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Original Article

Antibacterial activity of silver nanoparticles against methicillin-resistant *Staphylococcus aureus* synthesized using model *Streptomyces* sp. pigment by photo-irradiation method

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ABSTRACT

Background: In the past few years, the rates of morbidity and mortality are increasing due to methicillin-resistant *Staphylococcus aureus* (MRSA) infection so, studies in controlling this infection is gravely required. Silver nanoparticles antimicrobial activity were well known but rapid and green synthesis of silver nanoparticles having stupendous antimicrobial activity against MSRA is needed.
Aim: Synthesis and evaluation of antibacterial activity of silver nanoparticles against MRSA.
Methods: The pigment produced by *Streptomyces coelicolor* was used to reduce $AgNO_3$ in solution to yield silver nanoparticles using photo-irradiation. The synthesized silver nanoparticles were characterized by UV–visible spectroscopy; X-ray diffraction (XRD), transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). The synthesized silver nanoparticles were tested for antibacterial activity against MRSA, and the synergetic effects, of the combination of silver nanoparticles and antibiotics were evaluated.
Results: We developed rapid synthesis for silver nanoparticles using *S. coelicolor* pigment by photo-irradiation within 20 min. The UV–visible spectroscopy result show maximum absorption between 400 and 450 nm a preliminary confirmation for nanoparticles synthesis. The XRD result confirms the crystalline nature of silver nanoparticles. The TEM image shows the particles are irregular having the size in a range of 28–50 nm. The FTIR result indicates the pigment as the probable reducing agent. The silver nanoparticles alone and in combination with antibiotics, exhibited antibacterial activity against MRSA.
Conclusions: The rapid synthesis method developed in this study for the synthesis of silver nanoparticles has distinct advantages over traditional synthesis as the process is less time consuming. The kinetics of silver nanoparticles synthesis using *S. coelicolor* pigment with photo-irradiation indicates an appropriate way to develop green technology for the bulk synthesis. Furthermore, these biosynthesized silver nanoparticles, alone and in combination with antibiotics, exhibited excellent antimicrobial activity against a MRSA which could be an alternate drug of choice.

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1. Introduction

Staphylococcus aureus (*S. aureus*) resistant to methicillin is a major problem that the world is now facing.[1] The antibiotic era, barely 60 years old, is also threatened because of, increase resistance rhythm of this organism against different antibiotics.[2] So, studies are desperately required in finding out new antimicrobial agents against methicillin resistant *Staphylococcus aureus* (MRSA). Silver antimicrobial properties were known from antiquity, having the history with manhood dating back to 4000 BC.[3] Silver vessels were used to preserve water and wine. Hippocrates the father of medicine, promoted the use of silver for healing the wounds.[4] The mutation-resistant antimicrobial activities of silver are being used in different pharmaceutical formulations such as antibacterial clothing, burn ointments, and coating for medical devices.[5] With the present day understanding of nanoscience, one can clearly get enlightened that these formulations contained silver nanoparticles.[6] Keeping the knowledge of silver nanoparticles in mind, we made an attempt to use antimicrobial activity of silver nanoparticles against MRSA, isolated from Gulbarga region.

Generally, nanoparticles are prepared by several methods such as physical and chemical but these methods are not eco-friendly.[7] In contrast biological methods urged as safe, cost-effective, possible eco-friendly alternatives to physical and chemical methods.[8] Many non-toxic synthesis of silver nanoparticles using various fungi like *Aspergillus flavus*[9] *Rhizopus stolonifer*,[10] *Neurospora crassa*,[11] have been reported so far, but there is no report on synthesis of silver nanoparticles using pigment produced by *Streptomyces coelicolor* by photo-irradiation method. To our knowledge this is first report on synthesis of silver nanoparticles by this route.

S. coelicolor is a gram positive, well known blue pigment (actinorhodin) producer, widely used as a model for molecular genetics studies of secondary metabolism and differentiation in *Streptomycetes*.[12] The main reason for selecting this pigment is the antimicrobial property of the pigment (actinorhodin)[13] if it is used as reducing agent, the synthesized silver nanoparticles antimicrobial activity may be enhanced.

This paper deals with bio-based synthesis, characterization of silver nanoparticles using pigment produced by *S. coelicolor* by photo-irradiation method and assessment of antimicrobial activity of silver nanoparticles against MRSA.

2. Materials and methods

2.1. *Isolation and identification of MRSA*

S. aureus isolates have been isolated from different sources like pus, blood, and other exudates from different hospitals and health care centers of Gulbarga region. The preliminary identification of *S. aureus* was done using mannitol salt agar (differential media) which was detected by change in color of the medium from red to yellow due to mannitol fermentation Fig. 1a further, the *S. aureus* identified based on morphological, microscopic, and biochemical tests Table 1a among the identified *S. aureus* the MRSA was detected using antibiotic susceptibility test as per the guidelines recommended by Clinical and Laboratory Standards Institute (CLSI-2012).[14]

2.1.1. *Isolation and identification of S. coelicolor*

Soil samples from different areas of Gulbarga, Karnataka; India were collected and screened for pigment producing actinomycetes by serial dilution method using starch casein agar (SCA) medium and incubated at 27 °C for seven days. On the basis of the pigment production, the isolate klmp33 was selected and maintained on fresh SCA medium checked for its purity and stored at 4 °C for further work. The isolate klmp33 was identified as *S. coelicolor* based on 16S rRNA sequencing and the sequences were submitted to Gene Bank under the accession number JQ27722.

2.1.2. *Pigment analysis and synthesis of silver nanoparticles*

The blue pigment produced by *S. coelicolor* after 3 days of incubation was separated from the biomass by centrifuging the starch casein medium at 10,000 rpm for 10 min. The resultant supernatant was analyzed using Thin Layer Chromatography conducted on silica-gel 60 F_{254} plates (Merck) with benzene-acetic acid (9:1; v/v) as solvent. The pigment obtained a single spot with an Rf value of 0.28 indicating the presence of actinorhodin (now onwards the pigment is referred as actinorhodin).[15]

For the synthesis of silver nanoparticles, 15 ml of $AgNO_3$ (10^{-3} M) solution was treated with the 1 ml actinorhodin and exposed to direct sun light. A color change from colorless to brown took place within few minutes indicating the formation of silver nanoparticles. A yield about 1.4 g of silver nanoparticles per liter was obtained from the above method. Further, the same silver nanoparticles were used for antimicrobial studies.

2.2. *Characterization of silver nanoparticles*

2.2.1. *UV–visible spectroscopy*

The synthesis of silver nanoparticles was preliminary confirmed by UV–visible spectroscopy, which is an important technique to verify the formation of metal nanoparticles provided that surface plasmon resonance exists for the metal.[16] The UV–visible spectroscopy was analyzed for period of 20 min, conducted on Systronics double-beam UV–visible spectrophotometer 2200, operated at 0.1 nm resolution with scanning rate 270 nm/min.

2.2.2. *X-ray diffraction (XRD)*

The synthesis of silver nanoparticles was further confirmed using XRD. The X-ray diffraction patterns for the synthesized silver nanoparticles were recorded using a Rigaku Ultima 4 XRD instrument. The radiation used was Cu-Kα (0.154 nm) at 40 kV and 35 nm with scanning rate of 2°/min.

2.2.3. *Transmission electron microscopy (TEM)*

The TEM technique was employed to determine the size and shape of the silver nanoparticles. The TEM image was obtained using a Philips CM200 instrument. Sample for this analysis was prepared by coating carbon-coated copper grid with aqueous silver nanoparticles. After 5 min, the extra

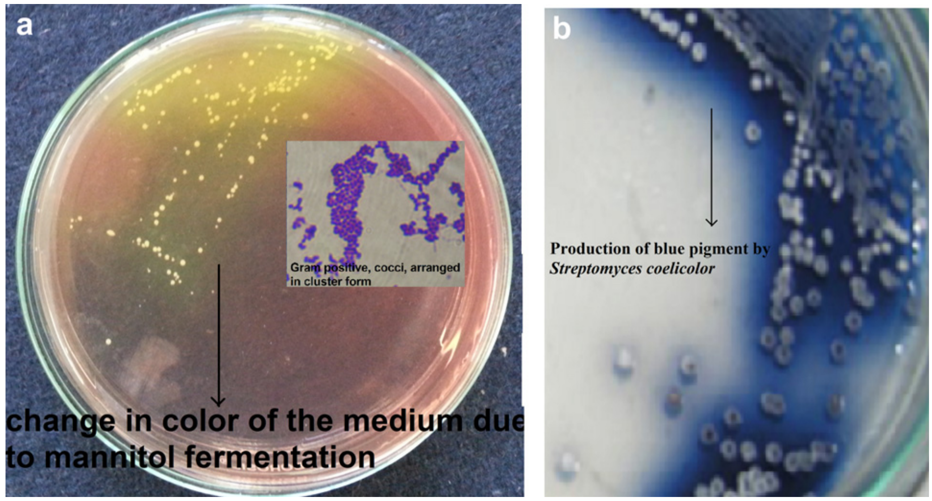

Fig. 1 – (a): *S. aureus* on mannitol salt agar. (b): *Streptomyces coelicolor* on starch casein agar.

solution was removed using blotting paper, and then the film on the grid was dried under IR light.

2.2.4. *Fourier transform infrared spectroscopy (FTIR)*

A powder of silver nanoparticles was prepared by centrifuging the solution of synthesized silver nanoparticles at 10,000 rpm for 20 min. The solid residue was then washed with distilled water to remove any unattached biological moieties from the surface of the nanoparticles. The resultant residue was then dried completely, and the powder was used for FTIR measurement, which was performed on a NICOLET iS5 with Diamond ATR. The FTIR peaks were identified and expressed in wave numbers (cm^{-1}). To know the probable reducing agent the actinorhodin was also analyzed.

2.3. *Antibacterial activity of silver nanoparticles against MRSA*

To evaluate antimicrobial property of silver nanoparticles against MRSA we determined the minimum inhibitory concentration (MIC). To determine MIC different volumes of synthesized silver nanoparticles (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μL) and MRSA culture (maintained at 10^6 CFU/ml) were added in to lactose broth medium and was incubated at 37 °C for 18 h. The MIC was determined by measuring the optical density at 625 nm.

2.4. *Synergistic effect of synthesized silver nanoparticles with antibiotics*

The synergistic effect of silver nanoparticles with antibiotics has proven to be beneficial[17] this effect against MRSA was determined by disk diffusion method. To assess the synergistic effect, each standard antibiotic disk was impregnated with 30 μL of freshly prepared silver nanoparticles, and then these disks was used in antibacterial activity assays.

Table 1a – Identification of *S. aureus* based on morphological, microscopic and biochemical tests.

Morphological characters	
1. Colony shape	Round
2. Colony size	Punctiform
3. Colony color	Golden yellow color
Microscopic characters	
1. Gram staining	Gram-positive (cocci, grape like arrangement)
2. Motility	Non-motile
3. Spore staining	Non-spore former
Biochemical tests	
1. Catalase	Positive
2. Coagulase	Positive
3. Phosphatase	Positive
4. β-hemolysis	Positive

3. Results and discussion

A number of approaches are available for the synthesis of silver nanoparticles, e.g., chemical synthesis, radiation-assisted synthesis, electrochemical sonication and biological synthesis.[18] Among these methods, biological synthesis are not only a good way to fabricate benign nano materials, but also reduce the use of substances hazardous to human health and the environment. Non toxic biological synthesis of silver nanoparticles using 5 days old biomass of *Aspergillus flavus* in 9 h was reported by Vigneshwaran et al[9] Similarly Binupriya et al synthesized silver nanoparticles using 3 days old *R. stolonifer* biomass within 72 h.[10] In this study, we synthesized silver nanoparticles in 20 min using *S. coelicolor* pigment (actinorhodin) by photo-irradiation method. Compared with the above biological methods our synthesis is rapid. Moreover, it is a bio-based synthesis so; it is advantageous over other methods, in being non toxic. To best of our knowledge this is the first report on synthesis of silver nanoparticles using *S. coelicolor* pigment by photo-irradiation.

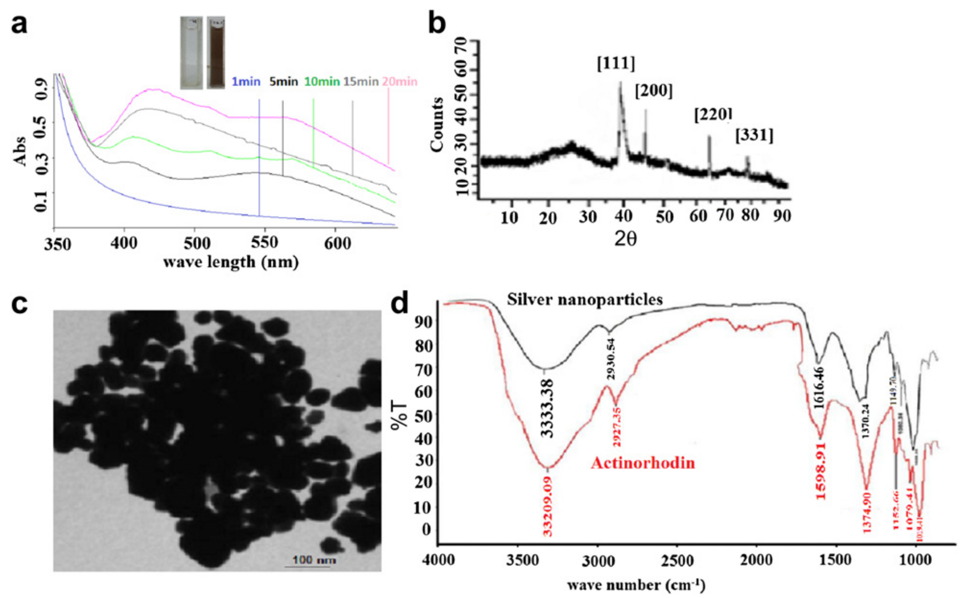

Fig. 2 – (a): UV–visible spectrum showing the photo-irradiated silver nanoparticles synthesis recorded as a function of time. (b): XRD pattern of synthesized silver nanoparticles. (c): TEM image of synthesized silver nanoparticles. (d): FTIR spectra of synthesized silver nanoparticles and actinorhodin.

3.1. *Synthesis and characterization of silver nanoparticles*

The actinorhodin produced by *S. coelicolor* was used for the synthesis of silver nanoparticles (Fig. 1b). For the synthesis, 15 ml $AgNO_3$ (10^{-3} M) solution was treated with 1 ml actinorhodin and the solution was exposed to sun light. A color change from colorless to brown took place within a few minutes indicating the formation of silver nanoparticles. The solution mixture also kept in dark (used as control). No change in color was observed indicating no synthesis of silver nanoparticles.

3.1.1. *UV–visible spectroscopy*

The synthesis of silver nanoparticles was preliminary confirmed by color change caused due to surface plasmon resonance of silver nanoparticles in the visible region.[19] The absorbance intensity of the brown color increased steadily as a function of reaction time. The absorption maximum between 400 and 450 nm (Fig. 2a) clearly indicates the formation of silver nanoparticles.

3.1.2. *XRD*

The crystalline nature of the synthesized nanoparticles was analyzed by X-ray diffraction. Fig. 2b shows a representative pattern of the synthesized nanoparticles after the reduction of $AgNO_3$. Intense peaks corresponding to (111), (200), (220) and (311) were observed. These peaks can be indexed based on the FCC structure of silver (JCPDS files no. 03–0921), confirming the crystalline nature of the silver nanoparticles.

3.1.3. *TEM*

A representative TEM image is shown in Fig. 2c. The size of the silver nanoparticles was in the range of 28–50 nm and they are irregular in shape.

3.1.4. *FTIR*

Fig. 2d shows the FTIR spectra of the purified silver nanoparticles and actinorhodin. The purified nanoparticles exhibited absorption peaks at 1149, 1616, 1645 and 3333 cm^{-1} due to cyclic C–O–C, C=O and OH functional groups respectively. The peaks obtained were compared with actinorhodin, less intense peaks with slightly shift were observed in the purified silver nanoparticles. From the FTIR spectra it may be inferred

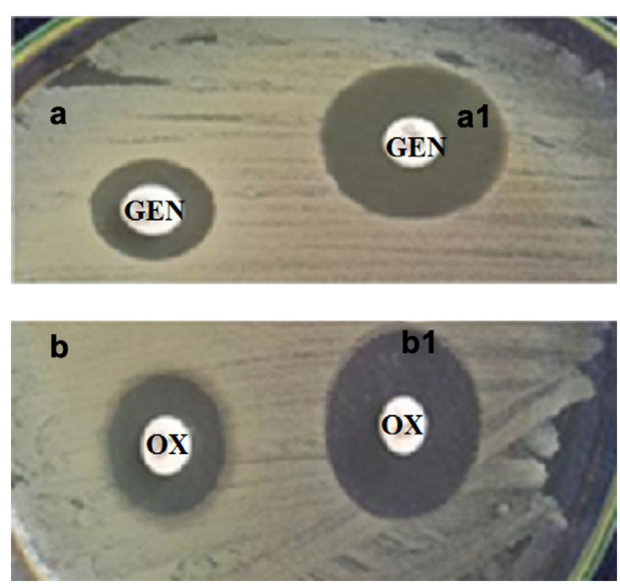

Fig. 3 – Zone of inhibition against MRSA: (a) Only with gentamicin, (a_1) with gentamicin and silver nanoparticles, (b) Only with oxacillin, (b_1) with oxacillin and silver nanoparticles.

Table 1b – Zone of inhibition (mm) of different antibiotics (with and without silver nanoparticles) against MRSA.

Antibiotics		Synergistic effect of antibiotics with silver nanoparticles	
Gentamicin (a) 10 mcg/disc	Oxacillin (b) 1 mcg/disc	Silver nanoparticles with gentamicin(a_1)	Silver nanoparticles with oxacillin(b_1)
14	10	22	20

that actinorhodin was the reducing agent which is involved in the synthesis of silver nanoparticles.

3.2. *Antibacterial activity of silver nanoparticles against MRSA*

To evaluate antibacterial effect of silver nanoparticles against MRSA we determined the MIC. The MIC of silver nanoparticles against MRSA was estimated (30 μL). The mechanism of the bactericidal effect of silver nanoparticles remains to be elucidated. Several studies have proposed that silver nanoparticles bind to the surface of the cell membrane, disrupting cellular permeability and the respiration functions of the cell. Smaller silver nanoparticles having a large surface area available for interaction have a greater bactericidal effect than larger silver nanoparticles.[20] It is also possible that silver nanoparticles not only interact with the surface of the membrane, but also penetrate inside the bacteria and inactivate DNA replicating ability[21] causing the devastation of the cell.

3.3. *Synergistic effect of synthesized silver nanoparticles with antibiotics*

To study the synergetic effect two antibiotics, gentamicin and oxacillin, with silver nanoparticles were selected against the MRSA isolate. The antimicrobial activity of the antibiotics (gentamicin and oxacillin) increased in the presence of silver nanoparticles Fig. 3 which may be caused due to interaction of active groups such as, hydroxyl and amide group present in the antibiotic molecules which chelates antibiotic silver nanoparticles interaction.[22] The fold increase in the antibacterial effect was greater for gentamicin than oxacillin when these antibiotics were combined with silver nanoparticles (Table 1b). From the results it is clear that the synthesized silver nanoparticles alone and in combination with antibiotics, exhibited excellent antimicrobial activity against MRSA. Furthermore, as this is bio-based synthesis they become safe, non toxic and alternate antibacterial agent for treatment.

Conflicts of interest

All authors have none to declare.

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REFERENCES

1. Awadh R, Al-Anazi. Prevalence of methicillin-resistant *Staphylococcus aureus* in a teaching hospital in Riyadh, Saudi Arabia. *Biomed Res*. 2009;20(1):7–14.
2. Richard P, Wenzel Michael BE. Vancomycin-resistant *Staphylococcus aureus*: infection control considerations. *Clin Infect Dis*. 1998;27:245–251.
3. Chen X, Schluesener HJ. Nanosilver: a nanoproduct in medical application. *Toxicol Lett*. 2008;176:1–12.
4. Wesley Alexander J. History of the medical use of silver. *Surg Infect*. 2009;10(3):289–292.
5. Ravishankar Bhat, Raghunandan Deshpande, Sharanabasava VG, Do Sung Huh, Venkataraman A. Photo-irradiated biosynthesis of silver nanoparticles using edible mushroom *Pleurotus florida* and their antibacterial activity studies, bioinorganic chemistry and applications. http://dx.doi.org/10.1155/2011/650979.
6. Kalishwaralal Kalimuthu, Venkataraman Deepak, Ram Kumar Pandian SureshBabu, et al. Biosynthesis of silver and gold nanoparticles using Brevibacterium casei. *Colloids Surf B Biointerfaces*. 2010;77:257–262.
7. Dubey Shashi Prabha, Lahtinen Manu, Sarkka Heikki, Sillanpaa Mika. Bioprospective of *Sorbus aucuparia* leaf extract in development of silver and gold nanocolloids. *Colloids Surf B Biointerfaces*. 2010;80:26–33.
8. Saifuddin N, Wong CW, Nur Yasumira AA. Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. *E-Journal of Chemistry*. 2009;6(1):61–70.
9. Vigneshwaran N, Ashtaputre NM, Varadarajan PV, Nachane RP, Paralikar KM, Balasubramanya RH. Biological synthesis of silver nanoparticles using the fungus *Aspergillus flavus*. *Materials Lett*. 2007;61:1413–1418.
10. Binupriya AR, Sathishkumar M, Yun SI. Biocrystallization of silver and gold ions by inactive cell filtrate of *Rhizopus stolonifer*. *Colloids Surf B Biointerfaces*. 2010;79:531–534.
11. Castro-Longoria E, Alfredo RVN, Avalos-Borja M. Biosynthesis of silver, gold and bimetallic nanoparticles using the filamentous fungus *Neurospora crassa*. *Colloids Surf B Biointerfaces*. 2011;83:42–48.
12. Bentley SD, Chater KF, Cerdeno-Tarraga AM, et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature*. 2002;417:141–147.
13. Wright F, Hopwood D. Actinorhodin is a chromosomally determined antibiotic in *Streptomyces coelicolor* A3 (2). *J Gen Microbiol*. 1976;96:289–297.
14. CLSI. *Performing Standards for Antimicrobial Susceptibility Testing: Twenty-second Informational Supplement*. CLSI document M100–S22. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.
15. Bystrykh LV, Fernandez-Moreno MA, Herrema JK, Malpartida F, Hopwood DA. Production of actinorhodin-related blue pigments by *Streptomyces coelicolor* A3 (2). *J Bacteriol*. 1996;178:2238–2244.
16. Basavaraja S, Balaji SD, Arunkumar L, Rajasab AH, Venkataraman A. Extracellular biosynthesis of silver

nanoparticles using the fungus *Fusarium semitectum*. *Mater Res Bull*. 2008;43:1164–1170.
17. Furno F, Morley KS, Wong B, et al. Silver nanoparticles and polymeric medical devices: a new approach to prevention of infection. *J Antimicrob Chemother*. 2004;54:1019–1024.
18. Devendra Jain, Daima Hemant Kumar, Kachhwaha Sumita, Kothari SL. Synthesis of plant mediated silver nanoparticles using papaya fruit extract and evaluation of their antimicrobial activities. *Dig J Nanomater Biostruct*. 2009;4:723–727.
19. Vadivel S, Suja S. Green synthesis of silver nanoparticles using *Coleus amboinicus lour*, antioxidant activity and in vitro cytotoxicity against Ehrlich's Ascite carcinoma. *J Pharm Res*. 2012;5(2):1268–1272.
20. Kvitek L, Panacek A, Soukupova J, et al. Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). *J Phys Chem*. 2008;112:5825–5834.
21. Morones JR, Elechiguerra JL, Camacho A. The bactericidal effect of silver nanoparticles. *J Nanotechnol*. 2005;16:2346–2353.
22. Fayaz Amanulla Mohammed. Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *J. Nanomed*. 2010;6:103–109.