Spring 5-10-2013

A New Class of Gold Nanoantibotics - Direct Coating of Ampicillin on Gold Nanoparticles

Dillon S. Pender
Western Kentucky University, dillon.pender472@topper.wku.edu

A NEW CLASS OF GOLD NANOANTIBIOTICS- DIRECT COATING OF AMPICILLIN ON GOLD NANOPARTICLES

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree of Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

By

Dillon S. Pender

* * * * *

Western Kentucky University
2013

CE/T Committee:

Professor Rajalingam Dakshinamurthy, Advisor

Professor Chad Snyder

Professor Leslie Baylis

Approved by

Advisor
Department of Chemistry

ABSTRACT

Need for novel, innovative strategies for developing antibiotics is becoming a necessity due to an increasing number of rapidly evolving micro-organismal threats. Antibiotic encapsulated gold nanoparticles (GNPs) are one such strategy showing promise. We report the development of ampicillin encapsulated gold nanoparticles (Amp-GNPs) that possess highly effective, dose dependant antibacterial activity. In this method, ampicillin molecules have been coated on individual GNPs which can then serve as drug carrier devices. Our method for synthesizing Amp-GNPs is an entirely ecofriendly, single step reaction taking place in an aqueous buffer. Following characterization of Amp-GNPs, we find them to be ~15 nm in diameter and spherical in shape. We have tested the antibacterial activity of Amp-GNPs against multiple strains of bacteria, both Gram-positive and Gram-negative, and have found Amp-GNPs to be highly efficient against all tested strains. By examining the mechanism of Amp-GNPs antibacterial activity, it was determined that Amp-GNPs disrupt the bacterial cells membrane when coming into contact with the cells, thus disturbing the cell equilibrium, leading to cell lysis or necrosis. Amp-GNPs have been shown to exhibit significant potential and ability to enter the medical field's arsenal to fight infectious disease.

Keywords: Ampicillin, gold nanoparticles, antibacterial, drug carrier, green synthesis

Dedicated to my family & friends

ACKNOWLEDGEMENTS

The completion of this project, my Baccalaureate degree, and my acceptance into medical school are all goals I could have not completed without the tremendous support of so many people. I would like to first thank my advisor Dr. Rajalingam Dakshinamurthy. Being highly knowledgeable, enthusiastic with his work, and genuinely insightful to all his students, Dr. Raja is a terrific mentor and I consider myself lucky to have worked under him and to have gained such a mentor, colleague and friend as worthwhile as he is.

I would like to thank Dr. Cathleen Webb chemistry department head. Her unending support pushes me to strive to become all that I can. I would like to thank the National Science Foundation Research Experience for Undergraduates. My project was financially supported by the foundation during my involvement in the WKU summer REU program. The program is one of the biggest factors to my involvement in undergraduate research at WKU. I am truly thankful for the opportunities the program and the program organizers provided. I have many warm memories from the summer REU's I have been a part of, where I have gained extensive knowledge about my research, other science related research, as well as made many friends some living hundreds of miles away.

I would like to also thank Dr. John Andersland for TEM analysis and my fellow researchers from Dr. Raja's lab. This project would not have been possible without their aid and teachings. Finally I would like to thank my family and friends for all of their support and encouragement. Having a supporting family is an invaluable blessing that has made my entire post secondary education experience all the more rewarding.

VITA

September 20, 1991……Born – Elizabethtown, KY

2009……Central Hardin High School, Elizabethtown, KY

2011……Elizabethtown Community & Technical College Graduate

Fall 2011……KAS Undergraduate Chemistry Department Oral Presentation Winner

Spring 2012……Posters at the Capitol

Spring 2012……...Oral Presentation: ACS National Meeting San Diego, CA

Spring 2012……Naff Symposium: UK

Fall 2012……KAS Undergraduate Chemistry Oral Presentation

Fall 2012……Size-dependent antimicrobial properties of sugar-encapsulated gold nanoparticles synthesized by a green method' Published: *Nanoscale Research Letters*

Spring 2013……...Posters at the Capitol

Spring 2013……...Oral Presentation: ACS National Meeting New Orleans, LA

Spring 2013…… 'A New Class of Gold Nanoantibiotics- Direct Coating of Ampicillin on Gold Nanoparticles' Published: *Pharmaceutical Nanotechnology*

Spring 2013……..... 'Bactericidal activity of starch-encapsulated gold nanoparticles' Under Review: *Frontiers in Bioscience*

Spring 2013…… 'Antibacterial Gold Nanoparticles-Biomass Assisted Synthesis and Characterization' Accepted: *Biomedical Nanotechnology*

FIELDS OF STUDY

Major Field: Chemistry & Biology

Concentration: Pre-medicine

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The fields of antibiotics and medicine are constantly evolving and changing in order to adapt to the threat of micro-organismal disease as well as numerous other medical complications [1, 2]. Ampicillin, a derivative of penicillin, introduced in 1961 has been an effective antibacterial agent against both Gram positive and Gram negative bacteria, remedying numerous bacterial infections and saving countless lives [3]. Recently, however, a number of microorganisms have developed strong resistance towards ampicillin and other antibiotics negating the antibiotics effectiveness as broad spectrum antibiotics [3-6]. With this rising threat, the need for antibiotics to be readily transported to the site of drug action has rapidly increased [7, 8].

Drug carriers are substances designed to aid in the transport of antibiotics throughout the body. With use of drug carriers to transport antibiotics it is far more likely the antibiotics will have greater efficacy, delivery and less toxicity [9]. Drug carriers have several properties giving them advantages over traditional antibiotic prescription alone [2, 10]. In this context, nanoparticles offer distinct advantages as drug carriers. Drug accuracy is possible due to the surface area to volume ratio of the nanoparticle allowing potentially millions of antibiotic molecules to be attached to a single nanoparticle [9, 11]. The use of nanoparticle based drug carriers can potentially save an exorbitant amount of

capital as well as drastically improve the quality of antibiotic administered [9]. Though many elements can be used for nanoparticle synthesis gold nanoparticles (GNPs) are most ideal, due to non-cytotoxicity, stability and biocompatibility in contrast to other metallic nanoparticles such as zinc and silver [11]. Potential drug carrier and target cell delivery capabilities of GNPs make their use as antimicrobial agents highly ideal and sought after [12-14]. Due to their high stability and small size GNPs possess the ability to extend the biological half life as well as bioavailability of administered antibiotics. This ability effectively decreases the amount of antibiotic required for administration and increases the likelihood of remediation of infectious disease. Being effective drug carriers GNPs would possess the capabilities mentioned previously of cost effectiveness, accurate drug delivery, and overall antimicrobial efficiency [9].

To obtain antibiotic conjugated NP, especially GNP, various strategies have been employed. Most of these strategies involve functionalization of GNP using different linkers such as amino acids, glutathione, polyethylene glycol [15, 16]. But sometimes addition of linker may either reduce the efficacy of the antibiotics or may also interfere with the stability of drugs [15-17]. Recently two different strategies have been shown to fabricate ampicillin conjugated gold nanoparticles without using linkers [18, 19]. Both of these strategies used either citrate or sodium borohydride for the formation of gold nanoparticles. However, a potential toxicity of GNPs produced by citrate method have been demonstrated, moreover the toxicity of sodium borohydride is also evidences [20-22]. Thus use of above mentioned chemicals for the synthesis of GNPs, not only makes the synthesis process nonecofriendly but also have potential toxicity on human health. To avoid all the disadvantages and potential risk, we have used a novel strategy of

completely green synthesis of ampicillin coated gold nanoparticles (Amp-GNP), in single step synthesis without using any linker or hazardous organic solvents.

In continuation of our research efforts centered on the development of innovative gold nanoparticles for use as antimicrobial agents, we report the completely green synthesis of gold nanoparticles (GNPs) individually coated with the antibiotic ampicillin. GNPs serve as drug carriers which might deliver the antibiotic to targeted locations minimizing the risk of over-dose and potentially revolutionizing medicine [9]. Synthesis of the Amp-GNPs is entirely eco-friendly involving a single-step synthesis method where the ampicillin serves as both the reducing and capping agent within an aqueous medium. Successful synthesis of Amp-GNPs has proven the effectiveness of the antibiotic ampicillin to serve as an efficient reducing agent in the reduction of Au^{3+} to Au^{0} within medium. The medium used in the synthesis is an aqueous medium. The novel synthesis process we have developed is advantageous over traditional GNP synthesis methods allowing the GNPs to engage in biomedical applications and is a single-step synthesis procedure further improving the GNPs' economical value and efficiency.

CHAPTER 2

MATERIALS AND METHODS

2.1. Reagents and material:

Chemicals including KAuCl4/HAuCl4, ampicillin, 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. Analytical grade chemicals were used.

2.2. Synthesis of Antibiotic Coated GNPs:

Ampicillin encapsulated gold nanoparticles (Amp-GNPs) were synthesized by the reduction of Au^{3+} ions to Au^{0} using the antibiotic ampicillin with environmentally friendly buffer solution [pH ~ 7.2 ± 0.2]. For the synthesis, 0.28 mM ampicillin solution and 400 PPM $KAuCl_4$ was mixed in buffer solution. Upon mixing, the container enclosing the solution was placed into an orbital shaker gyrating at ~150 rpm at room temperature for approximately 24 hours [23]. A color change resulted within 12 hours of incubation. The color change was from a clear solution to violet. The change in color denoted the formation of nanoparticles and hence the reduction of Au^{3+} to Au^{0} and the formation of the ampicillin encapsulated gold nanoparticles. For processing of the Amp-GNPs, centrifugation at 12000 rpm for 20 minutes allowed the separation of formed

Amp-GNPs from excess ampicillin solution and buffer located in the supernatant. After discarding the supernatant the remaining precipitate was thoroughly washed with nanopure water several times and used for further analysis [23].

2.3. Characterization of Antibiotic Coated GNPs:

With the use of PerkinElmer's LAMBDA 35 UV/Vis absorption spectra the Amp-GNPs were analyzed for their optical properties. JEOL-TEM Transmission electron microscopy (TEM) was used to determine the morphological characteristics of the Amp-GNPs. Samples for the TEM images were prepared by placing a 4 µL Amp-GNPs solution onto a 400 mesh size formvar coated copper grid and air drying them for 5 minutes. Excess water was removed using a filter paper wedge. In determination of the Amp-GNPs' elemental properties electron dispersive x-ray spectroscopy (EDS) was conducted using JEOL-JSM-S400 LV with IXRF system. Samples for EDS were placed onto an aluminum stub and allowed to dry in a desiccator before examination.

2.4. Antibacterial Activity of Amp-GNPs:

In determination of the Amp-GNPs efficacy as an antibacterial agent, both liquid broth turbidimetry and solid agar plate based assays were employed. These experiments were performed against multiple strains of bacteria representing both Gram-positive such as *Staphylococcus epidermidis*, *Streptococcus bovis* and Gram-negative strains such as *Pseudomonas aeruginosa*, and *Enterobacter aerogenes*. In the turbidimetry assay the Amp-GNPs were inoculated with 105 CFU/mL in a series of test tubes containing 4 mL

of sterile liquid media. A control was made by inoculating the media with bacterial cells in absence of Amp-GNPs. All tubes were incubated at 37 ºC for 12 hours. After every hour of incubation the optical density (OD) at a wavelength of 600 nm of both the control and all samples was examined and recorded. Any absorbance due to the Amp-GNPs or the medium was autocorrected using a blank of Amp-GNPs dispersed in the medium, in absence of bacterial organism. Following turbidimetry assays solid agar assays were performed [24].The bacterial suspensions were centrifuged at 4000 rpm, the pellet was resuspended in 500 μL DPBS and spread onto fresh, solid, nutrient rich agar plates (Luria-Bertani agar / tryptic soy agar). The plates were then allowed to incubate for 12 hours at 37 °C during which time the bacterial colonies became visibly populated. The bacterial colonies were then counted for examination of results [25, 26]. The number of colony forming units was established by taking average of counts from three or more independent experiments.

2.5. Amp-GNPs Antimicrobial Mechanism Examination:

To determine the mechanism of the Amp-GNPs antibacterial activity, propidium iodide testing was conducted using both Gram-positive and Gram-negative bacterial strains [14,27]. Propidium iodide is a chemical dye that fluoresces upon interacting and binding with nucleic acids. Notably, propidium iodide is impermeable to an intact, healthy cell wall. Therefore, organismal solutions with intact cell walls will yield a low level of fluorescence while lysed organismal solutions will fluoresce excessively. Bacterial organisms were cultured in liquid media samples in the presence of Amp-GNPs at minimum inhibitory concentrations, which was determined from turbidimetry and from

spread plate assay. Samples were collected and centrifuged at 6000 rpm for 3 minutes and washed twice with PBS saline buffer (pH~ 7.2). These bacterial cells were incubated with 10 mM propidium iodide in a dark environment for 30 minutes at room temperature. After incubation, the samples were washed using PBS to get rid of any excess, unbound dye for reducing the background fluorescence. For sampling, 10 µL of the bacterial suspension was placed on a glass slide, mounted with a cover slip and observed under a Leica fluorescence microscope. The bacterial suspension without Amp-GNPs was used as a control. The percentage of fluorescent cells was established by taking average counts from five or more fields of view from three independent experiments.

To visualize the morphology of the bacteria after exposure to Amp-GNPs, cross sectional analysis was performed with the aid of TEM. Bacterial cell samples exposed to minimum inhibitory concentrations of Amp-GNPs were collected at different time points and processed for sectioning. Controls were made by inoculating the media with bacteria in absence of Amp-GNPs. All tubes were incubated at 37 ºC for 12 hours. Sample of 1 mL was collected at different time points from the test tubes and centrifuged at 6000 rpm for 3 minutes. All the samples were thoroughly washed with nanopure water and then placed onto Formvar coated 400 mesh size copper grids and observed under JEOL-TEM. For cross-section analysis, bacterial cell cultures were fixed using 16 % w/v paraformaldehyde and 10 % w/v gluteraldehydeas primary fixing agents in 50 mM sodium cacodylatebuffer (pH ~ 7.4) and incubated for 2 hours in the hood. The fixed samples were washed thoroughly using the buffer solution and post fixed using 1 % osmium tetroxide (OsO4) and incubated for 1 hour at room temperature. The post-fixed samples were then thoroughly washed with nanopure water followed by the treatment of

graded ethanol series (25, 50,75, 95 and 100 %) for 5-10 minutes. The dehydrated samples were further infiltrated by incubation in a graded series of Spur's epoxy resin (33, 66, 95 and 100 %) for 1 hour and left overnight in a fresh 100 % resin. The samples were centrifuged through fresh resin in BEEM capsules and hardened at 70 °C for 18 hours. Ultra-thin sections of the pelleted samples were cut on an RMC MT-X ultra-microtome using glass knife. Sections were stained with 2 % aqueous uranyl acetate and Reynold's lead citrate for 15 minutes and 3 minutes respectively and examined using a JEOL-100CX TEM.

CHAPTER 3

RESULTS

3.1. Amp-GNP Synthesis and Characterization:

For Amp-GNP synthesis gold salt ($KAuCl_4$) concentration of 400 PPM and 0.28 mM ampicillin antibiotic solution were mixed within aqueous buffer. Upon mixing the contents the container in Erlenmeyer flask enclosing the solution was then placed into a gyrating orbital shaker at a temperature of 37 °C for 12 hours under normal atmospheric pressure. The solution of Amp-GNPs changed in color after 12 hours. With the use of UV/Vis absorption spectra the Amp-GNPs were analyzed for their optical properties. The antibiotic encapsulated GNPs were found to express a λ_{max} value at 540 nm as shown in **"Figure 1A"**. Transmission electron microscopy (TEM) was used to determine the morphological characteristics of the Amp-GNPs which showed formation of near spherical shaped nanoparticles with approximately 15 ± 5 nm in diameter, **"Figure 1B"**. In determination of the Amp-GNPs elemental properties, electron dispersive X-ray spectroscopy (EDS) was conducted. Results showed the anticipated high percent value of Au^0 (85 %) as well as a C (8%), O (5%), and S (2%) peak as shown in **"Figure 1C"**.

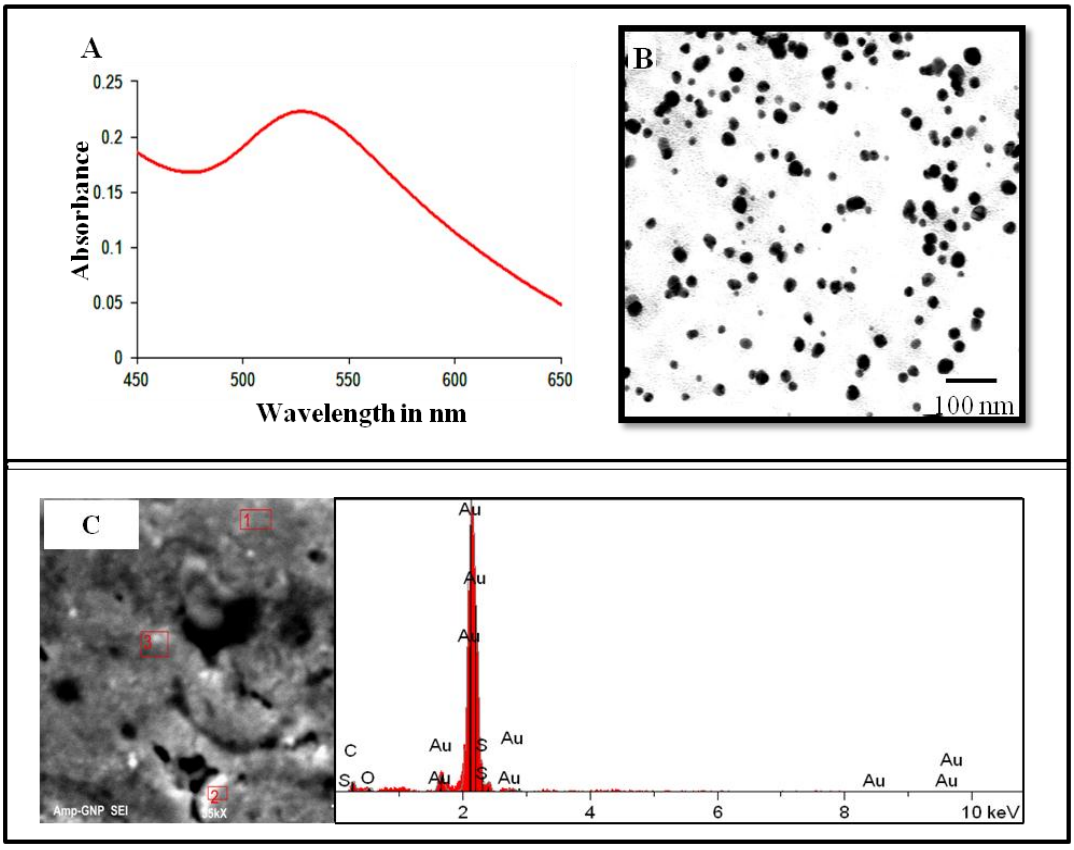


Figure 1. (A). UV-vis spectrum of Amp-GNPs with notable absorbance peak at ~540 nm. (B). Transmission electron microscope images of Amp-GNPs with average diameter of 15 nm. (C). Scanning electron microscope image and electron dispersion spectrum of Amp-GNPs. EDS shows the Amp-GNPs to be ~ 85% Au, ~ 8 % C, ~ 5 % O, and ~ 2 % S.

3.2. Antibacterial Activity of Amp-GNPs:

In assessment of the Amp-GNPs antimicrobial activity bacterial assays were performed against two strains of both Gram-positive bacteria (*Staphylococcus epidermidis,* and *Streptococcus bovis)* and Gram-negative (*Enterobacter*

aerogenes and Pseudomonas aeruginosa). Results from the liquid broth turbidimetry assay exhibited the Amp-GNPs to possess significant dose dependant bacteriostatic activity against *Staphylococcus epidermidis* and *Enterobacter aerogenes* as shown in **"Figure 2A & 2B"**, respectively. In addition, the turbidimetry assay allowed for the determination of the minimum inhibitory concentration (MIC) of each tested organism. For *Staphylococcus epidermidis*, a Gram-positive bacteria the MIC was found to be ~0.130 mg/mL while the MIC was ~0.510 mg/mL for *Enterobacter aerogenes*, a Gram-negative bacteria.

To discern whether or not the Amp-GNPs embody bactericidal activity alongside bacteriostatic activity, solid agar plate based assays were performed. The results from the solid agar plate based assay indicated the Amp-GNPs possess highly effective bactericidal activity. This was indicated by the abundance of bacterial colonies on control plates while plates treated with Amp-GNPs contained only scarce numbers of bacterial colonies as illustrated in **"Figure 3 I & II"**. In further pursuit of the Amp-GNPs broad spectrum efficacy as an antimicrobial agent the spread plate assay was repeated using additional strains. The additional bacterial strains were found to express similar results, Supplementary **"Figure 1A & 1B"**.

Additional antimicrobial tests were performed using just the antibiotic ampicillin. Spread plate assays against the same organisms tested against Amp-GNPs were used. Results from the experiment illustrated the effectiveness of ampicillin against these strains, **" Supplementary Figure 2A, B, C"** and the MIC of ampicillin against *Enterobacter aerogenes* was determined to be ~2.3 mg/mL, for *Pseudomonas aeruginosa*

was ~1.9 mg/mL, for *Staphylococcus epidermidis* was ~1.5 mg/mL, and for *Streptococcus bovis* was ~1.4 mg/mL respectively, as shown in **"Table 1"**.

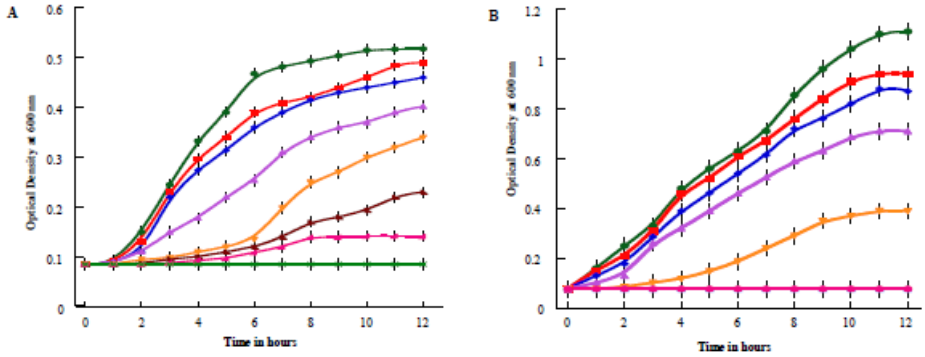


Figure 2. A. Growth curve of *Staphylococcus epidermidis* when exposed to various concentrations of Amp-GNPs. From top to bottom the concentrations are 0.008 mg/mL, 0.016 mg/mL, 0.033 mg/mL, 0.067 mg/mL, 0.08 mg/mL, 0.096 mg/mL, and 0.133 mg/mL. Minimum inhibitory concentration (MIC) was determined to be ~ 0.130 mg/mL. B. Growth curve of *Enterobacter aerogenes* when exposed to various concentrations of Amp-GNPs. From top to bottom the concentrations are 0.016 mg/mL, 0.033 mg/mL, 0.067 mg/mL, 0.133 mg/mL, 0.256 mg/mL, and 0.512 mg/mL. Minimum inhibitory concentration (MIC) was determined to be ~ 0.500 mg/mL.

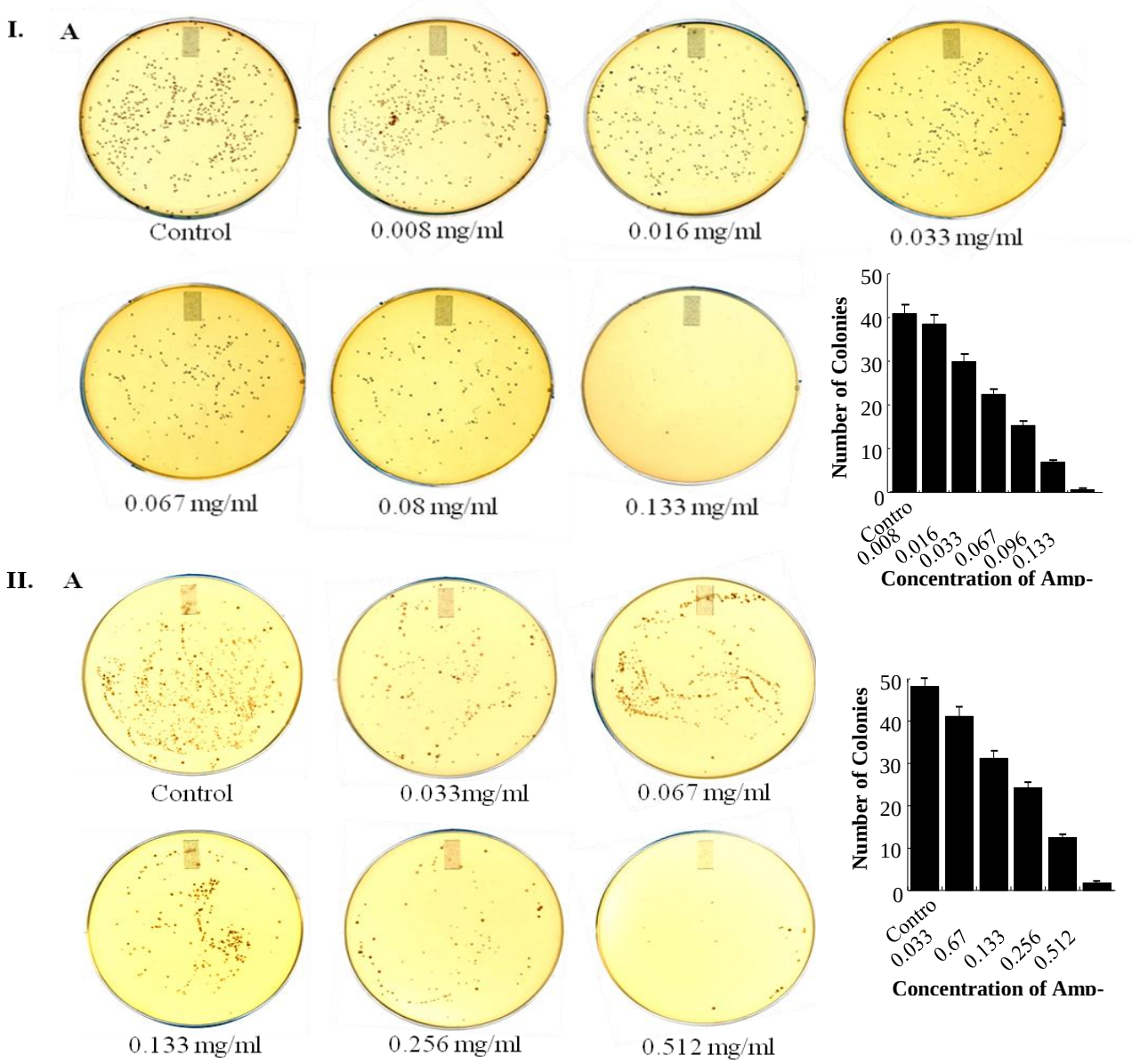


Figure 3. *Panel-I*-A. Spread plate assay for *Staphylococcus epidermidis* with (untreated) control and increasing Amp-GNPs concentrations. B. Graph of number of *S. epidermidis* colonies versus concentration of Amp-GNPs. *Panel-II*- A. Spread plate assay for *Enterobacter aerogenes* with (untreated) control and increasing Amp-GNPs concentrations. B. Graph of number of *E. aerogenes* colonies versus concentration of Amp-GNPs.

Bacterial Strains	Amp-GNPs MIC (mg/ml)	Ampicillin MIC (mg/ml)
Gram-negative strains		
Enterobacter aerogenes	0.512	0.65
Pseudomonas aeruginosa	0.496	0.8
Gram-positive strains		
Staphylococcus epidermidis	0.133	0.35
Streptococcus bovis	0.128	0.3

Table 1. Amp-GNPs and Ampicillin MIC Values MIC (Minimum Inhibitory Concentration) Table of Amp-GNPs and Ampicillin Against all Gram Positive and Gram Negative Strains Tested

3.3. Amp-GNPs Antimicrobial Mechanism Examination:

To determine the mechanism of the Amp-GNPs antibacterial activity, propidium iodide testing was conducted using both Gram-positive and Gram-negative bacterial strains. It was found that the solutions with Amp-GNPs fluoresced with far greater magnitude than samples without Amp-GNPs, **"Figure 4"**. To visualize the morphology of the bacteria after exposure to Amp-GNPs, cross sectional analysis was performed with the aid of TEM. Bacterial cell samples exposed to Amp-GNPs were collected at different time points and processed for sectioning. Bacteria untreated with Amp-GNPs were observed to have intact membranes even after 12 hours of incubation time. Amp-GNP treated bacteria, however, were found to show gradual morphological changes within six

hours time. After immediate Amp-GNP introduction (0 hour time point) the bacteria were observed to have the Amp-GNPs attached to their cell membrane at numerous locations. Within six hours time the bacterial cell began to exhibit deformations and perforations in its cellular membrane. At the twelve hour time point the bacterial cells were observed with their cell membranes completely lysed and leaking of the inner cellular components. All of these bacterial morphological changes were evident for both Gram-positive and Gram-negative bacteria, **"Figure 5"**.

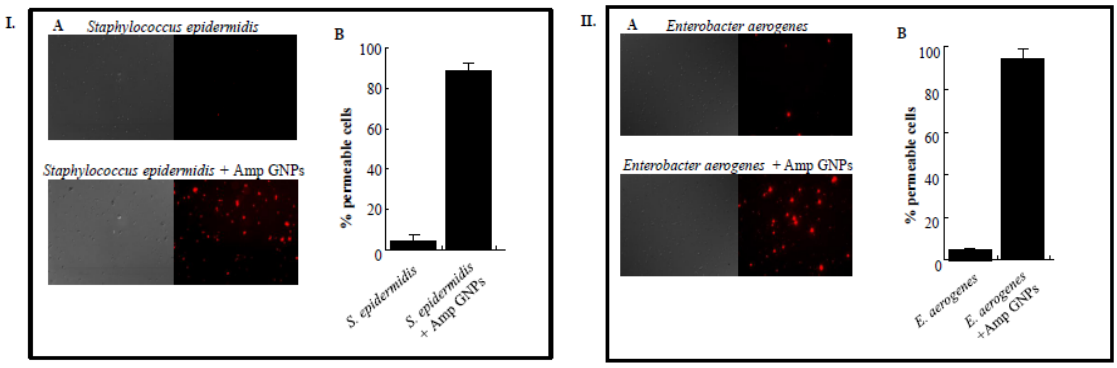


Figure 4. *Panel-I*- Monitoring Amp-GNPs induced permeability of *Staphylococcus epidermidis* cell membranes using propidium iodide dye. A. On each image, the left half shows an image in the differential interference contrast mode, while the right half shows the corresponding fluorescence image; B. Plot with percentage of permeable cells against concentration of Amp-GNPs. *Panel-II*- Monitoring Amp-GNPs induced permeability of *Enterobacter aerogenes* 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 percentage of permeable cells against concentration of Amp-GNPs.

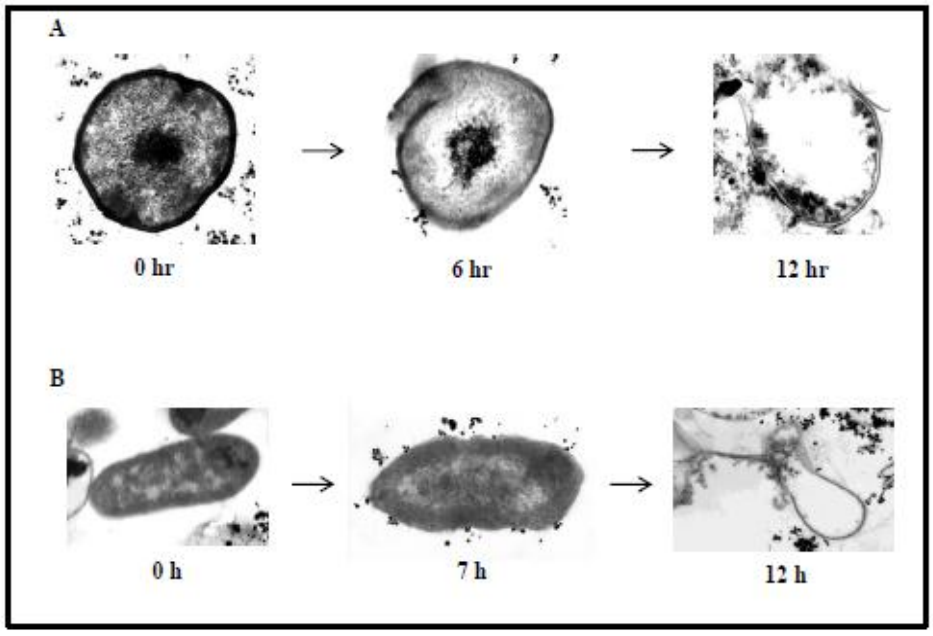


Figure 5. Visualization of bacterial morphological changes before and after Amp-GNPs exposure. A. Sequential analysis of Gram positive bacteria, *Staphylococcus epidermidis*. From left to right showing normal bacteria at 0 hour (pre Amp-GNP exposure), bacteria after six hours of Amp-GNP exposure showing cell membrane deformations, and after twelve hour of Amp-GNP exposure showing lysed cell. B. Sequential analysis of Gram negative bacteria, *Enterobacter aerogenes*. From left to right showing normal bacteria at 0 hour (pre Amp-GNP exposure), bacteria after six hour of Amp-GNP exposure showing cell membrane deformations, and after hour twelve of Amp-GNPs exposure showing lysed cell.

CHAPTER 4

DISCUSSION

4.1. Synthesis and Characterization of Antibiotic Coated GNPs

The objective was to synthesize antibiotic encapsulated GNPs that possess uniformity in both shape and size while still producing a yield of reasonable measure. The observed color change of the antibiotic encapsulated GNPs was from an approximately clear solution to darker shades of violet. Change in color denotes the formation of GNPs by reduction of gold (Au^{3+} to Au^{0}) and hence the formation of the antibiotic ampicillin encapsulated gold nanoparticles [23]. The reduction of the gold ions was possible due to the number of reducing groups such as hydroxyl or amino groups on the ampicillin molecule.

To examine the morphological, optical, and elemental properties of the Amp-GNPs a variety of instruments were employed. The UV-Vis experiments on the Amp-GNPs the maximum absorbance (λ max) value at 540 nm, the wavelength within the range of absorbance characteristic for GNPs, due to GNPs unique surface plasmon resonance [28,29]. It is the unique surface plasmon resonance of GNPs that allows this characterization to elucidate the approximate size of ~15 nm and spherical shape of GNPs. The TEM analysis of the Amp-GNPs was a critical step in assessing the morphological characteristics of the GNPs. The results eluded the approximate size and shape as previously mentioned and additionally, that the Amp-GNPs did not form

aggregates or any form of clusters. With the EDS results showing the elemental components of the Amp-GNPs, further proof of the composition of the Amp-GNPs was formed. The presence of the non Au peaks in the results aid in confirming the antibiotic ampicillin's presence on the Amp-GNP. This is evident as the antibiotic ampicillin possesses the additional elements of C, O, and S within its chemical structure.

4.2. Antibacterial Activity of Amp-GNPs

As a β-lactam antibiotic, ampicillin's mechanism of action against bacteria is through the binding of Penicillin Binding Proteins (PBPs) [30]. This binding in turn prevents the formation of the bacterial cell wall.

Without the capability to form a cell wall the organism and subsequent infection will subside. However, some bacterial strains have begun to form resistances towards ampicillin [3]. Through the enzyme beta lactamase which breaks down ampicillin, these evolved strains are capable of evading the ampicillin mechanism of antimicrobial action.

In determination of the Amp-GNPs efficacy as an antibacterial agent, both liquid broth and solid agar plate based assays were employed. These experiments were performed against multiple strains of both Gram-positive (i.e. *Staphylococcus epidermidis*) and Gram-negative bacteria (i.e. *Enterobacter aerogenes*). The liquid broth assay was conducted by absorbing the optical density at 600 nm because of the correlation between the optical density of the culture and the total number of cells it contains. This liquid broth assay provided evidence of the Amp-GNPs bacteriostatic activity as well as the minimum inhibitory concentration (MIC). The MIC is the lowest

concentration required in order to effectively halt bacterial multiplication. Gram-positive and Gram-negative bacteria differ in MIC however, the discrepancy in MIC between Gram-positive and Gram-negative bacteria is attributed to the naturally elevated resilience of Gram-negative bacteria [2, 31]. The greater resilience of Gram-negative bacteria is due to the structural differences between both types (Gram positive and Gram negative) of cellular membranes. Gram-negative bacteria are equipped with multiple layers of phospholipids and a smaller peptidoglycan region. This multilayered membrane provides the Gram-negative bacteria with added resilience when compared to Gram-positive bacteria [14, 32-34]. Gram-positive bacteria have a single phospholipid bi-layer and a larger peptidoglycan region. These differences in cellular anatomy explain the larger lack of Gram-positive bacteria ability to repel the Amp-GNP attack as effectively as Gram-negative bacteria [35, 36].

The bacterial cultures used in the liquid broth turbidimetry assay were spread onto fresh, solid, nutrient rich agar for conduction of the solid agar assay. The Gram-positive bacteria, *Staphylococcus epidermidis* was examined along with the Gram-negative bacteria, *Enterobacter aerogenes*. The plates were then allowed to incubate for 12 hours during which time the bacterial colonies became visibly populated. Results indicated the Amp-GNPs to possess bactericidal activity. Additionally the Gram-positive *Streptococcus bovis* and the Gram-negative *Pseudomonas aeruginosa* strains were examined in order to secure the premise of the Amp-GNPs being effective broad spectrum antimicrobial agents. With the results for the additional strains being similar to the preliminary tests the Amp-GNPs we are ever more confident in the Amp-GNPs efficacy as an antimicrobial agent.

In order to compare Amp-GNPs to ampicillin alone the spread plate assay was performed. The organisms tested were Gram-positive bacteria *Staphylococcus epidermidis* and *Streptococcus bovis* and the Gram-negative bacteria organisms were *Enterobacter aerogenes* and *Pseudomonas aeruginosa*. The results provided the MIC's for each strain. When comparing the MIC of ampicillin alone, however, it is evident that the Amp-GNPs are more potent in that a lower concentration is required to effectively neutralize bacterial growth.

The examined antimicrobial effects of Amp-GNPs have proven them highly effective against multiple bacterial organisms of both Gram-positive and Gram-negative type. Bacterial infection is one of the most basic yet highly deadly and severe forms of disease. It is therefore imperative that modern medicine is equipped with the adequate resources necessary to thwart such threats. With these data Amp-GNPs are shown to be a highly promising addition to modern medicine and a potential solution to bacterial infection.

4.3. Amp-GNP Antimicrobial Mechanism Examination

In assessment of the antimicrobial mechanism of Amp-GNPs the fluorescence microscopy experiments using propidium iodide were performed. Propidium iodide is a chemical dye that fluoresces upon interacting and binding with nucleic acids [14, 26]. Notably, propidium iodide is impermeable to an intact, healthy cell wall. Therefore, organismal solutions with intact cell walls will yield a low level of fluorescence while lysed organismal solutions will fluoresce excessively. A solution composed of liquid broth, bacterial organism, and Amp-GNPs were treated with the chemical dye. After

assessing the results it was found that the solution with Amp-GNPs fluoresced extensively more than the control. The increased fluorescence was caused by the amplified chemical dye, nucleic acid interaction. The interaction was possible because of the Amp-GNPs ability to lyse the bacterial cell membranes allowing the release of the bacterial nucleic acids. With these data we have been able to unveil the mechanism of Amp-GNP's bactericidal activity. The mechanism of Amp-GNP antibacterial activity is through the disruption of the bacterial cell wall, inhibiting the ability for the bacterial cell to regulate its homeostasis. Without the capability to regulate what materials enter and exit the cell the bacteria will inevitably die.

Cross sectional analysis of the tested bacteria (both Gram-positive and Gram-negative) before and after Amp-GNP exposure revealed a visual perspective of the destruction of the bacterial cells. Through the exposure time sequenced bacterial sectioned samples of initial exposure, six hours, and twelve hours it was possible to observe the morphological changes the bacteria underwent when confronted with the Amp-GNP antimicrobial attack. The gradual deformation resulting in complete lysis of the bacterial cell membrane provides further evidence of the mechanism behind the Amp-GNPs antimicrobial abilities of destruction of the bacterial cell wall.

The antibiotic ampicillin used in the green synthesis of the Amp-GNPs possesses a number of functional groups with chemical reduction capabilities such as hydroxyl and amino groups. Additionally the Amp-GNPs possess the capacity to act as both a hydrogen bond donor and as a hydrogen bond acceptor. This chemical property associated with hydrogen bonding is likely due to the electrostatic interaction that takes place between the antibiotic encapsulated GNP and the bacterial organism. This

interaction is what leads to the disruption of the bacterial cell membrane. It is speculated that the formation and deformation of chemical bonds associated with the bacterial cell membrane cause the cellular membrane breakage and the subsequent death of the bacterial organism.

The mechanism of Amp-GNP bactericidal action is entirely unique. Traditional antibiotics utilize mechanisms that share little similarity to Amp-GNPs. With traditional antibiotics the mechanism of action employed often involves the blocking of one of several possible bacterial cell functions. It is without the ability to carry out normal functions that the bacterial organisms die [37-39]. However, traditional antibiotic mechanisms' approach to warding off bacteria is the reason bacteria are capable of forming resistances or even immunities towards antibiotics. The ability of bacterial organisms to evolve and mutate in ways that allow the circumvention of traditional antibiotic attack is futile to the Amp-GNPs mechanism of action. In order to become resistant to the Amp-GNPs bacterial organisms would have to evolve in a way that essentially alters their entire anatomy in the form of the bacteria's cell membrane. Therefore, antibiotic encapsulated GNPs possess a truly unique and potentially revolutionary mechanism of bactericidal action that has the capabilities of transforming the age-old practice of fighting bacterial disease [40-42].

CHAPTER 5

CONCLUSION

In conclusion, we report the successful synthesis of antibiotic encapsulated gold nanoparticles using environmentally friendly methods with the antibiotic ampicillin. In addition, we report the effective use of ampicillin encapsulated gold nanoparticles (Amp-GNPs) as effective antibacterial agents. The antibacterial experiments were performed against multiple strains of bacteria representing both bacterial types, Gram-positive and Gram-negative. Amp-GNPs were found to possess highly effective, dose dependant antibacterial capabilities against all bacterial strains tested. The mechanism of antibacterial action was determined to be through the disruption of the bacterial cell membrane or necrosis. The lysing of the bacterial cell membrane resulted in the release of the bacterial cells inner components, the loss of bacterial cellular ability to regulate homeostasis, and the subsequent death of the bacteria. The antibiotic encapsulated gold nanoparticles have been shown to exhibit significant potential and ability to enter the medical field's arsenal to fight infectious disease. With the additional affect of being multifunctional and antibacterial themselves, the antibiotic coated GNPs are truly revolutionary for the fields of biomedicine and pharmaceuticals and are undoubtedly part of future nanomedicine.

BIBLIOGRAPHY

1. Nesse RM, Stearns SC, Omenn GS. Medicine Needs Evolution. Sci 2006; 311: 1071.
2. Prestidge LS, Perdee AB. Induction of Bacterial Lysis by Penicillin. J Bacteriol 1957; 74: 48-59.
3. Thornsberry C, Kirven LA. Ampicillin Resistance in *Haemophilus influenzae* as Determined by a Rapid Test for Beta-Lactamase Production. Antimicrob Agents Chemother 1974; 6: 653-54.
4. Donadio S, Maffiolo S, Monciardini P, Sosio M, Jabes D. Antibiotic discovery in the twenty-first century. Current trends and future perspectives. J Antibiot [Tokyo] 2010; 63: 423-30.
5. Levy SB. The challenge of antibiotics resistance. Sci Am 1998; 278: 46-53.
6. Wright GD. Antibiotic resistance in the environment. A link to the clinic? Curr Opi Microbio 2010; 13: 589-94.
7. Burke JP. Antibiotic Resistance-Squeezing the Balloon? JAMA 1998; 280: 1270-1.
8. Muller RH, Keck CM. Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles. Elsevier 2004; 151-70.

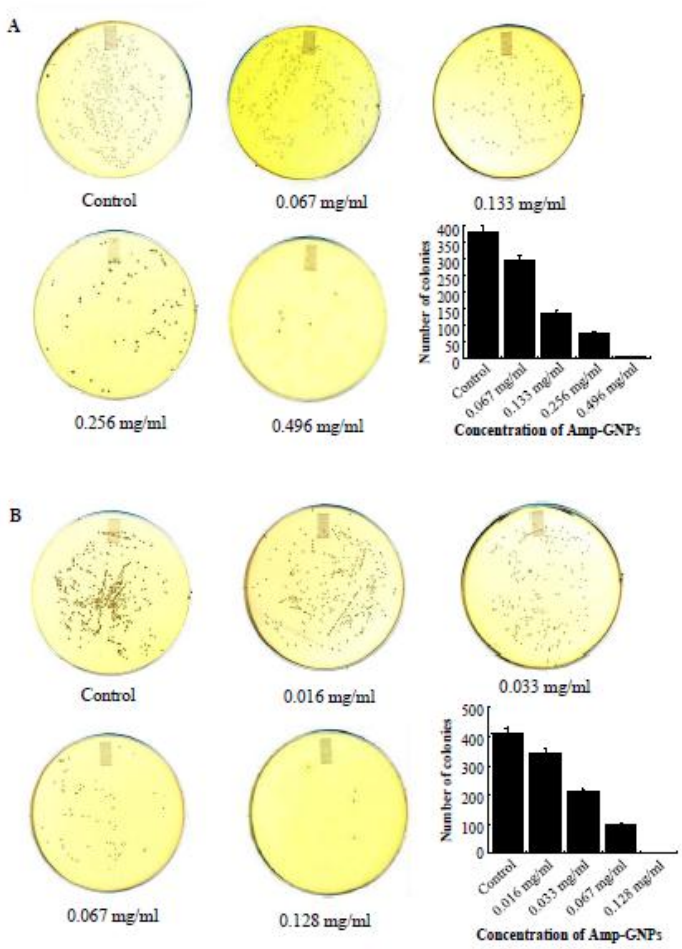
9. Giljohann DA, Seferos DS, Daniel WL. Gold Nanoparticles for Biology and Medicine. Angewandte Chemie International Edition 2010; 3280-94.
10. Pissuwan D, Niidome T, Cortie MB. The forthcoming applications of gold nanoparticles in drug and gene delivery systems. J Controlled Release 2011; 149: 65-71.
11. Brayner R, Ferrai-lliou R, Brivois N, Djediat S, Benedetti FM, Fernand F. Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Lett 2006; 6: 866-70.
12. Rosemary JM, MacLaren I, Pradeep T. Investigations of the antibacterial properties of ciprofloxacin@SiO2. Langmuir 2006; 22: 10125-9.
13. Wagner V, Dullaart A, Bock AK, Zweck A. The emerging nanomedicine landscape. Nat Biotechnol 2006; 24: 1211-7.
14. Zhao Y, Tian Y, Cui Y, Liu W, Ma W, Jiang J. Small moleculecapped gold nanoparticles as potent antibacterial agents that target gram-negative bacteria. J Am Chem Soc 2010; 132: 12349-56.
15. Shenoy D, Fu F, Li F. Surface functionalization of gold nanoparticles using hetero-bifunctional poly(ethylene glycol) spacer for intracellular tracking and delivery. Int J Nanomedicine 2006; 1: 51-4.
16. Gu YJ., Cheng J, Man CW, Wong WT, Cheng SH. Golddoxorubicin nano conjugates for overcoming multidrug resistance. Nanomedicine 2012; 8: 204-11.
17. Bhattacharya D, Saha B, Santra CR, Karmakar P. Gold nanoparticles conjugated antibiotics: stability and functional evaluation. Nanosci Nanotechnol 2012; 2: 14-21.

18. Brown AN, Smith K, Samueal TA, Lu J, Obare SO, Scott ME. Nanoparticles functionalized with ampicillin destroy multipleantibiotic resistant isolates of *Pseudomonas aeruginosa* and *Enterobacter aerogenes* and methicilling-resistant *staphylococcus aureus*. Appl Environ Microbiol 2012; 78: 2768-74.
19. Freese C, Uboldi C, Gibson MI. Uptake and cytotoxicity of citratecoated gold nanospheres: comparative studies on human endothelial and epithelial cells. Part fibre toxicol 2012; 9: 23.
20. Patra HK, Banerjee S, Chaudhuri U, Lahiri P, Dasgupta AK. Cell selective response to gold nanoparticles. Nanomedicine: nanotechnol, biolo, medicine 2007; 3: 111-9.
21. Chen YS, Hung YC, Liau I, Huang GS. Assessment of the *In Vivo* Toxicity of Gold Nanoparticles. Nanoscale Res Lett 2009; 4: 858-64.
22. Vecchio G, Galeone A, Brunetti V. Concentration-dependent, sizeindependent toxicity of citrate capped AuNPs in Drosophila melanogaster. PloS One 2012; 7: e29980.
23. Badwaik VD, Bartonojo JJ, Evans JW, Sahi SV, Willis CB, DakshinamurthyR. Single-step bio-friendly synthesis of surface modifiable, near-spherical gold nanoparticles for applications in biological detection and catalysis. Langmuir 2011; 27: 5549-54.
24. Chirea M, Freitas A, Vasile BS, Ghitulica C, Pereira CM, Silva F. Gold nanowires networks: synthesis, characterization and catalytic activity. Langmuir 2011; 27: 3906-13.

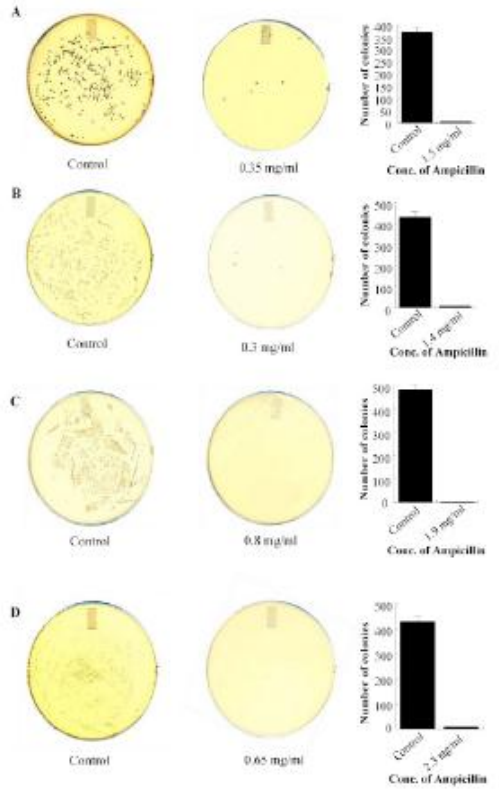
25. Burygin G. L., Khlebtsov B. N., Shantrokha A. N., Dykman L. A., Bogatyrev V. A, Khlebtsov N. G. On the enhanced antibacterial activity of antibiotics mixed with gold nanoparticles. Nanoscale Res Lett. 2009; 4:794-01.
26. Shrivastava S, Bera T, Roy A, Singh G, Ramachandrarao P, Dash D. Characterization of enhanced antibacterial effects of novel silver nanoparticles. Nanotechnol 2007; 18: 225103-12.
27. Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nature Protocols 2006; 1: 1458-61.
28. Easawi S, Negm K, Talaat HS. Shape and size dependence of the surface plasmon resonance of gold nanoparticles studied by Photoacoustic technique. Eur Phys J Spec Top 2008; 153: 361-4.
29. Hutter E, Fendler JH. Exploitation of localized surface plasmon resonance. Adv Mater 2004; 16: 1685-06.
30. Ghuysen JM. Serine Beta-Lactamases and Penicillin-Binding Proteins. Microbiol 1991; 45: 37-67.
31. Boisselier E. Astruc D. Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity. Chem Soc Res 2009; 38: 1759-82.
32. Fan L, Song J, Hildebrand PD, Forney CF. Interaction of ozone and negative air ions to control micro-organisms. J Appl Microbiol 2002; 93: 144-8.
33. Jiang W, Mashayekhi H, Xing B. Bacterial toxicity comparison between nano and micro-scaled oxide particles. Environ Pollut 2009; 157: 1619-25.

34. Premanathan M, Karthikeyan K, Jeyasubramanian K, Manivannan G. Selective toxicity of ZnO nanoparticles towards gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. Nanomedicine 2010; 7: 184-92.
35. Nair S, Sasidharan A, Rani VVD. Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. J Mater Sci Mater Med 2009; 20: 235-41.
36. Tsao N, Luh TY, Chou CK, Wu JJ, Lin YS, Lei HY. Inhibition of group A *Streptococcus* infection by Carboxyfullerene. Antimicrob Agents Chemother 2001; 45: 1788-93.
37. Hwang ET, Lee JH, Chae YJ. Analysis of the toxic mode of action of silver nanoparticles using stress-specific bioluminescent bacteria. Small 2008; 4: 746-50.
38. Ndeke M, Melusi T, Nomakhwezi N. The antibacterial effects of engineered nanomaterials: implications for wastewater treatment plants. J Enviorn Monit 2011; 13: 1164-83.
39. Su LH, Chou CC, Hung JD. The disruption of bacterial membrane integrity through ROS generation induced by nanohybrids of sliver and clay. Biomaterials 2009; 30: 5979-87.
40. Grace AN, Pandian K. Quinolone Antibiotic-Capped Gold Nanoparticles and Their Antibacterial Efficacy Against Gram Positive and Gram Negative Organisms. J ionanosci 2007; 1:96-105.

41. Pissuwan D, Cortie CH, Valenzuela SM, Cortie MB. Functionalized gold nanoparticles for controlling pathogenic bacteria. Trends Biotechnol 2010; 28: 207-13.
42. Rai A, Prabhuneb A, Perry CC. Antibiotic mediated synthesis of gold nanoparticles with potent antimicrobial activity and their application in antimicrobial coatings. J Mater Chem 2010; 20: 6789-98.



Supplementary Figure 1. Spread plate assay for (A) *Pseudomonas aeruginosa* and (B) *Streptococcus bovis* using Amp-GNPs. Images of plates show untreated control and increasing AMP-GNPs concentrations. Graph shows number of each respective organism's colonies versus concentration of AMP-GNPs.



Supplementary Figure 2. Spread plate assay for (A) *Staphylococcus epidermidis*, (B) *Streptococcus bovis*, (C) *Pseudomonas aeruginosa*, and (D) *Enterobacter aerogenes* using ampicillin. Images of plates show untreated control plate followed by ampicillin treated plate for all three strains. Graph shows the number of bacterial colonies versus control (untreated sample) and the MIC of ampicillin. Data confirms MIC of ampicillin against the various strains as being higher than the MIC of AMP-GNPs supporting the idea of AMP-GNPs being more potent and efficient than the standard antibiotic ampicillin.