O nanoparticles toxicity against Gram-positive and Gram-negative bacteria

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In view of antibiotic resistance among pathogens, the present study is to address the toxicity of Ag<sub>2</sub>O nanoparticles against the Gram-positive and Gram-negative bacteria through in vitro assays. The preliminary screening by agar diffusion assay confirms the antibacterial activity of Ag<sub>2</sub>O nanoparticles against all the test bacteria. Comparative antibacterial activity of Ag<sub>2</sub>O nanoparticles and respective antibiotics reveals their broad range of activity and lower inhibitory dose against the used bacterial strains. Further, they can inhibit *E. coli* with an effective dose of 0.036 mg/ml within 1 h of exposure time as determined by luciferin based ATP assay. Moreover, the Ag<sub>2</sub>O nanoparticles exhibit higher antibacterial efficacy against Gram-negative bacteria than Gram-positive bacteria, as revealed by their MIC & MBC values. Therefore, Ag<sub>2</sub>O nanoparticles pave the way for a new generation of antibacterial agents against the emerging multidrug resistant pathogens.

**Key Words**—Ag<sub>2</sub>O nanoparticles; antibacterial activity; ATP assay; *Bacillus circulans*; *Escherichia coli*; microdilution method

## Introduction

The discovery of antibiotics was considered to be a life-saving advance for humans against pathogens. However, in the past few decades several pathogens have been reported to develop resistance to the antibiotics that were believed to invincible, particularly methicillin-resistant *Staphylococcus aureus* (MRSA)

and vancomycin-resistant *Enterococci* (VRE) (Grundmann et al., 2011; Pfeifer et al., 2010). Further, the emergence of New Delhi Metallo  $\beta$ -lactamase (NDM-1), resistant to all commonly used recent antibiotics, leads to serious concern for their control (Moellering, 2010). These drug-resistant bacteria are emerging pathogens whose resistance profiles are a major challenge for their spread and impact on human health (Lara et al., 2010). The continuing emergence of antibiotic resistance in pathogenic and opportunistic microorganisms has compelled the scientific community to look forward for an alternative (Croswell et al., 2009; Martinez-Gutierrez et al., 2010).

One of the recent efforts in addressing this challenge lies in exploring antimicrobial nanoparticles. Inorganic nanoparticles offer many distinctive advantages in re-

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Abbreviations: Ag<sub>2</sub>O, silver oxide; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

ducing acute toxicity and overcoming resistance, as well as lowering cost, unlikely to conventional antibiotics (Pal et al., 2007). Thus, a great deal of attention has been devoted to inorganic nanoparticles for such potential applications as biological materials, and pharmaceutical and food packaging their (Costa et al., 2011). The advantage of using the nanoparticles for biomedical applications is due to their strong activity even in small concentrations (Premanathan et al., 2011).

Furthermore, a literature survey revealed the toxicity of a range of inorganic nanoparticles (Ag, TiO<sub>2</sub>, ZnO, clay, MgO, CeO<sub>2</sub>, etc.) which have been studied against different Gram-negative and Gram-positive bacteria (Alrousan et al., 2009; Chwalibog et al., 2010; Nangmenyi et al., 2009; Neal, 2008; Negi et al., 2011b; Shameli et al., 2011) as well as multidrug-resistant bacteria (Huh and Kwon, 2011). However, it is reported that the antibacterial effect of nanoparticles was found to be more pronounced on Gram-negative bacterial strains in virtue of their permeable cell membrane (Sinha et al., 2011). Thus, considering the above facts the present study was planned in continuation to an earlier study (Negi et al., 2011b) to further assess the dose and time-dependent toxicity of antibacterial Ag<sub>2</sub>O nanoparticles against avirulent strains of both Gram-positive (*Bacillus circulans* strain D1 and *Bacillus aerius* strain SPT2) and Gram-negative bacteria (*Escherichia coli* strain RGR13 and *Pseudomonas aeruginosa* strain PS1) through agar diffusion, microdilution and luciferin based ATP assay.

## Materials and Methods

**Starting materials.** The Ag<sub>2</sub>O nanoparticles used for this study were procured from the Department of Chemistry, College of Basic Sciences and Humanities, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India. All the chemicals and reagents used in the study were of analytical grade and bought from standard manufacturers viz., SRL Pvt. Ltd., Mumbai and Merck India Ltd., Mumbai, India. Growth media, i.e. Nutrient HIVEG Broth w/o NaCl, Agar and Antibiotic discs were procured from HiMedia Laboratories, India. The ATP assay kit was purchased from Calbiochem, EMD Biosciences, USA.

**Bacterial culture and inoculums preparation.** Four avirulent strains (Table 1) were procured from the Culture Collection, Department of Microbiology, College

Table 1. Bacterial strains used in the study.

Bacterial strain	Accession no.	T <sup>a</sup> (°C)
<i>Escherichia coli</i> strain RGR13	DQ 118017	37
<i>Pseudomonas aeruginosa</i> strain PS1	EU741797	28
<i>Bacillus circulans</i> strain D1	EU116046	37
<i>Bacillus aerius</i> strain SPT2	GU598257	37

<sup>a</sup> Optimum temperature for growth.

of Basic Sciences and Humanities, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India. These were used as the test bacteria for the antibacterial efficacy determination of the Ag<sub>2</sub>O nanoparticles. An aliquot of 500 µl was withdrawn from glycerol stocks and the cultures were revived by inoculation into 50 ml flasks containing 10 ml Nutrient HIVEG Broth w/o NaCl and incubated at their optimum pH (7 ± 0.2) and growth temperature (Table 1).

**Agar diffusion assay.** This is a qualitative assay designed to screen and detect the antimicrobial property of inorganic nanomaterials (Negi et al., 2011a). The test bacteria were incubated overnight in 50 ml flasks containing 10 ml Nutrient HIVEG Broth w/o NaCl. A 500 µl bacterial culture containing approximately 50 × 10<sup>7</sup> colony forming units (CFU) was then used to inoculate a 50 ml flask containing 10 ml nutrient HIVEG broth and then incubated for a further 4 h with continuous shaking at 120 rpm at their respective optimal growth temperature to attain test bacterial cells in mid-log phase. This active culture at mid-log phase was found to have approximately 10<sup>7</sup> CFU ml<sup>-1</sup>. Then, 1 ml of this culture was added to 10 ml lukewarm molten soft agar (0.7%) and poured over solidified nutrient HIVEG agar petriplates. This was followed by cutting of uniform cylindrical wells (1 cm diameter) into the solidified agar using a sterilized cork-borer. The wells were then filled with 10, 25 and 50 mg of sterile Ag<sub>2</sub>O nanoparticles. Wells without nanoparticles were used as a negative control. The plates were incubated overnight in an upright position at the respective temperature for each strain. The experiment was conducted in duplicate under the same set of conditions. The Ag<sub>2</sub>O nanoparticles used in the present study were characterized through X-ray diffraction (XRD), Fourier transform infra-red spectroscopy (FT-IR) and simultaneous differential thermal analysis (TG-DTG-DTA) and described in an earlier study by Negi et al. (2011b).

**Comparative inhibition assay of Ag<sub>2</sub>O nanoparticles and antibiotics.** This assay was to compare the anti-

bacterial activity of Ag<sub>2</sub>O nanoparticles and antibiotics against used test bacteria. One milliliter bacterial mid-log phase culture (as mentioned in section *Agar diffusion assay*) was added to 10 ml lukewarm molten soft agar (0.7%) and poured over solidified nutrient HIVE agar petriplates. This was followed by cutting of uniform cylindrical wells (1 cm diameter) into the solidified agar using a sterilized cork-borer. The wells were then filled by respective concentrations of Ag<sub>2</sub>O nanoparticles and the antibiotic discs (sensitive and resistant for the respective strain) were placed on the agar plates which were taken as positive control and negative control, respectively. The plates were incubated overnight in an upright position at the respective temperature for each strain. The experiment was conducted in triplicate under the same set of conditions.

**Microdilution method.** This was a quantitative assay to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ag<sub>2</sub>O nanoparticles against the test bacterial strains. For this purpose, bacteria were incubated with serial twofold dilutions of Ag<sub>2</sub>O nanoparticles. The inoculum used was mid-log phase bacterial culture containing approximately  $50 \times 10^7$  CFU ml<sup>-1</sup>. Nanoparticle-free broth inoculated with culture was used as a positive control. The flasks were incubated at optimum growth temperature of the respective bacterial strain with continuous shaking (120 rpm) for 16 h to allow the direct interaction of nanoparticles with bacteria from their exponential phase onwards. Following incubation, the MBC and MIC corresponding to the doses that inhibited 99.9% and 50% of bacterial growth, respectively, were determined based on the reduction in CFU ml<sup>-1</sup> in comparison to the control, using the serial dilution plating method.

**Cell viability determination using ATP assay kit.** This assay was to measure the dose- and time-dependent toxicity of Ag<sub>2</sub>O nanoparticles against the *E. coli* strain RGR13 by measuring the amount of ATP produced before and after the interaction with the nanoparticles. The Ag<sub>2</sub>O nanoparticles were added in increasing concentrations of 0.010, 0.018 and 0.036 mg/ml to 50 ml Erlenmeyer flasks containing 10 ml nutrient HIVE broth inoculated with approximately  $50 \times 10^7$  CFU ml<sup>-1</sup> bacteria. Nanoparticle-free broth inoculated with culture was used as a reference. The flasks were incubated at their optimum growth temperature with continuous shaking (120 rpm) to allow the direct exposure of nanoparticles to bacteria from the exponential

phase onwards. The aliquots were withdrawn from treatment and reference flasks at specific time intervals of 0, 1, 4, 6, 12, 16, 24 and 36 h. The aliquots are then measured for light generated and quantified as Reflective Light Units (RLU). The RLU corresponds to the ATP production by the metabolically active cells, quantified by the Luciferin-based ATP assay kit (Cat. No. 119107, Calbiochem, EMD Biosciences, USA) and subsequently the process was registered with a Lumimeter (BioTek FLx800). The O.D.<sub>600</sub> value of the aliquots was also measured using a spectrophotometer (Perkin Elmer, USA).

## Results and Discussion

The Ag<sub>2</sub>O nanoparticles used in the present study were characterized through X-ray diffraction (XRD), Fourier transform infra-red spectroscopy (FT-IR) and simultaneous differential thermal analysis (TG-DTG-DTA) and described in an earlier study by Negi et al. (2011b).

### Agar diffusion assay

The results illustrated that the effective antibacterial concentration of Ag<sub>2</sub>O nanoparticles against all the test bacteria is 10 mg/well. It also illustrated that the diameter of the inhibition zone of Ag<sub>2</sub>O nanoparticles increased with the increasing concentrations from 10 to 50 mg per well (Table 2), suggesting the concentration-dependent antibacterial activity of nanoparticles (Negi et al., 2011b; Shamli et al., 2011).

### Comparative inhibition assay of Ag<sub>2</sub>O nanoparticles and antibiotics

The antibacterial effect of the Ag<sub>2</sub>O nanoparticles was compared with the commercially used antibiotics by the zone of bacterial growth inhibition (Fig. 1). Ag<sub>2</sub>O

Table 2. Antibacterial activity of Ag<sub>2</sub>O nanoparticles against two Gram-positive and two Gram-negative bacteria using agar diffusion assay.

Bacterial strains	Inhibition zone dia (mm) at different concentrations of NPs		
	10 mg	25 mg	50 mg
<i>E. coli</i> strain RGR13	7	17	27
<i>P. aeruginosa</i> strain PS1	9	15	25
<i>B. circulans</i> strain D1	7	13	25
<i>B. aerius</i> strain SPT2	7	16	23

nanoparticles showed an antibacterial effect against all the test bacteria while the antibiotic discs were specific to a particular test bacteria. Hence, the nanoparticles showed a broad range of activity against all the test bacteria unlikely to antibiotics, which were group specific (Guzman et al., 2011). Further, metallic nanoparticles, including gold, silver, iron, zinc and metal oxide nanoparticles, have shown great promise in terms of biomedical applications, not only due to their large surface area-to-volume ratio, but also because they exhibit different biomedical activities (Hussain and Ferguson, 2006).

#### Microdilution method

The MIC and MBC of the Ag<sub>2</sub>O nanoparticles was determined against the test bacteria by reduction in their CFU ml<sup>-1</sup> after 16 h of exposure with nanoparticles (Table 2 and Fig. 2). In the earlier study the rate of survival of used Gram-negative bacteria was observed after a direct interaction with Ag<sub>2</sub>O nanoparticles (Negi et al., 2011b). In continuation, the MIC and MBC of Ag<sub>2</sub>O nanoparticles were determined by the microdilution method in virtue of its reproducibility, acceptability and accuracy (Luber et al., 2003). The results elucidated that in the case of Gram-negative bacteria, i.e. *E. coli* strain RGR13 and *P. aeruginosa* strain PS1, the minimum doses required for 50% and 99.9% reduction in a bacterial population were 0.009 and 0.018 mg/ml, respectively. For Gram-positive bacteria, viz. *B. circulans* strain D1 and *B. aerius* strain SPT2, the minimum doses required for the 50% reduction and 99.9% killing of a bacterial population were at 0.018 and 0.036 mg/ml, respectively (Table 3, Fig. 2). Based on the reduction in the CFU ml<sup>-1</sup> the MIC and MBC of Ag<sub>2</sub>O nanoparticles for respective test bacteria were

calculated (Table 3). It was observed that Ag<sub>2</sub>O nanoparticles were required in higher dose to inhibit Gram-positive bacteria in comparison to the Gram-negative (Table 3), and it may be because of the thick peptidoglycan layer of Gram-positive bacteria (Sinha et al., 2011).

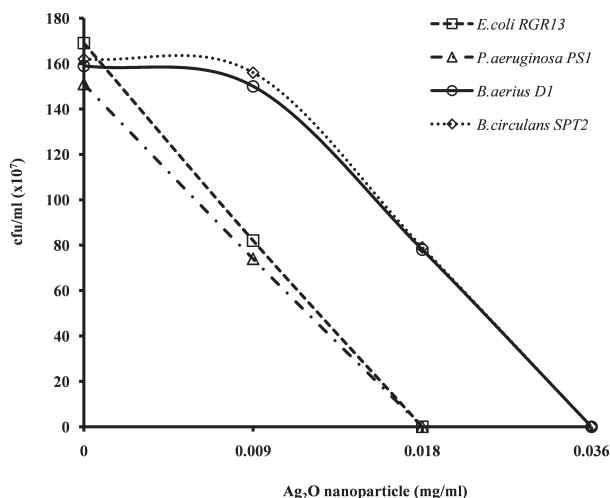


Fig. 2. Concentration-dependent antibacterial activity of Ag<sub>2</sub>O nanoparticles against used bacterial strains.

Table 3. Determination of Minimum Inhibitory Concentration (MIC) and Maximum Bactericidal Concentration (MBC) of Ag<sub>2</sub>O nanoparticles using a microdilution method against used bacterial strains.

Bacterial strains	MIC (mg/ml)	MBC (mg/ml)
<i>E. coli</i> strain RGR13	0.009	0.018
<i>P. aeruginosa</i> strain PS1		
<i>B. circulans</i> strain D1	0.018	0.036
<i>B. aerius</i> strain SPT2		

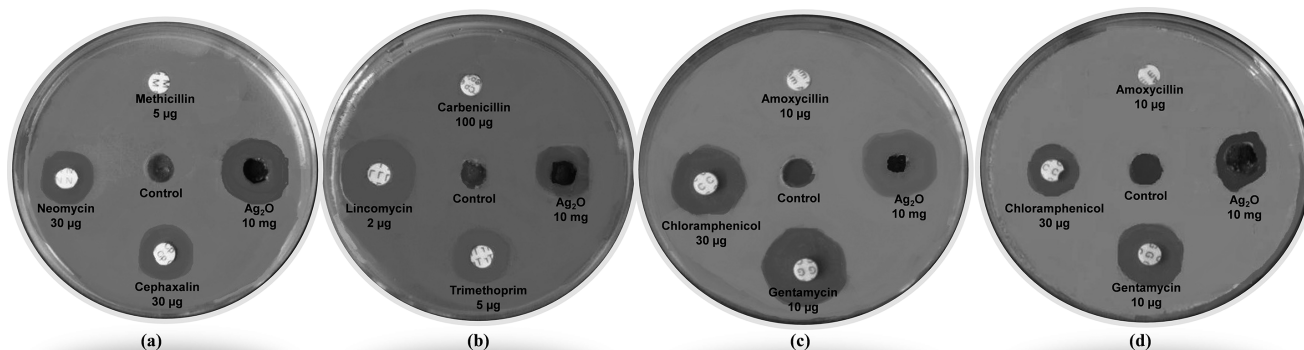


Fig. 1. Comparative inhibitory effect of antibiotics and Ag<sub>2</sub>O nanoparticles against (a) *E. coli* strain RGR13, (b) *P. aeruginosa* strain PS1, (c) *B. circulans* strain D1 and (d) *B. aerius* strain SPT2, respectively.



### Cell viability determination using ATP assay kit

The minimum effective dose of Ag<sub>2</sub>O nanoparticles and the interaction time required to inhibit about 99% bacteria was analyzed by observing the in dose- and time-dependent toxicity against *E. coli* strain RGR13 (Fig. 3). At 0.036 mg/ml, Ag<sub>2</sub>O nanoparticles show a 50% reduction in the bacterial RLU and ATP within 1 h of exposure, with about a 20% reduction at 0.018 mg/ml within the same time interval. Similarly, the O.D.<sub>600</sub> was also reduced after the exposure of nanoparticles (data not shown). Moreover, after the 24 h of incubation, almost complete reduction was observed in the ATP level at both doses tested, i.e. 0.018 and 0.036 mg/ml. On the other hand, there was a considerable increase in the ATP level at 0.010 mg/ml, up to 16 h of incubation (Fig. 3). Thus, the antibacterial activity of Ag<sub>2</sub>O nanoparticles was dose- and time-dependent, the higher dose being more effective in comparatively

smaller time interval (Fig. 3). The results illustrated the minimum time required to exhibit the bactericidal activity against *E. coli* at a low concentration of Ag<sub>2</sub>O nanoparticles is 24 h.

Moreover, the literature survey revealed that the nanomaterials are also used as molecular research tools and drugs for targeting antibodies. The paramagnetic nanoparticles, quantum dots, nanoshells and nanosomes are few of the nanoparticles used for diagnostic purposes. Improved methods of diagnosis and treatment of various diseases, especially cancer detection based on nanoparticles are being developed (Surendiran et al., 2009). A nanomedicine in the future will play a crucial role in the treatment of human diseases and also in enhancement of normal human physiology (Burgess, 2009). The safety of nanomedicine is not yet fully defined (Lewinski et al., 2008), as some inorganic nanoparticles absorbed and metabolized in soft tissue and may cause neurotoxic damage (Panyala et al., 2008). Results from ecotoxicological studies show that certain nanoparticles have effects on organisms under environmental conditions, but mostly at elevated concentrations (Nowack and Bucheli, 2007).

In conclusion, the present study illustrated the immense potential and toxicity of Ag<sub>2</sub>O nanoparticles against both Gram-positive and Gram-negative bacteria. By the virtue of their small size and large surface-to-volume ratio, they may interact closely with the bacterial cell, and require only a low concentration to inhibit bacteria (Neal, 2008; Shrivastava et al., 2007; Singh et al., 2009). Overall, this study paves the way for the use of Ag<sub>2</sub>O nanoparticles as an antibacterial agent to control the emerging pathogens of antibiotic resistance.

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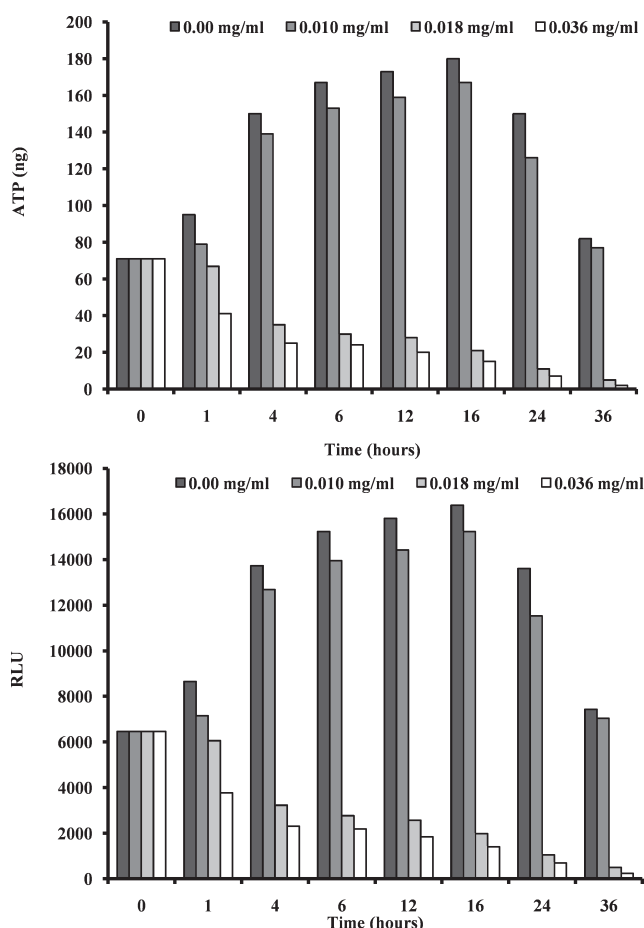


Fig. 3. Concentration- and time-dependent antibacterial activity of Ag<sub>2</sub>O nanoparticles against *E. coli* strain RGR13 as quantified in ATP (ng) (a), and Relative Light Units (RLU) (b), respectively.

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