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Antibiofilm efficacy of silver nanoparticles against biofilm forming multidrug resistant clinical isolates

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Abstract

Purpose: The purpose of this study was to assess the antibiofilm activity of silver nanoparticles (AgNPs) against multidrug resistant gram negative bacterial isolates. Different approaches have been used for preventing biofilm-related infections in health care settings. Many of these methods have their own difficulty, which include chemical-based complications; emergent antibiotic resistant strains, etc. Therefore, the aim of present study was to demonstrate the anti-biofilm activity of silver nanoparticles against the selected five strong biofilm forming multidrug resistant gram negative bacterial strains includes {*Escherichia coli* (ETEC12), *Klebsiella pneumoniae* (SKP7), *Pseudomonas aeruginosa* (ETPS11), *Proteus mirabilis* (PPM8) and *Acinetobacter baumannii* (SAB5)} were used in this study. **Methods:** The anti-biofilm activity of AgNPs with different concentration of 12.5 to 100 µg/ml was investigated by direct visualisation applying test tube method and congo red agar method along with scanning electron microscopy (SEM) techniques.

Results: The biofilm inhibitory concentration was found to be in the range of 12.5 – 100 µg/ml. The technique using SEM provides the visual evidence that AgNPs arrested the bacterial growth and prevent the exopolysaccharides formation. The AgNPs effectively restricted the biofilm formation of the tested bacteria. In our study, we could expose the complete anti-biofilm activity of AgNPs at a concentration as low as 100 µg/ml.

Conclusions: Our findings suggested that AgNPs can be defeated towards the development of potential anti-bacterial coatings for various biomedical and environmental applications. In future, the AgNPs may play major role in the coating of medical devices and treatment of infections caused due to extensively antibiotic resistant biofilm.

Keywords: Anti-biofilm, AgNPs, exopolysaccharide, congo red agar and SEM

Introduction

The first recorded observation pertaining to biofilm was probably given by Henrici in 1933, who observed that water bacteria are not free floating but grow upon submerged surfaces [1]. Biofilm consists of multifaceted cell clusters embedded in a matrix of extracellular polysaccharide (slime), which facilitates the adherence of these microorganisms to biomedical surfaces and protect them from host immune system and anti-microbial therapy [2]. Biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA), which mediates cell to cell adhesion and is the gene product of icaADBC [3]. Various reports attest to the presence of icaADBC gene in *Staphylococcus aureus* and *S. epidermidis* isolated from infections associated with indwelling medical devices [4].

It is now well documented that biofilms are exceptionally difficult to eradicate and are often resistant to systemic antibiotic therapy and removal of infected device becomes necessary [5, 6]. Biofilm organisms have a natural resistance to antibiotics, disinfectants and germicides. Unlike planktonic populations, bacterial cells entrenched in biofilms exhibit inherent resistance to antibiotics due to several specific defense mechanisms deliberated by the biofilm environment, including the inactivation of anti-microbial agents by exopolysaccharide (EPS), over expression of stress-responsive genes, oxygen gradients within the biofilm matrix and demarcation of a subpopulation of biofilm cells into resistant dormant cells [7, 8]. The natural resistance of bacterial cells within biofilms to conventional anti-microbials has motivated new approaches for the treatment of biofilm-associated infections, including the use of silver preparations. Several silver-containing dressings are recommended for long-term decontamination and wound healing based on silver's broad-spectrum, high-level anti-microbial activity [9]. The difficulty in eradicating a chronic infection associated with biofilm formation lies in the fact that biofilm bacteria are able to resist higher antibiotic concentration than bacteria in suspension [10].

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Nanotechnology may provide the remedy to penetrate such biofilms and reduce biofilm formation by the use of 'nanofunctionalization' surface techniques to prevent the biofilm formation. Silver nanotechnology can prevent the formation of life-threatening biofilms on medical devices. Silver is one of the former known anti-microbials. It has recently been established that AgNPs hydrogel hybrid with different sizes of AgNPs can be effectively employed as anti-bacterial agents [11]. Saxena *et al.* studied that propylene-based sutures immobilised AgNPs show anti-bacterial activity against *Staphylococcus aureus* and *Escherichia coli* [12]. Due to the strong anti-bacterial properties and low toxicity towards mammalian cells, AgNPs have been applied in a wide range of areas including wound dressing, coatings on medical devices to reduce nosocomial infection rates [13], protective clothing, anti-bacterial surfaces, water treatment, food preservation and cosmetics as biocidal and disinfecting agents [14].

In general, the ability of resistance to antimicrobial agents in biofilm is 10 to 1000 times higher than planctonic cells [15]. Considering the importance of biofilm in infectious diseases and increasing drug resistance, scientists are searching for appropriate ways to control and prevent biofilm. In general, therapy with a combination of antibiotics, novel cephalosporin, metal chelating agents, quorum sensing inhibitors, halogens, phage therapy and nanoparticles are used as antibiofilm agents [15].

Infections resulting from microbial biofilm formation remain a serious threat to patients worldwide. Particularly problematic are wound infections [16], with chronic wounds such as foot, leg and pressure ulcers being particularly susceptible to biofilm infections [17]. So as to kill or remove biofilms, anti-microbials must penetrate the polysaccharide matrix to increase access to the microbial cells. Developing AgNPs as a new generation of antimicrobial agents may be an attractive and cost-effective means to overcome the drug resistance problem seen with Gram-negative bacteria. The aim of this study involved systematically analyzing the antibiofilm potential of the biologically prepared AgNPs against a group of human pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Acinetobacter baumannii* by tube method, congo red agar (CRA) method and scanning electron microscopy (SEM).

Materials

Bacterial strains

Clinical isolates from different samples were procured from Rajah Muthiah Medical College and Hospital, Annamalai University and were subjected to biofilm formation and antibiotic susceptibility test. Biofilm formation was confirmed by microtiter well plate method [18, 19] and the biofilm producing bacterial strains were subjected to antibiotic susceptibility pattern to selected antibiotics was observed by Kirby Bauer disc diffusion method as per CLSI guidelines [20]. Thus the bacterial strains showed resistance to all drugs was chosen for this study. Thus the selected five strong biofilm forming multidrug resistant gram negative bacterial strains includes {*Escherichia coli* (ETEC12), *Klebsiella pneumoniae* (SKP7), *Pseudomonas aeruginosa* (ETPS11), *Proteus mirabilis* (PPM8) and *Acinetobacter baumannii* (SAB5)} were used in this study. The biosynthesized silver nanoparticles from *Euphorbia hirta* were characterized according to methods described previously [21]. The

subsequent dilutions of silver nanoparticles (AgNPs) were made in autoclaved Milli Q water and were used in this study.

Methods

(i). Antibiofilm assay of silver nanoparticles by tube method

{*Escherichia coli* (ETEC12), *Klebsiella pneumoniae* (SKP7), *Pseudomonas aeruginosa* (ETPS11), *Proteus mirabilis* (PPM8) and *Acinetobacter baumannii* (SAB5)} strains were used to study the antibiofilm activity of the biosynthesized silver nanoparticles (AgNPs) according to Hasan *et al.* [22] with a little modification in this method. Briefly, pre-sterilized narrow and small test tubes were taken and 2mL of sterilized trypticase soy broth (TSB) media was added to each tube. Then, 50 µl (microliter) of each freshly cultured strain was added to each respective labeled test tube. After that, five concentrations of AgNPs ranging from 12.5, 25, 50, 75 and 100 µg/ml (microgram per milliliter) were added to each strain. Both negative and positive controls in tubes containing without culture and without AgNPs respectively were maintained for each strain. Finally, all test tubes were incubated at 37°C for 24 h. Thus the formation of biofilm in all test tubes was monitored at 24 h interval. After specified incubation period, the culture broths from the tube were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were stained with crystal violet (0.1 %) and the contents were decanted gently. Excess stain was washed with deionized water and dried. Biofilm formation was considered positive when visible film lined the wall and bottom of the tube. Ring formation at the air-liquid interface was not indicative of biofilm formation.

(ii). Antibiofilm assay by congo red agar method

Freeman *et al.* [23], had described an alternative method for screening of biofilm formation, which requires the use of a specially prepared solid medium brain heart infusion agar (BHIA) supplemented with 5 % sucrose and congo red (BHIC) for screening the formation of biofilm. Following autoclave, the concentrated congo red solution was added to agar which was previously cooled to 55°C. With or without AgNPs treated inoculums from above stated tube method were used for congo red agar (CRA) method. After 24 h incubation of tube method, formerly inoculated and incubated cultures from each test tube containing different concentrations of AgNPs treated strains were inoculated into each labeled respective congo red agar medium and incubated aerobically for 24 h at 37 °C. The plates were inspected for color of the colonies at 24 h. On CRA, slime-producing strains develop black colonies, whereas non-slime-producing strains form red colonies. Positive result was indicated by black colonies with a dry crystalline consistency.

(iii). Antibiofilm activity of AgNPs by Scanning electron microscopy

Klebsiella pneumoniae (SKP7) strain was used as a model strain for antibiofilm efficiency of AgNPs by scanning electron microscopy (SEM) observation. Biofilms of *Klebsiella pneumoniae* (SKP7) strain were assessed as previously described and treated with or without AgNPs of different concentration (12.5, 25, 50, 75 and 100 µg/ml). Briefly, the cells were washed with PBS, fixed with 2.5 % glutaraldehyde, then fixed samples were subsequently washed again with PBS and dehydrated gently by washing in a series of ethanol alcohol (30%, 50%, 70%, 80%, 95% and 100%) for

10 min at room temperature, and critical point drying was performed [24]. Afterwards, cells were then oriented, mounted on the aluminium stubs and coated with gold before imaging. The topographic features of the biofilms were visualized with a SEM (JSM-5610, JEOL /EO, SCM version 1.1) with accelerating voltage of 15 kV.

Results

(i). Antibiofilm Activity of AgNPs by tube method

We analyzed the impact of AgNPs at various concentrations on the formation of biofilms by *Escherichia coli* (ETEC12), *Klebsiella pneumoniae* (SKP7), *Pseudomonas aeruginosa* (ETPS11), *Proteus mirabilis* (PPM8) and *Acinetobacter baumannii* (SAB5) strains. After 24 h of treatment, the silver nanoparticles inhibited the biofilm formation was observed as from strong biofilm (+++) producing to moderate biofilm (++) producing at both the concentration of 12.5 and 25 µg/ml of AgNPs and weakly (+) produced at both the concentration of 50 and 75 µg/ml of AgNPs and utterly inhibited the biofilm

formation at 100 µg/ml of AgNPs by *Escherichia coli* (ETEC12), *Klebsiella pneumoniae* (SKP7), *Pseudomonas aeruginosa* (ETPS11), *Proteus mirabilis* (PPM8) strains. The *Acinetobacter baumannii* (SAB5) strain was found no biofilm inhibition at the doses of 12.5 µg/ml concentration of AgNPs as such showed strong biofilm producer (+++) but the biofilm inhibition set off from 25 µg/ml as displayed as moderately produced (++) from strong biofilm producing (+++). The moderate and weak biofilm was produced at 50 and 75 µg/ml of AgNPs respectively and completely inhibited the biofilm formation at 100 µg/ml for *A. baumannii*. The biofilm formation was uniformly inhibited when treated with 100 µg/ml of AgNPs till 24 h for all strains and strong biofilm formation was observed in positive control tubes when treated without AgNPs (0 µg/ml) as well as no biofilm was observed in negative control for all the strains. Biofilm formation was progressively inhibited with increasing concentrations of AgNPs [Figure-1] [Table-1].

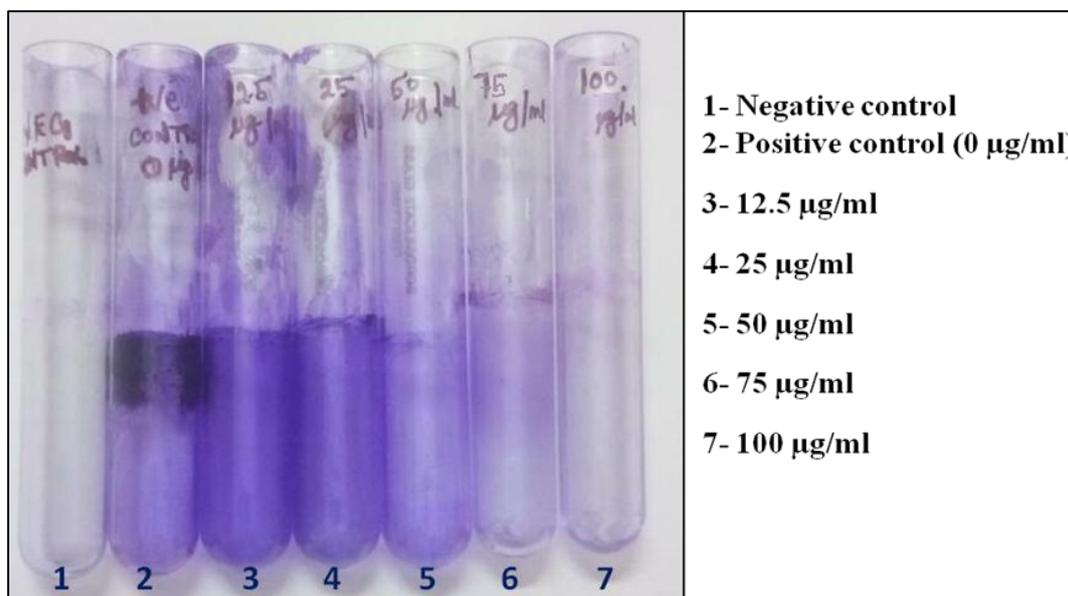


Fig 1: Antibiofilm activity of silver nanoparticles with different concentrations by tube method

(ii). Characterisation of anti-biofilm activity of AgNPs on CRA Plates

Biofilm formation is detected in many organisms synthesizing exopolysachharides. The biofilm is made up of microorganisms adhering to the surface coated with slime - the exopolysachharide matrix which protects the microbes from the unfavourable environmental factors. Biofilm formation by *Escherichia coli* (ETEC12), *Klebsiella pneumoniae* (SKP7), *Pseudomonas aeruginosa* (ETPS11), *Proteus mirabilis* (PPM8) and *Acinetobacter baumannii* (SAB5) strains was tested by growing the organism in brain-heart infusion agar supplemented with Congo Red (BHIC) with and without silver nanoparticles at 24 h of incubation period. When the colonies were grown without AgNPs in the medium, the organisms appeared as dry crystalline black

colonies, indicating the production of exopolysachharides, which is the precondition for the formation of biofilm. Whereas when the organisms were grown on BHIC with AgNPs, the organisms did not survive. During the treatment with reduced concentrations of AgNPs (12.5 µg/ml), the organisms continued to grow, but AgNPs treatment has inhibited the synthesis of glycocalyx matrix, indicated by the absence of dry crystalline black colonies. It was found that at higher concentration of AgNPs inhibited bacterial growth by more than 90%. When the glycocalyx matrix synthesis is arrested, the organism cannot form biofilm. It was also observed that 25 µg/ml of AgNPs significantly arrested biofilm formation without affecting viability, whereas 100 µg/ml completely blocked the biofilm formation and inhibited the growth of the organism itself [Figure-2] [Table-1].

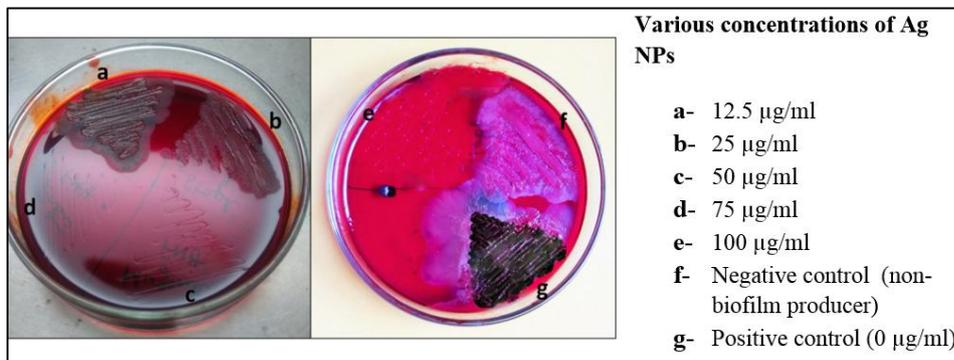


Fig 2: Antibiofilm activity of silver nanoparticles with different concentrations by congo red agar method

Table 1: Antibiofilm activity of AgNPs by tube and congo red agar method

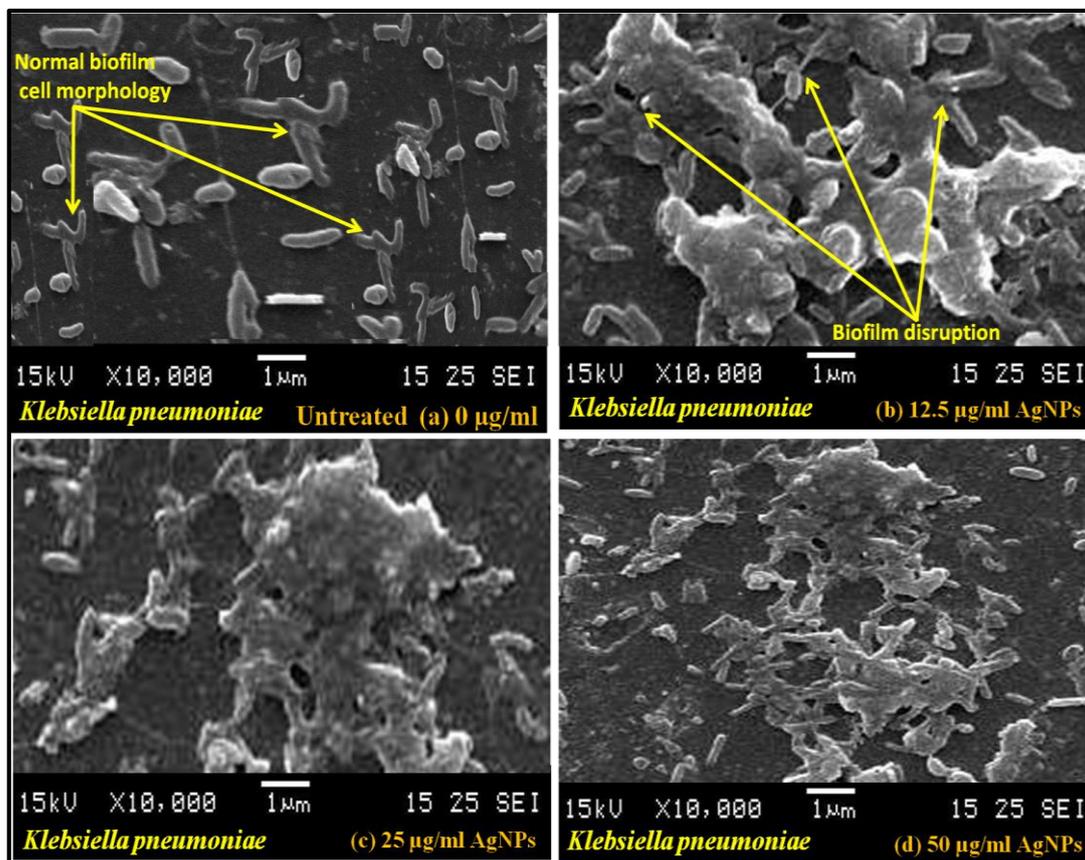
S. No	SBP-MDR strains	Negative control	Positive control (0 µg/ml)	Tube method					Congo red agar method				
				Various concentration of biosynthesized AgNPs in µg/ml									
				12.5	25	50	75	100	12.5	25	50	75	100
Strong biofilm producing multidrug resistant strains													
1	ETEC12	-	+++	++	++	+	+	-	B	B	AB	R	-
2	SKP7	-	+++	++	++	+	+	-	B	B	AB	R	-
3	ETPS11	-	+++	++	++	+	+	-	B	B	AB	R	-
4	PPM8	-	+++	++	++	+	+	-	B	B	AB	R	-
5	SAB5	-	+++	+++	++	++	+	-	VB	B	B	R	-

SBP- strong biofilm producing; MDR- multidrug resistant strains; µg/ml- microgram per milliliter; AgNPs- silver nanoparticles; VB- very black colonies; B- black colonies; AB- almost black colonies; R- red colonies; (+++/VB)- strong biofilm producer; (++/B)- moderate biofilm producer; (+/AB)- weak biofilm producer; (-/R)- Non biofilm producer.

(iii). Characterization of anti-biofilm activity of AgNPs by scanning electron microscopy

The biofilm grown on glass slide for 24 h was observed using scanning electron microscopy (SEM). The biofilm formed by the native strain have aggregated and clumped bacterial cells. The *Klebsiella pneumoniae* (SKP7) strains were selected as model biofilm for SEM examination. The SKP7 strain was

grown without AgNPs exhibit the expected normal cellular morphology with smooth cell surfaces [Figure-3(a)]. Under the same growth conditions but in the presence of different concentrations of AgNPs (12.5 - 100 µg/ml) *K. pneumoniae* cell shows changes in morphology and it was also examined that AgNPs inhibit bacterial colonisation on the surfaces [Figure-3(b-f)].



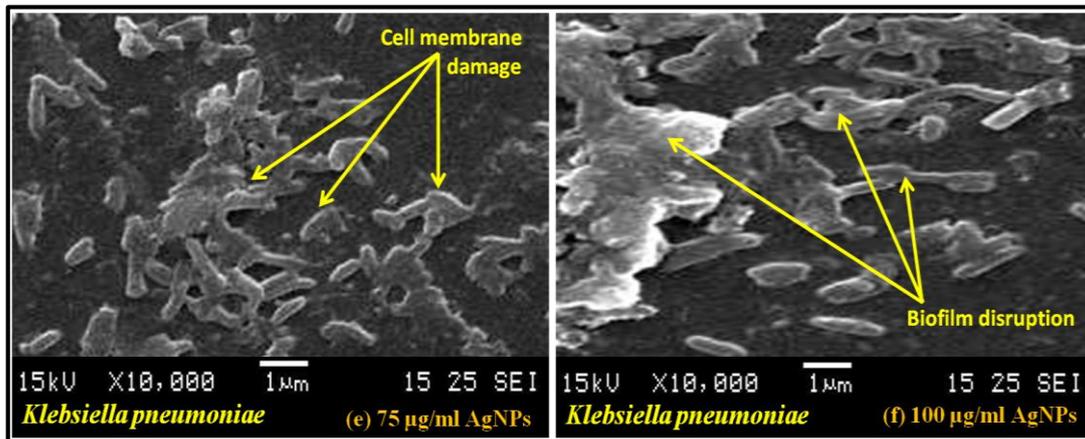


Fig 3: SEM micrographs of *Klebsiella pneumoniae* (SKP7) biofilms developed on the glass slide surface at 24 h of incubation; (a). Untreated; (b). Treated with 12.5, (c). 25, (d). 50, (e). 75 and (f). 100 µg/ml of silver nanoparticles (AgNPs).

The apparent biofilm formed by *K. pneumoniae* has very few cells individually scattered along the surface. The cells were arranged in pairs of bacilli or many bacilli with absence of exopolysaccharides matrix. The biofilm formed by the *K. pneumoniae* was very much erratic. More specifically, an obvious increase in the roughness of the cell surface suggested that it has been damaged by the nanoparticles. Microscopic evaluation of the surfaces clearly shows that the AgNPs treated glass surfaces do not allow bacterial colonisation and biofilm formation compared with the untreated control. Untreated glass surfaces supported a huge biofilm formation by *K. pneumoniae* [Figure-3(a)], while AgNPs treated glass surfaces shows dramatically restricted bacterial colonisation and biofilm formation [Figure-3(f)]. These results suggest that AgNPs are effective in limiting bacterial and biofilm colonization of the surface.

Discussion

Chemical antimicrobial agents are progressively more agreeable resistant to a wide spectrum of antibiotics. An alternative way to beat the drug resistance of various microorganisms is therefore urgently needed. Ag ions and silver salts have been used for decades [25] as antimicrobial agents in various fields due to their growth inhibitory abilities against microorganisms. However, there are some limitations in using Ag ions or Ag salts as antimicrobial agents. Probable reasons include the interfering effects of salts. This type of limitation can be removed by using silver in nano form.

In this study biofilm inhibition gradually decreased while increasing with AgNPs concentration. Biofilm was formed when the tubes were treated with AgNPs at 12.5, 25, 50 and 75 µg/ml concentrations of AgNPs [Figure-1(3-6)] but no biofilm was formed at 100 µg/ml containing tubes by all the strains we used [Figure-1(7)]. Similar results were also reported by Asaduzzaman *et al.* analyzed the impact of AgNPs at various concentrations on the formation of biofilm by *K. pneumoniae*. Biofilm was produced by *K. pneumoniae* at the doses of 5, 10 and 20 µg/ml and no biofilm was observed at 40 and 80 µg/ml concentration of AgNPs. On the other hand, biofilm was observed in the test tube containing no AgNPs [26].

However, the anti-biofilm efficacy of AgNPs was investigated by growing the organism on CRA supplemented with and without AgNPs. When the colonies were grown without AgNPs, the organisms appeared as dry crystalline black colonies, indicating the production of exopolysaccharides (EPS), which is the requirement for the formation of biofilm

[Figure-2(g)]. Whereas when the organisms were grown with AgNPs, the organisms did not survive. During the treatment with reduced concentrations of AgNPs (12.5 µg/ml), the organisms continued to grow, but AgNPs treatment has inhibited the synthesis of glycocalyx matrix, indicated by the absence of dry crystalline black colonies. However, at higher concentration of AgNPs (100 µg/ml) almost no growth was observed [Figure-2(c-e)]. Thus, when the exopolysaccharide synthesis is arrested, the organism cannot form biofilm. Similar results were also reported by Kalishwaralal *et al.* against *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms and found that 100 nM of AgNPs resulted in a 95-98 % reduction in biofilm [27]. It was also mentioned in literature that if the surface of medical devices has an AgNPs coating, then it was helpful in preventing bacterial adhesion and subsequent biofilm formation on the medical devices [28].

Based on these results we continued to address the mechanism of action of AgNPs on biofilm-forming bacterial species by applying SEM. SEM was used to examine cell morphologies following exposure to the nanoparticles. *K. pneumoniae* strain was grown without AgNPs exhibit the expected normal cellular morphology with smooth cell surfaces [Figure-3(a)]. Under the same growth conditions but in the presence of suspended AgNPs, *K. pneumoniae* cells showed changes in morphology [Figure-3(b-f)]. More specifically, an obvious increase in the irregularity of the cell surface suggested that it has been damaged by the AgNPs. EPS within *K. pneumoniae* biofilms was not detected by SEM. SEM observations clearly indicate that AgNPs reduced the surface coverage by *K. pneumoniae* biofilms [Figure-3(b-f)].

Our results are in agreement with previously reported anti-biofilm activity of nanocrystalline silver by Kostenko *et al.* They observed that nanocrystalline silver dramatically decreased viable cell numbers within the tested biofilms. SEM results shows that nanocrystalline silver reduced the surface coverage by *Pseudomonas aeruginosa* biofilms. Prolonged treatment with nanocrystalline silver provided a further reduction in the surface coverage by methicillin resistant *Staphylococcus aureus* (MRSA) biofilms. The surface coverage by *E. coli* biofilms was reduced by the tested dressings by approximately 20% after the first day [29]. The presence of biofilms on medical devices or surfaces can only be observed using a limited number of techniques. The reason why the demonstration of bacterial biofilms is challenging is because it is difficult to stain both the bacteria and

glycocalyx. Kostenko *et al.* reported that Acticoat nanocrystalline silver has the highest anti-biofilm efficacy compared with Aquacel silver and Silverlon and the silver concentration alone cannot account for the anti-biofilm efficacy of the silver dressings [29].

Ansari *et al.* proved that, *E. coli* and *K. pneumoniae* biofilms were treated with AgNPs when the exopolysaccharide synthesis is arrested, the organism cannot form biofilm on CRA medium and also in SEM observation by changing the morphology of biofilms as roughness of the cell surface damaged by 20 µg/ml of AgNPs [30]. Ansari *et al.* investigated that inhibitory effect of AgNPs on the existing biofilm may be due to the presence of water channels throughout the biofilm. Since all biofilms water channels (pores) that are present for nutrient transportation, AgNPs may directly diffuse through the exopolysaccharides layer by the way of pores and may impart antimicrobial function [30].

Chaudhari *et al.* investigated that the antibiofilm activity of AgNPs (51 nm) synthesized from *Bacillus megaterium* and reported that AgNPs showed enhanced quorum quenching activity against *Staphylococcus aureus* biofilm and prevention of biofilm formation. They concluded that AgNPs might be involved in neutralizing these adhesive substances, thus preventing the biofilm formation [31]. The reduction of the silver particle to the nanoscale level increases the relative surface area, which provides higher Ag⁺ release rates than for elemental silver particles [32]. Moreover, nanoparticles have a higher capacity to attach and to penetrate bacterial membranes and accumulate inside cells, providing a continuous release of silver ions inside the cell [30, 33, 34].

Another researcher reported that *Mukia maderaspatana* leaf extract mediated synthesized AgNPs were most effective against Gram-positive bacteria [35]. The mechanism of action of AgNPs against different bacteria is unknown. It has been reported that AgNPs cause formation of pores/pits in the bacterial cell wall [36] that may depend on the particle size. A small nanoparticle on bacterial surfaces increases the permeability [37], binds the functional groups of DNA and proteins, and destroys the cell [36]. Generally, biofilms are known to provide resistance against several antimicrobial agents [38] and biofilm can be one of the leading causes for a shift from acute-phase diseases to chronic diseases [39].

Franci *et al.* reported that AgNPs inhibit biofilm formation by altering the membrane of *K. pneumoniae* and causing irreversible damage on bacterial cells, alteration of membrane permeability, and respiration of *K. pneumoniae* [39]. Most biofilm-forming bacteria associated with human infections are *Enterococcus faecalis*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *K. pneumoniae*, *Moraxella catarrhalis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* [40]. Nowadays, researchers became interested in controlling the formation of bacterial biofilm using green AgNPs. Therefore, in the present investigation, multidrug resistance bacterial strain *K. pneumoniae* was cultured in the presence and absence of AgNPs and it was found that the growth of *K. pneumoniae* was completely inhibited in the presence of AgNPs at 100 µg/mL. Several studies reported the antibiofilm activity of silver nanoparticles [39, 41]. Palanisamy *et al.* also reported that AgNPs inhibited biofilm formation of *P. aeruginosa* [41]. Other researchers stated that *Calotropis procera* assisted AgNPs have antibiofilm activity against *Vibrio cholerae* and enterotoxigenic *Escherichia coli* [42].

In this study, silver nanoparticles had potent anti-biofilm effects. Antimicrobial effects of silver nanoparticles have

been previously studied [43, 44, 45], but there are a few studies on effects of silver nanoparticles against bacterial biofilm [46, 47]. A study from India reported that the production of biofilms in *E. coli*, *S. aureus*, *Salmonella typhi* and *Vibrio cholerae* were inhibited by silver nanoparticles [48]. Namasivayam *et al.* studied the effects of alone silver nanoparticles and also in combination with several antibiotics, and they concluded that silver nanoparticles made a complete inhibition of biofilm within 24 hours, as well as a good compatibility with combination of silver nanoparticles and antibiotics to inhibit biofilm [49].

Wood (2009) shown that several non-toxic antibiofilm (antivirulence) compounds exist for *E. coli* including brominated furanones, ursolic acid, indole derivatives and 5-fluorouracil [50]. The mechanism of resistance of bacterial biofilms has yet to be elucidated but a potentially important factor is production of the glycocalyx which enables cells growing within the biofilm to evade host defences and the activity of antimicrobial agents [51].

However, the exact mechanism of action of AgNPs in biofilm-related studies is yet to be demonstrated. Our findings suggested that AgNPs can be defeated towards the development of potential anti-bacterial coatings for various biomedical and environmental applications. In future, the AgNPs may play major role in the coating of medical devices and treatment of infections caused due to extensively antibiotic resistant biofilms. This study suggests that AgNPs have antibiofilm therapeutic potential, but further studies are still required namely regarding formulation and delivery means.

Conclusion

When assume the biofilm phenotype, these infections are often extremely difficult to treat. The biofilm infections may fail to respond to antibiotic therapy or it may initially respond only to relapse weeks or months later. In such cases, invasive treatments, such as surgical removal and replacement of the infected tissue or device, may be required. So for proper treatment of biofilm infections causing clinical isolates screening for biofilm production is necessary. The presence of biofilms on medical devices or surfaces can only be observed using a limited number of techniques. The reason why the demonstration of bacterial biofilms is challenging is because it is difficult to stain both the bacteria and glycocalyx.

The biofilm formation is associated with nature of clinical specimens. The small particles of silver nanoparticles are more antibiofilm activity depends on concentration. This research shows that silver nanoparticles have strong antibiofilm activity. The antibiofilm effect of silver nanoparticles against bacterial isolates is different and *K. pneumoniae* is more sensitive to nanoparticles. The silver nanoparticles can be used to inhibit bacterial biofilms, and may be useful for treatment of infectious diseases due to biofilms. We recommend conducting more studies concerning this issue and particularly conducting *in vivo* and clinical trial searches for toxicity level before the administration of silver nanoparticles in the treatment of biofilm infections.

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. O Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol.* 2000;

- 54:49-79.
2. O Gara JP, Humphreys H. Staphylococcus epidermidis biofilms: Importance and implications. J Med Microbiol. 2001; 50:582-7.
 3. Ammendolia MG, Rosa RD, Montanaro L, Arciola CR, Baldassarri L. Slime production and expression of slime-associated antigen by staphylococcal clinical isolates. J Clin Microbiol. 1999; 37:3235-8.
 4. Arciola CR, Baldassarri L, Montanaro L. Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter-associated infections. J Clin Microbiol. 2001; 39:2151-6.
 5. Schwank S, Rajacic Z, Zimmerli W, Blaser J. Impact of bacterial biofilm formation on *in vitro* and *in vivo* activities of antibiotics. Antimicrob Agents Chemother. 1998; 42:895-8.
 6. Souli M, Giamarellou H. Effects of Slime produced by clinical isolates of coagulase negative staphylococci on activities of various antimicrobial agents. Antimicrob Agents Chemother. 1998; 42:939-41.
 7. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett. 2004; 230:13-8.
 8. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol. 2005; 13:34-40.
 9. Leaper DJ. Silver dressings: Their role in wound management. Int Wound J. 2006; 3:282-94.
 10. Gristina AG, Hobgood CD, Web LX, Myrvik QN. Adhesive colonization of biomaterials and antibiotics resistance. Biomaterials. 1987; 8:423-6.
 11. Mohan YM, Lee K, Premkumar T, Geckeler KE. Hydrogel networks as nonreactors: A novel approach to silver nanoparticles for antibacterial applications. Polymer. 2007; 48:158-64.
 12. Saxena S, Ray AR, Kapil A, Pavon-Djavid G, Letourneur D, Gupta B, *et al.* Development of a new polypropylene-based suture: Plasma grafting, surface treatment, characterization, and biocompatibility studies. Macromol Biosci. 2011; 11:373-82.
 13. Chaloupka K, Malam Y, Seifalian AM. Nanosilver as a new generation of nanoparticle in biomedical applications Trends Biotechnol. 2010; 28:580-8.
 14. Moritz M, Geszke-Moritz M. The newest achievements in synthesis, immobilization and practical applications of antibacterial nanoparticles. Chem Engg J. 2013; 228:596-613.
 15. Czaczyk K, Myszka K. Mechanisms determining bacterial biofilm resistance to antimicrobial factors. Biotechnologia. <http://dx.doi.org/10.5772/33048>. 2007; 76(1):40-52.
 16. Percival SL, Bowler P, Woods EJ. Assessing the effect of an antimicrobial wound dressing on biofilms. Wound Repair Regen. 2008; 16:52-7.
 17. James GA, Swogger E, Wolcott R, deLancey Pulcini E, Secor P, Sestrich J, *et al.* Biofilms in chronic wounds. Wound Repair Regen. 2010; 16:37-44.
 18. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, *et al.* Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J. Clin. Microbiol. 1985; 22:996-1006.
 19. Christensen BE. The role of extracellular polysaccharides in biofilms. J Biotechnol. 1989; 10:181-202.
 20. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. In: 17th informational supplement. CLSI, Wayne, 2007, M100-S17.
 21. Gurunathan S, Kalishwaralal K, Vaidyanathan R, Deepak V, Pandian SRK, Muniyandi J, *et al.* Biosynthesis, purification and characterization of silver nanoparticles using *Escherichia coli*. Colloid Surf B. 2009; 74:328-335.
 22. Hasan Y, Ozeki, SR Kabir. Purification of a novel chitin-binding lectin with antimicrobial and antibiofilm activities from a Bangladeshi cultivar of potato (*Solanum tuberosum*), Indian Journal of Biochemistry and Biophysics. 2014; 51(2):142-148.
 23. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative Staphylococci. J Clin Pathol. 1989; 42:872-874.
 24. Ansari MA, Khan HM, Khan AA, Cameotra SS, Pal R. Antibiofilm efficacy of silver nanoparticles against biofilm of extended spectrum β -lactamase isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Appl Nanosci. 2014; 4:859-868.
 25. Silver S, Phung LT. Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol. 1996; 50:753-789.
 26. Asaduzzaman AKM, Byung-Soo Chun, Syed Rashed Kabir. *Vitis vinifera* Assisted Silver Nanoparticles with Antibacterial and Antiproliferative Activity against Ehrlich Ascites Carcinoma Cells. Journal of Nanoparticles, 2016; Article ID 6898926:1-9. <http://dx.doi.org/10.1155/2016/6898926>.
 27. Kalishwaralal K, Barath Mani Kanth S, Pandian SR, Deepak V, Gurunathan S. Silver nanoparticles impede the biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Colloids Surf B Biointerfaces. 2010; 79:340-4.
 28. Knetsch ML, Leo HK. New strategies in the development of antimicrobial coatings: The example of increasing usage of silver and silver nanoparticles. Polymers. 2011; 3:340-66.
 29. Kostenko V, Lyczak J, Turner K, Martinuzzi TJ. Impact of silver-containing wound dressings on bacterial biofilm viability and susceptibility to antibiotics during prolonged treatment. Antimicrob Agents Chemother. 2010; 54:5120-31.
 30. Ansari MA, Khan HM, Khan AA, Cameotra SS, Pal R. Antibiofilm efficacy of silver nanoparticles against biofilm of extended spectrum β -lactamase isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Appl Nanosci 2013. [In press]. DOI: 10.1007/s13204-013-0266-1.
 31. Chaudhari PR, Masurkar SA, Shidore VB, Kamble SP. Effect of biosynthesized silver nanoparticles on *Staphylococcus aureus* biofilm quenching and prevention of biofilm formation. Intern J Pharm Bio Sci. 2012; 3(1):222-229.
 32. Dunn K, Edwards-Jones V. The role of Acticoat with nanocrystalline silver in the management of burns. Burns. 2004; 30:1-9.
 33. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv. 2009; 27:76-83.
 34. Ansari MA, Khan HM, Khan AA, Cameotra SS, Saquib Q, Musarrat J. Gum arabic capped-silver nanoparticles inhibit biofilm formation by multi-drug resistant strains

- of *Pseudomonas aeruginosa*. J Basic Microbiol. 2014; 54:1-12.
35. Harshiny M, Matheswaran M, Arthanareeswaran G, Kumaran S, Rajasree S. Enhancement of antibacterial properties of silver nanoparticles-ceftriaxone conjugate through *Mukia maderaspatana* leaf extract mediated synthesis, Ecotoxicology and Environmental Safety. 2015; 121:135-141.
 36. Sharma VK, Yngard, Lin Y. Silver nanoparticles: green synthesis and RA their antimicrobial activities, Advances in Colloid and Interface Science. 2009; 30(1-2):83-96.
 37. Hamouda T, Baker JR. Antimicrobial mechanism of action of surfactant lipid preparations in enteric gram-negative bacilli, Journal of Applied Microbiology. 2000; 89(3):397-403.
 38. Lewis K. Multidrug tolerance of biofilms and persister cells, Current Topics in Microbiology and Immunology. 2008; 322:107-131.
 39. Franci G, Falanga A, Galdiero S. *et al.* Silver nanoparticles as potential antibacterial agents, Molecules. 2015; 20(5):8856-8874.
 40. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms, Clinical Microbiology Reviews. 2002; 15(2):167-193.
 41. Palanisamy NK, Ferina N, Amirulhusni AN *et al.* Antibiofilm properties of chemically synthesized silver nanoparticles found against *Pseudomonas aeruginosa*, Journal of Nanobiotechnology. 2014; 12:2.
 42. Salem W, Leitner DR, Zingl FG *et al.* Antibacterial activity of silver and zinc nanoparticles against *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, International Journal of Medical Microbiology. 2015; 305(1):85-95.
 43. Taylor EN, Webster TJ. The use of superparamagnetic nanoparticles for prosthetic biofilm prevention. Int J Nanomedicine. <http://dx.doi.org/10.2147/ijn.s5976>. 2009; 4:145.
 44. Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. Sensors. 2012; 12(3):2519-2538. <http://dx.doi.org/10.3390/s120302519>.
 45. Velázquez-Velázquez JL, Santos-Flores A, Araujo-Meléndez J, Sánchez-Sánchez R, Velásquillo C, González C, Martínez-Gutiérrez F. Anti-biofilm and cytotoxicity activity of impregnated dressings with silver nanoparticles. Materials Science and Engineering: C. 2015; 49:604-611.
 46. Guzmán MG, Dille J, Godet S. Synthesis of silver nanoparticles by chemical reduction method and their antibacterial activity. Int J Chem Biomol Eng. 2009; 2(3):104-111.
 47. Mathur T, Singhal S, Khan S, Upadhyay D, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol. <http://dx.doi.org/10.4103/0255-0857.19890>. 2006; 24(1):25.
 48. Kumar P, Senthamilselvi S, Lakshmi Praba A, Premkumar K, Muthukumar R, Visvanathan P, *et al.* Efficacy of bio-synthesized silver nanoparticles using *Acanthopora spicifera* to encumber biofilm formation. Dig J Nanomater Biostruct. 2012; 7(2):511-522.
 49. Namasivayam SKR, Preethi M, Bharani A, Robin G, Latha B. Biofilm inhibitory effect of silver nanoparticles coated catheter against *Staphylococcus aureus* and evaluation of its synergistic effects with antibiotics. Int J Biol Pharm Res. 2012; 3:259-265.
 50. Wood TK. Insights on *Escherichia coli* biofilm formation and inhibition from whole-transcriptome profiling. Environ Microbiol. 2009; 11(1):1-15.
 51. Costerton JW, Cheng KJ, Geesey GG, Ladd IT, Nickel JC, Dasgupta M *et al.* Bacterial biofilms in nature and disease. Ann Rev Microbiol. 1987; 41:435-464.