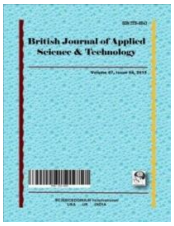

British Journal of Applied Science & Technology
15(6): 1-7, 2016, Article no.BJAST.25686
ISSN: 2231-0843, NLM ID: 101664541

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Silver Nanoparticles as Inhibition Agents of Cooling Tower Microorganisms

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAQ discussed microbiological results and wrote the manuscript. Author JJVD managed the analyses and literature searches. Author MORG designed the microbiological experiments and discussed data. Author ICA conducted the microscopy analyses and discussed data. Author GGCA analyzed results, managed literature searches and wrote the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJAST/2016/25686
Editor(s):
(1) Manoj Gupta, Department of Mechanical Engineering, NUS, 9 Engineering Drive 1, Singapore 117576, Singapore.
Reviewers:
(1) Erwan Rauwel, Tallinn University of Technology, Estonia.
(2) R. S. Rimal Isaac, Noorul Islam Centre for Higher Education, India.
(3) Aline Elesbão Do Nascimento, Universidade Católica De Pernambuco, Brazil.
Complete Peer review History: http://sciencedomain.org/review-history/14383

Original Research Article

Received 15th March 2016
Accepted 20th April 2016
Published 28th April 2016

ABSTRACT

Silver nanoparticles are widely studied and the antibacterial activity is well-known. Although several methods of synthesis have been described, this article reports the simplest procedure using low cost reagents and a low consumption of energy, where silver nitrate is reduced by nitrate ions in an

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aqueous solution from pH 10.60 to 11.02. A relatively controlled dispersion of sizes was achieved and particles presented sizes between 49 and 76 nm dependent on the pH.
Solutions of these particles were used to incubate a mixture of *Pseudomona* spp. ATCC and *Pseudomona* spp. recovered from a water well. All the particles were affective against inhibition of these bacteria after 3 hours of incubation. Specifically, 49 nm particles reached suitable efficiency after 120 minutes even if they were diluted 50 or 10%, whereas particles with 65 nm demonstrated having an efficient inhibitory effect on *Pseudomona* spp. at 10 or 100% of concentration after 140 minutes of incubation. Results obtained with both sizes proved that the contact time (140 minutes) to reduce the colony forming units (CFU) per milliliter ($x10^5$) to values lower than 1 is an adequate time for applications in water treatments of cooling towers. Additionally, the procedure here followed with low cost and low energy, facilitates the design of advanced antimicrobial treatments for cooling towers at industrial levels.

Keywords: Silver; nanoparticles; cooling tower; Pseudomona; biocide.

1. INTRODUCTION

Cooling towers are semi-open systems where water continuously circulates and some amount of mass is lost by evaporation, therefore, water must be fed to recover this loss of mass.

Some problems in cooling towers are associated to water, such as corrosion and growth of biofilm (i.e. microorganisms). Biofilms are formed because cooling towers are open circuits permanently exposed to microorganisms like algae, fungi and bacteria, which are transported through the water stream during the cooling process [1,2] and this produces a technical problem because microorganisms could form a bio film capable of retaining organic matter and inorganic powders (like sand and dust) from the atmosphere, thus limiting the flow rate of circulating water or reducing the heat transfer from the metallic tubes to the water due to the isolating characteristic of biofilms [2-4].

On the other hand, the large volume of water flowing is in contact with the air and the temperature of water is maintained between 25°C and 35°C. This provides a suitable environment to promote the fast growth of microorganisms, which receive nutrients from the atmosphere [3]. Among the large list of microorganisms detected in cooling towers, *Pseudomonas* spp., is a human pathogen group. This bacteria is responsible for producing diseases and is considered to be a public health problem. In cooling towers, *Pseudomonas* spp. has been clearly identified [4].

In order to control microbial action in cooling towers, biocide agents are added to water and these are classified as oxidizing and non-oxidizing biocides [5]. The former oxidize organic matter like cell walls, enzymes and proteins in relatively low concentrations (representatives of oxidizing agents include chlorine gas, calcium hypochlorite, bromide and potassium permanganate), however, these biocides produce side effects in the towers such as corrosion and the effectiveness have a dependence on the pH [6]. On the other side, the non-oxidizing agents interfere in cell metabolism pathways. Examples of these products are pentachlorophenate, sodium hypochlorite, copper salts and quaternary ammonium compounds. Generally, high concentrations of these agents are needed to eliminate microorganisms [6], thus environmental side effects are also involved. In several cases, a special combination of biocides must be considered in water treatments of cooling towers, but significant issues to take into account are the environmental risks as well as the toxicity for workers handling these compounds. Besides that, the evaluation of costs is required. Therefore alternative biocides overcoming these problems are needed.

Despite the fact that silver was empirically used as a water purifier by Greek civilizations [7], the first scientific report demonstrating the antiseptic effect was Crédé in 1881 [8]. Recent investigations revealed that silver possesses a biocide effect, which is increased when dimensions of the particles are in the nanometric scale due to the large surface/volume ratio. Furthermore, the biocide effect of the metallic form found in nanoparticles is more efficient than that of the cationic (Ag^+) form [7,9]. This biocide effect covers gram positive and negative bacteria, as well as viruses [8].

The aim of this work was to prepare silver nanoparticles with uniform size and to evaluate the efficiency for controlling the growth of *Pseudomona* spp., which is of interest for cooling towers maintenance.

2. MATERIALS AND METHODS

2.1 Synthesis of Silver Nanoparticles

Analytical grade reagents were used without special purification to prepare the following solutions: $6x10^{-4}$ M ascorbic acid, $3x10^{-3}$ M potassium citrate, 0.10 M sodium hydroxide and 0.10 M silver nitrate.

Silver nanoparticles were prepared by reducing silver nitrate with ascorbic acid in an aqueous medium [10] where citrate ions were added as passivating agents as follows: 50 mL of an ascorbic acid solution was mixed with 50 mL of potassium citrate, and sodium hydroxide was added to adjust the pH value between 10 and 11. The resulting solution was heated in a water bath to keep the temperature at 35°C, and then, 1.0 mL of silver nitrate was added at the rate of one drop per minute. Finally the mixture was kept at 35°C for 15 minutes under stirring. Thereafter, the resulting colored solutions were stored in dark sealed plastic flasks.

In order to verify changes influenced by pH, solutions were adjusted to pH values equal to 10.60, 10.65, 10.71, 10.82 and 11.02. These values were used as labels to discuss experimental data.

Characterization of nanoparticles was conducted by UV-vis spectroscopy and dynamic light scattering (DLS), by transferring silver nanoparticles solutions to the quartz cell of the ThermoScientific UV-Vis spectrometer, model GENESYS, and the Malvern equipment, model Zetasizer Nano ZS. Transmission electron microscopy (TEM) images were obtained with a JEOL JEM1010 microscope, and the powder XRD pattern of the particles was obtained in a PANalytical Empyrean difractometer. The sample for XRD was prepared by evaporating 50 mL of nanoparticles solution at 60°C until the volume decreased to approximately 2 mL, then three drops of the concentrated solution were deposited onto a glass slide for complete evaporation, and this process was repeated until the nanoparticles solution was over. The resulting thin film was then introduced in the difractometer.

2.2 Biocide Assays

Strains of *Pseudomona* spp. from ATCC and *Pseudomona* spp. extracted from from well water were grown separately in tryptic soy broth (TSB; BD) at 37°C from 14 to 18 h. Thereafter, 100 µL of each culture was transferred to screw-cap tubes containing 10 mL of TSB, incubated at 37°C for 4 to 6 hours and then transferred again to a fresh tube with TSB, and incubated at 37°C for 16 to 18 h. These cultures were then concentrated by centrifuging at 4°C at 4,506.6 g for 10 min by using a refrigerated centrifuge. The pellet was resuspended in 1 mL of TSB. These suspensions were used to prepare one inoculum with a mixture of both *Pseudomona* spp. strains.

To examine the bactericidal effect of silver nanoparticles on gram negative bacteria, approximately 3×10^9 colony forming units per mL (CFU/ mL) of a mixture of *Pseudomona* spp. strains were used to analyze different concentrations of silver nanoparticles freshly synthesized the same day when the antibacterial analyses were conducted. The analyses started with 1 mL of the mixed bacterial suspension inoculated into silver nanoparticles solution (10 mL) at concentrations of 10, 50, 100% and incubated at room temperature between 0 to 140 min. The negative control was prepared with the bacterial mixture incubated in phosphate saline buffer. After 1, 30, 60, 80, 100, 120 and 140 minutes of incubation 100 µL of suspensions were plated on tripticase soy agar and grown at 37°C between 14 and 18 h, then the numbers of colonies were counted.

3. RESULTS AND DISCUSSION

3.1 Nanoparticles Characterization

Solutions of silver NPs presented higher turbidity and darker color at lower pH values, whereas the solution prepared at the higher value (pH = 11.02) resulted in a translucent dark yellow solution (Fig. 1). The nominal content of silver in these solutions was calculated by relating the mass of silver in the $AgNO_3$ solution and final volume of the reaction solution. This value is 0.108 mg/mL and this content is also referred to as 100% in the antibacterial assays.

UV-vis spectra of these solutions are depicted in Fig. 1. A clear and intense absorption band in the visible region is detected. This signal is well-known and undoubtedly associated to the

surface plasmon resonance (SPR) of silver nanoparticles, which is dependent on the size and morphology [11]. For particles with spheroid shape, the visible absorption is near 414 nm, as it occurs in the spectrum here obtained at pH 11.02. Conversely, nanoparticles with cubic or pyramidal shapes absorb visible light at higher values and the absorption is higher for more defined (sharper) edges [11]. In our experiment, reactions between pH 10.60 and 10.82 absorb between 424 and 437 nm (see Fig. 1 and Table 1).

Although the increment in the wavelength can be associated to higher particle diameter [11,12] or to higher elongation (i.e. increase in the length to diameter ratio) [13], edges also promote this shift. In order to identify if the SPR shift from 414 nm to the 424 and 437 nm range is due to size changes or appearance of edges, a particle size analysis was conducted and revealed that diameters are between 49 and 76 nm (see Fig. 2 and Table 1).

Table 1. Relationship between pH of synthesis, maximum absorption wavelength and size of silver nanoparticles

pH	Maximum absorbance peak (nm)	Diameter (nm)	Percentage (%)
10.60	432	64	87
10.65	437	76	84
10.71	421	49	87
10.82	424	65	93
11.02	414	64	85

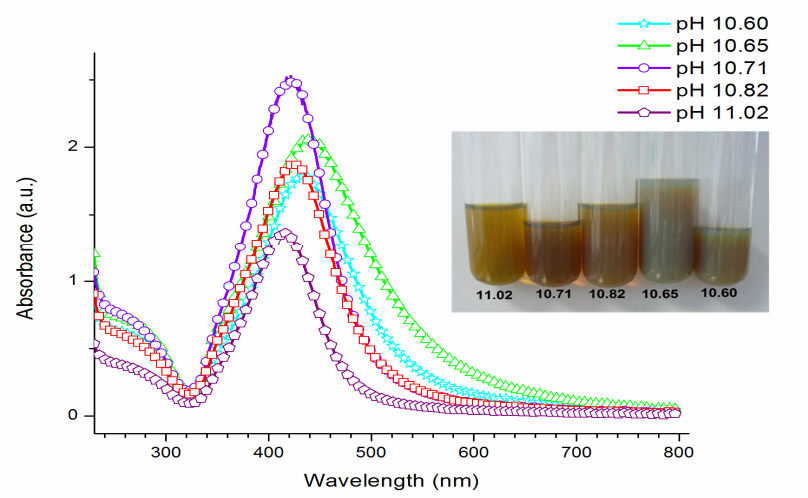

Fig. 1. UV-vis spectra of silver nanoparticle solutions obtained at different pH values. The inset shows the typical color of these solutions

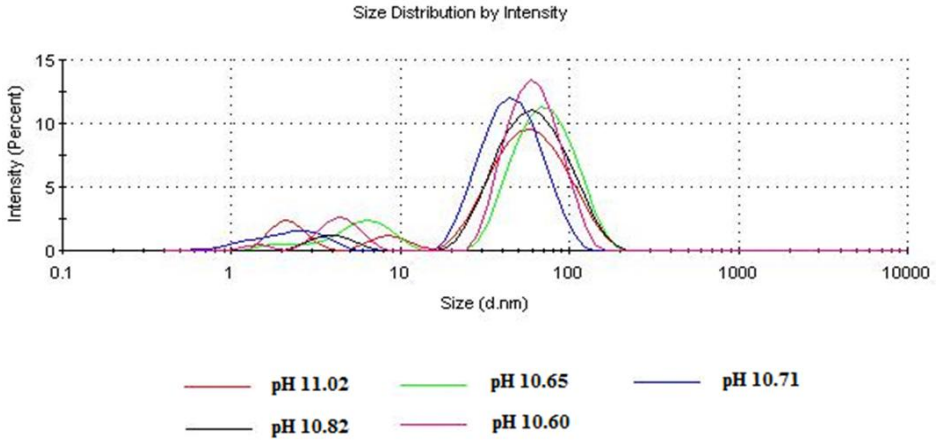

Fig. 2. Size distribution of silver nanoparticles synthesized at different pH values

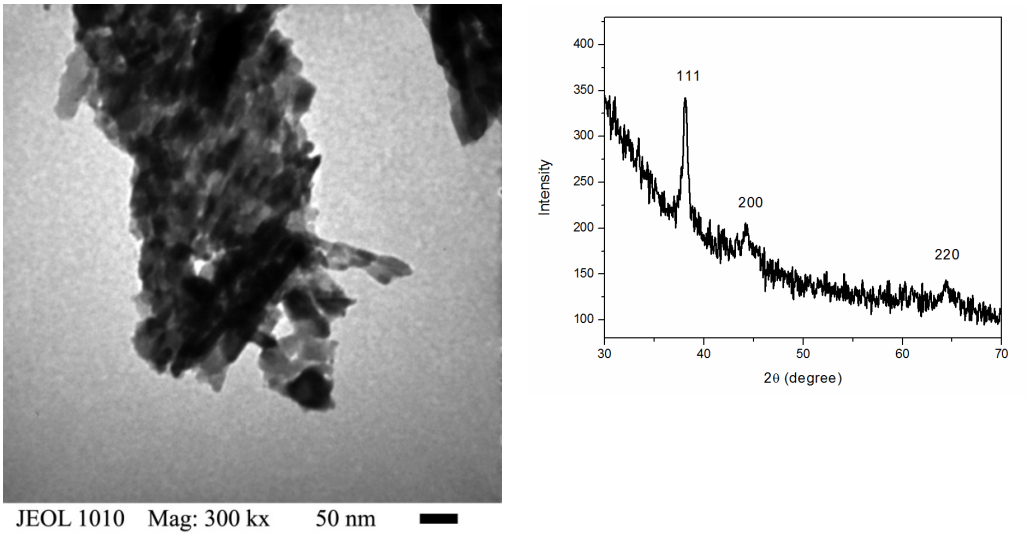

Fig. 3. TEM micrograph and powder X-ray diffraction pattern of silver nanoparticles synthesized at pH= 10.65

If the particle size caused the SPR to shift to the lowest absorption wavelength in the sample prepared at pH = 11.02, the particle size should be the lowest, however, this sample contains particles with 64 nm in size, which are larger than those prepared at pH 10.71 (with lower particle size: 49 nm); furthermore, this size is similar to the samples prepared at pH 10.60 and 10.82.

Then, the appearance or definition of edges in silver nanoparticles could be the reason to promote the shift to the 424 and 437 nm range. To clarify this, the sample with the highest SPR (at 437 nm) was analyzed by TEM. The micrograph of this sample (Fig. 3) presents aggregates of particles, which have elongated morphology and defined edges, and this can contribute to increasing the SPR wavelength. Additionally, the length to diameter ratio is larger than 2, and this elongation could also contribute to increasing the absorption wavelength. On the other hand, the X-ray diffraction analysis indicated that the obtained pattern is in agreement with a silver lattice recorded in the International Centre for Diffraction Data (ICDD) card number 04-0783 (Fig. 3). The noisy profile appeared due to the low content of nanoparticles and the intense background was produced by the amorphous glass sample support.

The synthesis here reported seems to be an adequate way for a large scale production of silver nanoparticles in terms of cost, effectiveness (demonstrated in the next section) and the low toxicity of reagents employed. A current trend to eliminate toxicity in nanoparticles production processes is green chemistry, however, the production times are large, for example, using plants as nanoparticles producers, common times are between 24 and 124 hours, while our method takes 20 minutes [14]. Additionally, green synthesis employing fungi, bacteria or plants does not finely control shape and size distribution as demonstrated by some authors who produced 2 nm particles with alfalfa or yeast, or 200 nm particles with *Pseudomonas stutzeri* AG259. Despite mean sizes obtained with our method range from 49 to 76 nm, dispersion is acceptable since the largest population percentage in the highest Gaussian curves in Fig. 2 are between 84 and 93% as listed in Table 1.

3.2 Biocide Assays

Antibacterial tests were performed against the gram negative bacterium *Pseudomona* spp. on tripticase soy agar plates after their incubation in different concentrations of silver nanoparticles. Fig. 4 shows the number of bacterial colonies grown on in the agar plates as a function of concentration and the size (related to the synthesis pH) of silver nanoparticles.

A previous experiment using solutions with 100% of nanoparticles (equal to 0.108 mg/mL) with size of 65 nm (prepared at pH = 10.82) provoked total inhibition of bacterial growth at 3 hours of incubation. This effect was detected even when

nanoparticles were diluted to 50 or 10% -due to this, the subsequent experiments were conducted at lower periods of incubation as recorded in Fig. 4, additionally, considering that the PBS negative control did not affect the bacterial growth after 3 hours of incubation, the antibacterial activity can be associated directly to silver nanoparticles.

The antibacterial effect from silver nanoparticles has been related with their size [15], in this way small nanoparticles would have more efficient antibacterial activity, therefore the nanoparticles selected for this assessment were those with sizes near to 65 nm (obtained at pH 10.60, 10.82 and 11.02 as presented in Table 1) and 49 nm (obtained at pH = 10.71).

Results in Fig. 4 showed that 1 hour of incubation significantly reduces the growth with 49 nm particles, whereas those of 65 nm has a lower effect. Solutions with 49 nm particles are generally the best with concentrations of 100 and 50%, however, after 120 minutes the 10% solution present the same observed at 50 and 100%. This is an important result considering that at an industrial level, if these particles were applied in cooling towers, the economic factor determines the usability. Regarding particles with 65 nm in size, a significant effect was observed after 100 minutes with 50% concentration, and the 10% concentration is only effective after 140 minutes.

The overall observation is that the smaller particles presented higher inhibition efficiency in a shorter time, however, the 65 nm particles—the larger particles studied—presented suitable antibacterial activity after 140 minutes, which is a suitable residence time for particles in cooling towers; additionally, this size can be obtained at diverse pH values as presented in Table 1. Then, particles prepared at any pH between 10.60 and 11.02 are capable to act as efficient biocide after 140 minutes at any concentration between 10 and 100%.

Another fact indicating viability to use silver nanoparticles in cooling towers is the stability of suspensions, at least, the stored suspensions do not precipitate after six months. However, it is recommended to study the effect of more realistic working conditions on silver nanoparticles before a final application. For example, the drastic temperature changes of cooling water, adhesion of nanoparticles on tower inner walls and removal of nanoparticles after the life-time.

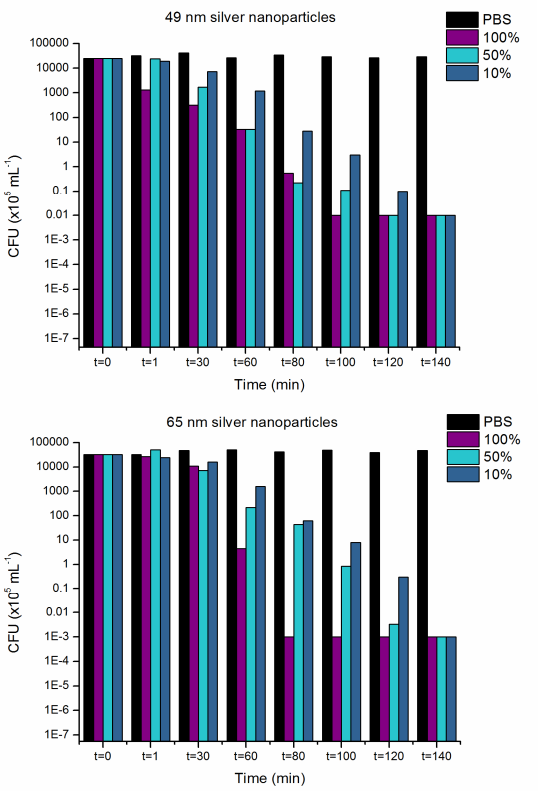

Fig. 4. Colony forming units counted as function of mean nanoparticles size, concentration and incubation time

4. CONCLUSION

Reduction of silver nitrate by citrate in aqueous solution between pH 10.60 and 11.02 produced particles ranging from 49 to 76 nm. The most recurrent size obtained in our experiment is near 65 nm; these particles demonstrated to have an efficient inhibitory effect on *Pseudomona* spp. at 10 or 100% of concentration after 140 minutes of incubation. Although particles with lower size reach a higher efficiency in a shorter time (120 minutes), the time observed for 65 nm particles is more than enough for applications as inhibitory agents of *Pseudomona* spp. in cooling towers. Therefore, the easy and fast synthesis of silver nanoparticles reported here is suitable to design advanced antimicrobial treatments for cooling towers.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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