One-Step Synthesis of Kanamycin Functionalized Gold Nanoparticles With Potent Antibacterial Activity Against Resistant Bacterial Strains

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ONE-STEP SYNTHESIS OF KANAMYCIN FUNCTIONALIZED GOLD NANOPARTICLES WITH POTENT ANTIBACTERIAL ACTIVITY AGAINST RESISTANT BACTERIAL STRAINS

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Hitesh Kumar Waghwani
May 2015
ONE-STEP SYNTHESIS OF KANAMYCIN FUNCTIONALIZED GOLD NANOPARTICLES WITH POTENT ANTIBACTERIAL ACTIVITY AGAINST RESISTANT BACTERIAL STRAINS

Date Recommended 4/15/2015

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Dean, Graduate Studies and Research Date
ACKNOWLEDGMENTS

The following project report is my master thesis which includes unpublished research data for my official conclusion of Master in Science (M.S.) program at Department of Chemistry, Western Kentucky University, Bowling Green, KY, USA. The thesis was defended in presence of my research mentor Dr. Rajalingam Dakshinamurthy, Thesis readers Dr. Bangbo Yan, and Dr. Moon-Soo Kim on Wednesday, April 15th, 2015.

I dedicate this thesis to my real god—my parents, Meenadevi and Ashok Kumar Waghwani, who are a great inspiration to me and who always supported me for higher education. Also, I dedicate this work to my grandmother, who died due to cancer and this project is an effort in a way to start my research for contributing towards healthcare society. I would also like to thank my brother and sisters for their constant support and creating me what I am today.

I express my gratitude for Dr. Cathleen Webb, Head of Chemistry Department for accepting my application into M.S. program in Chemistry. I want to thank my research mentor Dr. Rajalingam Dakshinamurthy for illuminating me with his presence throughout my tenure at Western Kentucky University. Working under Dr. Rajalingam Dakshinamurthy has definitely ended me into a strong platform wherein I can say that I am well prepared to do whatever task assigned to me and compete whole world. It was an inspiring journey under Dr. Rajalingam which includes being his research assistant, mentoring undergraduate students for research, being graduate student instructor for teaching CHEM 101 “Introduction to Chemistry” to a class of 90+ students for 1 year, being teaching assistant for conducting CHEM 330 and CHEM 106 labs, presenting my
research in various national and regional conferences and writing research proposals were the key attributes which I learnt under his guidance. Under his umbrella, I also gained professional experience by doing various certification programs such as “Biotechnologist Certification Program” and “Best Practice in Mentoring & College Teaching” program at WKU.

Handling multiple projects with proper planning and time management was one of the key for maximizing our research lab’s success. I thank Dr. Rajalingam for allowing me to work on various projects involving Kanamycin gold nanoparticles (GNPs), Phloridzin GNPs, Vancomycin GNPs, Ceftriaxone GNPs and Meropenam-GNPs and share my hands in Sugar GNPs project.

Apart from my research advisor, I would like to thank my thesis committee members Dr. Bangbo Yan and Dr. Moon-Soo Kim for their valuable comments.

For generously sharing their wisdom, I would like to thanks authors and co-authors of “Gold nanoparticles: various methods of synthesis and antibacterial applications” and “Nanotechnology’s Impact on medicinal chemistry”. It would not have been possible to publish mentioned publications without your support and team work.

Special thanks to Dr. Matthew Lawernz, Center for Predictive Medicine, University of Louisville School of Medicine for carrying out antibacterial assay against kan-resistant and MDR bacteria; Dr. John Andersland for sharing his valuable experience of working on electron microscope; Dr. Quentin Lineberry for teaching thermal analysis; Dr. Jerry Daday for his support in college teaching program; Ms. Naomi Rowland for providing extensive training on current biotechnology techniques
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ABBREVIATIONS

CFU  Colony forming units
CDC  Centers for disease control and prevention
CRE  Carbapenam-resistant enterobacteriaceae
°C  Degree celsius
DLS  Dynamic light scattering
DNA  Deoxyribonucleic acid
EDS  Energy dispersive spectroscopy
ESBLs  Extended spectrum β-lactamase producing enterobacteriaceae
ESKAPE  Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species

gram
Gold nanoparticles
Kanamycin capped gold nanoparticles
Liter
Luria Bertani
Molar
Microgram
Microliter
Miligram
Mililiter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum bactericidal concentrations</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistant</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>O.D</td>
<td>Optical density</td>
</tr>
<tr>
<td>PDR</td>
<td>Pan drug resistant</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermo gravimetric analysis</td>
</tr>
<tr>
<td>T.S</td>
<td>Tryptic soy</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>XTT</td>
<td>(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)</td>
</tr>
</tbody>
</table>
On the verge of entering the post-antibiotic era, numerous efforts are in place to regain the losing potential of antibiotics which are proving ineffective against common bacterial infections. Engineered nanomaterials, especially gold nanoparticles (GNPs) capped with antibacterial agents are proving to be an effective and novel strategy against multi-drug resistant (MDR) bacteria. In this study, we report a one-step synthesis of kanamycin-capped GNPs (20 ± 5 nm) utilizing the combined reducing and capping ability of the aminoglycoside antibiotic, kanamycin. Antibacterial assays showed dose-dependent broad spectrum activity of Kan-GNPs against Gram-positive (*Staphylococcus epidermidis* and *Enterococcus durans*), Gram-negative (*Escherichia coli* and *Enterobacter aerogenes*) and Kan-resistant and MDR bacterial strains. A significant reduction in the minimum inhibitory concentration (MIC) of Kan-GNPs was observed as compared to free kanamycin against all the sensitive and resistant bacterial strains tested. Mechanistic studies using TEM and fluorescence microscopy showed that Kan-GNPs exerted their bactericidal action through disrupting the cellular membrane resulting in leakage of cytoplasmic content and death of bacterial cells. Results of this study provide a novel method in the development of antibiotic capped GNPs as potent next-generation antibacterial agents.
1. INTRODUCTION

Bacteria belong to microorganisms which are part of earliest form of life existing on earth. There was a time when we humans were unaware of existence of tiny microorganisms. However, due to advancement in the field of microscopic and instrumental techniques we were able to provide proof that these tiny micrometer size organisms do exists and they are the causative agents for variety of diseases which were the ultimate cause of death in primitive days. Furthermore, researchers came up with more details regarding types of bacteria, their size and shapes, how they multiply and what their role is in life. It has been reported that there are at least 6 billion bacteria’s that reside on human body, but not all are pathogenic. With time, scientists started understanding how bacteria cause disease in a more sophisticated way and regarded pathogenic bacteria as “invisible enemies”.

The first antibiotic: penicillin was discovered in 1928 by Sir Alexander Fleming while he was studying and sorting culture of *staphylococcus* bacteria. During his research, he found an unexpected growth of colonies in one of his petri plates which he further identified as growth of mold of strain *Penicillium notatum*. He later had an idea that this mold is responsible for secretion of some unknown substance which inhibited the growth of bacteria. By the splendid discovery of antibiotic, every individual gained a ray of hope that “Yes! now we can treat bacterial infections and live longer!” With increase in other common infections and subsequent discovery of new generations of available antibiotics or new classes of antibiotics, the faith in modern medicine persisted and people enjoyed novelty of medicine. Hence, antibiotics were more commonly called as “Wonder drugs/Miracle drugs”.
Since the serendipitous discovery of the penicillin\(^3\), the process of developing resistance against antibiotics had been initiated in bacteria which was clearly evident from the presence of resistant bacterial strains in the early 1930's and 1940's.\(^{5,6}\) The reason behind bacterial resistance to the effect of antibiotic is attributed to mechanisms acquired or developed by bacteria such as “inactivation of drug by enzymes, alteration of drug target, activation of drug efflux pump and inhibition of drug uptake” etc.\(^7\) These resistant bacterial genes transfer from one generation to other and spread globally. Antibiotic resistance was also exaggerated by other contributing factors such as widespread use of antibiotics for livestock production, over the counter use for human consumption, improper diagnosis, leading to prescription of antibiotics in viral infections and dry new antibiotic pipeline, etc.\(^5–8\)

The bacterial resistance has increased from single infection to range of infections caused by bacteria, resulting in emergence of multi-drug resistant (MDR) bacterial strains more prevalently known as "superbugs".\(^8–13\) Common examples of such infections includes methicillin-resistant \textit{Staphylococcus aureus} (MRSA), carbapenam-resistant enterobacteriaeceae (CRE), extended spectrum β-lactamase producing enterobacteriaeceae (ESBLs), etc.\(^14,15\) Most of these infections are hospital acquired infections.\(^16\) According to World Health Organization (WHO), out of every 100 in-patients, at least 7 patients in high-income and at least 10 patients in low-/middle income countries may acquire a hospital acquired infection.\(^17\) The rate at which bacteria are becoming resistant to existing antibiotics is faster than the development of novel antibiotics.\(^18,19\) This bacterial resistance threatens the achievements of modern medicine,
such as organ transplantation, and common surgeries which are at higher risks of post-operative infections.\textsuperscript{14,18,19}

According to the recent report by Centers for Disease Control and Prevention (CDC), more than 23,000 people die annually from resistant bacterial infections in the United States.\textsuperscript{15} Hence, dominance of antibiotics are superseded by bacteria and we are failing to treat common bacterial infections. This current situation can be summed up in a single phrase where we are able to counteract big wars, but we are losing war to these tiny bugs.\textsuperscript{4} Therefore, it requires an urgent call to curb ill effects of MDR bacteria and prevent it from becoming pan-drug resistant (PDR) bacteria i.e., bacteria becoming resistant to all available antibiotics.\textsuperscript{14,15}

Several strategies are being employed to develop novel antibacterial agents. Aware of the exorbitant cost and time required to conduct research, scale up production, validate a process, and get an approval from regulatory bodies leading to success in a new chemical entity into the market, the current focus is shifting from developing new antibiotics to potentiating the activity of commercially successful antibiotics using alternative methods.\textsuperscript{20} Nanotechnology is one of the alternative route which is been under study for biological applications. It involves nanoparticles which deals with particles in size range of one billionth of a meter (1-100 nm).\textsuperscript{21} Nanoparticles possess unique optical properties when compared to bulk particles.\textsuperscript{21,22} They have been studied for various applications such as diagnosis,\textsuperscript{23} biosensors,\textsuperscript{24} catalysis,\textsuperscript{25} photothermolysis,\textsuperscript{26} drug delivery\textsuperscript{27} and gene therapy (Figure 1).\textsuperscript{28} Various researchers have reported successful capping of biomolecules on GNPs with similar or modified
pharmacological activity compared to bulk drug. For example, Gu et al have reported multiple fold in vivo potency for vancomycin-GNPs against vancomycin resistant enterobacteriaceae.  

Abraxane® (paclitaxel-albumin stabilized nanoparticle formulation) has been approved by USFDA in September 2013. Till date, no safety concern has been reported for the drugs in nano scale range.
Figure 1. Illustrates applications of gold nanoparticles. Gold nanoparticles are being studied for variety of applications such as diagnosis, biosensors, catalysis, photothermolysis, drug delivery and gene therapy.
One of the widely researched strategies involves use of metallic and metal oxide nanoparticles (1-100 nm) such as gold (Au), silver (Ag), zinc oxide (ZnO) and many more for their synergistic role in enhanced bactericidal activity.\textsuperscript{32,33} Among various inorganic nanomaterials, gold nanoparticles (GNPs) have gained immense attention in designing and developing new biomedical applications.\textsuperscript{21,22,34} Recently, ultra-fine, non-toxic GNPs are also being widely studied for combating endemic MDR bacteria.\textsuperscript{29,33,34} Some of the inherent features of GNPs such as biosafety,\textsuperscript{35,36} ease of functionalization,\textsuperscript{37} facile synthesis,\textsuperscript{22,38,39} and large surface/volume ratio allowing the release of high drug payload at the infected sites.\textsuperscript{40} GNPs also provides multiple-targets of bactericidal action, ability to penetrate biological membranes\textsuperscript{20,40} and many other relevant features that make GNPs the preferred candidates for developing novel antibacterial agents. Unlike the wide use of nanoparticles for cancer therapy,\textsuperscript{41-43} less extensive efforts have been made in the field of antibacterial agents as evident from a lack of FDA approved antibacterial formulation based on nanotechnology.\textsuperscript{33} Common methods that are employed for making GNPs involve multi-stepped use of organic chemicals and harsh reaction conditions, which could have detrimental effects on biological systems.\textsuperscript{44-49}

As an alternative route to prepare novel antibacterial nanoformulation by capping antibiotic onto GNPs in biofriendly manner, we selected kanamycin antibiotic. Kanamycin sulfate, derived from \textit{Streptomyces kanamyceticus}, is a broad spectrum aminoglycoside antibiotic discovered in 1956.\textsuperscript{50} Kanamycin is also classified under essential medicine list by WHO.\textsuperscript{51} Kanamycin is used in treatment of infections caused by pathogens such as \textit{E. coli}, \textit{Proteus species} (both indole-positive and indole-negative),
Enterobacter aerogenes, Klebsiella pneumoniae, Serratia marcescens, and Acinetobacter species.\textsuperscript{52–54} These organisms belong to a class of “ESKAPE” organisms which are primary cause for hospital acquired infections in U.S.\textsuperscript{55} Kanamycin acts by binding onto the 30S ribosomal subunit in prokaryotes thereby inhibiting protein synthesis.\textsuperscript{52,56} Bacteria have developed resistance to the antibacterial effects of kanamycin, thereby limiting its use in therapy.\textsuperscript{52}

We took an approach to develop antibiotic capped gold nanoparticles for potent antibacterial activity to curb the ill effects of resistant bacteria. Extending the scope of a method\textsuperscript{57,58} we developed to efficiently generate biomolecules capped-GNPs, in this study we report a simple kanamycin-mediated, bio-friendly synthesis and concomitant capping of GNPs, resulting in enhanced broad-spectrum antibacterial activity.

In this study, we synthesized Kan-GNPs and optimized its synthesis process. The synthesized Kan-GNPs were morphologically characterized using transmission electron microscopy (TEM), scanning electron microscopy, energy dispersive spectroscopy, UV-Vis spectroscopy, thermo gravimetric analysis, zeta potential and dynamic light scattering. Furthermore, Kan-GNPs were evaluated for their antibacterial efficiency against Gram positive, Gram negative, kanamycin sensitive, kanamycin resistant and multi-drug resistant (MDR) bacterial strains. Using TEM, we visualized morphological changes occurring in bacteria upon treating with minimum inhibitory concentration (MIC) concentration of Kan-GNPs. Our results demonstrate the successful development of a highly efficient Kan-GNPs against several infectious bacterial strains, suggesting a novel strategy to combat MDR bacteria.
2. MATERIALS AND METHODS

2.1. Materials

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Catalog Number</th>
<th>Vendor</th>
</tr>
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<tbody>
<tr>
<td>Formvar 15/95 resin, powder</td>
<td>63450-15-7</td>
<td>Electron Microscopy Sciences</td>
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<tr>
<td>Glycerol</td>
<td>GX0190-6</td>
<td>EMD Chemicals</td>
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<tr>
<td>Kanamycin sulfate</td>
<td>80058-286</td>
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<tr>
<td>L.B agar media</td>
<td>240110</td>
<td>BD Company</td>
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<tr>
<td>L.B media</td>
<td>71753-6</td>
<td>Novagen</td>
</tr>
<tr>
<td>Menadione</td>
<td>ME105</td>
<td>Spectrum Chemicals</td>
</tr>
<tr>
<td>Potassium aurochlorate</td>
<td>450235</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Potassium phosphate, dibasic (K$_2$HPO$_4$)</td>
<td>PX1570-1</td>
<td>EMD Chemicals</td>
</tr>
<tr>
<td>Potassium phosphate, monobasic (KH$_2$PO$_4$)</td>
<td>PX1565-5</td>
<td>EMD Chemicals</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>PX1595-1</td>
<td>EMD Chemicals</td>
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<tr>
<td>Propidium iodide</td>
<td>537059</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3624-05</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>Sodium phosphate, dibasic, 12-Hydrate</td>
<td>SX0718-1</td>
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<td>T.S agar media</td>
<td>236950</td>
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<td>T.S media</td>
<td>T0420</td>
<td>Teknova</td>
</tr>
<tr>
<td>XTT salt</td>
<td>10060</td>
<td>Biotium</td>
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</table>

**Table 1.** Represents list of all the chemicals used for the research project with catalog number and vendor details respectively.
## 2.2. Supplies

<table>
<thead>
<tr>
<th>Supply material</th>
<th>Catalog Number</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well Flat Bottom, Non-Treated, Sterile, Polystyrene, Microtiter Plates</td>
<td>25381-056</td>
<td>Costar</td>
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<tr>
<td>Microcentrifuge Tubes – 1.5 mL</td>
<td>87003-294</td>
<td>VWR</td>
</tr>
<tr>
<td>Falcon Tubes – 15 mL</td>
<td>89039-666</td>
<td>VWR</td>
</tr>
<tr>
<td>Falcon Tubes – 50 mL</td>
<td>89004-364</td>
<td>VWR</td>
</tr>
<tr>
<td>Glass Culture tubes</td>
<td>14-961-27</td>
<td>Fisher brand</td>
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<td>Micropipette Tips (1-20 µL)</td>
<td>53509-070</td>
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<td>Micropipette Tips (1-1000 µL)</td>
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<td>Nalgene™ Oakridge High speed PPCO centrifuge tubes</td>
<td>3119-0030</td>
<td>Thermo Scientific</td>
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<td>Sterile Polystyrene Petri Dish (100 mm x 15mm)</td>
<td>875713</td>
<td>Fisher Scientific</td>
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<td>TEM grids (400 square mesh, Oval Hole)</td>
<td>G400-Cu</td>
<td>Electron Microscopy Sciences</td>
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**Table 2.** Represents list of supply material used with catalog number and vendor details respectively.
## 2.3. Equipments

<table>
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<th>Equipment’s</th>
<th>Use</th>
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<tr>
<td>Autoclave Machine, GETINGE</td>
<td>Sterilize solutions and supplies</td>
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<tr>
<td>Centrifug litt (Model 228)</td>
<td>Concentrate gold nanoparticles (Medium scale)</td>
</tr>
<tr>
<td>Eppendorf Centrifuge</td>
<td>Concentrate gold nanoparticles (Small scale)</td>
</tr>
<tr>
<td>Excella E25 Incubator Shaker</td>
<td>Bacterial and nanoparticle incubation</td>
</tr>
<tr>
<td>-80 °C Freezer, Thermo scientific</td>
<td>Store bacterial stock culture</td>
</tr>
<tr>
<td>Hitachi U-3900 Spectrophotometer</td>
<td>Optical absorption of nanoparticle solution</td>
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<tr>
<td>JEOL JEM-1400 Plus Electron Microscope</td>
<td>Morphological characterization of kanamycin gold nanoparticles</td>
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<tr>
<td>JEOL JSM-5400 LV Scanning Microscope</td>
<td>Elemental composition of nanoparticles</td>
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<tr>
<td>Thermo scientific Bath Circulator (Haake A25,</td>
<td>Temperature dependent synthesis of nanoparticles</td>
</tr>
<tr>
<td>Haake SC150)</td>
<td></td>
</tr>
<tr>
<td>LABONCO Biological Safety Cabinet, Logic,</td>
<td>Working with bacteria (aseptic conditions)</td>
</tr>
<tr>
<td>Class II, Type A2</td>
<td></td>
</tr>
<tr>
<td>LABONCO Centrivap Cold Trap Lyophlizer</td>
<td>Freeze-drying of gold nanoparticles</td>
</tr>
<tr>
<td>METTLER TOLEDO New Classic MF Balance (Model:</td>
<td>Weighing of raw materials, nanoparticles etc.</td>
</tr>
<tr>
<td>ML 54/03)</td>
<td></td>
</tr>
<tr>
<td>MISONIX ultrasonic Liquid Processor XL-2000</td>
<td>Break the aggregation of nanoparticles</td>
</tr>
<tr>
<td>Probe Sonicator (Model: CML-4)</td>
<td></td>
</tr>
<tr>
<td>NANOpure infinity, Ultrapure water system,</td>
<td>Nano pure water (17.8-18.3 mΩ-cm)</td>
</tr>
<tr>
<td>Barnstead water machine</td>
<td></td>
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<tr>
<td>Petri Plate Scanner</td>
<td>Counting colonies on petri plates</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>Storing the solutions and reagents</td>
</tr>
<tr>
<td>SPECTRONIC 20D+ Thermo Spectronic</td>
<td>To check optical density of bacteria</td>
</tr>
<tr>
<td>Sorval RC-5B/5C Plus centrifuge</td>
<td>Concentrate gold nanoparticles (Large scale synthesis)</td>
</tr>
<tr>
<td>Synergy H1 Hybrid Plate reader, Biotek</td>
<td>Bacterial growth assay</td>
</tr>
<tr>
<td>TA Thermogravimetric Analysis, Q5000</td>
<td>Organic percent determination on gold nanoparticles</td>
</tr>
<tr>
<td>VWR Incubator</td>
<td>Growth of bacteria on agar plates</td>
</tr>
<tr>
<td>Zetasizer Nano S (Malvern Instruments Ltd.)</td>
<td>Particle size distribution</td>
</tr>
</tbody>
</table>

**Table 3.** Represents list of equipment’s used, their make and uses respectively.
### 2.4. Bacterial strains used for investigation

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Gram Bacteria Type</th>
<th>ATCC #</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Gram positive</td>
<td>12228</td>
</tr>
<tr>
<td><em>Enterococcus durans</em></td>
<td></td>
<td>6056</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>Gram negative</td>
<td>13048</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>67877</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> CO92</td>
<td>Kanamycin resistant bacteria</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia pestis</em> CO92::Km</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> PA01</td>
<td>Multi-drug resistant bacteria</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> UNC-D-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.** List of Gram positive, Gram negative, kanamycin resistant and multidrug resistant bacterial strains used in the research project.
2.5. Preparation of reagents

2.5.1. Cleaning protocol

All the glassware/apparatus/containers were thoroughly rinsed and washed with soap water followed by tap water and nanopure water and allowed to air dry. Furthermore, the apparatus were subjected to dry heat sterilization before use.

2.5.2. Preparation of sterile nanopure water

500 mL of nanopure water was collected in a sterile 1000 mL glass bottle. The cap was screwed loosely and the nanopure water was sterilized by moist heat sterilization using an autoclave.

2.5.3. Preparation of minimal media [M9 media without NH₄Cl] (1L, pH 7.2 ± 0.2)

Following cleaning protocol, a 1 L Erlenmeyer flask, two 1 L glass bottles, a 1 L graduated cylinder, a magnetic stirrer and a spatula was obtained. 13 g potassium phosphate, monobasic (KH₂PO₄), 10 g potassium phosphate, dibasic (K₂HPO₄), 9 g sodium phosphate, dibasic, 12-Hydrate (Na₂HPO₄), and 2.4 g potassium sulphate (K₂SO₄) were weighed accurately and transfered it to the 1 L Erlenmeyer flask. Approximately 800 mL of nanopure water was added to the Erlenmeyer flask. The contents of the Erlenmeyer flask were mixed to dissolve completely using a magnetic stirrer. In a large graduated cylinder, the volume was made up to 1000 mL using autoclaved nano pure water. 500 mL of solution was transferred to each 1 liter glass bottles. The bottles cap were screwed loosely and were sterilized by moist heat sterilization using an autoclave.
2.5.4. Preparation of kanamycin sulfate stock solution: (1.72 mM, 30 mL)

\[
Molarity = \frac{\text{Weight of kanamycin (g)}}{\text{Molecular weight of kanamycin} \times \frac{1000 \text{ mL}}{V \text{ in mL}}}
\]

\[
\text{Molecular weight of kanamycin} = 582.6 \frac{g}{\text{mole}}
\]

30 mg of kanamycin sulfate (see Appendix A for product specification) was weighed using microbalance and transferred to 50 mL falcon tube. 30 mL of M9 media (without \(\text{NH}_4\text{Cl}\)) was added to the falcon tube. The falcon tube was thoroughly vortexed in order to mix and dissolve the drug and a clear solution was obtained. 5 mL of drug solution was then transferred to six different 15 mL falcon tubes.

2.5.5. Preparation of potassium gold (III) chloride stock solution (KAuCl\(_4\)) (0.79 mM, 5.0 mL)

\[
Molarity = \frac{\text{Weight of gold salt (g)}}{\text{Molecular weight of gold salt} \times \frac{1000 \text{ mL}}{V \text{ in mL}}}
\]

\[
\text{Molecular weight of gold salt} = 377.8 \frac{g}{\text{mole}}
\]

250 mg of gold salt was weighed and transferred to 15 mL Falcon tube. 5 mL of sterile nanopure water was added to it. The mixture was vortexed to dissolve solute until clear, homogenous yellow solution was obtained. The falcon tube was covered with aluminum foil to protect from light. The tube was further stored at 2-8 °C in a refrigerator until further use.

2.5.6. Preparation of Luria-Bertani media (LB) (1L)

Following the cleaning protocol, 25 g of L.B media was weighed accurately and transferred to 2 L Erlenmeyer flask. Approximately 800 mL of autoclaved nanopure
water was added to it. The L.B media was completely mixed and dissolved in nanopure water using a magnetic stirrer. The volume was made up to 1000 mL using autoclaved nanopure water in a graduated cylinder. The resulting solution was then further transferred equally into two glass bottles (1 L capacity) and sterilized using autoclave. The solution was further stored in a refrigerator between 2-8 °C until further use.

**Note:** It is highly recommended to prepare fresh stock every time before use.

### 2.5.7. Preparation of Tryptic-Soy media (T.S) (1L)

Following the cleaning protocol, 30 g of T.S media was weighed accurately and transferred to 2 L Erlenmeyer flask. Approximately 800 mL of autoclaved nanopure water was added to it. The T.S media was completely mixed and dissolved in nanopure water using a magnetic stirrer. The volume was made up to 1000 mL with nanopure water using a graduated measuring cylinder. The resulting solution was then further transferred equally into two glass bottles (1L capacity) and sterilized using autoclave. The solution was further stored in a refrigerator at 2-8 °C until further use.

### 2.5.8. Preparation of L.B/T.S agar plates

Following the cleaning protocol, 40 g of L.B agar/T.S agar was weighed and transferred into a 2 L Erlenmeyer flask. Approximately 800 mL of autoclaved nanopure water was added to it. The agar media was completely mixed and dissolved in nanopure water using a magnetic stirrer. The volume was made up to 1000 mL in a graduated cylinder using autoclaved nanopure water. The flask was then covered with double folded aluminum foil and sterilized using moist heat sterilization. In the meantime, polystyrene petridishes were obtained and sterilized using UV light in a biological safety
cabinet. After sterilization of agar media, the flask was allowed to sit for approximately 5 minutes. 25 mL of sterilized media was poured in each petri dish using 50 mL falcon tube. At room temperature, the plates were allowed to solidify in biological safety cabinet by covering half portion of cap open. After solidification, the plates were wrapped using parafilm around its circumference, labeled and stored in refrigerator between 2-8 °C for further use. Note: The solidification of agar takes place at approximately 35 °C. Hence, care should be taken after autoclave for not allowing flask to sit for longer time.

2.5.9. Preparation of 10X phosphate buffer saline (PBS) (1L, pH 7.2 ± 0.2)

Following the cleaning protocol, 5.519 g sodium dihydrogen phosphate, 42.89 g disodium hydrogen phosphate and 87.66 g sodium chloride were weighed and transferred it to 2 L Erlenmeyer flask. Approximately 800 mL of autoclaved nanopure water was added and the solutes were mixed to dissolve. The volume was made up to 1000 mL in a graduated measuring cylinder using autoclaved nanopure water. The pH of solution was adjusted to pH 7.2 using calibrated pH meter. The 500 mL of solution was transferred to two reagent bottles, screw capped loosely and subjected to moist heat sterilization. After sterilization, the bottles were labeled, screw capped tightly and stored at room temperature until further use.

2.5.10. Preparation of 1X phosphate buffer saline (PBS) (0.5L, pH 7.2 ± 0.2)

Following the cleaning protocol, 50 mL of 10X PBS was diluted to 500 mL using autoclaved nanopure water in a graduated cylinder. The pH of solution was adjusted to pH 7.2 using calibrated pH meter. This solution was then transferred to 1 L
reagent bottle, screw capped loosely and subjected to moist heat sterilization. After sterilization, the bottles were labeled, screw capped tightly and stored at room temperature until further use.

2.5.11. Preparation of tetrazolium salt solution (XTT) (30 mL)

Following the cleaning protocol, 30 mg of XTT dye was weighed and transferred to 100 cc Erlenmeyer flask containing 30 mL of phosphate buffer saline (1X PBS, pH 7.2). The flask was covered with double folded aluminum foil (protected from light) and sterilized using moist heat sterilization method. After sterilization, 995 µL of XTT solution was aliquot (under aseptic conditions) into 1.5 mL eppendorf centrifuge tubes, labelled and stored at -4 °C freezer until further use.

2.5.12. Preparation of menadione solution (10 mM, 50 mL)

\[
Molarity = \frac{\text{Weight of menadione (g)}}{\text{Molecular weight of menadione}} \times \frac{1000 \text{ mL}}{V \text{ in mL}}
\]

\[
\text{Molecular weight of menadione} = 172.18 \frac{g}{\text{mole}}
\]

Following cleaning protocol, 86 mg of menadione was weighed accurately and dissolved in 50 mL acetone in a 100 mL glass bottle. The bottle was labelled and stored in a refrigerator at -4 °C for further use.

2.5.13. Preparation of 1 % formvar solution

Prepare a 1 % solution of formvar by dissolving 1 gram polyvinyl formvar desiccated resin powder in 100 mL ethylene dichloride solvent. Keep the reaction mixture overnight in order to completely dissolve resin in solvent.
2.5.14. Preparation of TEM grids

A 400 square mesh, oval hole copper grids were obtained and washed in 1 M HCl solution in order to remove any dust particles followed by air drying. The grids were further washed in water and air dried. A final wash with acetone followed by air drying was done for copper grids. The burette apparatus was set up and formvar coating solution was added into burette. A clean glass slide was dipped into the burette holding formvar solution and kept in contact for approximately 30 seconds. The glass slide was removed using tweezer and air dried. A surgical grade blade was used to scrap the edges of the glass slide. This process technically provides different films on each side of glass slide. The film coating was then further removed from glass slide by immersing it into a glass jar completely filled with distilled water. The formvar film so obtained was deposited onto light side of washed copper grids by holding copper grids inversely (light side facing downwards) and placing it onto formvar film at surface of water. Parafilm was used to obtain formvar film containing copper grids. The copper grids (with formvar film on top) were then removed from parafilm using sharp tweezer and kept separately in a petri plate containing a filter paper.
2.6. Experimental Methods

2.6.1. Synthesis of Kanamycin gold nanoparticles (Kan-GNPs)

Kan-GNPs was synthesized in a single step process (Figure 2) which involves a reaction of kanamycin drug in an aqueous buffer with an aqueous solution of KAuCl₄. Aiming at making monodisperse and stable Kan-GNPs with a uniform particle size, we carried out reactions at different concentrations of each reaction components. The combination which yielded desired morphology of Kan-GNPs with high percentage yield was selected. For a typical synthesis, 1.72 mM of kanamycin was dissolved in aqueous buffer (pH ~7.2 ± 0.2) and was preheated above room temperature. Furthermore, 0.79 mM of KAuCl₄ (30 µL from 50 mg/mL solution) was added and reaction mixture was incubated for 5 minutes. After incubation, the reaction tubes were removed and cooled at room temperature. The formation of Kan-GNPs was confirmed by visual change of a reaction mixture from colorless to pink. The Kan-GNPs suspension was then subject to repeated washing with autoclaved nanopure water and centrifugation (15,000 rpm for 20 minutes) to remove any traces of free reactants remaining in the reaction mixture. Sterile oakridge tubes were used for centrifugation in Sorval RC 5B/5C Plus Centrifuge set with F21 rotor. Supernatant solution was discarded at each centrifugation step and replaced with autoclaved nanopure water for further washing. The concentrated Kan-GNPs suspension was then obtained after final washing and purification procedure, freeze-dried and stored at room temperature for further analysis.
**Figure 2.** Illustrates a single step scheme for Kan-GNPs synthesis. In this process, kanamycin itself acts as reducing and capping agent.
2.6.2. Freeze-drying of synthesized Kan-GNPs

Freeze-drying of Kan-GNPs had various advantages such as ease in storage, weighing and handling and convenience in determining the dose of Kan-GNPs to be selected for further antibacterial testing. This also indicates robustness of manufacturing process with respect to percentage yields. For freeze-drying process, Kan-GNPs (100 mL batch) was synthesized, washed, centrifuged, and concentrated to a final volume of 2 mL in nanopure water. The LABONCO Centrivap Cold Trap Lyophlizer was switched on 2 hours prior to freeze-drying to attain -30 °C temperature. Concentrated Kan-GNPs suspension was then transferred to two different stainless steel cups, freezed in liquid nitrogen, and covered with miracloth which allowes loss of moisture but not permeable for loss of nanomaterials. The cups were then readily transferred to lyophilization chamber (previously cooled and maintained at -30 °C). The chamber was closed and vacuum was switched on. The samples specimen were kept overnight under freezing conditions. Temperature variation was performed the following day with increasing temperature by 5 °C at specific time interval. At 30 °C, the lyophlizer was switched off and the dried Kan-GNPs were collected, weighed accurately and stored in a glass vial at room temperature for further analysis.

2.6.3. Characterization of Kan-GNPs

Though synthesis of Kan-GNPs was physically noted by change in color of the reaction mixture, still it is very important to determine if kanamycin is actually capped onto gold nanoparticle surface. Thus, various electron microscopic and spectroscopic techniques were used to confirm presence of antibiotic kanamycin on GNP's surfaces.
**UV-Vis spectroscopy (UV-Vis)**

Gold nanoparticles possess a characteristic optical property, called surface plasmon resonance (SPR). This SPR band is observed in visible range of light and it depends on the size of particles, shape of particles and chemical environment in which nanoparticles (NPs) are present. Hence, UV-Vis spectroscopy was used as a qualitative analytical spectrometric technique to confirm the formation of Kan-GNPs. A dilute suspension of Kan-GNPs was probe sonicated for 30 seconds at 45 % amplitude. UV-Vis absorption spectrum of resulting suspension was recorded in the wavelength range of 400-850 nm using a Hitachi U-3900 spectrophotometer at a resolution of 0.5 nm and scanning speed of 600 nm/min while maintaining slit width at set value of 5 nm and path length 10 mm.

**Dynamic light scattering (DLS)**

DLS is a rapid and accurate technique used to characterize nanoparticles suspended in liquid medium. Diameter and average particle size distribution of synthesized Kan-GNPs were determined using a Zetasizer Nano S (Malvern Instruments Ltd.). The motion of nanoparticles in suspension leads to scattering of laser light at different intensity, causing intensity fluctuations, which is further recorded and analyzed, and particle velocity was determined. To analyze Kan-GNPs, dilute suspension of Kan-GNPs was probe-sonicated for 30 seconds at 45 % amplitude. The average of three individual measurements in total of 13 runs was recorded in 1 mL of sample. The data so obtained was plotted and average particle size distribution along with polydispersity was determined.
Transmission electron microscopy (TEM)

Transmission electron microscope possesses high image resolution at nanometer range.\(^{58}\) It works based on the principle of interaction of beam of an electron with a thin specimen sample, thus transmitted electrons produce an image of sample on phosphorus screen and the size and shape of nanoparticles were determined. To analyze Kan-GNPs, a dilute suspension of Kan-GNPs was probe sonicated for 30 seconds at 45\% amplitude. 10 µL of above suspension was loaded onto 400 square mesh, oval hole formvar coated copper grid and was allowed to air dry before observing under JEOL JEM-1400 Plus transmission electron microscope. The sample was allowed to be in contact with formvar film on grid. Excess sample was wiped off using filter paper wedge. The beam of an electron was maintained at 100 kv electron voltage and the image of desired region was taken at different magnifications using a built-in camera. The scale bar of the respective size of an image automatically was included in image based on magnification value.

Scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS)

Surface morphology of Kan-GNPs was studied using scanning electron microscopy (SEM) and elemental compositions including a gold and carbon was detected by high sensitive energy dispersive X-Ray spectroscopy (EDS) using a JEOL JSM-5400 LV with IXRF system. For a typical analysis, 50 µL of Kan-GNPs suspension (previously probe sonicated) was pipetted on rough side of clean silicon chip. The silicon chip containing sample was spin coated and placed in a petri plate and further dried under vacuum chamber for approximately 2 hours. The silicon chip was
then imaged onto SEM and elemental composition was determined using EDS. The SEM images were then exported into a built-in software and elemental composition of Kan-GNPs was determined at different sampling spots using IXRF system. The presence of organic compound was confirmed onto GNPs surface and the elements and their respective percent weights were then detected.

**Thermogravimetric analysis (TGA)**

Thermal analysis is an important quantitative method to determine amount of organic content capped on GNPs surface.\(^{61}\) To determine how much kanamycin is coated on GNPs, 5-10 mg of freeze dried powder of Kan-GNPs was placed in a platinum pan which was supported by a highly precise balance. The pan was introduced into furnace chamber in Q5000 TGA and subjected to thermal decomposition from room temperature till 850 °C. The sample environment is governed by sample purge gas used. Two types of methods (i.e., using nitrogen and air as purge gas) were employed to study the thermal degradation of Kan-GNPs (Appendix B: Method). In method I, Kan-GNPs were thermally degraded in inert conditions using nitrogen as purge gas till 650 °C followed by air till 850 °C to facilitate complete degradation of the organic content. In method II, air was used as a purge gas right from room temperature to 850 °C to facilitate complete oxidation of kanamycin. The weight loss of Kan-GNPs was monitored as a function of temperature throughout the experiment. A thermogram with percent weight on Y-axis and temperature on X-axis was plotted using universal TA software and thermal degradation pattern of kanamycin was studied. The thermal
analysis experiment of Kan-GNPs was repeated three times to confirm the percent weight of kanamycin on GNPs surface.

**Zeta potential**

Zeta potential provides a net electrical charge at the interface in colloidal system.\(^6\) For nanoparticles suspension, measuring charge of GNPs is a very key parameter for predicting its stability. It is also reported that negatively charged bacterial cell membrane attracts positively charged nanoparticles, thereby contributing to the bactericidal action of GNPs. Kan-GNPs were analyzed for zeta potential using Zetasizer Nano S (Malvern Instruments Ltd.) equipment. Kan-GNPs showed positive charge of +23.4 mV.

**2.6.4. Evaluation of antibacterial activity of Kan-GNPs**

Kan-GNPs were tested for *in vitro* antibacterial activity against Gram-positive (*Staphylococcus epidermidis* and *Enterococcus durans*), Gram-negative (*Escherichia coli* and *Enterobacter aerogenes*), kanamycin resistant (*Y. pestis* C092::km) and multi-drug resistance (*P. aeruginosa* UNC D-1) bacterial strains.

**Preparation of glycerol stock of bacterial strains**

A fresh glycerol stock of desired bacterial strains were prepared in respective nutrient media when required. A preculture of desired bacterial strain was grown by adding 200 µL of bacterial stock in 10 mL of respective nutrient media (L.B/T.S) in a 50 mL UV sterilized falcon tube. The bacterial suspension was mixed and incubated overnight in a incubator shaker at 37 °C, 150 rpm for 12-20 hours. After incubation interval, the growth of bacteria was determined by measuring the optical density (O.D)
of bacterial culture at 600 nm using 4 mL of culture in a clean sterilized glass testube in Spectronic-20 spectrometer corrected with a blank. When the O.D of bacterial culture reached to a value of ~1.2, a 1.5 mL (10%) of glycerol was added to the bacterial culture in falcon tube and mixed thoroughly. 250 µL of resulting suspension was aliquot into 1.5 mL sterilized labelled eppendorf centrifuge tubes. The culture containing tubes immediately kept in contact with liquid nitrogen and freezed. After all the tubes were completed for preparing an aliquot, the culture containing tubes were transferred to –80 °C freezer until further use. The entire process of preparing a glycerol stock was conducted in biological safety cabinet in the presence of blue flame using bunsen burner to ensure aseptic conditions.

**Preparation of preculture of bacterial strains**

A preculture of desired bacterial strain was prepared by thawing the glycerol stock of respective bacterial strain. 10 mL of nutrient media (L.B/T.S) was added into 50 mL UV sterilized falcon tube. 200 µL of thawed bacterial suspension from glycerol stock was transferred into falcon tube. The sample was mixed properly and then incubated overnight at 37 °C, 150 rpm for ~12-20 hours. The growth of bacteria was determined by testing O.D of bacterial culture. The desired O.D for testing is ~1.2. *Table 5* represents the preculture conditions for each bacterial strain used for antibacterial assay.
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Gram Bacteria Type</th>
<th>Nutrient Media Type</th>
<th>Incubation Time &amp; Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Gram positive</td>
<td>L.B/T.S Broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
<tr>
<td><em>Enterococcus durans</em></td>
<td>Gram positive</td>
<td>T.S Broth</td>
<td>37 °C, 150 rpm, 18-20 hrs</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>Gram negative</td>
<td>L.B Broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Gram negative</td>
<td>L.B/T.S Broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
</tbody>
</table>

Table 5. Represents incubation time and conditions for preparing small scale preculture of various Gram positive and Gram negative bacterial strains tested.

**In-vitro antibacterial assay**

*In-vitro* antibacterial activity of Kan-GNPs were tested on the basis of dose against Gram-positive (*Staphylococcus epidermidis* and *Enterococcus durans*), and Gram-negative (*Escherichia coli* and *Enterobacter aerogenes*) bacteria at Dr. Rajalingam Dakshinamurthy’s lab. The effect of Kan-GNPs against kanamycin resistance bacteria (*Y pestis* CO92::km) and MDR bacteria (*P. aeruginosa* UNC D-1) were tested at the Dr. Matthew Lawrenz lab at the Center for Predictive Medicine, University of Louisville School of Medicine. For the control, the cognate antibiotic kanamycin (i.e., kanamycin without GNPs) was included in all studies. The minimum inhibitory concentration (MIC) was calculated from the average MIC of three individual experiments. The MIC values obtained were utilized to establish experimental conditions for testing kanamycin resistant and MDR bacterial strains. The concentration of gram bacteria was maintained uniform at 1x10⁶ CFU per mL whereas concentration
of 3x10^5 CFU per mL of bacterial suspension was used for antibacterial testing for kanamycin resistant and MDR bacterial strains.

**Bacterial growth curve assay**

The bacteriostatic/bactericidal activity elucidation of Kan-GNPs was performed to measure cell viability after exposure to Kan-GNPs nanoformulation as a function of time.58,63 The lowest concentration of Kan-GNPs required to kill bacterial cells was determined as MIC against the particular bacterial strain.64,65 The *in vitro* antibacterial assay was performed in a 96 well microtiter plate (Appendix D). The circumference of microtiter plate was filled with sterilized nanopure water to prevent loss of sample due to evaporation from sample wells during incubation conditions. Each reaction plate contained three sample rows (comprising of Kan-GNPs + nutrient media and bacterial cells) to study the effect of Kan-GNPs on growth of bacteria, one row as positive control (comprising nutrient media + bacterial cells) to study growth of bacteria in the absence of Kan-GNPs and one blank row as a negative control (comprising of Kan-GNPs + nutrient media) to subtract the absorbance for Kan-GNPs and nutrients by themselves.

A standard stock solution of Kan-GNPs (1 mg/mL) was prepared using autoclaved nanopure water which was used to make various concentrations required for the antibacterial study. Growth of Gram-positive (*Staphylococcus epidermidis* and *Enterococcus durans*) and Gram-negative (*Escherichia coli* and *Enterobacter aerogenes*) bacterial strains in the presence of Kan-GNPs was measured using a microtiter broth method.64 Test samples contained serial dilutions of Kan-GNPs with a final volume of 100 μL in a UV sterilized 96-well microtiter plate. 10 μL of fresh
cultures of a bacterial strain grown overnight at 37 °C, 150 rpm were inoculated into the sample wells. A final volume of 250 µL was achieved in each well using the respective nutrient media. Wells containing the same concentration of Kan-GNPs (100 µL) with 150 µL of growth media were used as a negative control. For the positive growth control, the same volume of Kan-GNPs was replaced with equal amount of nanopure water. The plates were incubated at 37 °C, 150 rpm. Using a microtiter plate reader, the optical density at 600 nm (OD\textsubscript{600}) was measured every three hours for a period of 12 hours. A graph of O.D 600 nm was plotted against time (hrs). The same procedure was repeated for pure kanamycin and MIC values were compared with that of Kan-GNPs.

**Spread Plate Assay**

In addition to the growth curve assay, solid agar plate assay was performed to check the viable bacterial cells in the presence of different concentrations of Kan-GNPs.\textsuperscript{58,63} Spread plate assay was used to determine minimum bactericidal concentrations (MBC) i.e., the lowest concentration of Kan-GNPs that kills 99.9 % of bacteria upon treating with nanoparticles.

For this study, a respective bacterial strain was incubated in the presence and absence (control) of Kan-GNPs for 12 hours at 37 °C and 150 rpm. Following the incubation time, small volume of bacterial suspension samples were evenly spread onto a fresh, nutrient L.B/T.S agar plates using sterilized glass spreader in aseptic conditions. The plates were incubated for 12-16 hours followed by counting of viable numbers of bacterial colonies. The procedure was performed in triplicate and the average number of colonies from three measurements was used. The concentration which showed more
than 90 percent reduction in number of colonies was considered as MIC concentration. Hence, this assay was used to visibly confirm the MIC values obtained from growth curve assay.

2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay

XTT assay is a modern, simple, rapid, sensitive, and reliable colorimetric method used in cell proliferation, cytotoxicity and apoptosis studies. XTT, a yellow tetrazolium salt in the presence of a dehydrogenase enzyme in metabolically active cells is reduced to bright orange formazan derivative. Live cells have reducing environment due to electron transport chain mechanism (Figure 3). It measures the viable cell count by measuring the reducing activity of cells. Optimum cell concentration and incubation time were used based on the best suited culture conditions as observed in growth assays. The amount of the formazan produced is proportional to viable cells present and was quantified by measuring the absorbance at 492 nm using H1 Synergy plate reader.

To amplify the results of XTT, an activator called menadione was added. 50 µL of XTT tetrazolium salt stock solution containing menadione (995 µL XTT solution + 5 µL menadione) was added to the growth curve assay wells (test samples, positive and negative control) and plate was incubated for 2 hours at 37 °C, 150 rpm in the dark, followed by their absorbance measurement (at 0 and 2 hrs interval of adding XTT) at 492 nm. A graph of absorbance @ 492 nm against concentration of Kan-GNPs was plotted. The concentration of Kan-GNPs which resulted in no orange color formation or
90% reduction in absorbance as compared to the positive control was taken as the MIC.

All the MIC measurements were done in triplicate.

Figure 3. Illustrates bacterial cell viability detection mechanism by XTT assay. Metabolically active bacteria due to dehydrogenase enzyme reduces yellow tetrazolium salt (XTT) to orange-red colored formazan derivative.
Antibacterial assay against kanamycin resistant and MDR bacteria

For Kan resistant bacteria the minimum inhibitory concentration (MIC) was determined. Briefly, for *Yersinia pestis* 2-fold serially diluted Kan-GNPs concentrations were added to wells of a clear microtiter plate. 3x10^5 colony forming units (CFU) were then added and microtiter plates were incubated at 26 °C for 10 hours. Similar microtiter plates were prepared for *P. aeruginosa*, and incubated at 37 °C for 10 hours respectively. MICs were determined as Kan-GNPs concentration that completely inhibited bacterial growth (MIC\textsubscript{100}). The average MIC\textsubscript{100} from three independent experiments was calculated.

2.6.5. Visualization of bactericidal action of Kan-GNPs

Cellular imaging using propidium iodide (PI) was done to determine the antibacterial mechanism of Kan-GNPs.\textsuperscript{67} PI is a red-fluorescent dye which has high affinity towards bacterial DNA. Due to its inability to permeate living cells, it can be used to detect dead cells in a bacterial population.\textsuperscript{67} A respective bacterial strain was incubated in the presence of the MIC of Kan-GNPs for ~12 hours followed by which the samples were repeatedly washed with phosphate buffer saline (PBS) and centrifuged (6000 rpm, 3 min). Samples were further incubated with 5 µL of PI (10 mM) for 30 minutes in the dark. After incubation, unbound PI was removed by washing the sample with 1X PBS and 10 µL of the resulting suspension was placed on a glass slide and covered with a cover slip. The sample was viewed using a Leica fluorescence microscope. A sample of bacteria without Kan-GNPs was taken as control. The numbers of permeable cells (showing red fluorescence) were counted by taking an average from
three individual measurements and a graph was plotted in comparison to control for respective bacterial strains.

To visualize the morphological changes in bacteria after treating with Kan-GNPs, cross-sections of bacteria were prepared with the aid of ultramicrotome and observed under an transmission electron microscope (TEM).68,69 1 mL of sample containing bacterial culture incubated (37 °C, 150 rpm, and 12 hours) in the presence of MIC concentration of Kan-GNPs was centrifuged (4000 rpm, 3 min) and the pellet was re-suspended in 1 mL of primary fixing solvent (16 % w/v paraformaldehyde and 10 % w/v glutaraldehyde in 50 mM sodium cacodylate buffer (pH ~7.4)) and incubated for 2 hours. After incubation, the solution was centrifuged, washed twice with cacodylate buffer, re-suspended in 1 mL of 1% osmium tetroxide solution (OsO₄), and further incubated for 1 hour at 25 °C for post-fixation. After fixation, the bacterial samples were washed using sterile nanopure water. Samples were further treated with series of ethanol concentrations (25, 50, 75, 95 and 100 % respectively). The samples were dehydrated and further centrifuged. Spur’s epoxy resin in series of (33, 66, 95 and 100 %) was used to infiltrate pellets for one hour. The samples were left in contact with 100 % resin overnight. The samples were then centrifuged in BEEM® capsules, which were solidified by heating at 70 °C for 18 hours. Using glass knives for RMC MT-X ultramicrotome, ultra-thin sections of sample were cut and stained with 2 % aqueous uranyl acetate for 15 minutes and Reynold’s lead citrate for 3 minutes. The samples were then imaged under JEOL-100CX TEM.
3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of Kan-GNPs

One of the vital steps in the preparation of GNPs is the addition of chemical agents to reduce the ionic gold atoms \([\text{Au}^{3+}]\) to neutral gold atoms \([\text{Au}^0]\) which tends to result in aggregation upon reaching the saturation limit. Secondary capping agents are further added to stabilize and restrict the size of gold aggregates into the nano-range yielding gold nanoparticles. However, using such methods requires the need of multiple steps to purify the product from unwanted components, thus making the overall process highly expensive and labor intensive which limits scalability. Furthermore, due to the biological concerns of the chemical agents used in the synthesis process, emphasis to find biologically friendly methods for synthesizing GNPs is growing.

In this context, we developed a simple single step method for making antibiotic functionalized GNPs. The aminoglycoside kanamycin has electron rich hydroxyl and amine functional groups which could serve as a dual reducing and capping agent. Hence, kanamycin antibiotic was selected as a proof of concept to synthesize antibiotic capped GNPs. The optimum concentration of kanamycin and gold required for GNPs synthesis was determined by assessing varying concentrations of both kanamycin and KAuCl\(_4\) at different synthesis parameters. Based on these analyses, it was determined that 1.72 mM of kanamycin in aqueous buffer (pH ~7.2 ± 0.2) when incubated with 0.79 mM of KAuCl\(_4\) for 5 minutes yielded the optimal production of Kan-GNPs. The kanamycin solution was preheated above room temperature before adding KAuCl\(_4\).
Qualitative analysis of aggregation and morphological characteristics of Kan-GNPs was obtained under TEM at 100 kV showed the particles to be nearly spherical in shape and monodispersed with an average diameter of 20 ± 5 nm (Figure 4(a)). DLS is another widely used characterization technique for nanoparticles. DLS analysis of Kan-GNPs showed a sharp peak with an average size distribution of 20 ± 5 nm (Figure 4(b)) which is in agreement with TEM. A UV-Vis spectrum of Kan-GNPs recorded in the visible region (400-850 nm) showed a peak absorption ($\lambda_{\text{max}}$) value at 546 nm (Figure 4(c)) which is in compliance with the size of GNPs obtained from TEM.

Scanning electron microscopy coupled with energy dispersive spectroscopy was used further to determine the surface elemental composition of Kan-GNPs. Dense portions of the highly magnified SEM image of Kan-GNPs were selected where the electron beam was focused to obtain the localized elemental (atoms) composition using IXRF software. Multiple spots were selected for the analysis. Results of EDS spectral analysis revealed the presence of elemental peaks of carbon (C) and gold (Au) at ~0.2 keV and ~2.1 keV respectively (Figure 4(d)). The percent composition of carbon and gold was found to be 16.65 % and 66.55 %, respectively. The presence of a reduced gold peak ensured the formation of GNPs whereas the carbon peak confirmed the presence of the organic ligand (kanamycin) on the surface of GNPs.

Thermo gravimetric analysis of Kan-GNPs was used to quantify the amount of organic ligand (kanamycin) bound to the surface of GNPs as well as its precent weight which is essential in calculating the actual dose of drug for in-vivo studies. Method I (Appendix: B) was used in inert conditions using nitrogen as purge gas from room
temperature to 650 °C followed by air till 850 °C whereas, in method II (Appendix: C) air was used as purge gas throughout thermal degradation in order to facilitate complete oxidation. The weight loss for Kan-GNPs was observed to be 35 % of the total mass (Figure 4(e)). TGA of pure kanamycin was also performed for reference as shown in (Figure 4(e)). Zeta potential was used to determine surface charge of Kan-GNPs. Kan-GNPs showed zeta potential of +23.4 mV.
Figure 4. Illustration of the morphological characterization of Kan-GNPs. (a) TEM image showing formation of well-dispersed nearly spherical Kan-GNPs in the size range of 20 ± 5 nm. (b) Plot showing average particle size distribution of Kan-GNPs suspension obtained using DLS. (c) UV-Vis spectra of Kan-GNPs showing a strong absorption peak at 546 nm which is a characteristic of spherical GNPs. (d) Energy dispersive spectroscopy (EDS) spectra of Kan-GNPs showing the presence of elemental peak for carbon (C) and gold (Au) at 0.2 keV and 2.1 keV, respectively. Figure in the inset shows SEM image of spin coated sample of Kan-GNPs on silicon chip obtained at an accelerating voltage of 20 keV with a magnification of 5kX. (e) A comparison of thermo gravimetric (TGA) analysis showing loss of organic material for kanamycin (----) and Kan-GNPs (- - -) respectively. The samples were heated from room temperature to 650 °C at a rate of 10 °C min⁻¹ under nitrogen flow followed by heating till 850 °C under air.
3.2. Kan-GNPs have antibacterial activity

We evaluated the antibacterial efficacy of Kan-GNPs against a variety of Gram-positive (*Staphylococcus epidermidis* and *Enterococcus durans*) and Gram-negative (*Escherichia coli* and *Enterobacter aerogenes*) bacterial strains by observing the bacterial growth in the presence of different concentrations of Kan-GNPs. The MIC was determined as the lowest concentration of Kan-GNPs which showed complete inhibition of bacterial growth or at least 90% reduction in the absorbance of growth at 600 nm when compared to untreated bacteria.

Overnight culture of each bacterial strain was incubated (37 °C, 150 rpm) with different concentrations of Kan-GNPs and their growth was observed for a period of 12 hours in a 96-well microtiter plate. To verify the broad-spectrum activity of Kan-GNPs, antibacterial tests were performed on both Gram-positive and Gram-negative strains. A graph was plotted for O.D$_{600}$ nm against time (hrs). In general, dose-dependent inhibition of bacterial growth was observed for all bacterial strains. From the above assay MIC of 24.46 µg mL$^{-1}$ and 35.23 µg mL$^{-1}$ (Kan-GNPs) was observed against Gram-positive strains *S. epidermidis* and *E. durans* respectively (Table 6). Results for the Gram-negative strains *E. aerogenes* and *E. coli* were found to be 21.82 µg mL$^{-1}$ and 25.06 µg mL$^{-1}$, respectively. The MIC value observed in the bacterial growth assay was consistent with the spread plate assay as no visible growth of bacterial colonies were observed in presence of the Kan-GNPs at MIC (Figure 5).
Figure 5. Illustrates dose dependent inhibition of bacterial growth by Kan-GNPs against Gram positive, *S. epidermidis* (a, b) and Gram negative *E. aerogenes* (c, d) bacteria. (a and c) Monitoring growth in the presence of increasing concentration of Kan-GNPs by measuring the OD at 600 nm every 3 hours for a period of 12 hours. MIC of Kan-GNPs was found to be 24.46 µg/mL and 21.82 µg/mL against *S. epidermidis* and *E. aerogenes* bacteria, respectively. (b and d) Visualizing the growth of *S. epidermidis* and *E. aerogenes* bacteria on a solid agar plate in presence of MIC of Kan-GNPs obtained from the growth assay. Untreated sample of bacteria was taken as control.
To check the precision of the results, XTT, a cell viability assay was performed. Viable or metabolically active cells have potential to convert the water-soluble XTT to a water-soluble, orange colored formazan product which can be easily quantified colorimetrically. The lowest concentration in the well which showed no orange color formation or 90% reduction in the absorption at 492 nm (characteristic for XTT) was taken as the MIC. The MIC values of XTT assay for Gram-positive strain S. epidermidis (Figure 6 (a and b)) and Gram-negative strain E. aerogenes (Figure 6 (c and d)) were in compliance with the previous assays.

A comparison of results showed approximately two fold reduction in the MIC of Kan-GNPs for all the bacterial strains tested when compared to the pure kanamycin (Table 6). Therefore, kanamycin conjugated on to GNPs surface is more efficient in combating the bacteria than the free antibiotic.
Figure 6. Colorimetric illustration of dose dependent inhibition by Kan-GNPs against Gram-positive, *S. epidermidis* (a, b) and Gram-negative *E. aerogenes* (c, d) bacteria. (a and c) Susceptibility testing against varying concentrations of Kan-GNPs by colorimetric assay which involves reduction of a yellow tetrazolium salt (XTT) to orange formazan product by metabolic active bacterial cells. The MICs were similar to the MIC obtained using growth assay and spread plate. (b and d) A plot corresponding to XTT assay obtained by measuring absorbance of wells at 492 nm, which shows peak absorption for orange formazan derivative. Wells with 90% reduction in absorbance compared to positive control or no orange color formation were taken as MIC.
Table 6

<table>
<thead>
<tr>
<th>Gram Positive</th>
<th>Kan</th>
<th>Kan-GNPs</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em></td>
<td>40</td>
<td>24.46</td>
<td>1.64</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>100</td>
<td>35.23</td>
<td>2.85</td>
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<table>
<thead>
<tr>
<th>Gram Negative</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>60</td>
<td>25.06</td>
<td>2.39</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>40</td>
<td>21.82</td>
<td>1.83</td>
</tr>
</tbody>
</table>

Table 6. Illustration of MIC of Kan-GNPs against Gram-positive and Gram-negative strains in comparison to pure kanamycin. The growth in presence of increasing concentrations of Kan-GNPs was monitored by measuring the optical density (O.D) at 600 nm every 3 hours for a period of 12 hours to determine the MIC. Decrease in the MIC of Kan-GNPs compared to pure kanamycin proved Kan-GNPs enhanced potential in combating bacteria.
Next we tested whether Kan-GNPs have greater efficacy than kanamycin against kanamycin resistant and MDR bacteria. Two kanamycin resistant bacterial strains were used, a genetically engineered *Y. pestis* kanamycin resistant strain (CO92::Km) and a multidrug resistant *P. aeruginosa* clinical isolate (UNC-D). The MIC\(_{100}\) of kanamycin against both of these strains is significantly higher than equivalent kanamycin sensitive strains (WT *Y. pestis* CO92 and *P. aeruginosa* PA01; Table 7). When kanamycin sensitive bacteria were incubated with Kan-GNPs a 2.88- and 7.50-fold decrease in kanamycin concentration was required when bound to GNPs to inhibit the growth of *Y. pestis* CO92 and *P. aeruginosa* PA01 respectively. In the case of the kanamycin resistant and MDR bacterial strains, we observed even higher impact of GNPs on the kanamycin MICs, with a 13.50- and 41.88-fold decrease in MICs for *Y. pestis* CO92::Km and *P. aeruginosa* UNC-D, respectively. Importantly, GNPs linkage to kanamycin resulted in kanamycin MICs similar to the kanamycin alone MICs of the kanamycin sensitive strains. Taken together, our data demonstrate that GNPs linkage to kanamycin reduces the kanamycin MIC against drug resistant strains.
Table 7

<table>
<thead>
<tr>
<th>P. aeruginosa</th>
<th>Kan</th>
<th>Kan-GNPs</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA01</td>
<td>50</td>
<td>6.67</td>
<td>7.5</td>
</tr>
<tr>
<td>UNC D-1 (MDR&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>139.46</td>
<td>3.33</td>
<td>41.88</td>
</tr>
<tr>
<td>Y. pestis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO92</td>
<td>4.81</td>
<td>1.67</td>
<td>2.88</td>
</tr>
<tr>
<td>CO92::Km</td>
<td>180</td>
<td>13.33</td>
<td>13.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Concentration of Kan

<sup>b</sup>Multidrug resistant

Table 7. Illustration of MIC of Kan-GNPs against genetically engineered Y. pestis kanamycin resistant strain (CO92::Km) and a multidrug resistant P. aeruginosa clinical isolate (UNC-D). The growth in presence of increasing concentrations of Kan-GNPs was monitored by measuring the optical density (OD) at 600 nm every 3 hours for a period of 12 hours to determine the MIC. The MIC<sub>100</sub> of kanamycin against both of these strains is significantly higher than equivalent kanamycin sensitive strains (WT Y. pestis CO92 and P. aeruginosa PA01; Table 7). When kanamycin sensitive bacteria were incubated with Kan-GNPs a 2.88- and 7.50-fold decrease in kanamycin concentration was required when bound to GNP to inhibit the growth of Y. pestis CO92 and P. aeruginosa PA01, respectively. For kanamycin resistant strains, a higher impact of GNP on the kanamycin MICs, with a 13.50- and 41.88-fold decrease in MICs for Y. pestis CO92::Km and P. aeruginosa UNC-D was observed respectively.
3.3. Kan-GNPs treatment alters the morphology of bacteria

The morphology of Gram-positive (*S. epidermidis*) and Gram-negative (*E. aerogenes*) bacteria against Kan-GNPs was determined by examining ultra-thin sections of the samples under electron microscope (TEM), obtained using an ultra-microtome, collected at various time intervals (0, 6 and 12 hours) of bacterial growth. A series of morphological changes were observed for bacterial cells treated with nanoparticles. Based on TEM observation, after 6 hours of incubation, Kan-GNPs were found to be localized on the cell membrane, covering most of the surface of Gram-positive *S. epidermidis* bacteria. Nanoparticles were also observed in the cytoplasm which shows the ability of Kan-GNPs to permeate the bacterial cell wall. As a result of perforations into the cell wall, disruption and leakage of cytoplasmic contents were observed after 12 hours resulting in complete cell lysis (Figure 7(a)). Cross-sectional TEM images showed localization of Kan-GNPs on the surface as well as entry into the cytoplasm, suggesting bactericidal action of Kan-GNPs resulting in the dual killing effect. A similar pattern of lysis of bacterial cell wall by Kan-GNPs was observed against Gram-negative *E. aerogenes* (Figure 7(b)).
Figure 7. TEM images for visualizing the morphological changes in bacteria upon treating with Kan-GNPs at different intervals of time. Panel (a) represents sequential images (from left to right) of Gram positive, *S. epidermidis* bacteria treated with Kan-GNPs (24.46 µg mL⁻¹) after 0 hour, 6 hours and 12 hours of incubation. Panel (b) Represents sequential images (from left to right) of Gram negative, *E. aerogenes* bacteria treated with Kan-GNPs (21.82 µg mL⁻¹) after 0 hour, 6 hours and 12 hours of incubation. After 6 hours of exposure, Kan-GNPs were found to adhere and penetrate the bacterial cell wall which resulted in disruption of cellular environment leading to lysis of cell due to leakage of cellular components as observed after 12 hours of exposure.
Permeability of Kan-GNPs into the bacterial cytoplasm is crucial for the delivery of attached kanamycin to inhibit the bacterial protein synthesis. To assess the permeability of Kan-GNPs, fluorescence imaging using propidium iodide (PI) was used. PI dye which shows fluorescence after binding to nucleic acids in membrane compromised cells, was used to detect dead cells. Kan-GNPs treated samples of *S. epidermidis* (24.46 µg mL\(^{-1}\)) and *E. aerogenes* (21.82 µg mL\(^{-1}\)), after incubating for 12 hours at 37 °C were stained with PI dye followed by further incubation in the dark for 2 hours (Figure 8(a)). Fluorescence images of the samples showed 75 ± 10% permeability of Kan-GNPs for *S. epidermidis* and *E. aerogenes* respectively when compared to control which showed only 15 ± 10% permeability for *S. epidermidis* and *E. aerogenes* respectively (Figure 8(b)).
Figure 8. Fluorescence images of Kan-GNPs induced cell membrane permeability using propidium iodide (PI) dye which has strong binding affinity towards nucleic acids. Upper panel represents Gram-positive, *S. epidermidis* and lower panel represents Gram-negative, *E. aerogenes*. (a) The image represents differential interference contrast mode with corresponding fluorescence image. Untreated samples of respective bacteria without Kan-GNPs were taken as control. (b) Represents a plot showing percentage permeability of *S. epidermidis* and *E. aerogenes* bacterial cells in presence (MIC) and absence (control) of Kan-GNPs.
These results supported the observation from TEM cross-section images confirming bactericidal action of Kan-GNPs resulting in lysis of bacterial cell. Kan-GNPs binds to the surface of the bacterial cell wall, making perforations which disrupts cellular equilibrium. In addition, free kanamycin existing on GNPs surface may also bind to the 30S ribosome and inhibit protein synthesis, thereby retaining its inherent pharmacological activity.\textsuperscript{70} We also hypothesize that due to the presence of amine groups in kanamycin, which can easily be protonated ($\text{RNH}_2 + \text{H}_2\text{O} \rightarrow \text{RNH}_3^+$) imparting a partial positive charge to the Kan-GNPs, could bind to the negatively charged DNA (due to presence of phosphate) and unwind the two strands, resulting in disruption in DNA.\textsuperscript{62} The positive amine groups could also facilitate in binding with the negatively charged bacterial cell wall as observed from the TEM pictures.\textsuperscript{71,72} Correlating the hypothetical antibacterial mechanism of Au@TiO\textsubscript{2} systems,\textsuperscript{73} Kan-GNPs present on the surface of bacteria could also extract electrons from the bacterial respiratory proteins, thereby starving bacteria to death. In addition, the internalized Kan-GNPs could affect important biological processes in bacteria such as ATP synthesis, protein synthesis and many more.\textsuperscript{74} As a result of multiple routes of bactericidal action, it becomes relatively problematic for the bacteria to resist the action of antibiotic GNPs, thereby making them more favorable for clinical use as an effective antibacterial agent. One of the major concerns regarding the use of GNPs for clinical applications is its cytotoxicity which is still under debate. In a previous study, spherical gold nanoparticles (20 ± 5 nm) were shown to be nontoxic\textsuperscript{75} which can be used to predict the nontoxicity of Kan-GNPs.
4. CONCLUSION

Our results illustrate a novel and efficient antibacterial strategy involving the use of a commercial antibiotic, kanamycin capped onto the surface of gold nanoparticles through a single-step process without the use of any toxic chemicals. Monodisperse, spherical GNPs in the size range of $20 \pm 5$ nm were formulated which showed dose-dependent bactericidal activity against both Gram-positive and Gram-negative bacteria. Significant decrease in the MIC of Kan-GNPs in comparison to pure kanamycin proved Kan-GNPs enhanced potential in combating Kan-resistant and MDR bacteria. Kan-GNPs were found to kill bacteria by causing perforations in the bacterial cell wall, as well as probably due to the protein inhibition activity of free kanamycin on the surface, causing loss of cellular integrity leading to leakage of cell constituents, which eventually resulted in lysis of the bacterial cell. In conclusion, we report a simple, bio-friendly process using combined reducing and capping ability of kanamycin to produce stable and efficient GNPs which holds a promising future for its clinical use as a potent antibacterial agent against multi drug resistant bacteria. This could facilitate biomedical application of Kan-GNPs and can open a new route for other commercially available antibiotics to which bacteria have gained resistance.
5. FUTURE STUDIES

*In vivo* interaction of Kan-GNPs in animal model

In order to use a nanoformulation for biological applications, the Kan-GNPs should be tested for *in vivo* activity in experimental animal models. This will be done by studying the physiological changes occurring in animal systems upon acute and chronic exposure to Kan-GNPs. The MIC concentration of Kan-GNPs will be administered intravenously to the experimental animal model and animals will be studied for various parameters such as change in body temperature, body weight and behaviour before and after treatment with Kan-GNPs. The blood and urine samples will be collected at specific time intervals and tested for hematological and urinary analysis. For detailed interaction studies, serum biochemical and histo-pathological analysis will also be done. All the toxicity analysis will be done for Kan-GNPs and will be compared with pure kanamycin.

**Pharmacokinetic (PK) and pharmacodynamic (PD) studies of Kan-GNPs**

After successful qualitative analysis of Kan-GNPs for *in vivo* applications, we will study the Absorption Distribution Metabolism Excretion (ADME) profile of kanamycin nanoformulation which will give us pharmacokinetic information, whereas efficacy and toxicity studies in pre-clinical models will provide us pharmacodynamic information of Kan-GNPs. The ADME, PK and PD studies of Kan-GNPs will be done by collecting samples from blood, tissues, plasma, bile and urine upon treating animal model with Kan-GNPs and will be tested for drug concentration, half life, clearance, toxicity and release profile of drug.
6. REFERENCES


(9) ANTIBIOTIC RESISTANCE: AN ECOLOGICAL PERSPECTIVE ON AN OLD PROBLEM, SEPTEMBER 2009; General Microbiology; American Academy of Microbiology: USA; p 38.


In Vivo PK/PD Studies | Nanomedicines Characterization Core Facility.
7. APPENDIX

7.1. Appendix A: Product specification of kanamycin sulfate, *Streptomyces kanamyceticus*

Product Name: Kanamycin sulfate, *Streptomyces kanamyceticus*

Chemical Formula: \( C_{18}H_{36}N_{4}O_{11} \cdot H_2SO_4 \)

Formula Weight: 582.6 g/mole

Description: White odorless crystalline powder

Solubility: Soluble in water (10 mg/mL)

Turbidity: Clear

Storage: 15 – 30 °C

Supplier: EMD Millipore

Specifications: Contains more than 98 % kanamycin

**Figure 9.** Illustrates structure of kanamycin sulfate. a) Represents chemical structure of kanamycin sulfate. b) Represents molecular model of kanamycin sulfate constructed using Gausview 5.0.
7.2. **Appendix B**: Thermo gravimetric analysis protocols of Kan-GNPs

**Method log (Nitrogen atmosphere)**

- Step 1. Select purge gas “Nitrogen”
- Step 2. Isothermal for 30 minutes
- Step 3. Mark end of cycle 0
- Step 4. Ramp 10 °C/min to 100 °C/min
- Step 5. Isothermal for 60 minutes
- Step 6. Ramp 10 °C/min to 650 °C/min
- Step 7. Mark end of cycle 0
- Step 8. Isothermal for 60 minutes
- Step 9. Mark end of cycle 0
- Step 10. Select purge gas “Air”
- Step 11. Ramp 10 °C/min to 850 °C/min
- Step 12. Mark end of cycle
- Step 13. Isothermal for 30 minutes
- Step 14. Mark end of cycle 0
- Step 15. End of method

**Figure 10.** Represents method for thermal degradation of Kan-GNPs in presence of nitrogen as purge gas.
Method log (Air atmosphere)

Step 1. Select purge gas “Air”

Step 2. Ramp 10 °C/min to 100 °C/min

Step 3. Isothermal for 60 minutes

Step 4. Ramp 10 °C/min to 850 °C/min

Step 5. Mark end of cycle 0

Step 6. Isothermal for 30 minutes

Step 7. Mark end of cycle 0

Step 8. End of method

Figure 11. Represents method for thermal degradation of Kan-GNPs in presence of air as purge gas. In this method, air was selected as purge gas for complete sample run in order to determine percent weight loss of Kan-GNPs upon complete oxidation.
7.3. **Appendix C**: Thermo gravimetric analysis of Kan-GNPs using air as purge gas

![Thermal degradation plot](image)

**Figure 12.** Represents thermal degradation plot showing weight loss of organic material for kanamycin (---) and Kan-GNPs (- - -) respectively using air as purge gas. The samples were heated from room temperature till 850 °C using air as purge gas in order to facilitate complete oxidation of organic compound. Kan-GNPs showed weight loss of 35.05 % which is comparable to percent weight loss of Kan-GNPs obtained using nitrogen as purge gas.
7.4. Appendix D: Microtiter plate schematic for growth curve assay and XTT assay

Figure 13. Represents design of 96 wells microtiter plate. All sample reactions were done in triplicate. Sample row contained varying concentration of Kan-GNPs concentrated to 100 μL as final volume, 140 μL nutrient media and 10 μL of bacterial culture (1 x 10⁶ CFU per mL). Blank wells contains same concentrations of drug as in sample rows and 150 μL nutrient media. Control wells contained 100 μL sterilized nanopure water, 140 μL nutrient media and 10 μL of bacterial culture. For XTT assay, 50 μL of XTT solution (previously activated with menadione) was added to each sample, blank and control wells.