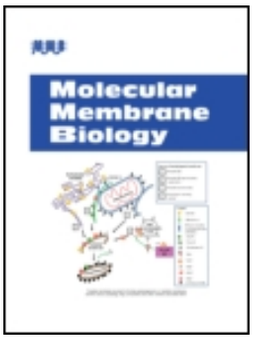

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Nano silver entrapped in phospholipids membrane: Synthesis, characteristics and antibacterial kinetics

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Abstract

The antimicrobial property of stabilized silver nanoparticles (AgNPs) with phospholipid membrane was investigated on both Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacterial strains. The influence of phospholipid concentrations on antibacterial kinetics actions of AgNPs was studied with two different methodologies in order to understand the bactericidal and bacteriostatic effects. The bacterial inactivation of synthesized AgNPs fitted well to the Chick-Watson model with a high regression coefficient, $R^2 > 0.91$. The antibacterial properties of AgNPs depend on the particle size, stabilizer and lecithin concentrations. Only the stabilized AgNPs that have the $K_{lec/Ag}$ values of 1 and 2 presented the inhabitation zone, while unstabilized AgNPs agglomerated quickly, settled on the wells and did not diffuse in agar. In addition, the specific coefficient of lethality depends on the lecithin concentration. An increase in lecithin concentration caused multilayer creation on the AgNPs' surface and reduced the release of AgNPs which led to low bacterial killing rate.

Keywords: *Silver nanoparticles, phospholipids membrane, antibacterial, antibacterial kinetic*

Introduction

As physical, chemical and biological properties of metal nanocomposites can be improved in nanoscale, many researchers have paid great attention on their synthesis and application for specific purposes. Nano-sized metals have special characteristics that can be used for several advanced applications (Carotenuto and Nicolais 2004, Majewski and Thierry 2007, Dong et al. 2009).

Recent studies have shown that metal nanocomposites are effective biocides against bacteria (Chen and Chiang 2008, Falletta et al. 2008, Hernández-Sierra et al. 2008, Dastjerdi et al. 2010, Marambio-Jones and Hoek, 2010), fungi (Kim et al. 2007, 2009), and viruses (Elechiguerra et al. 2005, Zodrow et al. 2009). The antibacterial properties of silver nanocomposites (Bakumov et al. 2007, Dastjerdi et al. 2009), silver ion (Woo et al. 2008, Barani et al. 2010), and silver compounds (Fisher et al. 2003, Silver et al. 2006) have been thoroughly investigated.

Silver is an 'oligodynamic' agent because of its capacity to have a bactericidal effect at very low concentrations (Collart et al. 2006). Results of studies on antibacterial properties of silver nanoparticles proposed that silver nanoparticles may attach to the cell membrane surface, which causes change in cell permeability and finally cell death (Rai et al. 2009). Moreover, there is a possibility of their penetration into the bacteria, resulting in cell death (Morones et al. 2005). Thus, the smaller silver nanoparticles have the larger available surface area for binding to the cell membrane surface of bacteria.

The presented models for disinfection are empirically (Hassen et al. 2000) and many of them have been proposed for disinfection kinetics (Hom 1972, Selleck et al. 1978, Severin et al. 1984, Haas and Joffe 1994). The simplest disinfection model was proposed by Chick and Watson. In the Chick-Watson model, the rate of inactivation of a microorganism is dependent on the silver nanoparticles concentration and contact time (Cho et al. 2003).

Several methods have been employed to synthesize silver nanoparticles among which, the chemical-reduction method has been extensively investigated

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because of its simplicity of performance and mild conditions (Zhang et al. 2007). Usually, the chemical reduction is composed of silver nitrate as a precursor, reducing agent, and stabilizer. The stabilizing agents are used to separate the particles to prevent aggregation (Chou and Lai 2004). Moreover, phospholipids as a stabilizer are used to improve the biocompatibility of synthesized metal nanoparticles (Chung et al. 2008, Barani et al. 2010). Park et al reported the membrane fluidity of loaded nanoparticles to the liposome (Park et al. 2005, 2006), while the synthesis of nanoparticles in phospholipid membrane had been reported already. In this study, the effect of phospholipid concentrations on the antibacterial and killing kinetic of two Gram-positive and Gram-negative bacteria was studied and the Chick-Watson model was adopted for evaluation of AgNPs disinfection kinetics.

Materials

All the chemicals and reagents used were of analytical grade. Distilled water was used throughout the work. Silver nitrate ($AgNO_3$ extra pure, >99.8%), sodium borohydride ($NaBH_4$, 99.9%), sodium chloride (99.99%), CASO agar, Mueller-Hinton agar, and CASO broth were purchased from Merck Company (Germany). Lecithin was used as a biocompatible agent for stabilizing silver nanoparticles and was received as a gift from Lipoid Company (Lipoid® S 75; Germany).

Bacteria and culture condition

Staphylococcus aureus ATCC 6538 as a Gram-positive bacterium and *Escherichia coli* ATCC 8739 as a Gram-negative bacterium were used in this study. The test strains were maintained as frozen stocks at −70°C in PBS (phosphate buffered saline) containing 20% (v/v) glycerol. Fresh cultures were prepared by inoculating 100 µl aliquots of the thawed microbial stock suspensions into CASO agar (Merck, Germany) plates followed by overnight incubation at 35°C. The bacteria inocula were prepared by suspending overnight colonies from CASO agar medium in 0.9% saline. The inocula were adjusted photometrically at 600 nm to a cell density equivalent to 0.5 McFarland standard (1.5×10^8 CFU/ml) before use.

Synthesis of AgNPs

Preparation of AgNPs was carried out according to our previous reported method (Barani et al. 2010). Nano liposome was prepared by heating method. The prepared lecithin solution (200 ppm, 50 ml) was stirred vigorously (approximately 1500 rpm) in a special designed bottle (Colas et al. 2007) for 30 min at 70°C and then cooled to room temperature. Then, the reducing agent with an amount of twice the concentration of silver nitrate solution was added, while stirring on a hotplate stirrer for 15 min. The mixture of reducing agent and liposome colloidal solution was cooled to 0°C, because the reducing agent is only activated by the reaction of silver salt according to Equation (1), and not by heating (Zhang et al. 2006).

$$NaBH_4 + 4H_2O \rightarrow NaB(OH)_4 + 4H_2 \quad (1)$$

The complete hydrolysis of the sodium borohydride can be rapidly achieved by the rise of temperature or by adding acids in aqueous solution (Schlesinger et al. 1953). Finally, the 10 ml of prepared silver nitrate (100 pm) was added to colloidal solution dropwise at a rate of 1 ml/min while stirring at 0°C. Adding silver nitrate to the reducing agent solution leads to the reaction in Equation (2) and changes the colour of the solution from clear transparent to dark yellow indicating synthesis of silver nanoparticles.

$$AgNO_3 + NaBH_4 \rightarrow Ag^{\bullet} + 1/2H_2 + 1/2B_2H_6 + NaNO_3 \quad (2)$$

The $K_{Lec/Ag}$ ([Lecithin]/[$AgNO_3$]), the lecithin to silver nitrate molar concentration ratio, is the influencing factor in synthesis of silver nanoparticles. The $K_{Lec/Ag}$ values were 2×10^{-3}, 0.2, 1 and 2 while the micelles concentration (CMC) of lecithin is 5×10^{-9} molar (Barenholz and Lasic 1996). To obtain particles with small size (Petit et al. 1993), concentration of the reducing agent was chosen twice as the precursor ($K_{NaBH_4}/Ag = 2$). The remaining reducing agent in the solution of synthesized silver nanoparticles was decomposed by heating the colloidal nanoparticles solution to about 50°C (Wang et al. 1999).

Characterization of AgNPs

The UV absorption spectra of all AgNPs solutions were determined on an Optizen 2120UV spectrophotometer with 2 nm resolution at room temperature. The spectrum wavelengths were recorded between 200 and 700 nm and a glass cuvette with 1 cm optical path was used. The studies of size and morphology of the nanoparticles were performed by transmission electron microscopy (TEM) using Philips CM120 microscope operating at up to 120 KV. Samples for TEM studies were prepared by placing drops of the silver nanoparticles solutions on carbon-coated TEM grids and allowing the solvent to slowly evaporate under vacuum for 30 min at room temperature.

Antibacterial studies

Minimum inhibitory concentration (MIC). MIC was determined by both agar well diffusion and broth dilution methods. In the well diffusion method, the surface of Petri dishes containing 25 ml of Mueller-Hinton agar was seeded individually with bacterial suspensions (10^8 CFU/ml) with a sterile cotton swab. Wells with 7 mm diameter were created by punching a stainless steel cylinder on to the agar plates and removing the agar to form a well. Finally, 80 µl aliquots of each prepared sample (100, 50, 25, 12.5, and 6.25 ppm) were placed individually in two wells. The prepared Petri dishes were incubated at 35°C for 20 h, and diameters of inhibition zones (i.e., areas surrounding the test samples where bacteria growth is inhibited or not) were determined.

In the broth dilution method, the bacterial suspension was added to the tubes containing various concentrations of silver nano composites solutions (100, 50, 25, 12.5, and 6.25 ppm) in Mueller-Hinton broth to reach the final concentration of about 10^6 CFU/ml. Tubes were incubated at 35°C for 20 h. Then, 1 ml of each tube was added to the 9 ml of neutralizing solution (0.6% sodium thioglycolate and 1% sodium thiosulphate) and the total bacterial count was determined by pour plate method using CASO agar medium. The minimum inhibitory concentration (MIC) was defined as the minimum concentration of the antibacterial agent that inhibits the growth of tested microorganism. The minimum bactericidal concentration (MBC) was defined as the minimum antibacterial concentration that kills more than 99.9% of the first bacterial inocula.

Bacterial killing kinetic. It was assessed without nutrient medium. For this purpose, 40 ml of AgNPs solutions (100 ppm) were inoculated with bacterial suspension at a final concentration of about 10^5 CFU/ml. Then 1 ml aliquots were removed at different intervals of 0, 5, 10, 20, 30, 45, 60, 90, and 120 min which were added to 9 ml of neutralizing solution. Total bacterial count was determined by pour plate method using CASO agar medium.

Results and discussion

UV-visible spectrum analysis

Localized surface plasmon resonances of AgNPs depend on the size, shape, as well as the surrounding dielectric medium. Usually the spherical nanoparticles in the range of 10–50 nm show an extinction peak at ~400 nm, leading to a characteristic yellow colour (Cobley et al. 2009) and changing the size of spherical particles can induce small shifts in the peak position. Figure 1 shows the UV absorption of synthesized AgNPs, which is capped with phospholipids membrane. The fresh synthesized silver nanoparticles have a yellow colour which has higher absorption than the capped AgNPs. But they have a short lifetime and their colour changes to dark yellow, and then black suspension and slowly turn to macro particle and settle. AgNPs have a localized peak at 405 nm and their colour shift to darker by adding lecithin which causes their absorption to reduce (Barani et al. 2010). This is because of the lack of absorption for lecithin suspension at this position.

Transmission electron microscopy studies

The morphology of AgNPs was further proved by recording TEM of prepared silver colloidal suspension. The TEM images of synthesized AgNPs are presented in Figure 2. The silver nanoparticles which were not stabilized by phospholipids membrane agglomerated and created large particles, which

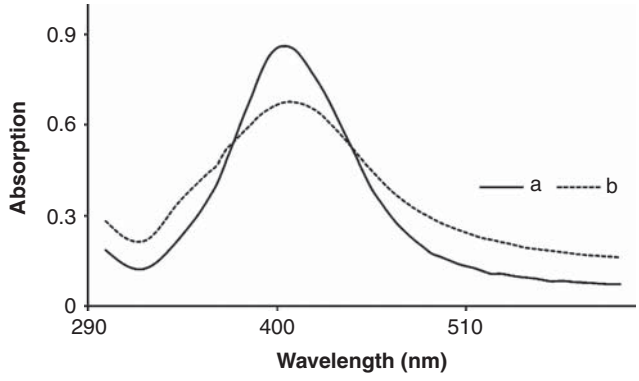

Figure 1. UV spectra of synthesized AgNPs solution (10 ppm) at two different lecithin concentration: (a) $K_{Lec/Ag} = 1$, and (b) $K_{Lec/Ag} = 2$. AgNPs are capped by lecithin molecules and lead to a reduction in their maximum absorption.

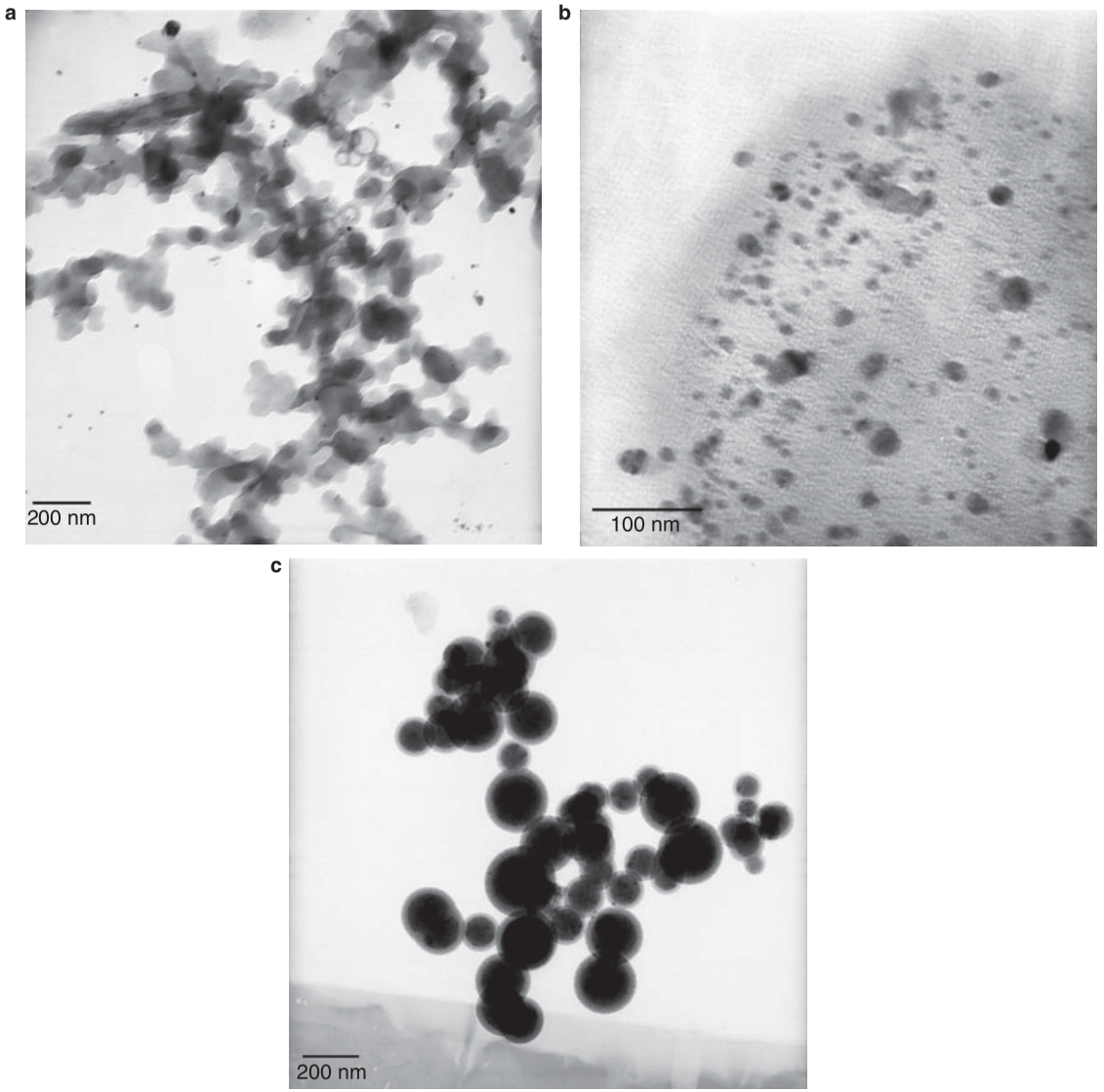

Figure 2. TEM images of prepared AgNPs with various lecithin concentrations: (a) $K_{lec/Ag} = 0$; synthesized AgNPs have a short life without stabilizer and linked one to another and appear agglomerated silver particles; (b) $K_{lec/Ag} = 0.2$, and (c) $K_{lec/Ag} = 2$; lecithin molecules capped AgNPs and prevent them to link to one another.

linked to one another and created sediments in range of micrometer (Figure 2a). By adding the lecithin stabilizer ($K_{Lec/Ag}$ = 0.2) spherical AgNPs (Figure 2b) with a size of 5–19 nm and mean value of 12.06 nm were synthesized.

Figure 3a presents the size distribution of these AgNPs, which have a narrow size distribution. The spherical and narrow size distribution of AgNPs were also confirmed by the narrow and sharp UV absorption peak at 405 nm. Higher amount of stabilizer led to synthesis of AgNPs in a range of 75–110 nm and mean value of 83.84 nm (Figure 3b). With a high lecithin concentration, liposome nanoparticles were created in the mean size of 84 nm, and AgNPs were entrapped in them (Figure 2c).

Antibacterial of AgNPs

As well as the synthesis of AgNPs, the antibacterial property of AgNPs against two Gram-positive and Gram-negative bacteria was also analyzed. Table I shows MIC and MBC of synthesized AgNPs with $K_{Lec/Ag} = 2$. The ratio of MBC/MIC is a feature which can show the bactericidal ability of antibacterial agents (Ayala-Núñez et al. 2009). The synthesized AgNPs in the phospholipids membranes with $K_{Lec/Ag} = 2$ had a ratio of 1.6 and 2 for *E. coli* and *S. aureus*, respectively. The smaller ratio suggests that a little amount of antibacterial agent is needed to reach the bactericidal effect. Therefore, the synthesized AgNPs with a ratio lower than 2 could be considered as a bactericidal agent (Ayala-Núñez et al. 2009).

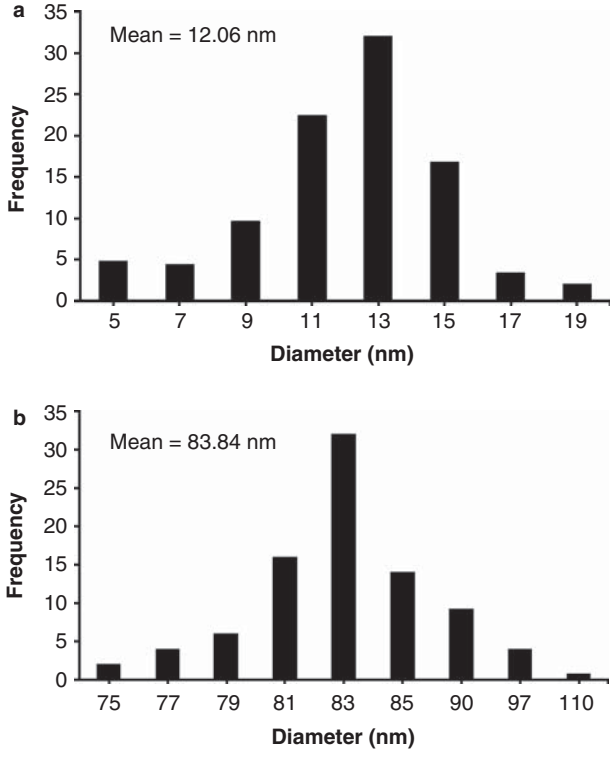

Figure 3. Size distribution histogram of AgNPs at two different lecithin concentrations: (a) $K_{Lec/Ag}$ = 0.2, and (b) $K_{Lec/Ag}$ = 2, increased lecithin concentration causes to increase size complex which indicates the presence of multilayer of lecithin molecules on AgNPs surface.

Table I. Mean value of MIC and MBC for synthesized AgNPs at phospholipid membrane with the ratio of $K_{Lec/Ag}$ = 2 and determined by broth dilution method. Experiments were performed in duplicates.

Bacterial strain	MIC (μg/ml)	MBC (μg/ml)
E. coli	25	40
S. aureus	12.5	25

Table II shows the bacterial inhibition zones at various silver contents for two samples with $K_{lec/Ag}$ values of 1 and 2, while the other ratios did not show any bacterial inhibition zones. Results indicated that AgNPs demonstrate higher inhibition zone diameter against *S. aureus* than *E. coli* which was confirmed by other researchers (Cho et al. 2005, Prema and Raju 2009, Barani et al. 2010). So, it can be suggested that the antibacterial effects of AgNPs can be associated with types of bacterial species. It is hypothesized that the AgNPs with smaller particles size can easily penetrate to the agar medium and can absorb to the cell surface of bacteria which result in higher antibacterial activity (Morones et al. 2005). However, the large AgNPs at ratio lower than 1 did not show the antibacterial properties. The size of nanoparticles ensures that a significantly large surface area of the particles is in contact with the bacterial cell membrane. Considering a spherical particles of uniform size on a theoretical case, a decrease in the particle size from 10 μm to 10 nm will increase the contact surface area by 10^6 times. Also, inhibition depends on the AgNPs concentrations, which the stabilized AgNPs at lower 6.25 ppm did not prove the bacterial inhibition zones (Prema and Raju 2009).

When the $K_{Lec/Ag}$ value was lower than 1, the inhibition zone was not remarkable in both agar plates containing *E. coli* and *S. aureus* (Figure 4). The results suggested that the AgNPs were not stable at lower concentrations of lecithin which leads to agglomeration and sedimentation of AgNPs and not to diffuse to the agar plates (Lee et al. 2010). Figure 4 shows that silver nanoparticles were settled in the wells and cannot diffuse to the agar medium. According to the results, it can be assumed that the antibacterial property of AgNPs depends on silver concentration, lecithin concentration, and bacterial species.

Table II. Mean value of inhibition zone diameter of AgNPs with various amounts of lecithin. Experiments were performed in duplicates.

		Mean of inhibition zone diameter (mm)					
AgNPs concentration (ppm)		100	50	40	25	12.5	6.25
	Bacterial strain						
$K_{lec/Ag} = 1$	*S. aureus*	13	12	11.25	10.25	9	—[a]
	E. coli	10.5	9.75	9.25	9	—	—
$K_{lec/Ag} = 2$	*S. aureus*	12.75	11.75	11	9	9	—
	E. coli	10.75	9.25	9	9	—	—

[a]No antibacterial activity was found with the concentrations tested in this work.

Bacterial killing kinetics:

The antibacterial kinetics of AgNPs were determined in the aqueous media and nutrition broth by counting the number of colony units. The kinetics reduction of bacterial colonies was calculated according to Equation (3):

$$\text{Reduction } \% = (N_0 - N)/N_0 \times 100 \quad (3)$$

where N is the number of CFU per milliliter of solution at any defined time, and N_0 is the initial number of CFU per milliliter of solution at the beginning of test. Figure 5 shows the percentage of *E. coli* and *S. aureus* reduction at various $K_{Lec/Ag}$ values at 100 ppm concentrations during 120 min. It shows that increasing of $K_{Lec/Ag}$ value led to lower bacterial killing rate. This means that higher value of lecithin causes slow release of silver nanoparticles to the medium which leads to longer bacterial inactivation time.

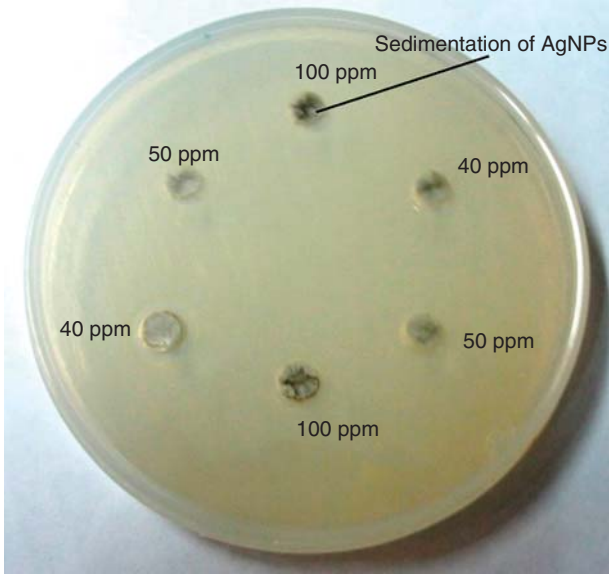

Figure 4. Photographic images of unstabilized AgNPs sedimentations ($K_{lec/Ag}$ = 0.002) in the CASO agar wells at various concentrations.

The buffer testing media does not support the bacterial growth. The bacterial suspension at all conditions has a decline trend in the number of bacteria during 120 min. Furthermore, the bacterial suspension without AgNPs also showed a decline trend, which was not significant.

Lecithin molecule is a safe biological agent which capped the AgNPs with a bilayer structure. Figure 6, shows the formation of phospholipids membrane on the surface of silver nanoparticles. The presence of lecithin molecule bilayer leads to reduce the direct contact of AgNPs to the bacterial cell membrane which results in lower killing rate of AgNPs. The silver nanoparticles should diffuse through the bilayer and contact to the bacterial cell membrane and cause the cell death. The killing rate is more decreased by an increase in lecithin concentration which leads to formation of multilayer structure on the AgNPs surface and prolong reaching time to the bacterial cell membrane.

The higher amount of $K_{lec/Ag}$ resulted in the lowest killing rate because of slower release rate of AgNPs. The slow release of AgNPs from phospholipid membranes can be achieved by high amount of lecithin which is due to larger nanocomposites (Barani et al. 2010) and leads to slow release. In comparison, the silver nanoparticles without stabilizer killed all bacteria within 20 min at $K_{Lec/Ag} = 2$; this time was prolonged to 90 and 120 min for *S. aureus* (Figure 5a) and *E. coli* (Figure 5b), respectively, because the AgNPs had direct contact to the bacterial cell membrane and could easily disturb its permeability.

The killing kinetic of bacteria can be shown according to the Chick-Watson equation (Kwok-Keung and LeChevallier 2004):

$$\frac{dN}{dt} = KC \quad (4)$$

$$ln\frac{N}{N_0} = -KCt \quad (5)$$

where N is the number of CFU per milliliter of solution at the time t, N_0 is the initial number of

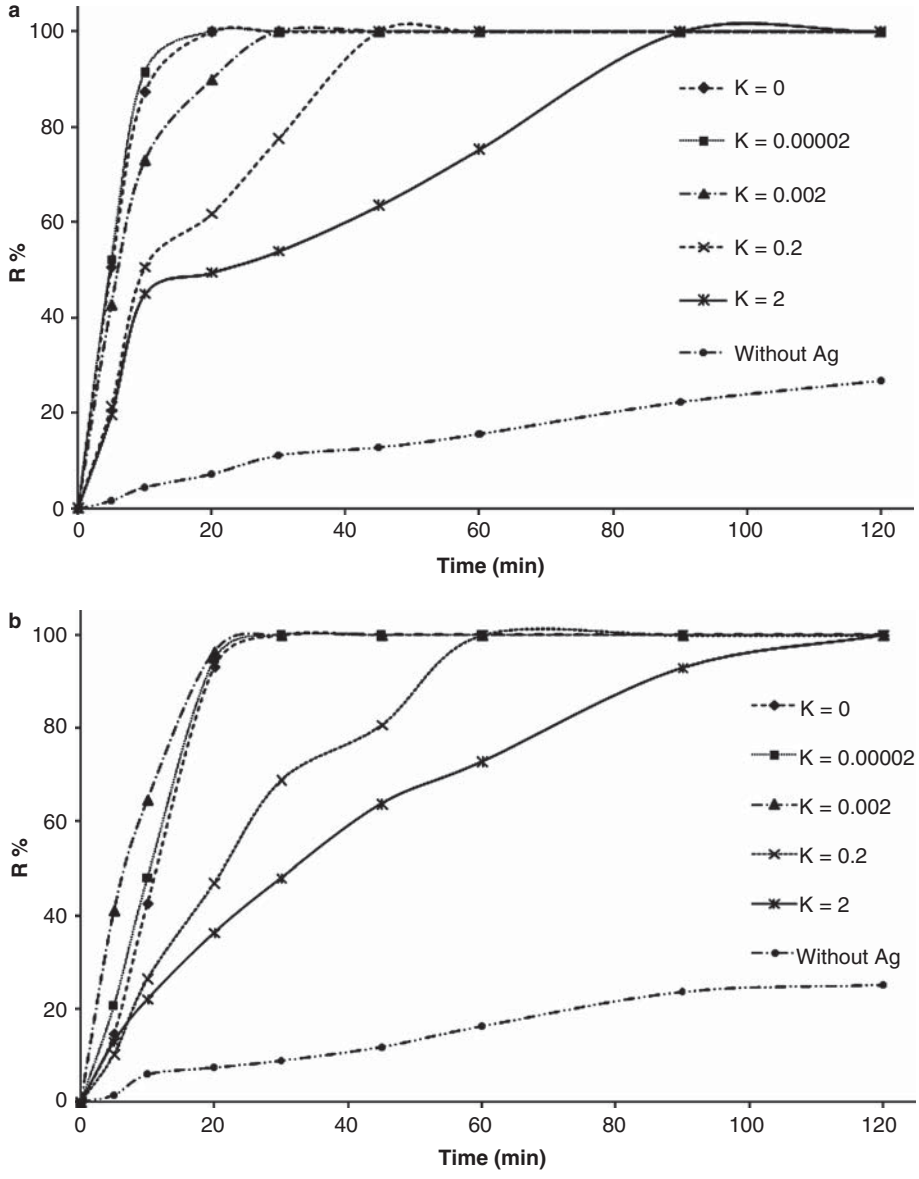

Figure 5. Bacterial inactivation Kinetics of (a) *S. aureus* and (b) *E. coli* which is calculated according to $R\% = (N_0-N)/N_0 \times 100$ during 120 min, in which N, and N_0 are the number of CFU per milliliter of solution at the defined and initial time, respectively.

CFU per milliliter of solution at the beginning of the test, C is the concentration of applied AgNPs colloidal solution and K is the specific coefficient of lethality. The killing rate at specific concentrations can be shown as Equation (4). The specific coefficient of lethality was calculated according to Equation (4) for 100 ppm concentration of AgNPs colloidal solution and presented in Figure 7. The linear regression was applied for fitting a mathematical model to the data. The existed models have a good correlation to the experimental data with a high regression coefficient ($R^2 > 0.91$) for all samples.

The specific coefficient of lethality of AgNPs increases with a decrease in lecithin concentration which increases their direct contact to the bacterial cell membrane. The AgNPs without lecithin had the highest specific coefficient of lethality because of high direct contact of AgNPs to bacterial cell membrane. In addition, this coefficient on *S. aureus* is higher than *E. coli,* which shows that these bacteria are inactivated quicker than *E. coli* in presence of AgNPs.

Conclusion

The synthesized AgNPs colloidal solution had a localized peak at 405 nm indicating the presence of spherical structure. Moreover, this was also confirmed

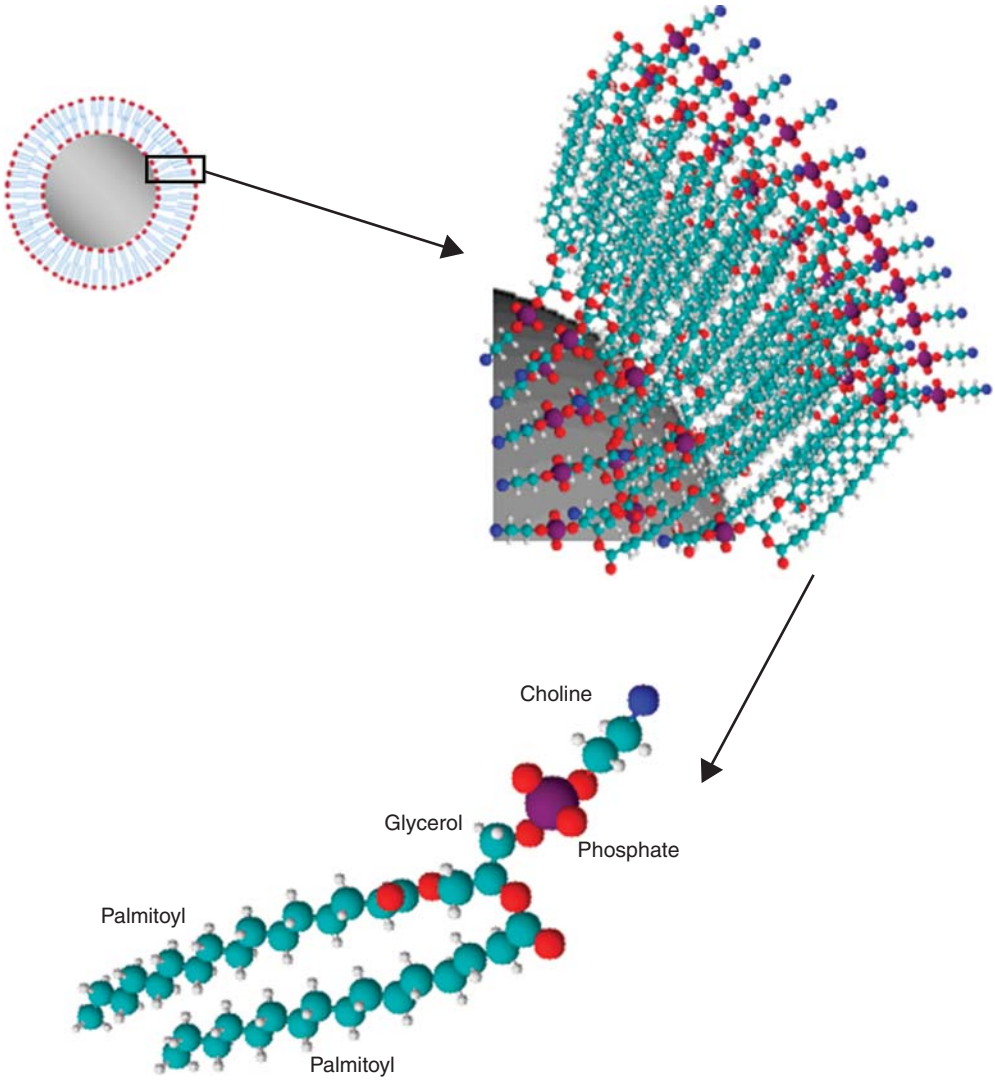

Figure 6. Schematic molecular of lecithin which is formed as a bilayer membrane on the AgNPs surface.

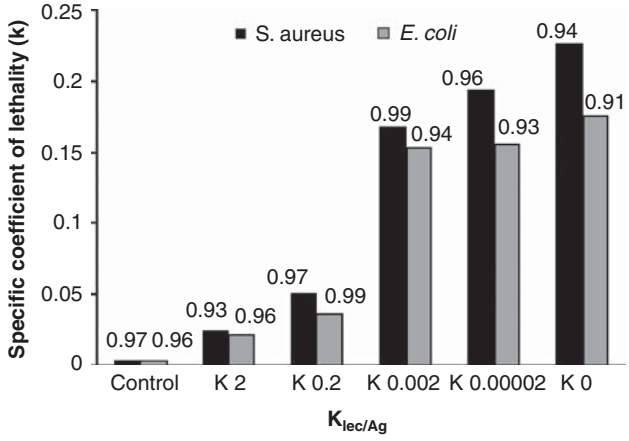

Figure 7. The specific coefficient of lethality of colloidal AgNPs solution (100 ppm) on *S. aureus* and E. *coli* is obtained by adopting the bacterial inactivation data to the Chick-Watson model by linear regression. The inset numbers on each bar show the linear regression coefficient.

by the TEM micrographs. The synthesized AgNPs without stabilizer had a short shelf life and agglomerated quickly, linked to each other and created large particles. Lecithin molecules as a stabilizer separated the AgNPs and prevented their agglomeration. The stabilized AgNPs only presented antibacterial properties in agar method due to their small size. The MBC/MIC ratio of stabilized AgNPs was low which indicated the capacity of bactericidal effects of these nanoparticles. The sample with lower $K_{Lec/Ag}$ did not show antibacterial properties at various concentrations of silver in the well diffusion test because of less stability of colloidal silver nanoparticles. The bacteria reduction by AgNPs in the buffer solution was well described by the Chick-Watson model. The specific coefficient of lethality was calculated from this model with a high regression coefficient. The specific coefficient of lethality was dependant on the lecithin concentration. An increase in lecithin concentration, as a phospholipids membrane stabilizer, caused slower release of silver and prolonged the time for total bacterial inactivation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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