Bactericidal activity of starch-encapsulated gold nanoparticles

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1. ABSTRACT

We report the bactericidal applications of eco-friendly starch encapsulated gold nanoparticles (St-AuNPs). The mechanism of interaction of the properly characterized St-AuNPs with both Gram negative and Gram positive bacteria were investigated using spread plate assay, transmission electron microscopy (TEM), and fluorescent propidium iodide (PI) exclusion assay. The St-AuNPs were found to possess significant dose dependent antibacterial activity against both types of bacteria. St-AuNPs at 1.2 mg/mL caused 98 % eradication of Gram positive bacteria that was exposed over a period of 12 h. Similarly, 4.8 mg/mL St-AuNPs caused 98 % eradication of Gram negative bacteria over a period of 12h. The St-AuNPs are biocompatible and present a useful solid porous carbohydrate-based polymer vehicle with excellent antimicrobial activity against both Gram negative and positive bacteria.

2. INTRODUCTION

Disinfection in hospitals and clinics is a growing concern in the medical industry because of the increasing appearance of antibiotic-resistant strains of pathogenic bacterial species (1,2). In addition, traditional antibiotics are no longer effective against such pathogens which is aggravated by a decline in the development of new antibiotics (3) .To solve these challenges advanced therapeutic strategies are required. A lot of anticipation is placed in the promises of nanotechnology to advance new strategies in nanomedicine including the development of antibacterial nanomaterials (4-6).

Nanomedicine holds great promise for major improvements in health care (7). The potential medical application of nanomaterials is attributed to their very small size which offers the ability to interact with complex biological molecules. This creates enormous opportunities

for novel nanomaterial applications including diagnosis, antibacterial activity, medical imaging, targeted drug delivery, gene therapy, tissue engineering, and immune therapy (8). Current advances in material sciences allow nanotechnology processes to focus on the synthesis of environmentally friendly, biodegradable, and multipurpose nanoparticles (9). However, successful achievement of these goals are limited because of the following reasons: a) use of non-ecofriendly synthesis method, b) complication in the method of synthesis, c) uncontrolled disparity of nanoparticles, d) non-biocompatibility, e) non-convenient surface bioconjugation, f) irregular sizes, and g) low stability (10-12).

In spite of the existing limitations, gold nanoparticles (AuNPs) have been employed most extensively because of its unique properties such as stability, rigidity, non- toxic nature, remarkable plasmon-resonant optical properties, biocompatibility, imaging ability, and multi-functionality. These properties render the AuNPs useful for a wide variety of environmental, biomedical and industrial applications (13-15).

In this study, we report the antibacterial activity of starch encapsulated gold nanoparticles. These multifunctional St-AuNPs were synthesized using starch as the reducing and stabilizing agent (16-18). The synthesis of near spherical St-AuNPs in the size range 15 ± 5 nm is aqueous, single step, single phase, and does not require the addition of any external secondary capping agent making it a green process. The eco-friendly St-AuNPs were tested for antibacterial activity against Gram-negative (*Escherichia coli, Enterobacter aerogenes, and Pseudomonas aeruginosa)* and Gram-positive bacteria (*Staphylococcus epidermidis, Enterococcus durans, and Streptococcus bovis)*. The results of these studies that were evaluated with spread plate techniques, TEM, and Fluorescent PI Exclusion Assay (FPIE) are discussed below.

3. MATERIALS AND METHODS

3.1. Reagents and material

Chemicals including $KAuCl_4/HAuCl_4$, Lysogeny Broth (LB) agar, and Tryptic Soy (TS) agar were purchased from Aldrich, St. Louis, MO. The various bacterial strains were either purchased from Invitrogen, Carlsbad, CA or obtained from the biological specimen collection at Western Kentucky University. Starch was extracted from potatoes by a liquid-solid extraction process using water as a solvent. Analytical grade chemicals were used.

3.2. St-AuNPs

St-AuNPs were synthesized by the reduction of Au^{3+} ions using starch, in buffer solution (pH $\sim 7.2 \pm 0.2$). For a typical synthesis, 1.5 mM $KAuCl_4$ was mixed with buffer containing 2 mM starch solution. The mixture was incubated in an orbital shaker at the speed of 150 rpm for 12 hours. Change in the color of solution from white to yellow indicates the reduction of Au^{3+} to Au^0 i.e., the formation of St-AuNPs. To collect the nanoparticles, the samples were centrifuged at 12000 rpm for 20 min, and the supernatant was discarded. The precipitate was thoroughly washed with nanopure water several times and used for further analysis. The absorption spectrum of the St-AuNPs was measured using PerkinElmer's LAMBDA 35 UV/vis spectrophotometer. The size and shape of the synthesized St-AuNPs was investigated using a transmission electron microscope (JEOL-TEM). Samples for the TEM images were prepared by placing a drop of St-AuNPs solution onto a 400 mesh size Formvar coated copper grid. Excess water was removed by using a filter paper wedge and air dried for 5 minutes. The elemental composition of the nanoparticles was determined by energy dispersive X-ray spectroscopy (EDS) using JEOL-JSM-S400 LV with IXRF system.

3.3. Effect of St-AuNPs on various microorganisms

Various microorganism, including Gram-negative (*Escherichia coli, Enterobacter aerogenes, and Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus epidermidis, Enterococcus durans, and Streptococcus bovis*) strains were exposed to various concentrations of St-AuNPs. A 24 hours liquid broth culture of each organism containing 10^5 cells/mL was exposed to various concentrations of the St-AuNPs over a period of 12 hours in a 37 ^{0}C incubator. Controls consisted of microorganisms in the absence of the St-AuNPs. After 12 hours, the bacterial suspensions were centrifuged at 4000 rpm, the pellet was resuspended in 500 uL DPBS and spread on fresh LB/TS agar plates. The plates were incubated for 12 hours at 37 ºC and observed for the growth of colonies (19,20) .The number of colony forming units was established using three or more plates from independent experiments.

3.4. Morphology of the St-AuNPs treated bacteria

Morphology was used to establish the effect of the St-AuNPs on the bacterial cell wall. To determine the morphology of the St-AuNPs treated bacteria, ultra-thin cross-sections of bacterial cells were observed under TEM. Bacterial preparations from the St-AuNPs treatment and controls were made from 1 mL bacterial cell cultures that were collected at various time points. The bacteria were pelleted by centrifugation at 4000 rpm. The bacterial pellets were resuspended in a 1 mL of primary fixing solvent mixture containing 16% w/v paraformaldehyde and 10 % w/v glutaraldehyde in the presence of 50 mM sodium cacodylate buffer (pH ~ 7.4). After 2 hours of incubation the secondary fixation was carried out by washing the samples with 50 mM sodium cacodylate buffer followed by centrifugation. This step was repeated twice. The pelleted cells were resuspended in 1 mL of 1 % osmium tetraoxide solution (OsO_4) and incubated for 1 hour at 25º C for post fixation. The post-fixed samples were thoroughly washed with nanopure water followed by treatment with graded ethanol series (25, 50, 75, 95 and 100%). The dehydrated samples were centrifuged and the pelleted cells were further infiltrated with 100 % Spur's epoxy resin overnight. The samples were centrifuged through fresh resin in BEEM capsules and baked at 70°C for 18 hours. The baked pelleted samples were used to prepare several ultra-thin sections that were cut on an RMC MT-X ultra microtome using glass knives. Sections were stained with 2 % aqueous uranyl acetate and Reynold's lead citrate for 15 minutes
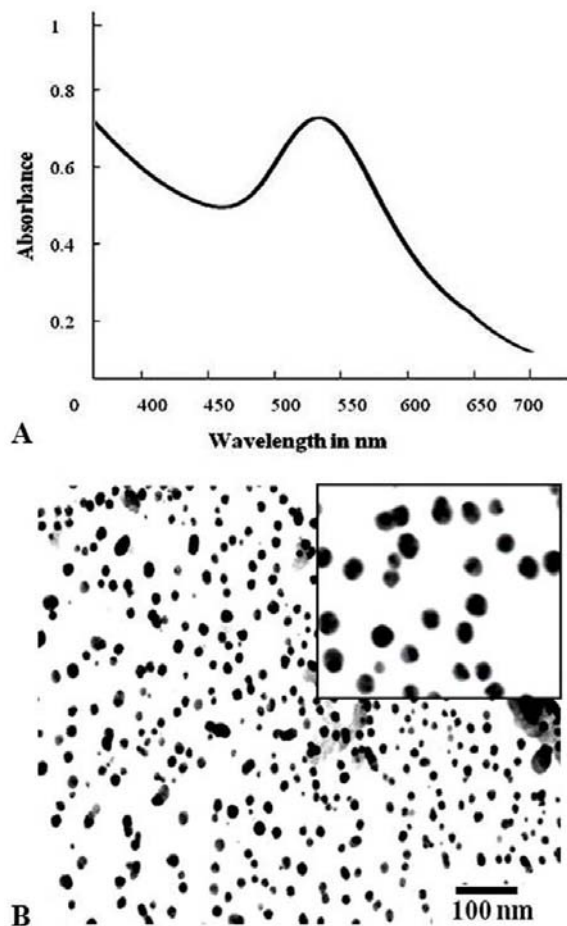
Figure 1. (A) UV/ vis spectra for St-AuNPs with absorbance peak at ~ 540 nm; and (B) TEM image of St-GNPs with average diameter 15 ± 5 nm.

and 3 minutes respectively. Each preparation was examined using a JEOL-100CX TEM (21,22).

3.5. Fluorescent propidium iodide permeability assay

Propidium iodide, PI, is a nucleic acid binding dye that can permeate damaged cell membrane but is impermeable to normal, healthy bacterial cell membrane (4). Hence, by treating the cells with PI the proportion of viable to non-viable cells can be determined based on the observed fluorescence.

To establish the PI permeability, liquid media culture of the bacterial cells in the presence of St-AuNPs at different concentrations were prepared. Samples at different time points from these preparations (using unexposed bacteria as control) were collected and centrifuged at 6000 rpm for 3 minutes and washed twice with PBS buffer (pH ~ 7.2). The bacterial cells were resuspended in 1X DPBS in the presence of 10 mM PI was incubated in the dark for 30 minutes at room temperature. At the end of this period, 5 μL of the bacterial suspension was placed on a glass slide, mounted with a cover slip and observed under a fluorescence microscope. The percentage of fluorescent cells was established using five or more fields of view from three independent experiments.

4. RESULTS AND DISCUSSION

Nanomedicine promises new hope for diseases that currently do not have a cure with its potential for major improvements in health care. Gold nanoparticles hold a lot of potential in nanomedicine because it is significantly non-toxic compared with other nanomaterials. But fabrication of eco-friendly AuNPs for biomedical applications is a need as well as a challenge. In particular, one of the growing areas of AuNPs in nanomedicine involves its possible use in antibiotics (1-6).

In this study, eco-friendly St-AuNPs that were synthesized using a novel process involving starch "Figure 1" were evaluated for bactericidal properties (23-27). To determine the effect on the cell viability, number of colony forming units was established using spread plate technique. The antibacterial mechanism of the St-AuNPs with Gram positive and Gram negative microorganisms were evaluated microscopically with TEM and Fluorescent PI Exclusion Assay.

4.1. Bactericidal activity of St-AuNPs

To quantify the effect of St-AuNPs on the viability of the bacterial cells, Spread plate technique was employed to determine the number of viable cells that were recovered after treating the bacteria with different doses of St-GNPs. In these studies, both Gram-negative and Gram-positive bacterial strains were treated with increasing doses of St-GNPs in sterile liquid media. After the control reached its stationary phase (i.e., 12 hours), all the samples were cultured in solid agar plate. Upon complete incubation (~ 12 hours), the number of colonies was counted. The number of colonies was plotted against the increasing dose of St-GNPs "Figure 2, 3 & 4". The result for both strains indicated consistent decrease in number of recovered viable bacterial cells with increasing doses of St-GNPs "Figure 2, 3 & 4". "Table 1" summarizes the minimum inhibitory concentrations (MIC) of the tested St-GNPs sample against three Gram-positive and three Gram-negative bacteria. The MIC against the Gram-negative bacterial strains such as *Escherichia coli, Enterobacter aerogenes, and Pseudomonas aeruginosa* was found to be 4.8 ± 0.2 mg/mL which is more than the MIC obtained against different Gram-positive bacterial strains (1.2 ± 0.2 mg/mL) such as *Staphylococcus epidermidis, Enterococcus durans and Streptococcus bovis*. As shown in part-B of "Figure 2, 3 and 4", the results demonstrate that the bacterial growth inhibition of St-GNPs against both types of bacterial strains is dose-dependent. At a given point of growth, it is important to note that viable cells recovered significantly decreased with increasing concentration of St-GNPs "Figure 2, 3 & 4".

4.2. St-AuNPs route of antibacterial activity

Determining the mechanism of action of an antibacterial agent is very important in advancing its proper use and application. In this report, the route of action of St-

Table 1. MICs (mg/mL) of St-AuNPs against different strains of Gram-negative and Gram-positive bacteria

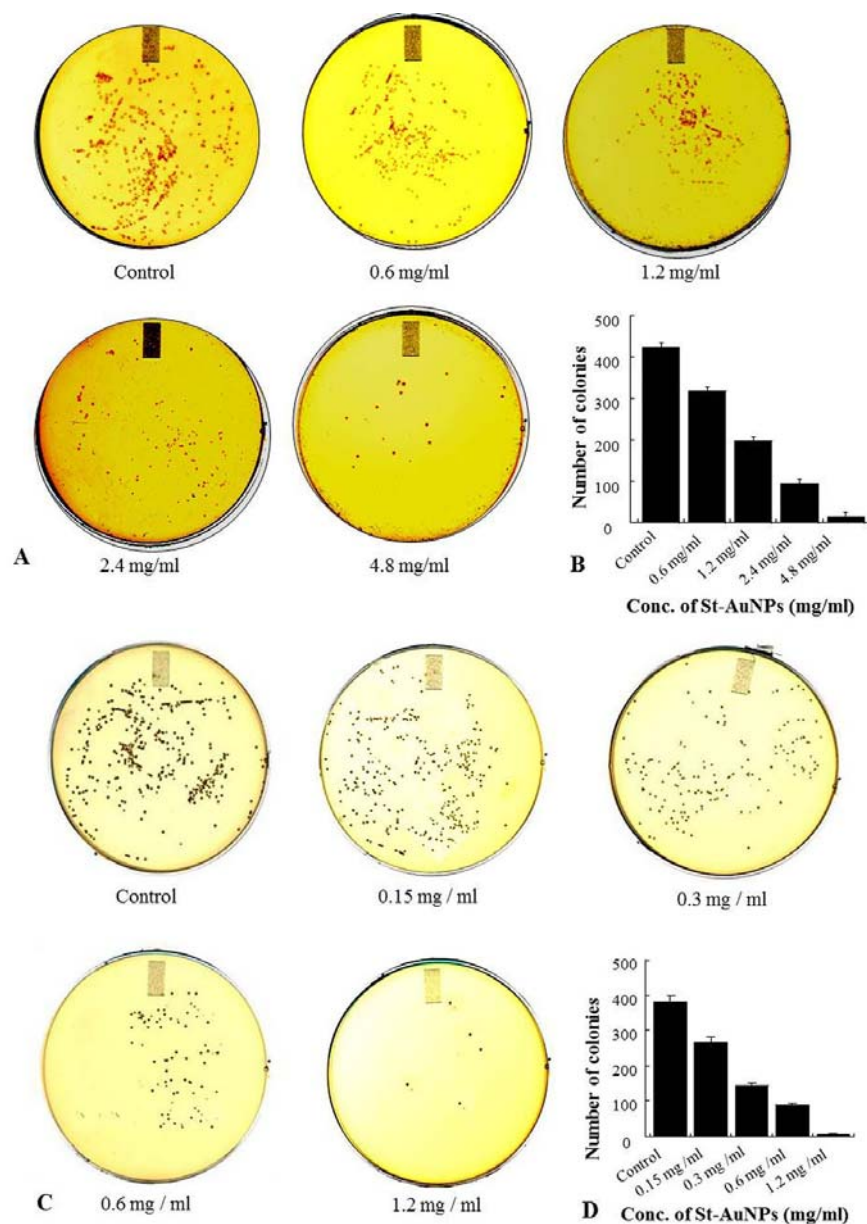| Bacterial Strains	St-AuNPs MIC ± 0.2 mg/ml
Gram-negative strains	
Escherichia coli DH5α	4.8
Enterobacter aerogenes ATCC 13048	4.8
Pseudomonas aeruginosa ATCC 27853	4.65
Gram-positive strains	
Staphylococcus epidermidies ATCC 12228	1.2
Streptococcus bovis ATCC 9809	1.4
Enterococcus durans ATCC 6056	1.2

Figure 2. Spread plate assay for *E. coli* (A &B) and *S. epidermidis* (C&D) treated with different concentrations of St-AuNPs. (A) Agar plates with control (untreated sample) and the St-AuNPs treated samples showing the anti-bacterial effect; (B) Graph plotted with number of *E. coli* colonies grown on each agar plate against different concentrations of St-AuNPs (mg/mL); (C) Agar plates with control (untreated sample) and the treated samples showing the anti-bacterial effect; (D) Graph plotted with number of *S. epidermidis* colonies grown up on each agar plate against different concentrations of St-AuNPs (mg/mL).
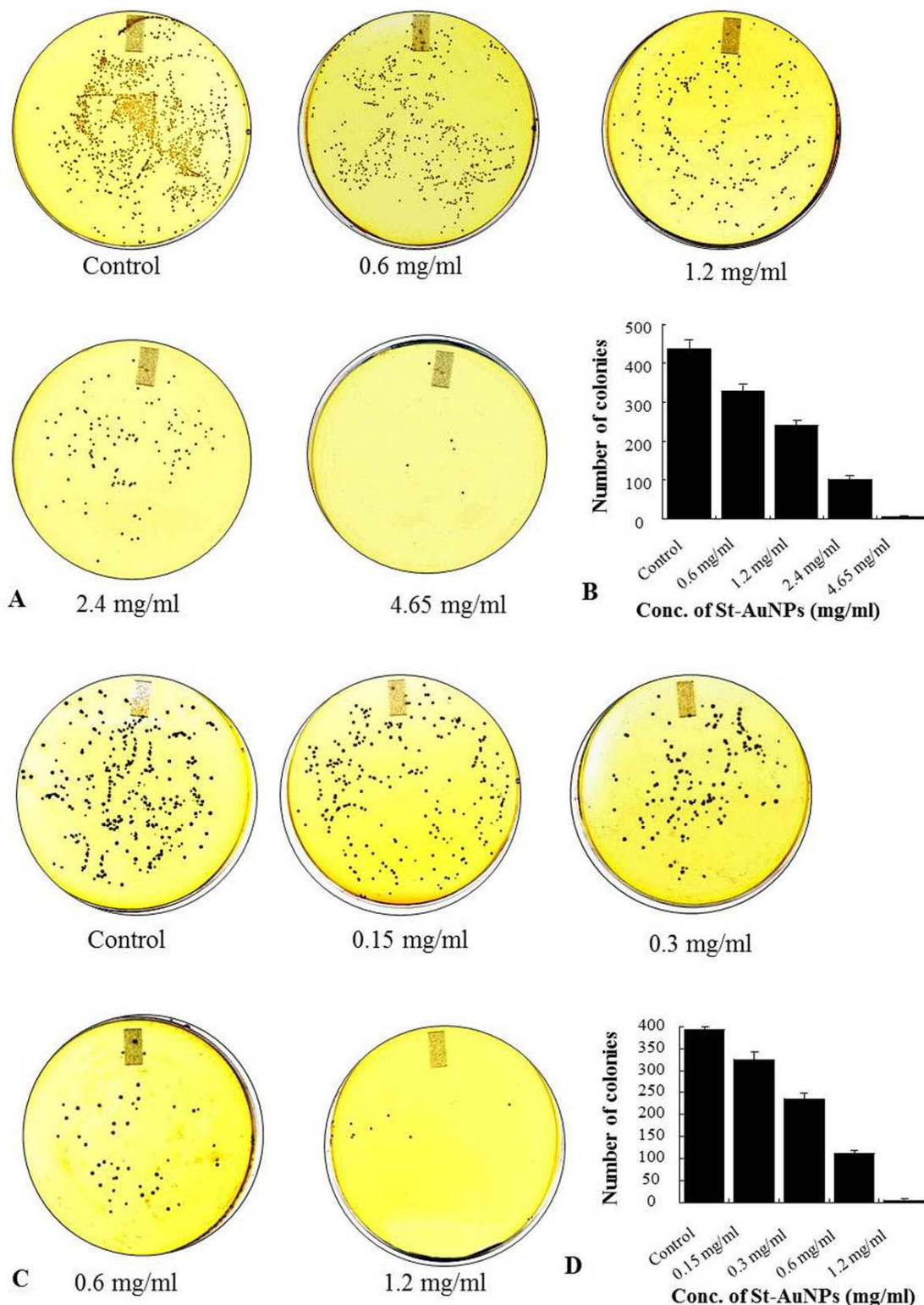
Figure 3. Spread plate assay for *S. bovis* (A&B) *and E. durans* (C&D) treated with different concentrations of St-AuNPs. (A) Agar plates with control (untreated sample) and the St-AuNPs treated samples showing the anti-bacterial effect; (B) Graph plotted with number of *S. bovis* colonies grown on each agar plate against different concentrations of St- AuNPs (mg/mL); (C) Agar plates with control (untreated sample) and the St-AuNPs treated samples showing the anti-bacterial effect; (D) Graph plotted with number of *E. durans* colonies grown up on each agar plate against different concentrations of St-AuNPs (mg/mL).
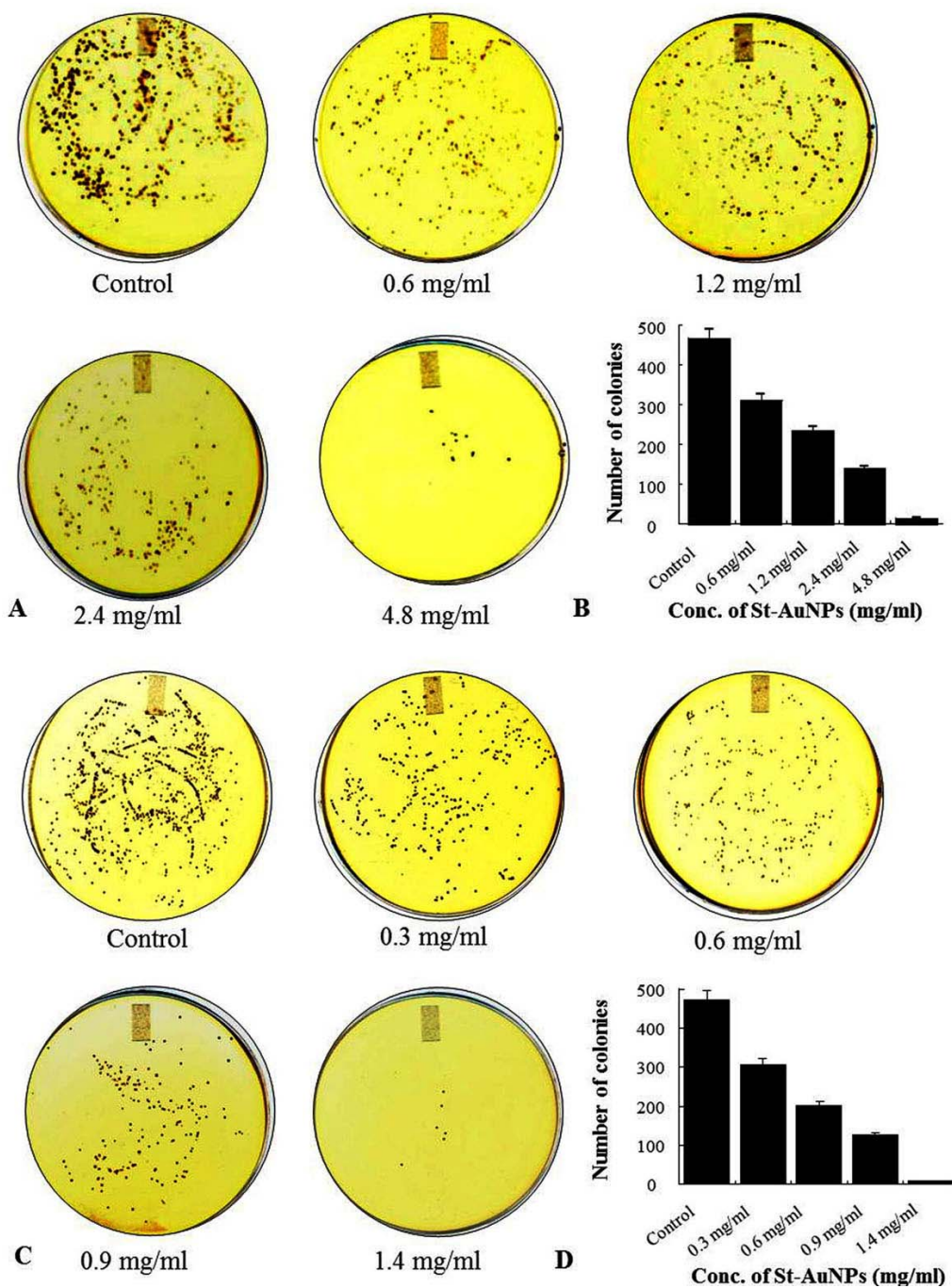
Figure 4. Spread plate assay for *E. aerogenes* (A&B) and *P. aeruginosa* (C&D) treated with different concentrations of St-AuNPs. (A) Agar plates with control (untreated sample) and the St-AuNPs treated samples showing the anti-bacterial effect; (B) Graph plotted with number of *E. aerogenes* colonies grown on each agar plate against different concentrations of St-AuNPs (mg/mL); (C) Agar plates with control (untreated sample) and the St-AuNPs treated samples showing the anti-bacterial effect; (D) Graph plotted with number of *P. aeruginosa* colonies grown up on each agar plate against different concentrations of St-AuNPs (mg/mL).

AuNPs was investigated by following the morphological changes in the bacterial cells. Changes in morphology were established by preparing cross-sections of the bacterial cells at various time points during the St-AuNPs treatment. Cross sections of native bacterial cells (unexposed bacterial cells) were used as control.
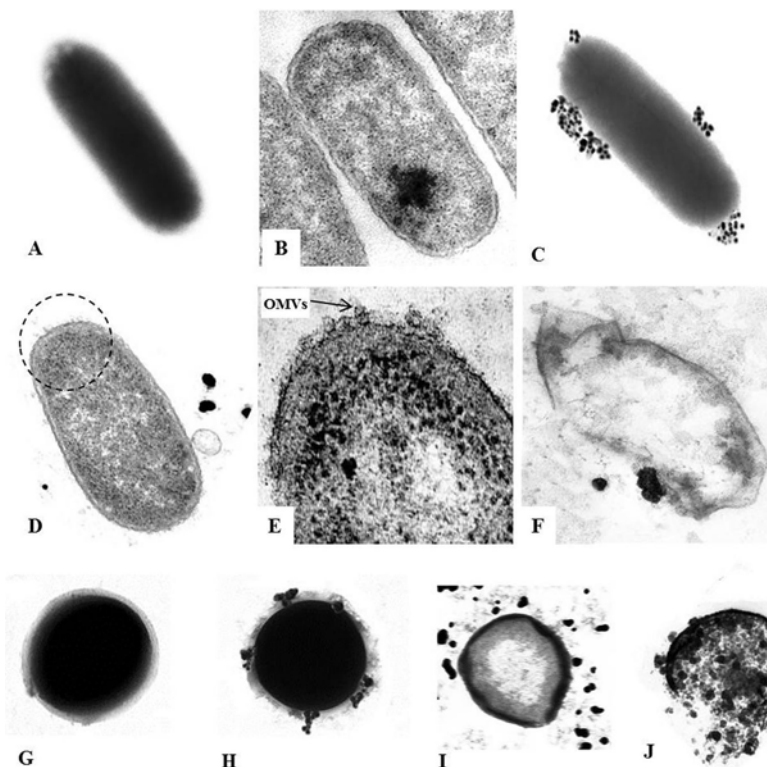
Figure 5. Visualizing St-AuNPs induced morphological changes in the bacterial cell. **(A)** TEM image of the normal *E. coli* cell at 0 hour; (B) Cross-section of normal *E. coli* cell; (C) TEM image of the *E. coli* cell treated with St-AuNPs at 0 hour; (D) *E. coli* cell showing perforations at 6 hours when treated with St-AuNPs; (E) Magnification of the area showing perforation in the 6 hours sample; (F) Lysed *E. coli* cell at 12 hours when treated with St-AuNPs; **(**G) TEM image of the normal *S. epidermidis* cell at 0 hour; (H) TEM image of the *S. epidermidis* cell treated with St-AuNPs at 0 hour; (I) *S. epidermidis* cell treated with St-AuNPs at 6 hours; (J) Lysed *S. epidermidis* cell at 12 hours when treated with St-AuNPs.

As seen in "Figure 5", TEM elucidated the interaction of the nanoparticles with the bacterial cell wall. Upon addition of the St-AuNPs to *E. coli*, the NPs are observed to attach on the surface of the bacterial cell wall. This is consistent with published observations regarding interaction of proteins on cell membranes upon exposure to nanoparticles (4,6,13,27,28). Bacterial cross sections from the 6-hour time point showed perforations on the cell membrane which were not observed on the naïve cells "Figure 5". This observation is highly significant because this exhibited the damaging effect of the St-AuNPs on the bacterial cell membrane. The St-AuNPs induction of damage on the bacterial cell was further verified with the cross section preparations from the 12-hour time point. These showed complete loss of integrity of the bacterial cell through the rupture of the bacterial cell wall "Figure 5". Cell membrane rupture, consequently led to loss of metabolic control that caused homostatic imbalance, leading to cellular metabolic discharge and ultimately cell death (27-29).

The TEM observations of the effect of exposure to St-AuNPs of *S. epidermidis (Gram-positive)* cells also suggested similar mechanism of bactericidal action. The gradual damage to the cell membrane at increased time-

points was also apparent which eventually led to the rupture of the bacterial cell. Just like in the case of the Gram negative bacteria, the cell membrane rupture, led to loss of metabolic control resulting in homostatic imbalance that caused cell death (27-29). Based on these observations, we concluded that the bactericidal action of St-AuNPs started with adherence to the cell membrane followed by membrane perforations that disturbed osmotic balance in the cell and its surroundings leading to cell death "Figure 5".

To further confirm the mechanism of anti-bacterial activity, fluorescence microscopy experiments were performed. Propidium iodide (PI) dye was used to monitor the permeability of the cell membrane. PI is an intercalating agent and a fluorescent molecule which can be used to stain cells through its binding with DNA as well as RNA emitting red fluorescence (4). Cell wall of live cells is impermeable to PI but not to damaged or dead cells. Hence, it was used to differentiate between viable and non-viable cells. As seen from "Figure 6", after treatment with St-AuNPs for 12 hours, the *E. coli* (Gram-negative) and *S. epidermidis* (Gram-positive) exhibited fluorescent signals which were > 90% higher than the control. This indicated that the St-AuNPs exposed cells loss their cell membrane
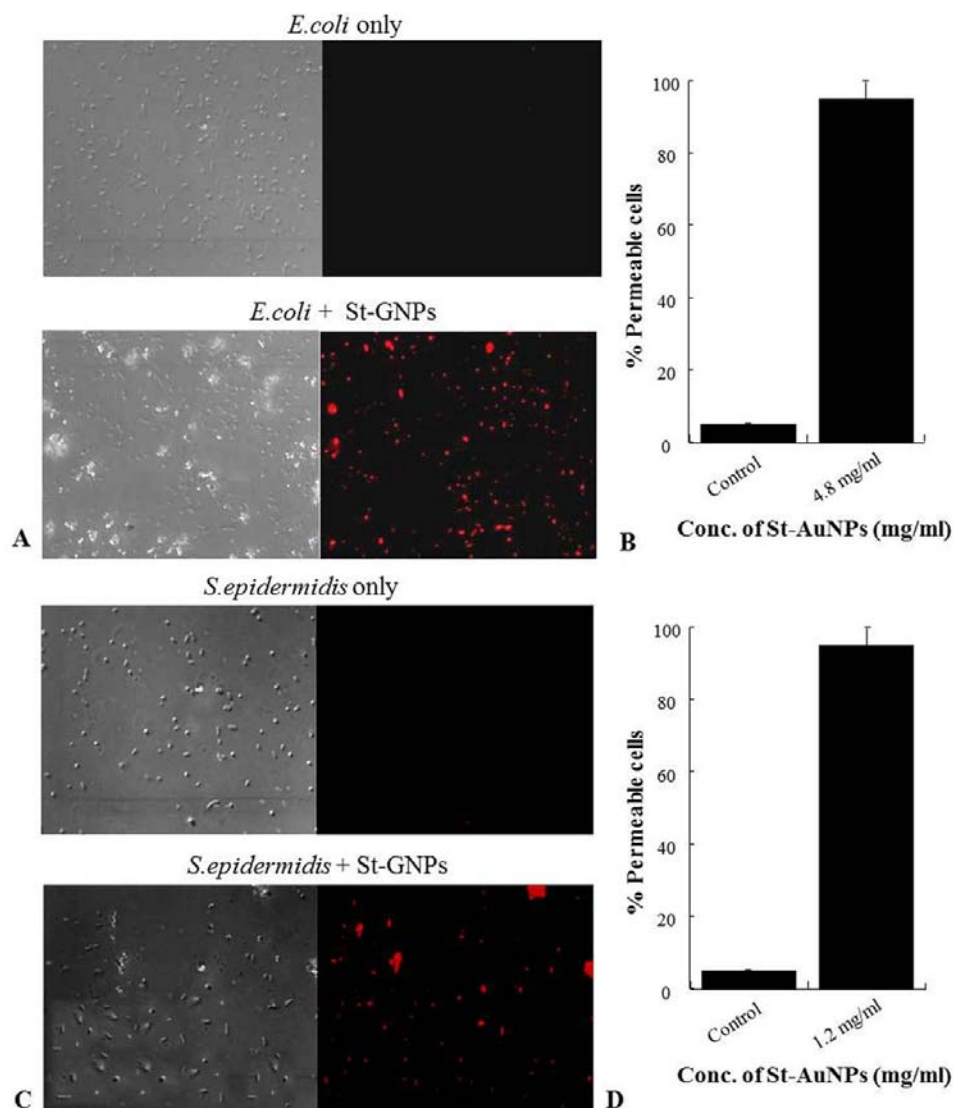
Figure 6. Monitoring St-AuNPs induced permeability of *E. coli (A&B) and S. epidermidis(C&D)* cell membranes using propidium iodide dye. (A) On each image, the left half shows an image in the differential interference contrast mode, while the right half shows the corresponding fluorescence image; (B) Plot with permeable cells percentage against concentration of St-AuNPs; (C) On each image, the left half shows an image in the differential interference contrast mode, while the right half shows the corresponding fluorescence image; (D) Plot with permeable cells percentage against concentration of St-AuNPs.

integrity which allowed the PI to permeate the cells, thereby causing the cells to fluoresce. This confirmed the effect of bacterial exposure to St-AuNPs that resulted in the disruption of the bacterial cell wall which was not observed in the control cells.

The results obtained through TEM analysis and fluorescent assay clearly suggested that the bactericidal action of St-GNPs occurs through lysis of bacterial cell wall. Being a chain of large number of glucose units, starch is a polyhydroxylated molecule. Thus, we believe that the starch molecule acted as a hydrogen bond donor as well as a hydrogen bond acceptor (which has to be proven in future studies). With this in mind, theoretically, strong electrostatic interaction between St-AuNPs and Gram-

negative or Gram-positive bacteria can be attributed to the presence of hydroxyl group, which in turn lead to the disruption of cell wall. The disruption of cell wall essentially disturbed the cell equilibrium, leading to the possible leakage of the cytoplasmic content including nucleic acid that was observed as cell lysis or necrosis (30).

The difference in potency of St-GNPs against both Gram-negative and Gram-positive bacteria can possibly be explained considering their different cell wall composition (31,32). The cell wall of Gram-negative bacteria consists of multiple layers; consisting of a thin middle peptidoglycan layer, and more largely an outer and inner membrane comprised of lipopolysaccharide, phospholipids, and protein molecules, which provide an

effective resistance barrier against nanoparticles (4,33,34). On the other hand the cell wall of Gram-positive bacteria mainly consists of several layers of peptidoglycan, which is unable to resist the nanoparticle action. Thus, this difference in cellular structure caused the decreased activity against Gram-negative bacteria (35,36).

In summary, we have shown that eco-friendly synthesized St-AuNPs using starch as reducing/capping agents have potent antimicrobial activity on both Gram-negative and Gram-positive bacteria. The St-GNPs showed a dose dependent bactericidal activity that was shown to be caused by the formation of cell perforations which eventually led to cellular rupture. The cell wall rupture disturbed the cell equilibrium, leading to the possible leakage of the cytoplasmic content including nucleic acid which eventually resulted in cell lysis or necrosis. Further studies are on-going to study the chemistry based-mechanism of cell wall rupture using the St-AuNPs. The use of renewable polysaccharide/solvent, plus the continuing development of porous polysaccharide-derived materials, presents a number of opportunities particularly with regard to applications in antimicrobial textile fibers and film surfaces. Results from this study anticipated to provide a potential for a wide range of applications in medicine, biology, and industry.

5. ACKNOWLEDGEMENT

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Abbreviations: AuNPs : gold nanoparticles, St-AuNPs : starch-AuNPs, LB Agar : Lysogeny Broth, *E. coli* : *Escherichia coli*, *S. epidermidis* : *Staphylococcus epidermidis*, EDS : energy dispersive x-ray spectroscopy, PI : propidium iodide, MIC : minimum inhibitory concentration

Key Words: Starch, gold nanoparticles, multifunctional gold nanoparticles, antibacterial activity.

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