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Synthesis and evaluation of bactericidal properties of Ag_2O nanoparticles against *Aeromonas hydrophila*

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ABSTRACT

The nanomaterials have important application in different field of science such as biology and pharmacology, which draws the attention of biologists towards this field of study more than before. As the worldwide mortality rate is high due to the pathogens and especially because of the bacteria associated *Aeromonas* and the antibacterial effect of metal nanoparticles is well known for centuries, these materials can be used to annihilate *Aeromonas hydrophila*. In this study, silver oxide nanoparticles were synthesized by sol-gel procedure and antibacterial activity of silver oxide nanoparticles as a function of particle concentration against gram-negative bacterium *Aeromonas hydrophila* ATCC 7966T was carried out in liquid as well as solid growth media. Synthesized Ag_2O nanoparticles (NPs) were characterized by X-ray diffraction (XRD), scanning electron micrograph (SEM) and transmission electron microscopy (TEM). The average size of the Ag_2O NPs determined through transmission electron microscopy (TEM) 15 nm. The bactericidal effect of silver oxide nanoparticles was compared based on the diameter of inhibition zone in well diffusion tests in nutrient culture media. Minimum bactericidal concentration (MBC) of nanoparticles dispersed in peptone water, liquid cultures in 22-25 ºC for 24 h were determined.

Keywords: *Ag_2O nanoparticle; Aeromonas hydrophila; Synthesis; Bactericidal effects; Pathogens.*

INTRODUCTION

The unique, unusual and interesting physical, chemical, and biological properties of nanometer sized materials have recently attracted a great deal of interest in the scientific community. As the size of materials is reduced to the nanometer regime the resulting properties change noticeably. Considerable efforts have been made to characterize and describe the physical and chemical properties of metal oxide nanomaterials because of their significant applications in numerous technological fields [1–4].

The oxides of transition metals are an important class of semiconductors that has wider applications in magnetic storage media, solar energy transformation, electronics, and catalysis [5–12]. Ag_2O particles are commonly used as water cleaning agent, colorant and catalyst. Because of the mild catalytic reaction condition, high catalytic activity, selectivity and production yield, Ag_2O particles become one of the most popular catalysts in organic reactions in recent years [13]. There are a few reports on the synthesis of Ag_2O nanoparticles with special morphology, for example, Murray et al. [14] synthesized flower-like Ag_2O nanoparticles by an electrochemistry method, but the preparation process was somewhat complex. Motile *Aeromonas* of the *Aeromonas hydrophila* complex cause a hemorrhagic septicemia in numerous species of cultured and wild freshwater fish such as rainbow trout, brown trout, Coho salmon, eels, carp, channel catfish, tilapia, ayu and goldfish. Although classically three species, *A. hydrophila*, *A. sobria* and *A. caviae*, were included within the motile *Aeromonas*, further taxonomic data, including genetic studies allowed the identification of at least 10 new motile *Aeromonas* species.

However, still *A. hydrophila* is regarded as the predominant fish pathogen, although its importance may have been overestimated in the past. Isolates differ greatly in their pathogenicity with some strains being highly virulent and others non-virulent. Although motile *Aeromonas* species are typically recognized as opportunistic pathogens or secondary invaders, cases have been reported of *A. hydrophila* acting as a primary fish pathogen. In fact, *A. hydrophila* is widely distributed in the intestinal tract of fish as well as in the water and sediment of freshwater ponds, which are rich in organic matter. Virulent strains of *A. hydrophila* in these environments are a possible source of infection [15-21].

In the current work, we wish to synthesis, characterization and evaluation of silver oxide nanoparticles as a new class drug against Gram-negative bacteria, *Aeromonas hydrophila* ATCC 7966T, and compare the effects with Tetracycline as a reference antibacterial drug.

EXPERIMENTAL

Chemicals

All chemicals used in the experiment were analytical reagent grade. Silver nitrate ($AgNO_3$, 99.99%), Sodium hydroxide, NaOH, (pellets) were purchased from Merck, Germany. De-ionized water is used throughout the experiment. Nutrient agar and peptone water medium were purchased from E. Merck Co; Darmstadt Germany. Tetracycline antibiotic was purchased from Fluka Germany.

Synthesis of Ag_2O NPs

All reagents were purchased from Merck Germany and used without further purification. The reactions were carried out under an atmosphere of air. In a typical synthesis procedure, $AgNO_3$ was dissolved in de-ionized water. The solution was stirred with a magnetic stirrer at 100°C. About 0.8 g of NaOH was added to solution till pH reaches to 8. With increase pH to 8 large amounts of AgOH precipitate was formed immediately. The precipitate was filtered and washed 4 times with de-ionized water. The obtained precipitate was dried in air for 24 h. Then, powders were annealed for 1 hour at a temperature of 400 °C, to obtain the highly crystalline Ag_2O NPs.

Characterization of Ag_2O NPs

Synthesized Ag_2O NPs were characterized by X-ray diffraction (XRD), scanning electron micrograph (SEM) and transmission electron microscopy (TEM). Crystalline, structure, and crystallite size of Ag_2O NPs was determined by XRD technique using a Rigaku-Miniflex X-ray diffractometer (Rigaku Corporation, Tokyo, Japan) with Cu-Kα radiations (λ = 0.15406 nm). TEM analysis was carried out using a 200 kV JEOL transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Disk diffusion test

Antimicrobial activity of the synthesized Ag_2O NPs was determined using Gram-negative bacteria (*Aeromonas hydrophila* ATCC 7966T) following a modified Kirby Bauer disc diffusion method [22]. A lawn of bacterial culture was prepared by spreading 100 µl culture broth, having1.5×108 CFU/ml of test organism on solid nutrient agar plates. The plates were allowed to

stand for 10–15 minutes, to allow for culture absorption. After culture, four wells were made at equal distance by using a sterile steel cork borer (8mm diameter). Wells were sealed with 1 ml of molten agar (0.8% nutrient agar) to prevent leakage from the bottom of the plate. The bacteria were plated onto solid nutrient agar plates. Using a micropipette, 20-100 % (V/V) of 100 µl (50 µg) of the nanoparticles solution sample was poured into each of four wells on all plates. After incubation at 22-25± 2 °C for 24 hours, the size of the zone of inhibition was measured with a ruler with up to1 mm resolution. Each experiment was repeated three times, and the resulting bacterial growth on three plates corresponding to a particular sample were averaged and reported ($p<0.05$). A solvent blank was run as a negative control, whereas the antibiotic (tetracycline) was used as a positive control.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC), defined as the lowest concentration of material that inhibits the growth of an organism [23], was determined based on batch cultures containing varying concentrations of silver oxide nanoparticles in suspension (0–300 µg/ml). Sterile Erlenmeyer flasks (500 ml), each, containing 100 ml peptone water medium were sonicated for 10 min after adding the nanoparticles to prevent aggregation of the nanoparticles. Subsequently, the flasks were inoculated with 1 ml of the freshly prepared bacterial suspension in order to maintain initial bacterial concentration 1.5×108 CFU /ml. Bacterial growth was measured as increase in absorbance at 625 nm determined using a spectrophotometer (Thermo Spectronic, Helios Epsilon, USA). The experiments also included a positive control (flask containing nanoparticles and peptone water medium, devoid of inoculums) and a negative control (flask containing inoculums and peptone water medium, devoid of nanoparticles). The negative controls indicated the microbial growth profile in the absence of nanoparticles. The absorbance values for positive controls were subtracted from the experimental values (flasks containing peptone water media, inoculums and nanoparticles). All the experiments were carried out in triplicate. Silver oxide nanoparticles were tested for the bactericidal effect using the microbial culture selected for the study. The minimum bactericidal concentration (MBC) [23], the lowest concentration of nanoparticles that kills 99.9% of the bacteria was also determined from the batch culture studies. For growth inhibitory concentration (MIC) the presence of viable microorganisms was tested and the lowest concentration causing bactericidal effect was reported as MBC.

RESULTS AND DISCUSSION

XRD, SEM and TEM study

Figure 1 shows the SEM image of prepared Ag_2O NPs. It shows that the Ag_2O NPs are in spherical shape. XRD pattern of silver nanoparticles produced by sol-gel technique is shown in Figure 2. All diffraction peaks correspond to the characteristic face centered cubic (FCC) silver lines. XRD patterns were analyzed to determine peak intensity, position and width. Full width at half-maximum (FWHM) data was used with the Scherer's formula to determine mean particle size. Scherer's equation is given by

$$d = \frac{0.9\lambda}{\beta \cos\theta}$$

Where d is the mean diameter of the nanoparticles, λ is wavelength of X-ray radiation source, β is the angular FWHM of the XRD peak at the diffraction angle θ [24]. The mean size of nanoparticles estimated by XRD is 15 nm.

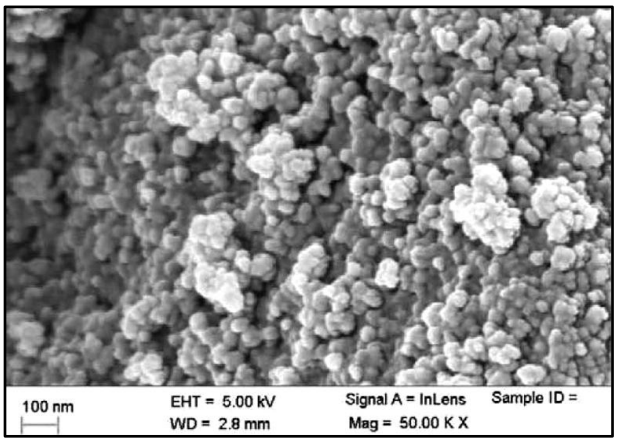

Fig. 1. Scanning electron micrograph (SEM) of Silver Oxide (Ag_2O) nanoparticles.

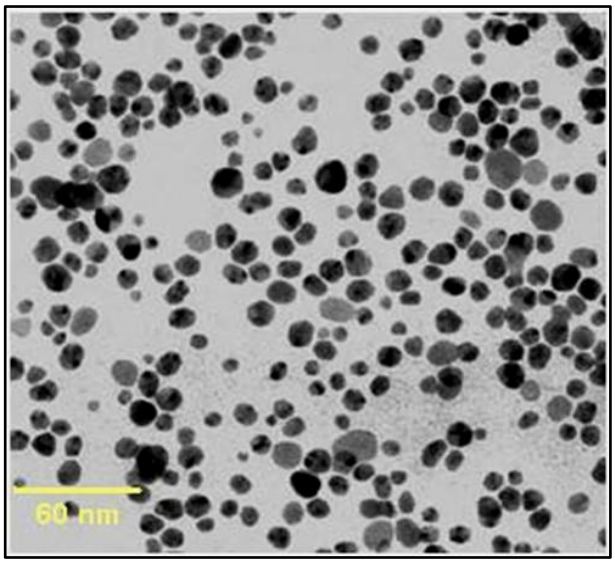

Fig. 2. TEM image of prepared Ag_2O nanoparticles.

TEM micrograph of silver oxide nanoparticles is shown in Figure 3, which demonstrates a spherical shape and narrow particle size distribution. The mean particle size estimated by TEM is 16 nm, which compares well with the size estimated from XRD data.

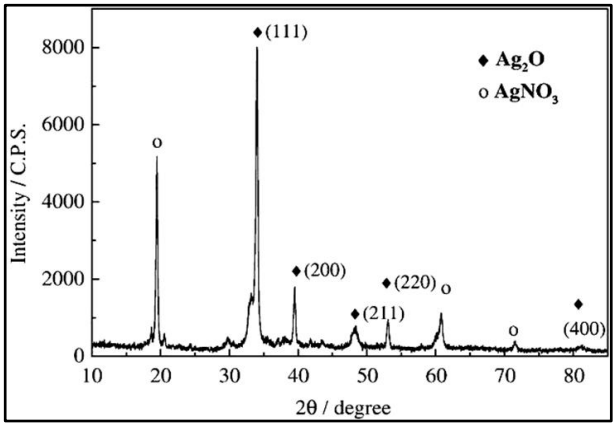

Fig. 3. XRD pattern of Ag_2O nanoparticles.

Antimicrobial properties

In batch studies, a greater lag phase and lower maximum absorbance (at 625 nm) were observed as the concentration of nanoparticles increased. As the concentration of nanoparticles increased to MIC of the respective strain, no growth was observed in the flask. The bactericidal effect of nanoparticles is dependent on the concentration of nanoparticles and the initial bacterial concentration [25]. In this study, the initial bacterial concentration was almost constant at 1.5×108 CFU /ml irrespective of nanoparticles concentration and microbial strain. The MIC observed in this study for Silver oxide nanoparticles is 80 µg/ml for *Aeromonas hydrophila* ATCC 7966T and MBC value for Ag_2O NPs is 300 µg/ml (Table 1).

Table 1. The diameter of inhibition zone (DIZ) and MBC of silver oxide nanoparticles (annealed at 400 °C temperature) against *Aeromonashydrophila*

Ag_2O Nanoparticles Concentration (µg/ml)	DIZ (mm)	MBC (Log CFU/ml)
Negative Control[a]	0	6.7±0.0
60	2	5.7±0.0
120	6	3.1±0.0
180	7	1.1±0.0
240	9	NCD[b]
300	9	NCD[b]

[a] Negative Control: flask containing inoculum and peptone water medium, devoid of nanoparticles
[b] NCD = No culturable cells detected

Figure 4 (4a, 4b and 4c) exhibit the zone of inhibition of Ag_2O NPs synthesized and positive control, a known antibiotic tetracycline, against Gram-negative bacteria (*Aeromonas hydrophila*).

We have successfully synthesized Ag_2O NPs using the aqueous precipitation method. XRD spectra confirmed the formation of single phase Ag_2O NPs. From SEM and TEM study, it is found that particles are spherical in shape with average size of 15 nm. TEM results corroborate well with XRD results. The silver oxide nanoparticles showed remarkable antibacterial activity against *Aeromonas hydrophila* as Gram-negative bacteria. The antimicrobial effects of Ag_2O NPs, which have been used widely in antimicrobial applications, are attributed to electrical changes that occur during their interactions with bacterial membranes. These changes are thought to contribute to the enhancement of reactivity of silver nanoparticles surfaces [26].

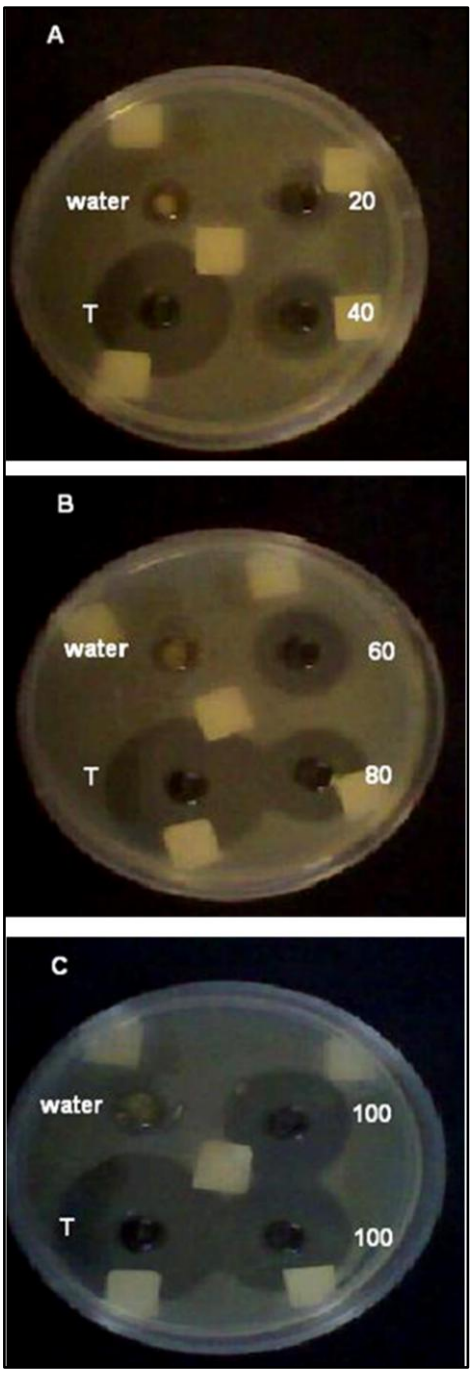

Fig. 4. Theinhibition zone of Silver Oxide nanoparticles in various concentrations (a, b and c) against *Aeromonas hydrophila* ATCC 7966T (A-C).

As well as this, the stability of metal nanoparticles in culture conditions and their properties of bringing effective for a long time without decomposition; improve their in vitro bactericidal efficacy. The penetration mechanism of Ag_2O NPs has not been clearly understood. Nevertheless, when *Escherichia coli* was exposed to these nanoparticles, owing to the disruption of the transport mechanism, morphological changes in bacterial membrane and a considerable increase in membrane permeability were observed [27]. Ag_2O NPs that penetrate into bacteria are believed to interact strongly with molecules containing sulfur and phosphorus groups like DNA [28]. It has been suggested that DNA loses its replication ability when bacteria are exposed to Ag_2O NPs. The cell cycle halts at the G2/M phase owing to the DNA damage [29, 30]. The cells get affected by oxidative stress, which is caused by inhibition of ATP synthesis and occurrence of reactive oxygen species (ROS). As a consequence, apoptosis are included [31]. Another reason for bacterial cell death after the exposure to Ag_2O NPs may be the release of silver ions from the nanoparticles. It is believed that after the penetration, Ag_2O NPs make the bacterial enzymes inactive by releasing atomic Ag° and ionic Ag^{+} clusters, and cause cell death by producing hydrogen peroxide and the other free radicals [32, 33]. Gram-negative bacteria like *Aeromonas hydrophila* have a special cell membrane structure which possesses an important ability to resist antimicrobial agents [34].

CONCLUSIONS

This study reports the successful synthesis of silver oxide nanoparticles. The antimicrobial screening studies were also performed in the study. The antimicrobial screening suggests that synthesized Ag_2O NPs exhibited moderate activity toward *Aeromonas hydrophila*. One unique observation was that Ag_2O NPs synthesized at 400 °C demonstrated the maximum zone of inhibition in the case of *Aeromonas hydrophila*.

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