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Effect of reducing and protecting agents on size of silver nanoparticles and their anti-bacterial activity

Amany A. El-Kheshen[1,2*] and Sanaa F. Gad El-Rab[1,3]

[1]Taif University, Faculty of Science, Kingdom of Saudi Arabia
[2]Glass Research Department, National Research Centre, Dokki, Cairo, Egypt
[3]Biotechnology Department, Assuit University, Assuit, Egypt

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ABSTRACT

Silver nanoparticles (AgNPs) with different shapes and sizes were chemically prepared and characterized by transmission electron microscope (TEM), UV-vis spectra and Fourier transmission IR (FTIR). Their antibacterial activities against gram positive bacteria (Staphylococcus aureus ATCC 6538, Staphylococcus epidermidis ATCC 12228) and gram negative bacteria (E. coli ATCC 8739) were studied. Antimicrobial activities of Ag nanoparticles had been increased with their larger surface area to volume ratio. Best antibacterial activity was observed on using AgNPs prepared from sodium hypoborite ($NABH_4$) as reducing agent and polyvinyl pyrrolidone (PVP) as stabilizer or protecting agent. It exhibit zone of inhibition about 25 mm against S. aureus ATCC 6538, 19 mm against S. epidermidis ATCC 12228 while the Cefoperazone antibiotic had inhibition zone of 15 mm. The same silver nano particles showed inhibition zone against E. coli ATCC 8739 of about 15 mm while the antibiotic had 12 mm inhibition zone. TEM revealed small size of silver nanoparticles (ranging from 1.5-3 nm) which stimulate biofilm production and aggregate within this biofilm. They bind closely to the surface of microorganisms causing visible damage to the cells, and demonstrating good self-assembling ability.

Keywords: silver nanoparticles**,** reducing agents, protecting agent, TEM, antibacterial effect, Cefoperazone as antibiotic, *S. aureus* (ATCC 6538), *S. epidermidis* ATCC 12228, *E. coli* ATCC 8739.

INTRODUCTION

In the past decades, the synthesis of nanocrystals, crystalline particles ranging in size from 1 nm to 100 nm, has been intensively studied not only due to their fundamental scientific interest but

also because of their many useful applications [1]. Nanoparticles (NPs) exhibit outstanding electrical, optical, magnetic, etc. properties that cannot be revealed by their bulk counterparts [2]. These fascinating properties of nanomaterials strongly depend on size, shape of NPs, their interactions with stabilizers and surrounding media and also on the manner of their preparation. Therefore the controllable synthesis of nanocrystals is a key challenge to achieve their better applied characteristics.

Nanoparticle synthesis and the study of their size and properties is of fundamental importance in the advancement of recent research [3,4,5]. It is found that the optical, electronic, magnetic, and catalytic properties of nano particles depend on their size, shape and chemical surroundings [4,5]. In nanoparticle synthesis it is very important to control not only the particle size but also the particle shape and morphology as well.

Among the noble metals, silver nanoparticles have become the focus of intensive research due to its wide ranges of application for many sectors of life and industry [6]. This led to a rapid increase in the number of scientific publications devoted to the development of the techniques of silver colloids preparation and further understanding of their properties [7–9].

Silver is a safe and effective anti-bactericidal metal because it is non-toxic to animal cells and highly toxic to bacteria such as *Escherchia coli* (*E. coli*) and *Staphylococcus aureas* [10]. Silver based compounds have been used in recent years to prevent bacterial growth in applications such as burn care. Nano silver in the form of powders as well as suspensions, due to the high surface to volume ratios, has been used as anti-bacterial because it enables the loading of small quantities of silver and thus makes the product cost effective.

Silver nano particles have received considerable attention due to their attractive physical and chemical properties. Metallic silver colloids were first prepared more than a century ago. Ag nanoparticles can be synthesized using various methods: chemical, electrochemical, γ-radiation etc. The most popular preparation of silver colloids is chemical reduction of silver salt by sodium citrate or sodium borohydride. This preparation is simple, but the great care must be exercised to make stable and reproducible colloid. The purity of water and reagents, cleanliness of the glassware are critical parameters. Solution temperature, concentrations of the metal salt and reducing agent and reaction time also influences particle size. Controlling size and shape of metal nanoparticles remains a challenge [11].

In this study, Silver nanoparticles with controlled particle size were prepared using different reducing and protecting agents. The antibacterial activities of AgNPs were also studied to show their highly action on bacteria according to their particle size.

MATERIALS AND METHODS

II.1. Preparation of Ag nano particles

II.1.1. Preparation of Ag nanoparticles by glucose

Silver nano particles of the size 40–80 nm are formed in the process of oxidation of glucose to gluconic acid by amine in the presence of silver nitrate, and the gluconic acid caps the nano

silver particle. The nano silver particle is encapsulated by gluconic acid, there was no surface oxidation, the $AgNO_3$: glucose molar ratio was 1:0.5.

II.1.2. Preparation of Ag nano particles by $NaBH_4$ and polyvinyl pyrrolidone (PVP) [12]
Dissolve 0.0285 gm of $NaBH_4$ in 10 ml distilled water (ice-cold), and then add 0.4 gm of PVP as stabilizer (protecting agent). Dissolve 0.0214 gm of $AgNO_3$ in 10 ml ice-cold distilled water. Put the flask on magnetic stirrer for 1 h. at 50-60°C at 1500 rpm, the prepared sample was black.

II.1.3. Reduction of Ag nanoparticles by trisodium citrate [13]
The silver colloid was prepared by using chemical reduction method. All solutions of reacting materials were prepared in distilled water. In typical experiment 0.0849 gm of $AgNO_3$ was dissolved in 500 ml distilled water, and then the solution was heated to boiling.

Then1 gm of tri sodium citrate was dissolved in 100 ml distilled water and 5 ml of trisodium citrate were added to 500 ml of $AgNO_3$ after boiling (drop by drop). During the process, the solution was mixed vigorously.

The solution was left on hot plate for 2 hours at 90°C for heating only, then it was cooled to room temperature, the color was reddish green.

II.1.4. Preparation of Ag nanoparticle using ascorbic acid as reducing agent [14]
Two samples of AgNPs were prepared using ascorbic acid as reducing agent; one of them was prepared by adding polyvinyl alcohol (PVA) as stabilizer and the other by adding polyvinyl pyrrolidone (PVP) as stabilizer (Table 1).

The first sample was prepared by dissolving 1.01922 gm of $AgNO_3$ in 100 ml distilled water and then 0.5 gm of PVA was added. Dissolve 1.0567 gm of ascorbic acid in 100 ml distilled water, then added to 0.5 gm of PVA. The solution was green after the addition of 10 ml ascorbic acid and turned to red when all reducing agent was added.

The second sample was prepared by dissolving 1.528 gm of $AgNO_3$ in 150 ml distilled water, and then 5 gm of PVP was added. Dissolve 1.585 gm of ascorbic acid in 150 ml distilled water, ascorbic acid was added drop by drop to $AgNO_3$ solution (Table 1).

Table 1: Composition of prepared AgNPs

Sample No.	Reducing agent	Protecting agent)
1	Glucose	
2	$NaBH_4$	PVP
3	Sodium citrate	------
4	Ascorbic acid	PVA
5	Ascorbic acid	PVP

II.2. Characterization of prepared AgNPs
The absorption optical spectra of these silver colloids were recorded using Jasco Ubest 570 UV–vis–NIR spectrophotometer. All the spectra were recorded in air at room temperature. The morphology of silver nanoparticles in silver colloids was measured with a JEOL-JSGM T1230

transmission electron microscopy (TEM) operating at 200 kV. Those samples were prepared by dropping the colloid onto a carbon coated Cu grid underlying tissue paper, leaving behind a film.

The IR spectrum was conducted using FTIR (Fourier Transformation Infrared Spectroscopy) by means of the FT-IR spectrometer Jasco FT/IR-430 (Japan).

II.3. Antibacterial activity studies

II.3.1. Disc diffusion method

The modified disc diffusion method [15] was used to evaluate the antimicrobial activity of Ag NPs and a 75 mcg Cefoperazone diffusion disk as antibiotic against *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228) and *E. coli* (ATCC 8739) This method was performed in Luria Bertani (LB) medium solid agar Petri dish. Briefly, 6 mm sterile paper discs impregnated with 100 µg/ ml of silver nanoparticles samples and was placed on Staphylococcus *aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228) and *E. coli* (ATCC 8739) cultured agar plate. Agar plate which were then incubated for 24 h at 37 ºC and inhibition zone was monitored After incubation the presence of bacterial growth inhibition halo around the samples were absorbed and their diameter in millimeters was measured .

II.3.2. Measurement of minimum inhibitory concentration (MIC)

AgNPs was added in LB medium, respectively. Each bacterium culture [*Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228) and *E. coli* (ATCC 8739)] was controlled at 10^5-10^6 CFU/mL and incubated at 37ºC To establish the antimicrobial activity of silver nanoparticles on the bacterial growth, the minimum inhibitory concentration of nanosilver shapes for *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228) and *E. coli* (ATCC 8739) were determined by optical density of the bacterial culture solution containing different concentration of Ag NPs after 24h. All of the experiments (MIC) were triplicated, on three different days.

II.3.3 Bacterial Growth Curve

To study growth of bacteria in broth media, inoculations were given from fresh colonies on agar media into 10 ml broth (Luria Bertani). These media was supplemented with silver and silver nanoparticles 150 µg/ml and the bacterial cultures were incubated at 37°C temperature with rapid shaking at 150 rpm. The growth of *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228) and *E. coli* (ATCC 8739) in broth media was indexed by measuring the optical density (OD) at λ=600nm at regular intervals using UV-Vis Spectrophotometer. The control culture was treated in a similar fashion but without any exposure to the silver nanoparticles. The growth curve was plotted between optical density and time.

RESULTS AND DISCUSSION

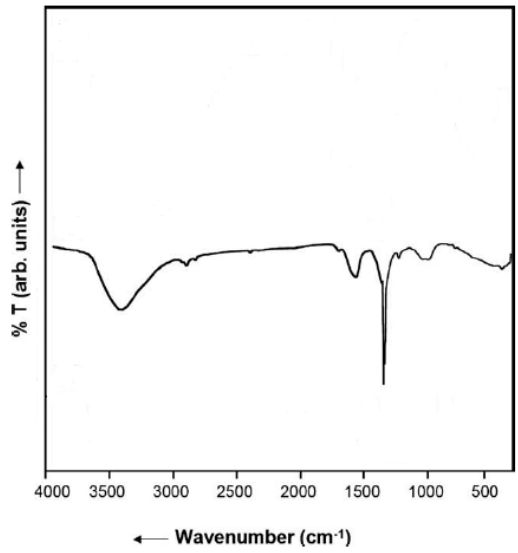
III.1. Morphological characterization of Ag nano particles

III.1.1. AgNPs prepared by glucose

Fig. 1 shows the FTIR spectra recorded for silver nanoparticles. The attempt made here is to do only a qualitative study. The spectrum for the silver nanoparticles shows various peaks. The peaks at 3423 and 1605 cm^{-1} are very broad and strong, and can be assigned to the hydroxyl groups [10], either from glucose/gluconic acid, from adsorbed moisture or both. A prominent and

very sharp peak is observed at 1384 cm^{-1} which was concluded to be due to the nitrate ions. The gluconic acid shows peaks at 1740, 1638, 1412, 1230, 1100, 1036 and 875 cm^{-1}. Almost all these peaks appears at the nano silver sample. Thus a small amount of gluconic acid remains in the sample, even after repetitive washings of the silver precipitate.

Fig. 1: FTIR Spectra for silver nano-particle prepared by glucose



TEM images of the prepared silver nano particles are shown in Fig.2. The Ag nano particles are spherical in shape with a smooth surface morphology. The diameter of the nano particles is found to be ranging from 30 nm to 7 nm. TEM image also shows that the produced nano particles are more or less uniform in size and shape. The agglomeration of the particles is clear (Fig.2).

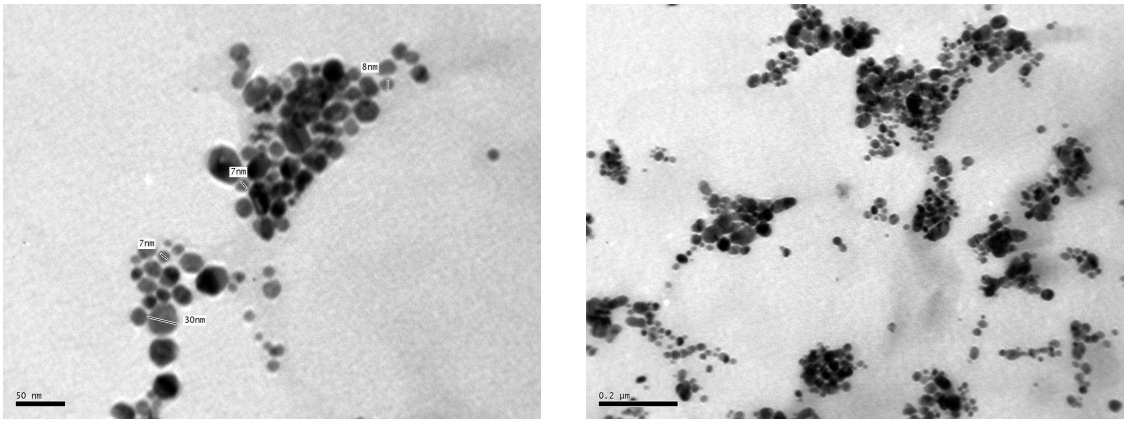


Fig. 2: TEM images of silver nano particles prepared by glucose

Janardhanan et al [10] found that, the gluconic acid caps the nano silver particle. As the nano silver particle is encapsulated by gluconic acid, there was no surface oxidation and hence prevents the oxidation/ sulfidisation of the nano silver particle.

III.1.3. AgNPs powders prepared from $NaBH_4$ and Polyvinylpyrrolidone (PVP)

The Uv-Vis spectra of the powder sample (Fig. 3) did not show any clear absorption peaks in UV–Visible range. This may be due to the agglomeration of the particles, observed in the TEM (Fig.4).

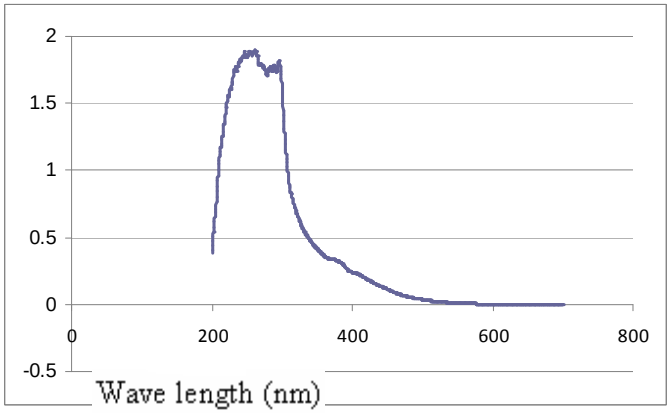


Fig. 3: UV–Visible absorption spectra for AgNPs (sample No. 1)

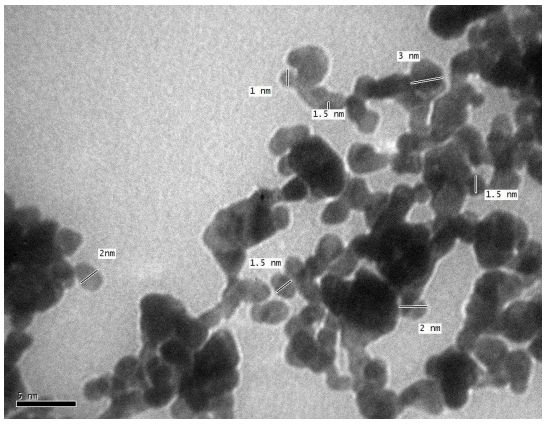


Fig. 4: TEM image of AgNPS (sample No.1)

A broad emission peak was centered at about 320 nm. The presence of this peak was the result of the surface plasmon resonance of silver nanoparticles [12]. The small particle size (Fig.4) is reflected in the optical absorption studies. The shift observed in silver peak Fig (3) may be due to the decrease in silver concentration [16].

During the chemical reduction, the reducing agent donates electrons to the silver ions (Ag^{+}), causing silver to revert to its metallic form (Ag^{0}). In case of using PVP as protecting agent, the stabilization of Ag^{+} was highly effective. AgNPs, known to possess inhibitory and bactericidal

effects, have a high surface area to volume ratio along with high fraction of surface atoms that elicits elevated antimicrobial activity compared to the silver metal as a whole [17]**.**

III.1.4. Ag nanoparticles prepared by trisodium citrate

The mechanism of reaction could be expressed as follows:

$$4Ag^{+} + C_6H_5O_7Na_3+2H_2O \rightarrow 4Ag^{0}+C_6H_5O_7H_3+3Na^{+}+H^{+}+O_2\uparrow \text{ [18]}$$

The structure of prepared silver nanoparticles has been investigated by X-ray diffraction (XRD) analysis. Typical XRD pattern of the prepared sample by the present chemical method is shown in the Fig. (5) which confirms the formation of silver with coarse grain size which have a low surface area to volume ratio.

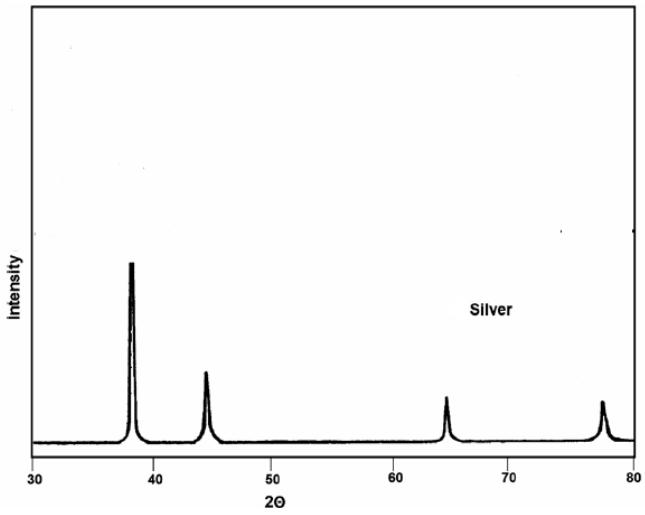


Fig.5: XRD of silver nano particles (sample No.2)

Fig. 6 exhibits the TEM of the prepared sample with its rod and spherical shapes with coarse grain size which have a low surface area to volume ratio.

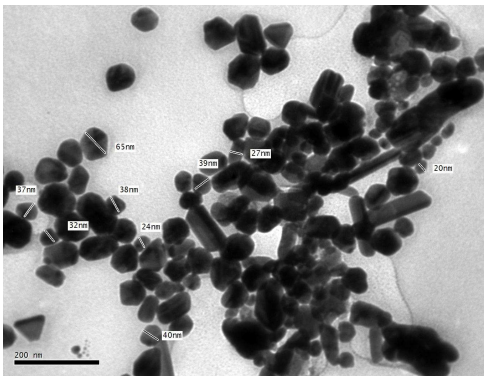


Fig. 6: TEM image of silver nanoparticles (sample No.2)

III.1.5. Ag nanoparticles prepared using ascorbic acid

Fig. 7 (a) shows the TEM micrograph of silver nanoparticles prepared using ascorbic acid as reducing agent with polyvinyl alcohol (PVA) as stabilizer. From the Figure, the agglomeration of the particle is clear also, the particle size is ranging from 55 nm to 68 nm.

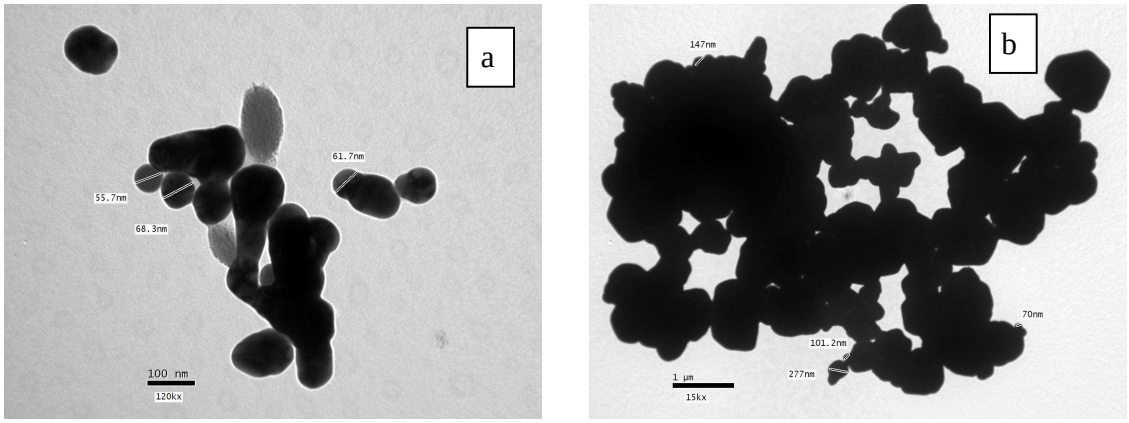


Fig. 7: TEM image of silver nanoparticles prepared by ascorbic acid as reducing agent and (a) sample 3 (b) sample 4

Fig. 7 (b) exhibits the TEM photo of silver particles prepared by ascorbic acid as reducing agent and polyvinyl pyrrolidone as protecting agent. These materials gave the silver particles bigger size ranging from 101 nm to 227 nm.

Due to the growth process of nanocrystallites controlled by the stabilizers, it is possible to manipulate the shape and size of silver nanoparticles by choosing different protecting agents. Thus, the stabilizer employed may be different with different shapes of silver nanoparticles required in the targeted application [19]. The particle size and morphology can be modified due to changing the concentration of reducing agent and stabilizer. The strongest reducing agent produced larger particles.

III.2. Agar disks diffusion test

In this work, the antibacterial effect of the prepared AgNPs samples was studied on different types of bacteria (*S. aureus* ATCC 6538 and *S. epidermidis* ATCC and *E. coli* ATCC 8739).

Table 2 and Fig. 8 exhibit the inhibition zone of different Silver nanoparticles (prepared from glucose) sample No. 1 and the samples Nos. 2, 3, 4 and 5 showed zone of inhibition (mm) of about 0, 25, 23, 19 and18 mm in diameter for *S. aureus* ATCC 6538 respectively; while the antibiotic (sample No. 6) showed 15 mm that means the last four nano silver particles have greater antibacterial effect than antibiotic for *S. aureus* ATCC 6538. In case of *S. epidermidis* ATCC 12228, the zone of inhibition (mm) of about 0, 19, 18, 16 and 13 mm for last four nanoparticles respectively; while the antibiotic (sample No. 6) showed 15 mm diameter. So, the antibacterial effect of the samples Nos. 2, 3, 4 is greater than antibiotic.

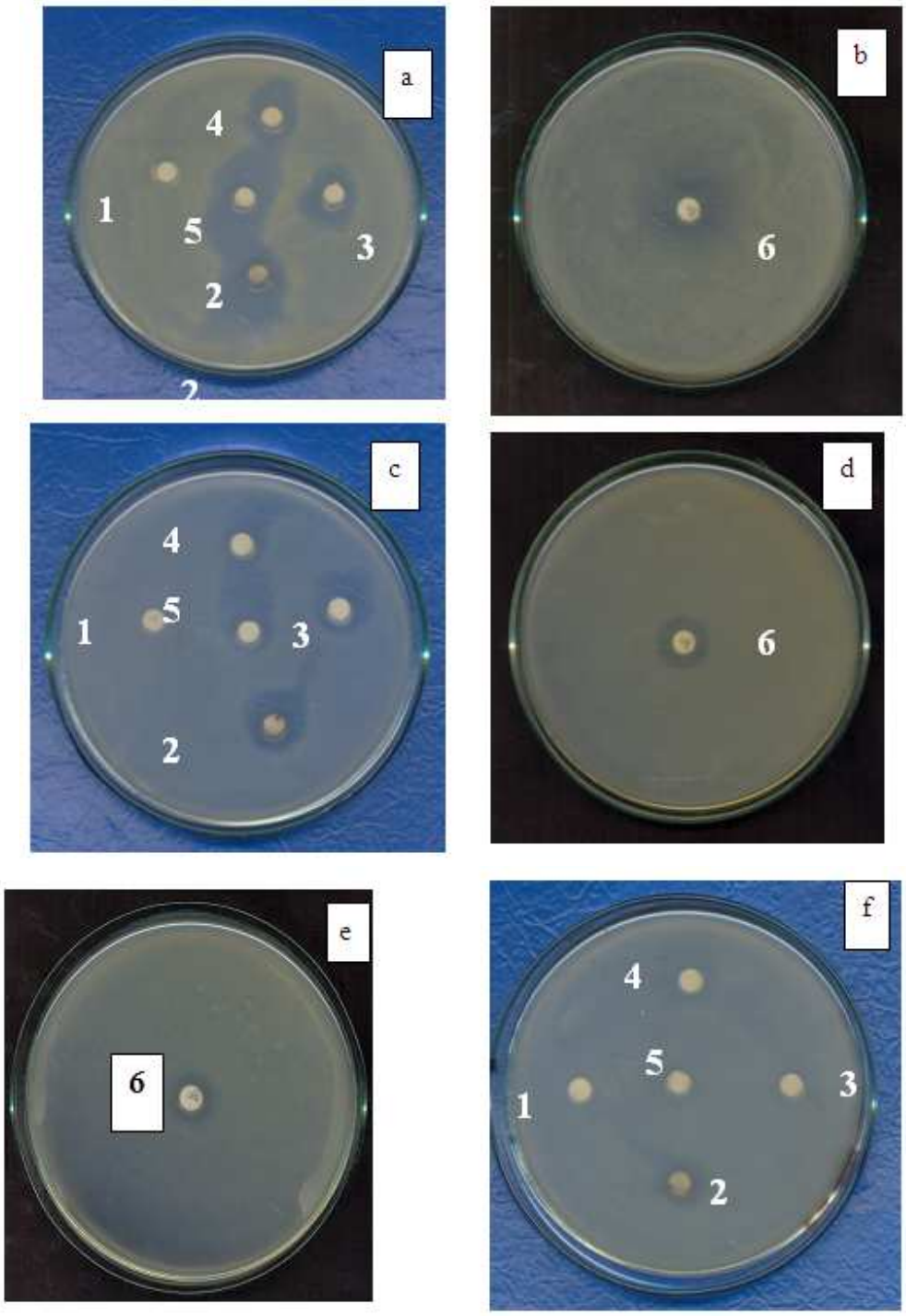


Fig.8: The diameter of inhibition zone (DIZ) surrounding silver nanoparticle impregnated disks (1, Ag ; 2, Ag+NaBH4 + PVP ; 3, Ag + ascorbic acid + PVA ; 4, Ag + ascorbic acid + PVP ; 5, Ag tri sod. Citrate ; 6, antibiotic) (6 mm diameter) in presence of *S. aureus* (a–b), *S. epidermidis* (c–d) and *E. coli* ATCC 8739

Silver nanoparticles are harmful to bacteria [20]. Samples 2, 3, 4 and 5 stimulate biofilm production and aggregate within this biofilm. They bind closely to the surface of microorganisms causing visible damage to the cells, and demonstrating good self-assembling ability. Sample 2 showed inhibition zone about 15 mm against *E. Coli* ATCC 8739 while antibiotic has 12 mm inhibited zone. Chamakura et al [21] found that Ag-NPs react with cell walls and cytoplasmic membranes of *Escherichia coli*, resulting in pits in the cell wall of bacteria, and finally killing them. Last result explained that Ag+$NaBH_4$ + PVP showed greater antibacterial effect than antibiotic. These silver nanoparticles which have antibacterial effect can be used against resistant antibiotic bacteria. This clearly demonstrates that the antimicrobial activity is only due to nanosilver shapes impregnated inside bacterial and not due to individual bacterial. The mechanism for antibacterial action of silver nanoparticles is bacterial membrane disruption by the silver ions released from the PVP. Ag ions form insoluble compounds with sulphydryl groups in the cellular wall of the microorganism. This result can be explained in terms of the presence of amino groups in the PVP chain and it's easy to induce Ag^+ motility. The Ag^+ release mechanism is not elucidated. However, it is possible that the amino group improve the Ag^+/ H^+ ionic exchange [22].

Although silver ions have somewhat better antimicrobial effect, they are inactive when we used glucose as reducing agent because of the encapsulation effect of glucose and trapping the AgNPs inside it and leads to none antibacterial effect for silver particles. Finally smaller particles for sample No.2 having larger surface area for interaction and have efficient bactericidal effect than the larger particles for samples Nos. 3, 4 and 5 [23]. This antibacterial properties can been used to prevent and reduce the bacteria colonization on catheters [24] prostheses vascular grafts, dental materials and human skin.

Table 2: Zone of inhibition (mm) of nanoparticles against bacteria tested

Sample	Bacteria		
	***S. aureus* ATCC 6538**	***S. epidermidis* ATCC 12228**	***E. coli* ATCC 8739**
AgNPs + Glucose	**0**	**0**	**0**
1	**25**	**19**	**15**
2	**23**	**18**	**0**
3	**19**	**16**	**0**
4	**18**	**13**	**0**
Cefoperazone	**15**	**15**	**12**

III.3. Minimum Inhibitory concentration (MIC)

Table 3 shows the MICs of AgNPs prepared from glucose , and samples Nos. 1, 2, 3 and 4 against the individual tested bacterial strains. These results tend to indicate that the samples Nos. 1, 2, 4 had higher anti-bacterial activity than sample No. 3 against *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228 while AgNPs prepared from glucose and sample No. 1, had higher anti-bacterial activity than the others NPs against E. *coli* ATCC 8739; this is due to its nanoparticles size that is smaller than the particle size of other samples. The MIC observed in this study for silver nanoparticles prepared from glucose and, (Ag + $NaBH_4$ + PVP), (Ag + ascorbic acid + PVA), (Ag + ascorbic acid + PVP) and Ag + tri sod. citrate are 30 µg/mL, 40 µg/mL, 40 µg/mL, 20µg/mL respectively against *S. aureus ATCC 6538*. In case of *S. epidermidis ATCC 12228*. In this study, the observed MIC for Ag + $NaBH_4$ + PVP, Ag + ascorbic acid + PVA, Ag + ascorbic acid + PVP and Ag + tri sod. citrate are 20 µg/mL, 40

µg/mL, 40 µg/mL, 30µg/mL respectively, While are 80 µg/mL, 160 µg/mL, 160 µg/mL, 125µg/mL respectively against *E. coli* ATCC 8739. From the latter results, it is clear that *Staphylococcus aureus* ATCC 6538 and *S. epidermidis ATCC 12228* were sensitive to all silver NPs because the formation of biofilm surrounded the bacterial cell and accumulation of nanopartiles inside it, while *E. coli* ATCC 8739 was resistance to silver ions (PVP, Ag + ascorbic acid + PVA, Ag + ascorbic acid + PVP, Ag tri sod. Citrate) is due to their large size.

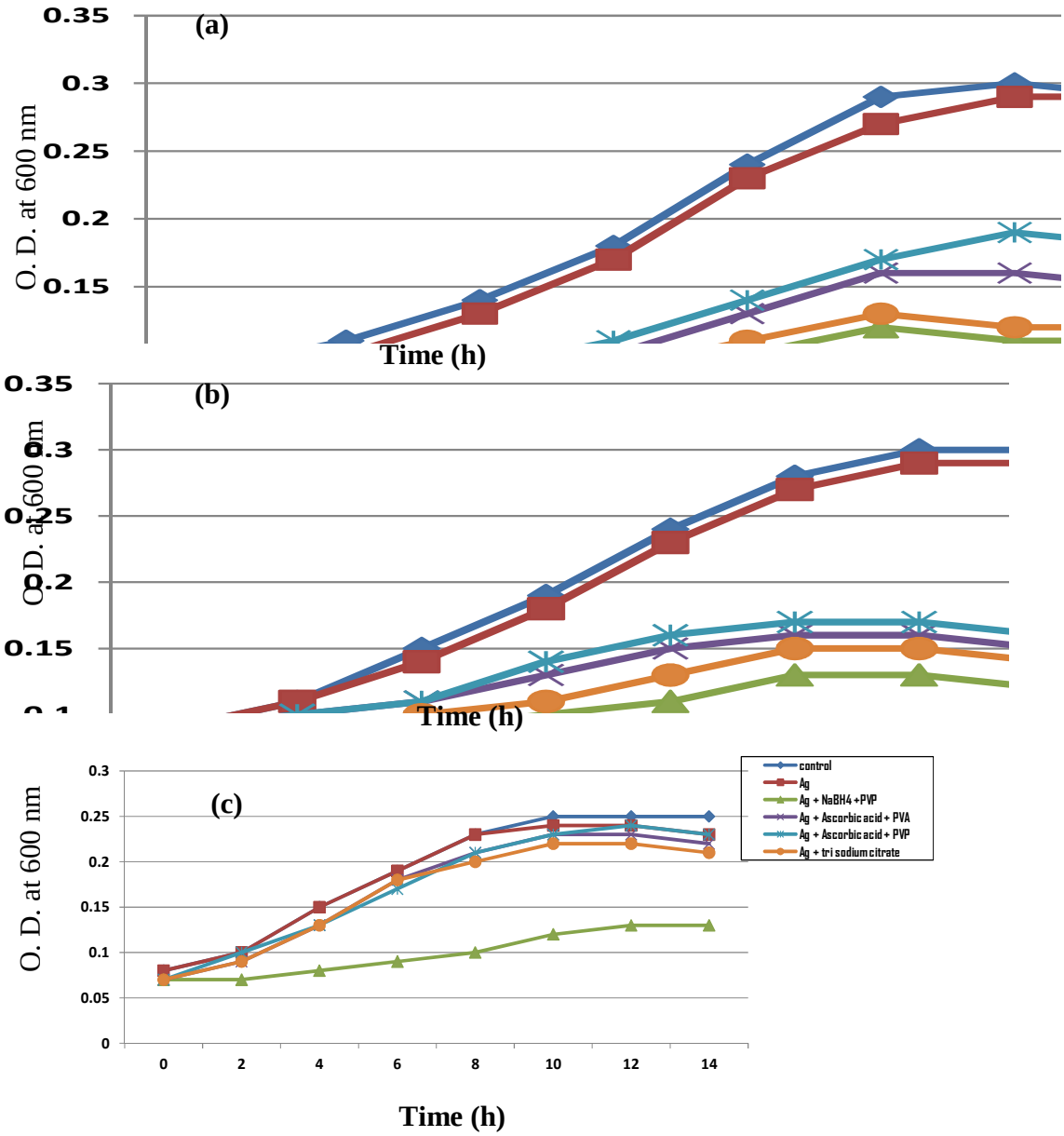


Fig. 9. Representative batch growth profile in the absent and in the presence of 150µg /ml nanoparticles (Ag+NaH4 + PVP, Ag + ascorbic acid + PVA, Ag + ascorbic acid + PVP and Ag tri sod. Citrate) for (a) *S. aureus* (ATCC 6538), (b) *S. epidermidis* (ATCC 12228) and (c) *E. coli* (ATCC 8739).

Table 3: Minimum inhibition concentrations of Ag nanoparticles

Sample	Minimum inhibition concentration (μg/mL)		
	Bacteria		
	S. aureus ATCC 6538	*S. epidermidis ATCC 12228*	*E. Coli* ATCC 8739
1	20	20	80
2	30	30	125
3	40	40	160
4	40	40	160

III.4. Analysis of Growth Curve

In batch studies, a greater lag phase and lower maximum absorbance (at 600 nm) were observed at the concentration of nanoparticles 150 μg/ml for the strain of *S. aureus* and the strain of *S. epidermidis*. In case of AgNPs, it capsulated with glucose so optical density of bacterial growth does not decrease. For *E.coli* only Ag + $NaBH_4$ + PVP observed a greater lag phase and lower maximum absorbance (at 600 nm). Similar observation was reported by Sondi and Salopek-Sondi [25] in their studies on effect of silver nanoparticles on a single strain of *E. coli*. Optical densities were measured and plotted as a function of time at regular intervals with different types of silver nanoparticles as shown in Fig. 9.

CONCLUSION

The antibacterial activity of silver nano-particles was studied. Nano particles materials show highly active properties due its large surface area. Silver has harmful action on different kinds of gram –ve and gram +ve bacteria. By examination of surface morphology, it was found that the particle size can be ranged from very small size to bigger size, by changing both reducing and protecting agents used for silver preparation.

The AgNPS prepared by using glucose gave zero inhibition zones, because of the encapsulation of silver inside the glucose so, there are no free silver particles to damage the bacteria.

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