Antimicrobial Nanoparticles: A Green and Novel Approach for Enhancing Bactericidal Efficacy of Commercial Antibiotics

Monic Shah
Western Kentucky University, monic.shah598@topper.wku.edu

Follow this and additional works at: http://digitalcommons.wku.edu/theses

Part of the Analytical Chemistry Commons, Medicinal-Pharmaceutical Chemistry Commons, and the Organic Chemistry Commons

Recommended Citation
http://digitalcommons.wku.edu/theses/1389

This Thesis is brought to you for free and open access by TopSCHOLAR®. It has been accepted for inclusion in Masters Theses & Specialist Projects by an authorized administrator of TopSCHOLAR®. For more information, please contact topscholar@wku.edu.
ANTIMICROBIAL NANOPARTICLES: A GREEN AND NOVEL APPROACH FOR ENHANCING BACTERICIDAL EFFICACY OF COMMERCIAL ANTIBIOTICS

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
Monic Shah
August 2014
ANTIMICROBIAL NANOPARTICLES: A GREEN AND NOVEL APPROACH FOR ENHANCING BACTERICIDAL EFFICACY OF COMMERCIAL ANTIBIOTICS

Date Recommended 7/9/2014

Dr. Rajalingam Dakshinamurthy
Director of Thesis

Dr. Chad Snyder, Thesis Reader

Dr. Kevin Williams

Dean, Graduate Studies and Research 7-25-14
ACKNOWLEDGEMENT

There were a bunch of people who helped and supported me for my success at Western Kentucky University (WKU). Firstly, I would like to deeply acknowledge my wonderful family who believed in my potential and made courageous efforts to send me for higher education in the United States, often considered as the land of opportunities. Initially, it wasn't so easy for me as well as my parents to digest the fact of me living miles away from home. Western Kentucky University was truly a university with international reach. I sincerely thank each and every individual at WKU, who never allowed me to feel homesick. I am grateful to Dr. Cathleen Webb, Head, Department of Chemistry for accepting my application for an admission in the MS in Chemistry program at WKU. I was amazingly fortunate that all the efforts and hard work I had put for my research paid off well in the form of 3 peer-reviewed publications with 4 more in the drafting stage, more than 15 research presentations at scientific conferences including 246th ACS, 41st NATAS and 99th KAS, multiple university awards that includes Ogden College of Science and Engineering Outstanding Graduate Student (2014), Carl P. McNally Chemistry Fellowship Award (2013), Nell Skean Outstanding Laboratory Assistant Award (2014) and Kentucky Innovation Entrepreneur Award (2014) and an opportunity to serve as Graduate Student Instructor (Super GA) to teach CHEM-101 to 100+ students for two semesters. In addition, I was also assigned Teaching Assistantship for CHEM 447 and CHEM 121. The entire credit for all my above achievements goes to my research adviser Dr. Rajalingam Dakshinamurthy whose endless support, patience and guidance throughout my graduate tenure helped me to nurture my skills and bring my talent to work. Despite many rough moments, his belief and faith turned me from a mere
graduate student to outstanding graduate student. I wouldn't have been what I am today without his advice and encouragement. He enforced strict validations and maintained a high research standard which helped me and my lab colleagues to learn how to do research. Beside my research mentor, I also thank my other thesis committee members, Dr. Kevin Williams and Dr. Chad Snyder for their insightful comments and interesting questions. I am also thankful to all my lab peers including Yogesh Kherde, Rammohan Paripelly, Hitesh Waghwani and Tulsi Modi for all their help and cheerful moments. All the days I spent with them in lab would always remain one my beloved memories. I am extremely grateful to Dr. John Andersland for all his advice and help with the microscopic analysis of our samples. In addition, I would also like to extend my deepest gratitude to Dr. Quentin Lineberry, Mrs. Pauline Norris, Mrs. Naomi Rowland, Mr. Vivek Badwaik and Mr. Ryan Vincent for their help in my research. I also thank Mrs. Alicia Pesterfield for arranging, providing and ordering all the necessary chemicals and solvents on time. Most of the times, the research demanded staying late night which was quite troublesome for janitors as they had to skip their routine work due to our presence in lab. I sincerely owe a big thank you for their patience. I also thank WKU for providing an excellent platform to quick start my career in the field of biomedical research and development. Last but not the least, I would like to acknowledge all my friends here and back in India who were with me in all my good and bad times.
PREFACE

The following report is my master thesis for the official conclusion of my Master in Science (MS) degree at the Department of Chemistry, Western Kentucky University – Bowling Green, KY-USA. The project was defended in presence of Dr. Raja, Dr. Chad Snyder and Dr. Kevin Williams on Wednesday, July 9th, 2014. This report presents the preliminary unpublished data of one of the projects currently being studied in Dr. Rajalingam Dakshinamurthy’s lab. I joined Dr. Raja’s lab in the Fall of 2012 with an ambition to pursue research in the field of protein and nanotechnology. Since then, I have worked on multiple projects with a primary focus on this project. I feel extremely proud to start the project when it was in its budding stage. Initial training from lab colleagues (Dillon Pender and Rammohan Paripelly) helped me to get a hands-on expertise on various methods and instrumental techniques used for the study. The data presented in this report is the fruitful result of our long days spent in lab working on the synthesis, characterization and antibacterial evaluation of our product. It was a lot of fun initially using the humongous looking electron microscope for the analysis. The most tiresome part of the entire project was the antibacterial assays where I had to devote almost 12 hours a day, periodically checking the absorbance of sample. Moreover, it was very frustrating when the results weren’t as expected. Each failed attempt aided me to improve my skills and techniques which eventually ended up in good data. I would like to sincerely thank my advisor Dr. Raja for his immense support and encouragement by giving me all the possible opportunities in research and academics. During the course of research, I got a wonderful chance to present my research in various prestigious scientific conferences and meetings for absolutely no cost. I would also like to thank all my previous and current lab colleagues who made me feel happy during my stay in lab.
# TABLE OF CONTENTS

1. INTRODUCTION ........................................................................................................... 1

2. MATERIALS AND METHODS .................................................................................. 14
   2.1. MATERIALS ........................................................................................................ 14
   2.2. PREPARATION OF REAGENTS ........................................................................... 18
      2.2.1. Wash protocol .............................................................................................. 18
      2.2.2. Preparation of sterilized nanopure water ..................................................... 18
      2.2.3. Preparation of ceftazidime stock solution ................................................... 18
      2.2.4. Preparation of potassium gold (III) chloride (KAuCl₄) stock solution ...... 19
      2.2.5. Preparation of L.B media .............................................................................. 19
      2.2.6. Preparation of tryptic Soy (T.S) media ....................................................... 20
      2.2.7. Preparation of L.B/T.S agar plates ............................................................. 20
      2.2.8. Preparation of 10X phosphate buffer saline (PBS) ....................................... 21
      2.2.9. Preparation of XTT solution ........................................................................ 21
      2.2.10. Preparation of menadione solution .......................................................... 22
      2.2.11. Preparation of TEM copper grids .............................................................. 22
      2.2.12. Preparation of Cef-GNPs stock solution ................................................... 23
   2.3. EXPERIMENTAL METHODS ................................................................................. 24
      2.3.1. Synthesis of ceftazidime gold nanoparticles (Cef-GNPs) ......................... 24
      2.3.2. Determining the yield of ceftazidime gold nanoparticles (Cef-GNPs) ...... 25
      2.3.3. Characterization of ceftazidime gold nanoparticles .................................... 26
      2.3.4. Assessing the stability of Cef-GNPs ........................................................... 29
      2.3.5. Evaluating antibacterial activity of Cef-GNPs ........................................... 29
      2.3.6. Visualizing mechanism of bactericidal action of Cef-GNPs ....................... 36
3. RESULTS AND DISCUSSION ................................................................. 38
   3.1. Synthesis of ceftazidime gold nanoparticles (Cef-GNPs) .................. 38
   3.2. Characterization of ceftazidime gold nanoparticles (Cef-GNPs) ........... 43
   3.3. Evaluation of *in-vitro* antibacterial activity .................................... 53
   3.3.1. Cef-GNPs against Gram-negative bacteria ................................. 53
   3.3.2. Cef-GNPs against Gram-positive bacteria ..................................... 57
   3.4. Visualizing bacterial permeability of Cef-GNPs .................................. 61
4. CONCLUSION .................................................................................... 63
5. REFERENCES ..................................................................................... 64
6. APPENDIX ........................................................................................... 69
   6.1. Appendix A: Product specification of ceftazidime hydrate ..................... 69
   6.2. Appendix B: Step-wise process involved in TGA analysis of Cef-GNPs ...... 70
   6.3. Appendix C: Petri plates images for calculating bacterial CFU/mL ........... 71
   6.4. Appendix D: Microtiter plate design for bacterial growth and XTT assay..... 73
   6.5. Appendix E: Additional TEM image of Cef-GNPs ................................. 74
   6.6. Appendix F: TGA of Cef-GNPs under air flow ..................................... 75
   6.7. Appendix G: Interaction of nutrient media with Cef-GNPs ..................... 76
7. ABBREVIATIONS .................................................................................. 77
LIST OF FIGURES

Figure 1: Representative images showing advantages of using gold nanoparticles........ 9
Figure 2: Schematic showing the steps involved in synthesis of gold nanoparticles...... 13
Figure 3: Schematic illustrating the process involved in the calculation of bacterial CFU/mL .................................................................................................................................................. 33
Figure 4: Image showing color of test tubes for various concentrations of ceftazidime and gold after 12 hours of incubation................................................................................................................... 41
Figure 5: Representative images showing morphological characterization of Cef-GNPs........................................................... 45
Figure 6: An overlay IR spectrum of Cef-GNPs and pure ceftazidime............................ 47
Figure 7: Represents qualitative and quantitative analysis of organic ligand (ceftazidime) on GNP surface............................................................................................................................... 50
Figure 8: Illustrates assessment of stability of Cef-GNPs.............................................. 52
Figure 9: Illustrates dose dependent inhibition by Cef-GNPs against Gram-negative, *P. aeruginosa*.......................................................................................................................... 55
Figure 10: Illustrates dose dependent inhibition by Cef-GNPs against Gram-negative, *E. aerogenes*.......................................................................................................................... 56
Figure 11: Illustrates dose dependent inhibition by Cef-GNPs against Gram-positive bacteria.......................................................................................................................... 58
Figure 12: Illustrates a plot of MIC comparison for pure ceftazidime and bound ceftazidime (Cef-GNPs) against multiple strains of Gram-positive and Gram-negative bacteria.............................................................................................................................. 60
Figure 13: Illustrates fluorescence images of Cef-GNPs induced cell membrane permeability using propidium iodide (PI) dye which has strong binding affinity towards nucleic acids....................................................................................................................... 62
LIST OF TABLES

Table 1: List of all the reagents or chemicals used for the study with their respective catalogue number and manufacturing company ................................................................. 14

Table 2: List of all the supplies required for the study with their respective catalogue # and manufacturing company .......................................................... 15

Table 3: A detailed list of most of the instruments used for the study along with their specific use .......................................................................................................................... 16

Table 4: List of all bacterial strains with their ATCC number and characteristic features used for the antibacterial assay .......................................................... 17

Table 5: Incubation conditions for the preparation of preculture of various bacterial strains under study. .......................................................... 31

Table 6: A list showing the color and UV observation of all the different combinations of ceftazidime and gold tried for the synthesis of Cef-GNPs ................................................. 42
On the verge of entering the post-antibiotic era, numerous efforts are in place to regain the waning charm of antibiotics which are proving ineffective against most “Superbugs”. 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 antibiotic-capped GNPs (25 ± 5 nm) utilizing the combined reducing and capping ability of a cephalosporin antibiotic, ceftazidime. No signs of aggregation or leaching of ceftazidime from GNP surface was observed upon its storage. Antibacterial testing showed dose-dependent broad spectrum activity of Cef-GNPs against both Gram-positive (S. bovis and E. durans) and Gram-negative (P. aeruginosa and E. aerogenes) bacteria. A significant reduction in the minimum inhibition concentration (MIC) of Cef-GNPs was observed as compared to the ceftazidime by itself against Gram-negative bacteria. The MIC of Cef-GNPs were 0.1 mg mL$^{-1}$ (P. aeruginosa and E. aerogenes) and 1.2 mg mL$^{-1}$ (E. durans and S. bovis). Cef-GNPs exerted bactericidal action on both P. aeruginosa and E. durans by disrupting the cellular membrane resulting in leakage of cytoplasmic content and death of bacterial cell. Our investigation and results provides an additional step in the development of antibiotic capped GNP as potent next generation antibacterial agents.
1. INTRODUCTION

Bacteria, the most primitive organisms, on earth have remained one of the greatest fears of human beings. Until 1940’s, infections caused by pathogenic bacteria were regarded as the major cause of deaths amongst humans.¹ These tiny little organisms, ubiquitous in nature, which can be seen only under super powerful microscopes, have continued to be a topic of intensive scientific research all over the world. It is estimated that over billions of distinct types of bacteria are always present on, in, and around us at any moment of time. Among those, only a handful of bacteria are beneficial to mankind in terms of health and economy since they are employed in a variety of industrial processes such as fermentation to make a variety of food products (alcohol, dairy products etc.), genetic engineering for a large scale production of recombinant drugs and vaccines and so on.²⁻⁴ Unlike mammals, the exponential growth of bacteria is in number rather than size with relatively a short generation period, thereby turning them into micro-factories engaged in large-scale manufacturing of desired products.⁵ In addition, a huge number of pathogenic bacteria are also known for causing mild to lethal infectious diseases, necessitating medical intervention.⁶ Plants, animals and humans serve as the prime targets of bacteria. Viruses are the only organisms that have the potential to infect a bacterial cell. Bacteria are often classified by their shape, atmosphere and their structural make up.⁷ One of the most striking features of bacteria is their structural make-up of outer cell wall which is regarded as the main armor of bacteria. Bacteria, whose cell/plasma membrane is covered by only a thick layer of peptidoglycan are called Gram-positive and the ones with an additional lipoprotein layer surrounding the thin peptidoglycan layer are known as Gram-negative bacteria.⁸
layer in Gram-negative bacteria confers them more strength and resistance towards hydrophobic drugs and biomolecules.

Human beings can become more prone to bacterial infections which are aggravated by poor sanitary conditions and health habits. Infections are also observed during surgical operations, organ transplantations and conditions of war milieu where the open wounds serve as an entry-point for the bacteria into the body.\(^9\) To safeguard from the ill effects of pathogenic bacteria, nature has provided the human body with a protection mechanism called the immune system to kill all the foreign agents which are not beneficial for our body. This system works as the first-line defense against all the infections caused by pathogenic agents including bacteria. The immune system produces different cells, with a primary function to recognize and kill foreign pathogens entering the body. For most of the infections, the human body has natural immunity while for other infections, the body acquires immunity after repeated exposure to the specific antigens.\(^10\) Despite lacking a brain, bacteria seemed very clever as they continuously alter their genetic make-up in order to become unrecognized and resistant to attack by immune system and continue to spread its ill effects. This was one of the main reasons for a humongous number of deaths among people during World War I and II due to the unavailability of appropriate medication to treat bacterial infections. This period was regarded as the pre-antibiotic era, one of the most dreadful eras in the history of medicine.\(^11\) In 1928, a miracle happened when Sir Alexander Fleming, a biologist, accidentally discovered penicillin from the mold *Penicillium notatum* which inhibited the growth of the *Staphylococcus* colonies.\(^12\) Soon after the discovery, penicillin became the world’s first antibiotic and thus was the dawn of the antibiotic era. The structure and
function of penicillin were elucidated, resulting in discovery of other potent structurally related classes of antibiotics which were used against a range of serious bacterial infections. In a nutshell, all the classes of antibiotics either kill or inhibit bacteria by four major mechanisms i.e. disrupting the cell wall, blocking the protein synthesis, inhibiting the DNA replication, or by affecting important bacterial cellular processes. With the advent of antibiotics, the mortality rate due to bacterial infections dropped drastically and the infections were very much under control.

Despite the fact medical practitioners were well aware of the capability of bacteria to develop resistance, improper care and irrational use of antibiotics was practiced worldwide which gradually enabled bacteria to develop defense mechanisms to render the potent antibiotics ineffective. The bacteria which became resistant to the antibiotics were called as resistant bacteria. This was a clear suggestion that the process to downfall antibiotics was initiated in bacteria. From a single antibiotic, bacteria started developing resistance towards different classes of antibiotics, making them more powerful and difficult to treat. Such bacteria were grouped under a new class called multi-drug resistant bacteria (MDR). Some of the common examples include Methicillin-resistant Staphylococcus aureus (MRSA) USA 300, Escherichia coli ST131, Klebisella ST258, Carbapenem-resistant Enterobacteriaceae etc. Resistance is not localized; instead it is spreading worldwide as a result of gene transmission, poor sanitary conditions in hospitals and communities in which people live, global travel, bioterrorism etc. This is evident from multiple cases such as the spread of antibiotic-resistant gonorrhea to the Philippines and USA from Vietnam where it was first emerged in 1967 and New Delhi
metallo-beta-lactamase 1 (NDM-1), first discovered in 2008 in New Delhi is now a global problem.\textsuperscript{17}

Similar to conditions during the pre-antibiotic era, bacterial infections are now regarded as one of the major health concerns all over the world.\textsuperscript{18–21} This concern relates to the soaring increase in cases of bacterial resistance towards front-line antibiotics. Bacteria which were once susceptible to an antibiotic, have now developed some kind of defense mechanism making them invulnerable to the effects of antibiotic. Development of resistance in bacteria is inevitable and occurs due to genetic manipulation. Among the normal population of bacteria, some inherit resistant genes which make them survive the attack from antibiotics. As a result, the resistant bacteria that survived multiply rapidly and produce a large number of bacterial populations containing the antibiotic resistant gene. Treating infections with sub-therapeutic concentrations of antibiotics serves as the driving force for developing resistance in bacteria. The root of rapid development of resistance in bacteria can be attributed to human activities which includes the irrational and overuse of antibiotics in hospitals, farms, and communities.\textsuperscript{22} In under-developed and developing countries, due to the exorbitant cost of consulting a doctor, self-medication has been practiced for many decades. It has become a common practice to prescribe antibiotics without any proper diagnosis when a person falls sick. Common people relate all infections to bacteria ignoring the fact that the infection can also be caused by other pathogenic microorganisms like viruses or fungi. Prescribing antibiotics for flu and other infections which are caused by virus is still practiced in many parts of the world.\textsuperscript{23} Antibiotics are also used abundantly in hospitals. Nearly 7.7 million pounds of antibiotics are prescribed every year in United States.\textsuperscript{24} Use of antibiotics for preventing infections
and improving the growth of farm animals is another growing concern which causes and spreads the resistant bacterial strains among local community population.\textsuperscript{25,26} Until recently, this problem was never taken seriously which made the antibiotic resistant bacteria to become multi-drug resistant (MDR) bacteria thereby making it very difficult to cure infections caused by such resistant bacteria.\textsuperscript{27} The first antibiotic to which bacteria had gained resistance was penicillin in 1943. Over the years, various antibiotics such as tetracycline, erythromycin, methicillin, vancomycin, and more. were identified to be no longer effective against the bacteria against which it was potent before.\textsuperscript{28} Among the various resistant bacteria, \textit{Enterococcus faecium}, \textit{Staphylococcus aureus}, \textit{Klebsiella pneumoniae}, \textit{Acinetobacter baumannii}, \textit{Pseudomonas aeruginosa} and \textit{Enterobacter species} (known as “ESKAPE” organisms) are regarded as the most harmful of all.\textsuperscript{29} Today, MDR has become one of the most talked and researched topic in scientific world with magazines and newspapers warning about a future without any effective antibiotic to treat bacterial infections.\textsuperscript{26} Some authors have described antibiotic resistance as being ‘as big a risk as terrorism’ whereas some author describes it as the ‘return of our old enemies in an untreatable form’.\textsuperscript{30,31} This pandemic condition must be addressed immediately to avoid the MDR bacteria becoming pan-drug resistant (PDR), making our arsenal of antibiotics ineffective. It is very essential to take an action today in order to have cure tomorrow. Some of the major modern achievements of medicine, such as organ transplants, are at stake because they cause a high risk of post-operative infections which would be untreatable and fatal in many cases. In a recent 114 page report from CDC, it was estimated that more than 2,049,442 Americans fall sick due to antibiotic-resistant bacterial infections, killing 23,000 people annually. The report also categorized harmful
pathogens into different threat levels ranging from URGENT THREAT (C. difficile, Carbapenem-resistant Enterobacteriaceae, Drug-resistant Neisseria gonorrhea), SERIOUS THREAT (Multidrug-resistant Acinetobacter, Drug-resistant Campylobacter, Fluconazole-resistant Candida (a fungus), Extended spectrum β-lactamase producing Enterobacteriaceae (ESBLs), Vancomycin-resistant Enterococcus (VRE), Multidrug-resistant Pseudomonas aeruginosa, Drug-resistant Non-typhoidal Salmonella, Drug-resistant Salmonella Typhi, Drug-resistant Shigella, Methicillin-resistant Staphylococcus aureus (MRSA), Drug-resistant Streptococcus pneumoniae, Drug-resistant tuberculosis) and CONCERNING THREAT (Vancomycin-resistant Staphylococcus aureus (VRSA), Erythromycin-resistant Group A Streptococcus, Clindamycin-resistant Group B Streptococcus). This report calls for an urgent effort to find novel approaches for novel antibiotic research and mobilizing the development pipeline to prevent the world from entering post-antibiotic era. Organizations like the WHO and the CDC have released various reports and infographics to educate people about the need for antibiotic stewardship for preventing the current situation getting worse. Well aware of the time and capital involved in developing new drug moieties, the United States Food and Drug Administration (US-FDA) introduced Generating Antibiotics Incentives Now (GAIN) under food and safety act in 2012 with a goal to boost the novel antibacterial research in pharmaceutical and research organizations. Under this program, the FDA assigns Qualified Infectious Disease Product (QIDP) status to novel antibacterial and antifungal drugs which are found effective against qualifying pathogens (ESKAPE organisms, Clostridium difficile etc.). QIDP drugs benefits from a quick review process and upon approval get an additional 5 years of market exclusivity rights. So far, 46 investigational
drugs have been assigned QIDP status by FDA.\textsuperscript{35} Recently, two of the QIDP status drugs, namely Dalvance (Durata Therapeutics)\textsuperscript{36} and Sivextro (Cubist Pharmaceuticals)\textsuperscript{37} were approved by the FDA for the treatment of infections caused by MRSA. New Drugs for Bad Bugs (ND4BB), a part of Europe’s Innovative Medicines Initiative program introduced their third project called ENABLE (European Gram-Negative Antibacterial Engine) which fosters 13 countries and 32 partners to develop new antibacterial agents against Gram-negative resistant bacteria.\textsuperscript{38,39} There is a great need in developing next-generation antibacterial agents active against Gram-negative bacterial infections. In addition, funding organizations all over the world are financially supporting various research facilities who are involved in developing alternative strategies against antibiotic resistant bacteria. Recently, the National Institute of Health (NIH) awarded $2 million to Duke University, USA to start a new clinical research network which focuses on antibacterial resistance.\textsuperscript{40} Similarly, the U.S. military under the Defense Threat Reduction Agency (DTRA) is funding a whopping $13.5 million on a research for developing new systemic antibiotics to fight pathogens showing resistance to current antibiotics. The research is being done jointly by Anacor Pharmaceuticals, Colorado State University (CSU) and the University of California at Berkeley.\textsuperscript{41}

In an alternate approach, ultra-fine particles in the size range of 1-100 nm called nanoparticles are being widely exploited for their role as antibacterial agent.\textsuperscript{42} Nanotechnology has brought a revolution in science and technology. It has become one of the most intense fields of scientific research, and has grown enormously over the last ten years as evident from the soaring increase in the number of research articles published on nanoparticles in various journals.\textsuperscript{43} Nanotechnology finds its applications in various
fields such as medicine, electronics, biomaterials and energy storage production. The variety of applications of nanoparticles are attributed to its unique physic-optical properties which depend upon the size, shape, and environment in which they are present. Nanoparticles can be easily manipulated into various sizes and shapes by changing the concentration of reactants and experimental conditions. Nanoparticles have a long history. They were used by various artisans for making bright colored glass cups such as Lycurgus cup in Rome and more. Modern interest towards nanotechnology started after a famous talk entitled “There’s a plenty room at the bottom” by physicist Richard Feynman in 1959. Through the years, technology gave us different techniques and methods to characterize and understand the nanoparticles in a much better way. In general, the nanoparticles are classified into five distinct groups such as semiconductor quantum dots, magnetic nanoparticles, polymeric particles, carbon based nanostructures and metallic nanoparticles. Each class of nanoparticles has its unique features and applications. For instance the semiconductor quantum dots are used for biological labeling due to their size dependent fluorescence properties. Polymeric nanoparticles such as dendrimers and liposomes acts as good carriers to load different hydrophobic and hydrophilic drugs which can then be used for targeted drug delivery. Carbon based nanoparticles are mostly used in batteries and as hydrogen storage. Most flexible among all are the metal nanoparticles because of their synthetic control over size, shape, composition, structure and assembly resulting in fine tunability of surface optical properties. This project emphasizes metal nanoparticles. Gold nanoparticles (GNPs) due to their non-toxic and biocompatibility nature have been widely researched for their use
in biomedical applications involving targeted drug delivery, biological imaging, and diagnostics\textsuperscript{44,51–53} (Figure 1).

**Figure 1:** Representative images showing advantages of using gold nanoparticles. (a) Gold nanoparticles exhibit different colors owing to its unique optical properties known as surface plasmon resonance. (b) Gold nanoparticles can be easily tuned to a variety of sizes and shapes as required. (c) Gold nanoparticles can be decorated with various functional groups as required for the specific application. (d) Gold nanoparticles are known to be highly stable in nature. (e) Biocompatibility of gold nanoparticles is evident from many marketed drug formulations involving gold for the medical treatment such as rheumatoid arthritis. (f) Gold nanotechnology continues to attract researchers worldwide as evident from more than 2000 patents registered in FY 2012.
One of the emerging applications of GNPs is their use as nano-carriers for improved antibacterial activity. There are many papers in the literature which show enhanced antibacterial effect of either naked GNPs or antibiotic conjugated GNP’s.\textsuperscript{42,43} The methods used for the synthesis of GNPs in above studies involves the use of toxic chemicals which can reduce and stabilize the ionic gold Au\textsuperscript{3+} to neutral gold atoms \( \text{Au}^{0} \) which aggregates resulting in GNPs (Figure 2(a)). Citrate, sodium borohydride, hydroquinone and hydroxyl radicals are the common reducing agents used whereas thiolates, amines, phosphanes, carbonyls, dendrimers and surfactants are used as stabilizing agents. Lately, the use of all these chemicals have raised both biological and environmental toxicity concerns, demanding a need for finding a cleaner and more green method of synthesis for GNPs.\textsuperscript{54} In response to this, we have successfully developed and patented (U.S. Patent No. 8257670) a unique method for the biofriendly synthesis of GNPs wherein dextrose was used as both reducing and capping agent in a buffered aqueous solution at moderate temperature to synthesize monodisperse, stable, water-soluble GNPs with significant yield.\textsuperscript{55} Due to structural resemblance of dextrose with most of the commercial antibiotics, we hypothesized and successfully synthesized GNPs using ampicillin as a reducing and capping agent to yield ampicillin capped GNPs (Amp-GNPs) by a green process. Antibacterial testing revealed greater activity of Amp-GNPs when compared to equivalent concentrations of free ampicillin. In order to make sure ampicillin is not an isolated case, various other antibiotics are currently being studied for enhanced antibacterial activity upon conjugating with GNPs. In the present study, ceftazidime was used as the investigational antibiotic. Ceftazidime, a third generation cephalosporin is widely used as a broad spectrum antibiotic for treating bacterial
infections. It inhibits a step involved in the synthesis of the bacterial cell wall by binding to penicillin binding proteins (PBP) which inhibits crosslinking within the cell wall. The activity is due to the presence of a β-lactam ring. Ceftazidime is used intravenously or intramuscularly to treat lower respiratory, urinary tract, joint, skin and blood-stream infections. It is prescribed as a first-line drug for the treatment of meliodosis. Ceftazidime is mostly used against infections caused by Gram-negative bacteria. Ceftazidime is also listed in the WHO List of Essential Medicines. Currently, ceftazidime is marketed with a brand name of Fortaz and Tazicef. Ceftazidime still remains as the investigational drug for research against Gram-negative infections. Recently, a ceftazidime/avibactam combination which is jointly researched by AstraZeneca and Forest Laboratories Inc. against Complicated Intra-Abdominal Infections (cIAI) and Complicated Urinary Tract Infections (cUTI) received QIDP status from US-FDA. The drug combination is currently under phase III clinical trials. Keeping all the above facts in mind, ceftazidime was selected to increase its efficacy against Gram-negative bacterial infections. In terms of synthesis, the presence of hydroxyl and carbonyl groups which can reduce ionic gold were hypothesized to lead to GNP synthesis. People have written in the literature about the involvement of amine groups in stabilizing gold nanoparticles. Since the ceftazidime structure contained hydroxyl, carbonyl, and amine groups, we hypothesized the ability of ceftazidime to act as dual agent (reducing and capping) for making antibiotic capped GNPs. The overall objective of the study was to design and develop a unique therapeutic formulation involving ceftazidime capped GNPs for enhanced antibacterial activity against normal and resistant bacterial strains.
The design of the study involved finding optimum concentration to synthesize ceftazidime gold nanoparticles (Cef-GNPs) using a single step method followed by its characterization using various analytical techniques to obtain morphological and elemental properties of Cef-GNPs. The schematic involved in the synthesis of Cef-GNPs is shown in Figure 2(b). \textit{In-vitro} antibacterial efficiency of synthesized Cef-GNPs was tested against Gram-positive and Gram-negative bacterial strains with a more emphasis on Gram-negative bacteria. Antibacterial assays used for the study include bacterial growth assay, spread plate assay, XTT assay, and propidium iodide assay. Efficiency of Cef-GNPs was determined by comparing its MIC, obtained from the above assays with the MIC of pure ceftazidime.
Figure 2: Schematic showing the steps involved in synthesis of gold nanoparticles. (a) Represents a conventional method of synthesis with the use of a reducing agent which reduces ionic gold to neutral gold atoms followed by addition of capping agent which stabilizes the gold aggregates to form nanoparticles. (b) Illustrates our single-step biofriendly process for making gold nanoparticles using the dual reducing and capping nature of the antibiotic, ceftazidime. (c) Represents the various stages involved in the synthesis of gold nanoparticles, starting from nucleation which grows until stabilized by a capping agent.
2. MATERIALS AND METHODS

2.1. MATERIALS:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime Hydrate</td>
<td>C3809</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Glycerol</td>
<td>GX0190-6</td>
<td>EMD Chemicals</td>
</tr>
<tr>
<td>L.B Agar Media</td>
<td>240110</td>
<td>BD Company</td>
</tr>
<tr>
<td>L.B Media</td>
<td>71753-6</td>
<td>Novagen</td>
</tr>
<tr>
<td>Menandione</td>
<td>ME105</td>
<td>Spectrum Chemicals</td>
</tr>
<tr>
<td>Petri Dishes</td>
<td>875713</td>
<td>Fisher Brand</td>
</tr>
<tr>
<td>Potassium aurochlorate</td>
<td>450235</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>537059</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>T.S Agar Media</td>
<td>236950</td>
<td>BD Company</td>
</tr>
<tr>
<td>T.S Media</td>
<td>T0420</td>
<td>Teknova</td>
</tr>
<tr>
<td>XTT Salt</td>
<td>10060</td>
<td>Biotium</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>3624-05</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>Sodium dihydrogen</td>
<td>SX0711-1</td>
<td>EMD Chemicals</td>
</tr>
<tr>
<td>phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disodium hydrogen</td>
<td>1.06573.0503</td>
<td>EMD Chemicals</td>
</tr>
<tr>
<td>phosphate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: List of all the reagents or chemicals used for the study with their respective catalogue number and manufacturing company
<table>
<thead>
<tr>
<th>Supply</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well Microtiter Plates</td>
<td>25381-056</td>
<td>Costar</td>
</tr>
<tr>
<td>Ependorff’s – 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>
</tr>
<tr>
<td>Micropipette tips – 1-1000 µL</td>
<td>83007-382</td>
<td>VWR</td>
</tr>
<tr>
<td>Micropipette tips – 1-20 µL</td>
<td>53509-070</td>
<td>VWR</td>
</tr>
<tr>
<td>Micropipette tips – 1-200 µL</td>
<td>53503-606</td>
<td>VWR</td>
</tr>
<tr>
<td>Parafilm</td>
<td>PM-996</td>
<td>Bemis Flexible Packaging</td>
</tr>
<tr>
<td>Spreaders</td>
<td>420201-786</td>
<td>VWR</td>
</tr>
<tr>
<td>TEM grids</td>
<td>0400-CU</td>
<td>Electron Microscopy Science</td>
</tr>
<tr>
<td>TEM Negative Films</td>
<td>74100</td>
<td>Electron Microscopy Science</td>
</tr>
</tbody>
</table>

**Table 2**: List of all the supplies required for the study with their respective catalogue # and manufacturing company
<table>
<thead>
<tr>
<th>Instrument</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>-80 °C Freezer</td>
<td>Store bacterial stock culture</td>
</tr>
<tr>
<td>Autoclave Machine</td>
<td>Sterilize solutions and supplies</td>
</tr>
<tr>
<td>Bio Microtiter Plate Reader</td>
<td>Bacterial growth assay</td>
</tr>
<tr>
<td>Biological Safety Cabinet</td>
<td>Working with bacteria (aseptic conditions)</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Concentrating gold nanoparticles</td>
</tr>
<tr>
<td>DLS</td>
<td>Particle size distribution</td>
</tr>
<tr>
<td>Excella 625 Shaking Incubator</td>
<td>Bacterial and nanoparticle incubation</td>
</tr>
<tr>
<td>Hitachi UV-Vis Spectrometer</td>
<td>Optical absorption of nanoparticle solution</td>
</tr>
<tr>
<td>JEOL SEM</td>
<td>elemental composition of nanoparticles</td>
</tr>
<tr>
<td>JEOL TEM</td>
<td>Morphological characteristics of nanoparticles</td>
</tr>
<tr>
<td>Lyophlizer</td>
<td>Freeze drying of gold nanoparticles</td>
</tr>
<tr>
<td>Nanopure water machine</td>
<td>Nano pure water (free from impurities)</td>
</tr>
<tr>
<td>Perkin Elmer FTIR</td>
<td>Organic confirmation of nanoparticles</td>
</tr>
<tr>
<td>Petri Plate Scanner</td>
<td>Counting colonies on petri plates</td>
</tr>
<tr>
<td>Probe Sonicator</td>
<td>Breaking the clusters and aggregates of nanoparticles</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>Storing the solutions and reagents</td>
</tr>
<tr>
<td>TA Thermogravimetric Analysis</td>
<td>Organic % determination on gold nanoparticles</td>
</tr>
<tr>
<td>VWR Incubator</td>
<td>Growth of bacteria on agar plates</td>
</tr>
<tr>
<td>VWR Magnetic Stirrer</td>
<td>Mixing and dissolving solutes in solution</td>
</tr>
</tbody>
</table>

**Table 3:** A detailed list of most of the instruments used for the study along with their specific use.
<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterobacter aerogenes</strong></td>
<td>• Gram –ve bacterium belonging enterobacteriaceae family*</td>
</tr>
<tr>
<td><em>(ATCC # 13048)</em></td>
<td>• Bacilli (rod) shape bacteria with flagella</td>
</tr>
<tr>
<td></td>
<td>• Causes opportunistic infections</td>
</tr>
<tr>
<td></td>
<td>• Multi-resistant to antibiotics * requires quick and urgent action</td>
</tr>
<tr>
<td></td>
<td>according to CDC 2013 report</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>• Gram –ve bacterium belonging pseudomonadaceae family*</td>
</tr>
<tr>
<td><em>(ATCC # 27853)</em></td>
<td>• Coccobacillus shape</td>
</tr>
<tr>
<td></td>
<td>• Causes healthcare associated infections</td>
</tr>
<tr>
<td></td>
<td>• Some strains are found to be pan-drug resistant * possess serious</td>
</tr>
<tr>
<td></td>
<td>concern and requires steps to prevent the spread</td>
</tr>
<tr>
<td><strong>Enterococcus Durans</strong></td>
<td>• Gram +ve bacterium belonging enterococcaceae family*</td>
</tr>
<tr>
<td><em>(ATCC # 6056)</em></td>
<td>• Cocci shape</td>
</tr>
<tr>
<td></td>
<td>• Major cause of urinary tract, bloodstream and surgical infections</td>
</tr>
<tr>
<td></td>
<td>• Some strains show resistance to antibiotic of last resort, vancomycin</td>
</tr>
<tr>
<td></td>
<td>* possess serious concern and requires prompt action to prevent the</td>
</tr>
<tr>
<td></td>
<td>spread</td>
</tr>
<tr>
<td><strong>Streptococcus Bovis</strong></td>
<td>• Gram +ve bacterium belonging streptococcaceae family*</td>
</tr>
<tr>
<td><em>(ATCC # 9809)</em></td>
<td>• Cocci shape, lactic acid bacterium</td>
</tr>
<tr>
<td></td>
<td>• Strains of Group A and B causes pharyngitis, necrotizing fasciitis,</td>
</tr>
<tr>
<td></td>
<td>rheumatic fever, meningitis, sepsis etc.</td>
</tr>
<tr>
<td></td>
<td>• Group A and B are proven resistant to clindamycin * Streptococcus A</td>
</tr>
<tr>
<td></td>
<td>and B group strains resistance against macrolides is concerning and</td>
</tr>
<tr>
<td></td>
<td>needs careful monitoring</td>
</tr>
</tbody>
</table>

**Table 4:** List of all bacterial strains with their ATCC number and characteristic features used for the antibacterial assay
2.2. PREPARATION OF REAGENTS:

2.2.1. Wash protocol:

All the apparatus and containers were thoroughly rinsed and washed with soap water, tap water and nanopure water and are allowed to air dry. The containers were then sterilized in an autoclave machine at 121 °C for ~1 hour and stored until further use.

2.2.2. Preparation of sterilized nanopure water:

Following the wash protocol, roughly ~500 mL of nanopure water from the nanopure machine was transferred into 1000 mL sterilized reagent bottle. The caps were screwed loosely and the solution was autoclaved using the liquid cycle. Once the process was finished, the caps were screwed tightly and stored in 2-8 °C until further use.

(Note: For all the liquid cycles, the volume of the solution must be half of the container capacity)

2.2.3. Preparation of ceftazidime stock solution: (5 mM, 500 mL)

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

\[
\text{Molecular weight of ceftazidime hydrate} = 546.58 \frac{g}{\text{mole}}
\]

Following the wash protocol, 1.365 g of ceftazidime hydrate (see Appendix A for product specification) was weighed using microbalance and transferred into a 500 mL reagent bottle. Around 250 mL of sterilized nanopure water was added to the flask and the contents were thoroughly mixed using a magnetic stirrer until a homogenous solution was obtained. The resulting solution was transferred into a 500 mL measuring cylinder.
and water was added to make up to the desired volume (500 mL). The solution was transferred back into the reagent bottle and caps were screwed tightly. The bottle was labelled appropriately and stored at 2-8 °C until further use.

2.2.4. Preparation of potassium gold (III) chloride (KAuCl₄) stock solution: (132 mM, 50 mL)

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

\[
\text{Molecular weight of gold salt} = 377.88 \frac{g}{\text{mole}}
\]

Following the wash protocol, 2.5 g of potassium gold (III) chloride was weighed using the microbalance and transferred into a 100 mL reagent bottle. The solute was dissolved by adding 50 mL of sterilized nanopure water to obtain a homogenous solution. The bottle was labelled appropriately and stored at 2-8 °C until further use.

2.2.5. Preparation of L.B media: (1 Liter, 25g/L)

Following the wash protocol, 25 g of L.B media was accurately weighed using the weighing balance and transferred into a 2000 mL reagent bottle. ~500 mL of sterilized nanopure water was then added and the contents were mixed thoroughly using a magnetic stirrer until all the contents are properly dissolved. The contents were then transferred into a 1000 mL measuring cylinder and made up to the desired volume (1000 mL) using sterilized nanopure water. The solution was retransferred into 2000 mL reagent bottle and with the loosely screwed caps. The solution was sterilized and stored at 2-8 °C in refrigerator until further use.
2.2.6. **Preparation of tryptic Soy (T.S) media: (1 Liter, 30g/L)**

Following the wash protocol, 30 g of T.S media was accurately weighed using the weighing balance and transferred into a 2000 mL reagent bottle. ~500 mL of sterilized nanopure water was then added and the contents were mixed thoroughly using a magnetic stirrer until all the contents are properly dissolved. The contents were then transferred into a 1000 mL measuring cylinder and made up to the desired volume (1000 mL) using sterilized nanopure water. The solution was retransferred into 2000 mL reagent bottle and with the loosely screwed caps. The solution was sterilized and stored at 2-8 °C in refrigerator until further use.

2.2.7. **Preparation of L.B/T.S agar plates: (40 plates)**

(Note: 25 mL of solution is required for each petri plate. Make sure to prepare extra in case of any spills of improper pouring)

Following the wash protocol, 40g of L.B agar/ T.S agar was weighed and transferred into a 2000 mL Erlenmayer flask. ~500 mL of sterilized nanopure water was added and the contents were mixed thoroughly using magnetic stirrer until all the solute was dissolved. The solution was transferred into a 1000 mL measuring cylinder and made up the volume to the desired level (1000 mL) using sterilized nanopure water. The flask was covered using two fold aluminum foils and sterilized in an autoclave using liquid cycle. In the meantime, the disposable petri plates were UV-sterilized under a biological safety cabinet. After the autoclaving was done*, 25 mL of the solution was poured using a 50 mL falcon tube into each petri plate without forming bubbles. The plates were allowed to solidify by keeping them half open in the hood. Once the plates were solidified, the lid was covered and the petri plates were wrapped using parafilm all
around the rim to avoid contamination. The plates were labelled appropriately and stored at 2-8 °C in refrigerator until further use.

* Do not allow the autoclaved solution sit for a long time. The solidifying temperature for the agar solution is ~36 °C

2.2.8. **Preparation of 10X phosphate buffer saline (PBS) (1 liter):**

Following the wash protocol, 6.084 g of sodium dihydrogen phosphate, 57.28 g of disodium hydrogen phosphate and 87.66 g of sodium chloride was accurately weighed and transferred into a 2000 mL reagent bottle. ~800 mL of sterilized nanopure water was then added and the contents were mixed until all the solutes were uniformly dissolved. The volume was made up to 1000 mL using sterilized nanopure water and the pH was adjusted to ~7.2 using a pH meter. The caps were loosely screwed and the solution was sterilized using liquid cycle. After the autoclaving was done, the caps were tightly screwed and labelled appropriately. The solution was stored at room temperature until further use.

2.2.9. **Preparation of XTT solution: (1mg/mL, 30 mL)**

Following the wash protocol 30 mg of XTT dye was weighed and transferred into 100 mL conical flask. 30 mL of 7.2 pH phosphate buffer saline (PBS) was added and contents were mixed vigorously. The flask was covered with two folds aluminum foils and sterilized using liquid cycle. After the autoclaving was done, 1mL of the solution was aliquot into each 1.5 mL ependorff’s under aseptic conditions. The ependorff’s were labelled appropriately and stored in -4 °C refrigerator until further use.
2.2.10. Preparation of menadione solution: (10mM, 50 mL)

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

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

Following the wash protocol, 86 mg of menadione was accurately weighed and transferred into a 100 mL reagent bottle. 50 ml of acetone was added and mixed thoroughly. The bottle was labelled appropriately and stored it in -4 °C until further use.

2.2.11. Preparation of TEM copper grids:

The 400 mesh size copper grids were washed and cleaned by dipping them into 1M HCL, followed by water with intermittent drying using a filter paper. Finally, the grids were dipped into a solution of acetone and dried by dabbing on a filter paper. The formvar coating solution was assembled which includes the glass burette attached to a stand. The burette was washed by running a wash solution through it. The formvar solution was poured into the burette with the knob in close position. A clean glass slide was kept into the formvar solution for roughly 30 seconds after which the solution was drained by turning the knob. The glass slide was then removed from the apparatus using tweezers and placed it in a closed jar for drying. The edges of the slide were scrapped using a razor in order to release the film coating from the slide smoothly. With the help of a jar filled with water up to the rim, the film coating was gently released from the slide by immersing it into the water slowly. The light sides of the washed grids were deposited onto the silver/gold interface of the floating film. With the help of parafilm, the grids were carefully removed and cut using tweezers and placed it in a petri dish containing filter paper. Once dried, the grids were used immediately for TEM analysis.
2.2.12. Preparation of Cef-GNPs stock solution: (1mg/mL, 10 mL)

For the antibacterial assays, a stock solution of Cef-GNPs was prepared by accurately weighing 10 mg of Cef-GNPs lyophilized powder and adding it into 15 mL sterilized falcon tube. 10 mL of sterilized nanopure water was added and the solution was mixed vigorously using a vortex. For monodisperse GNPs, the solution was sonicated using a probe sonicator at 45% amplitude for 10 minutes with pulse on for 10 sec and pulse off for 5 sec. The solution was labelled and stored at 2-8 °C until further use.
2.3. EXPERIMENTAL METHODS:

2.3.1. Synthesis of ceftazidime gold nanoparticles (Cef-GNPs):

Unlike the conventional method which uses various harmful chemical agents to reduce and stabilize GNPs, we designed and developed a self-patented synthesis method for making monodisperse GNPs whose size can be easily varied by changing the concentration of either nucleating or stabilizing agent. For the synthesis, a range of concentrations in different ratios were tried to determine the optimum concentration which yielded the GNPs of desired morphology. Some of the properties which were taken into consideration for determining the optimum concentration include yield, monodispersity, the concentration of reactants, as well as the size and shape of GNPs. The fabrication process was a unique, single step method which followed most of the 12 principles of green chemistry. Multiple concentrations involving ceftazidime (0.5, 1, 3 and 5 mM) and potassium aurochlorate (50, 100, 200 and 300 ppm) were screened by monitoring their color and UV-Vis spectrum. Among all the concentrations, 5 mM of ceftazidime with 200 ppm of gold salt resulted in nanoparticles with high yield and desired morphology. For a typical synthesis (120 mL) of ceftazidime gold nanoparticles (Cef-GNPs), 12 mL of 5 mM pre-prepared ceftazidime stock solution was mixed with 108 mL of sterilized nanopure water in a 250 mL Erlen Mayer flask. To the mixture, 480 µL of pre-prepared gold salt solution was added and mixed using a vortex. 30 mL of solution was aliquot in four 50 mL falcon tubes respectively. Test tubes were labelled appropriately and incubated at 37 °C for 12 hours with a shaking speed of 150 rpm. Shaking was very essential to prevent the aggregation of nanoparticles. After the incubation time, the contents of each falcon tube were transferred into a clean and
sterilized oakridge tubes (centrifuge tubes) and the solution was centrifuged at 15,000 rpm for 15 minutes using F21 rotor in Sorval RC 5B centrifuge. After the centrifugation was done, the supernatant was carefully discarded using a pipette and was replaced with equal volume of sterilized nanopure water. The tubes were centrifuged again as mentioned above. The process was repeated thrice to ensure no unreacted reagents (antibiotic or gold salt) was present in the resulting nanoparticle. After the last cycle, the solution was concentrated to a final volume of 12 mL using a microcentrifuge and stored in a 50 mL falcon tube at 2-8 °C until further use.

2.3.2. Determining the yield of ceftazidime gold nanoparticles (Cef-GNPs):

One of the major factor is any manufacture process is the yield obtained from the reaction. Yield of the product helps in evaluating the efficiency of synthesis process and also gives an insight about the extent of reactants that are getting used up in the reaction. The yields of synthesized Cef-GNPs were also evaluated using freeze drying process. For each batch of 120 mL, the concentrated solution obtained after repeated washing and centrifugation was added into aluminum cups, freezed under liquid nitrogen and kept for drying at 30 °C overnight under vacuum. The cups were covered with a thin cloth to avoid loss of product during the drying process. The lyophilized powder of Cef-GNPs was carefully extracted from the cups and weighed using a highly precise analytical balance. The process was repeated for multiple batches of synthesis to determine the yield and percentage variation.
2.3.3. **Characterization of ceftazidime gold nanoparticles:**

Before the synthesized Cef-GNPs were evaluated for antibacterial activity, it was necessary to confirm the formation of GNPs as well as the presence of antibiotic on its surface. For this purpose, a range of spectroscopic and microscopic techniques were used to characterize Cef-GNPs. 61,62

*UV-Vis Spectroscopy*

One of the inherent features of gold nanoparticles is its ability to show a plasmon resonance peak in the visible region due to its surface properties. GNPs show a characteristic UV-Vis peak which is dependent on the size, shape, and nature of environment in which the GNPs are present. Therefore, UV-Vis was used as a qualitative tool to evaluate the formation of GNPs. For the analysis, a dilute solution of synthesized Cef-GNPs was sonicated at 45 % amplitude for 5 minutes. A UV-Vis absorption spectrum of the resulting solution was recorded from 400-900 nm using Hitachi U-3900 with a scan speed of 600 nm/min and slit width of 5 nm.

*Dynamic Light Scattering (DLS)*

This is a simple technique to determine the particle size distribution of synthesized GNPs. The principle behind analysis is the scattering of light emitted from laser by the suspension of GNPs which are in constant motion called the Brownian motion. The scattered light from the sample falls onto the detector which is processed by the software to give the average size distribution and polydispersity of the sample. For the analysis, 1 mL of diluted sample of Cef-GNPs was probe sonicated and analyzed
using Zetasizer Nano S, Malvern Instruments Ltd. at 25 °C with a scattering angle of 90°. Sampling was done in triplicate with 13 runs in each measurement.

*Transmission Electron Microscope (TEM)*

In order to observe things which are really small, super powerful high resolution microscopes are required. TEM analysis of Cef-GNPs was performed to determine its morphological characteristics. A 10 µL of dilute sample of Cef-GNPs was loaded on 400-mesh formavar coated copper grids. Excess liquid was wiped off by dabbing the grid onto a filter paper. The grids were air-dried at room temperature and imaged on a JEOL-TEM. The sample was viewed at different magnifications on a phosphorous screen by shooting 120 kV of electron beam. Images of the desired region of the grid were taken using the in-built camera which was later processed, developed, and scanned for future reference. The scale marker (size of the grid X magnification = size on negative film) of 100 nm (obtained from the ruler) was placed on the TEM negative film.

*Scanning Electron Microscope- Energy Dispersive Spectroscopy (SEM-EDS)*

Elemental composition of Cef-GNPs was determined using low resolution SEM-EDS technique. Our primary interest was to check the presence of organic elements like carbon (C) which confirms the presence of ceftazidime bounded onto GNPs surface. For the analysis, 50 µL of Cef-GNPs solution was pipetted on a clean aluminum stub and air-dried at room temperature. The stubs were then imaged on a JEOL JSM-5400 LV microscope with IXRF system. The samples were thoroughly washed with sterilized nanopure water to get pure GNPs, free from any traces of impurities. Using the IXRF system, the SEM images were exported into the software and elemental composition was
determined at different spots on the images. The elements and their corresponding weight were recorded.

*Fourier Transform Infrared Spectroscopy (FTIR)*

One of the widely used techniques by organic chemists is FTIR which gives an insight about the nature of functional groups present in an unknown compound. FTIR was used as a qualitative technique to determine the presence of organic ceftazidime on GNPs, as well as to determine the changes in the IR peak of functional group which helps in understanding the bonding interactions between ceftazidime and GNP. For the analysis, first a background (air) spectrum was taken to avoid any unwanted signals. For the sample spectrum ~5 mg of lyophilized powder of Cef-GNPs was placed on the highly sensitive Perkin-Elmer Spectrum 100 FT-IR spectrophotometer fitted with a universal ATR accessory. Force was applied using the pressure gauge onto the sample to ensure better contact with the IR rays. IR spectrum was run from 4000-650 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) using the Spectrum One software. 128 scans were taken for each spectrum. The process was repeated for pure ceftazidime and the IR spectrums were overlapped for data interpretation.

*Thermogravimetric Analysis (TGA)*

After confirming the presence of organic content onto GNPs surface, the amount of ceftazidime present onto GNPs was qualitatively determined by studying thermal decomposition using TA Q5000 TGA technique. Roughly ~7 mg lyophilized sample of Cef-GNPs was placed on a platinum pan and heated from room temperature to 650 °C under nitrogen gas flow followed by air from 650 °C to 850 °C. TGA was also performed
under total air flow from room temperature to 850 °C. A thermogram of decomposition and decrease in the weight percentage over temperature of organic ceftazidime was obtained using universal TA software. Analysis is done multiple times to get an average composition of organic weight percentage. TGA of pure ceftazidime was also done to compare the weight loss pattern of ceftazidime upon capping onto GNP surface. (see Appendix B for detailed methodology)

2.3.4. Assessing the stability of Cef-GNPs:

One of the prime factors that is considered for any drug formulation is its storage stability under normal conditions. A formulation which is stable for a long period of time is generally preferred over less-stable formulations. In that context, the stability of Cef-GNPs was studied over a fixed period of time. Any change in the morphology of GNPs, directly affects its UV-Vis peak. For that reason, UV-Vis spectrum of two Cef-GNPs samples, one kept at room temperature and another in refrigerator were monitored periodically (0 days, 30 days, and 60 days). The data from this study helps in understanding the stability of Cef-GNPs at room temperature and under proper storage conditions. In addition, binding interactions of organic ceftazidime with GNP surface was also evaluated over a period of 60 days by comparing the TGA thermogram of lyophilized Cef-GNPs samples maintained at room temperature and in the refrigerator.

2.3.5. Evaluating antibacterial activity of Cef-GNPs:

Preparation of glycerol stock of bacterial strains:

Fresh glycerol stock of bacterial strains was prepared as and when required. Firstly, a preculture or a small scale culture of the desired bacterial strain was grown by adding 200 µL of bacterial stock into 10 mL of nutrient media (L.B/T.S) taken in a 50
mL sterilized falcon tube. The solution was mixed and incubated at 37 °C, 150 rpm for 12 hours in a shaking incubator. After the incubation period, the optical density (O.D) of the culture was measured using Spectronic-20 spectrometer. When the O.D of culture reached ~1.2, 1.5 mL (10%) of viscous glycerol was added into the falcon tube and mixed properly. 250 µL of resulting solution was aliquot into 1.5 mL sterilized ependorff’s, labelled and stored in -80 °C freezer until further use. The entire process was done in a biological safety cabinet in order to ensure aseptic conditions.

**Preparation of preculture of bacterial strains:**

A small culture of desired bacterial strain referred to as preculture was prepared by the following method. Thaw an ependorff, stored in -80 °C freezer containing the glycerol stock of desired bacterial strain. Add 10 mL of nutrient media (L.B/T.S) into a 50 mL sterilized falcon tube and transfer 250 µL from glycerol stock into the falcon tube. Screw the cap tightly and incubate the mixture at 37 °C, 150 rpm for 12-14 hours in a shaking incubator until the O.D of the culture reaches to ~1.2. **Table 5** lists the preculture conditions required for the strains that are used for the antibacterial assays.
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Staining</th>
<th>Glycerol Stock for 10 mL preculture</th>
<th>Nutrient media used</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacter aerogenes</td>
<td>Gram-negative</td>
<td>200 µL</td>
<td>L.B broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
<tr>
<td>Enterococcus durans</td>
<td>Gram-positive</td>
<td>200 µL</td>
<td>T.S broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Gram-negative</td>
<td>200 µL</td>
<td>L.B broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
<tr>
<td>Streptococcus bovis</td>
<td>Gram-positive</td>
<td>200 µL</td>
<td>T. S. broth</td>
<td>37 °C, 150 rpm, 12-14 hrs</td>
</tr>
</tbody>
</table>

**Table 5:** Incubation conditions required for the preparation of preculture of various bacterial strains under study.
Ennumeration of bacterial colony forming units (CFU/mL):

For all antibacterial assays, the concentration of bacteria was maintained uniform at $1 \times 10^6$ CFU per mL of solution. In order to obtain the desired CFU for different strains, serial dilution was performed and the resulting solution was plated onto agar plates to get countable colonies (300-400) as shown in Figure 3. Using the serial dilution and number of colonies on agar plate, number of colony forming units was calculated using the below formula (see Appendix C more images of petri plates)

$$\frac{\text{No. of } \text{CFU}}{\text{ml}} = \text{No. of colonies} \times \text{Dilution factor}$$

$$\text{Dilution factor} = \frac{1}{\text{Serial Dilution}}$$
Figure 3: Schematic illustrating the serial dilution process involved in the calculation of bacterial CFU/mL.
**Bacterial Growth Curve:**

Growth of bacteria follows a definite pattern starting from a lag phase, exponential phase, stationary phase and death phase. In the lag phase, the bacterial cells get accustomed to the new nutrient conditions. In the exponential or growth phase, the bacterial cell starts multiplying by binary fission and grows in number. In the stationary phase, the growth of new bacterial cells becomes equal to the death of old cells. Finally in the death phase, due to unavailability of nutrients and accumulation of waste, the bacterial cells start dying. Therefore by measuring the growth of bacterial cells in presence of various concentrations of Cef-GNPs, its efficiency in killing/inhibiting bacteria can be evaluated. The primary goal of the assay was to determine the lowest concentration of Cef-GNPs required to inhibit the growth of bacterial cells which is more often called the minimum inhibition concentration (MIC). The assay was performed in a 96-well microtiter plate. The perimeter of the plate was filled with nanopure water to prevent evaporation of sample during incubation. Each plate contained three rows of sample (Cef-GNPs + nutrient media + bacterial cells), positive control (nutrient media + bacterial cells) and negative control (Cef GNPs + nutrient media). For the sample, 100 µL of sterilized nanopure water was added into all the working wells. Double the concentration of the desired highest concentration of Cef-GNPs was prepared from the Cef-GNPs stock and concentrated down to a final volume of 100 µL which was then added into the first sample well. A serial dilution was performed by transferring 100 µL from first well into the succeeding well. Serial dilution was continued until the last well and 100 µL from the last well was discarded. The above steps were repeated for all the sample and blank rows. For the control well, 100 µL of sterilized water was added
instead of Cef-GNPs. To all the sample and control wells, 150 µL of bacterial solution having 1 X 10^6 CFU/ml was added. In the blank well, 150 µL of nutrient media was added. (see Appendix D for microtiter plate design) The micotiter plate was covered with the lid and incubated at 37 °C and 150 rpm. Absorbance of the solution was measured at 600 nm at every 2 hours for a period of 12 hours. Using the data from excel, a graph of absorbance vs. time was plotted for different concentrations of Cef-GNPs. The above procedure was repeated for all the bacterial strains and also for the pure ceftazidime drug.

_Spread plate assay^61_:  

As an extension to the bacterial growth assay, a small volume of solution from the sample well used in the growth assay was diluted appropriately and spread onto a L.B/T.S agar plate respectively using a sterilized glass spreader. The plates were then kept in the incubator for the colonies to grow for 12-16 hours at 37 °C. After the incubation time, the numbers of colonies in each plate were counted to determine the bactericidal concentration. In general, the MIC concentration of Cef-GNPs against a particular bacterial strain won’t show growth of any viable colonies after the incubation period. In a way, the assay was used as a confirmatory test to the bacterial growth assay.

_XTT assay^63,64_:  

It is a colorimetric method for determining MIC of antibiotic formulation. It is generally considered as more convenient and robust than the conventional plate count method which is associated with many drawbacks such as cell clumping, giving an inaccurate number of colonies resulting in improper results. This technique is more accurate as it measures the viable cell count by measuring the reducing activity of the
cells. Respiration occurs in all live/active cells through a reducing environment involving electron transport system. In this method, an artificial tetrazolium dye (yellow colored) is reduced to formazan (orange color product) by active cells which has a characteristic absorbance at 492 nm. Since formazan is a color product, it can be quantitatively determined using visible spectrometer which gives the number of viable cells. To amplify the results of XTT, an activator is added called menadione. Therefore formation of orange color indicated growth of bacterial cells and MIC is taken as the lowest concentration which does not show formation of orange color formazan product. For the assay, the bacterial cells were grown in a 96-well microtiter plate against various concentrations of Cef-GNPs as mentioned in the above assay. After the 12 hour incubation period, 50 µL of XTT stock (995 µL of XTT solution + 5µL of menadione solution) was added to the entire working wells and the plate was covered with an aluminum foil and incubated in dark for more 2 hours. After 2 hours of incubation, absorbance of the plate was read at 492 nm. Using the excel data; a graph of XTT absorbance vs. concentration of Cef-GNPs was plotted. The assay was done for all the bacterial strains.

2.3.6. Visualizing mechanism of bactericidal action of Cef-GNPs

*Propidium Iodide (PI) Assay*:

PI was used to determine the antibacterial mechanism of MIC of Cef-GNPs obtained from the growth assays. PI is a red-fluorescent staining dye which has high affinity towards bacterial DNA. Due to its inability to permeate live cells, it can be used in detecting the dead cells in a bacterial population. For the assay, one Gram-positive and Gram-negative bacterial strain was incubated in presence of the MIC of Cef-GNPs for ~
12 hours followed by which the samples were centrifuged (6000 rpm, 3 min) and washed multiple times with pH ~7.2 phosphate buffer saline (PBS). Samples were further incubated with 5 µL of PI (10 mM) for 30 minutes in dark. After incubation, unbound PI was removed by washing the sample with PBS and 10 µL of the resulting suspension was sandwiched on a glass slide covered with cover slip. Sample was viewed using Leica fluorescence microscope. A sample of bacteria without Cef-GNPs was taken as control. Numbers of permeable cells (showing red fluorescence) were counted by taking average from three individual measurements and a graph was plotted in comparison to control for respective bacterial strains.
3. RESULTS AND DISCUSSION

3.1. Synthesis of ceftazidime gold nanoparticles (Cef-GNPs):

Gold nanoparticles are formed when $\text{Au}^{3+}$ of $\text{KAuCl}_4$ is reduced to $\text{Au}^0$ in presence of a reducing agent. $\text{Au}^0$ being unstable, settles down in the course of time and clusters with other $\text{Au}^0$ atoms to form gold colloids/aggregates. In order to prevent further aggregation of gold colloids beyond the nanoscale, a secondary agent is added which stabilizes the nucleation of gold aggregates by capping its surface resulting in gold nanoparticles. To synthesized GNPs are then functionalized with desired ligands based on the application of interest. In the conventional methods of GNP synthesis, a range of reducing agents such as sodium borohydride, sodium citrate, superhydride, hexadecyl aniline were used whereas thiols, xanthates, disulfides, trithiols, phosphine, amine, carboxylate ligands, iodine, acetone etc. were used as stabilizing agents. Looking at the wide biological applications of GNPs, assessing the risk and toxicity of GNPs synthesized using the above methods to the environment and other living organisms has become a crucial topic of research. The use of organic chemicals and solvents in the above chemical synthetic methods might prove to be toxic to the environment and biological species. In response, there is a great interest in finding alternate methods which are safer and biofriendly. Biocomponents derived from plant and animals are being widely studied for their role in biosynthesis of GNPs.\textsuperscript{43,65} The advantages of using biological sources include cost-effective process and non-toxicity but practically it becomes quite a laborious process due to the presence of many impurities for which additional purification steps must be performed to obtain pure GNPs. In addition, large scale production requires a huge biomass which must be acquired and processed to be
used in the synthesis process. As an alternate approach, use of substances which can acts as ‘dual agents’ in reducing and concomitant capping of GNP are gaining a lot of focus. Advantages of using dual agents include single step process, they are cost-effective, they can have applicability for large-scale production, they avoid waste and byproducts, and are non-laborious, as well as others. In our previous studies, we had developed a green and biofriendly process for making monodispersed GNPs using dextrose as dual agent. Dextrose was efficient in reducing gold ions due to the presence of electron rich hydroxyl groups which can readily be oxidized. Extending the scope of our method, we have been trying a range of antibiotics which can be used as dual agents to form antibiotic capped gold nanoparticles, a formulation that can potentiate the activity of functionalized antibiotic against various bacterial strains.

The molecular structure of ceftazidime contains many electron rich groups such as amine, hydroxyl and carbonyl which can donate their electrons in reducing the ionic gold to form GNPs. It is very crucial for ceftazidime to have enough electrons that can be used to reduce gold ions (Au$^{3+}$) derived from KAuCl$_4$ to form Au$^0$ atoms. The exact mechanism for reducing and simultaneous capping of gold by antibiotic is still under debate. Therefore, one of the preliminary steps in our study was to determine the optimum concentration of ceftazidime (reducing/capping agent) and gold salt (nucleating agent) required which was achieved by trying different combinations. Small scale synthesis involving a combination of ceftazidime (0.5, 1, 3 and 5 mM) and gold (50, 100, 200 and 300 ppm) were tried by incubating the mixture in a culture tube at 37 °C for 12 hours with a shaking speed of 150 rpm. The solution was kept on continuous stirring to prevent aggregation of GNPs. The synthesis protocol was adhered to the ‘12 principles of
Green Chemistry’. The color of solution and its respective UV peak were used as an indicator for determining the nature of GNPs. Color of tubes after 12 hours of incubation for different concentration is shown in Figure 4. Detailed information about the different trials and their respective UV observation is listed in Table 6. Among all the concentrations, 0.5 mM ceftazidime with 200 ppm of gold salt gave a high yield of GNPs with a significant UV peak and thus was selected as the concentration required for all the further synthesis. For a typical synthesis, an aqueous solution of 0.5 mM of ceftazidime is mixed with 200 ppm of aqueous solution gold salt and the resulting mixture is incubated at 37 °C for 12 hours at a shaking speed of 150 rpm. The color of solution changes from colorless to light brownish upon addition of gold salt to ceftazidime solution. An indication of the formation of GNPs was from the color change of the reaction mixture from light brownish to clear pink after 12 hours of incubation. After multiple washings, the resulting solution containing Cef-GNPs was concentrated and subjected to lyophlization to determine the yield. The average yield of Cef-GNPs from multiple batches of 120 mL was found to be 15 ± 0.63 mg.
Figure 4: Image showing color of test tubes for various concentrations of ceftazidime and gold after 12 hours of incubation. (a) Ceftazidime concentration of 0.5 mM with 50, 100 and 200 ppm of gold. Ceftazidime concentration of 1 mM (b), 3 mM (c) and 5 mM (d) with 100, 200 and 300 ppm.
<table>
<thead>
<tr>
<th>Concentration of Reactants</th>
<th>Observation</th>
<th>Yield/supernatant</th>
<th>UV peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime</td>
<td>Gold salt</td>
<td>0 hour</td>
<td>12 hour</td>
</tr>
<tr>
<td>5 mM</td>
<td>300 ppm</td>
<td>Brown; clear</td>
<td>Brown; clear</td>
</tr>
<tr>
<td>5 mM</td>
<td>200 ppm</td>
<td>Less brown; clear</td>
<td>Light yellowish; clear</td>
</tr>
<tr>
<td>5 mM</td>
<td>100 ppm</td>
<td>Light yellowish; clear</td>
<td>Yellowish; clear</td>
</tr>
<tr>
<td>3 mM</td>
<td>300 ppm</td>
<td>Light brown; clear</td>
<td>Greenish; clear</td>
</tr>
<tr>
<td>3 mM</td>
<td>200 ppm</td>
<td>Light brown; clear</td>
<td>More brownish; clear</td>
</tr>
<tr>
<td>3 mM</td>
<td>100 ppm</td>
<td>Light brown; clear</td>
<td>Light brownish; clear</td>
</tr>
<tr>
<td>1 mM</td>
<td>300 ppm</td>
<td>Brown; turbid</td>
<td>Settled; pinkish</td>
</tr>
<tr>
<td>1 mM</td>
<td>200 ppm</td>
<td>Brown; clear</td>
<td>Light pink; settled</td>
</tr>
<tr>
<td>1 mM</td>
<td>100 ppm</td>
<td>Brown; clear</td>
<td>Light brownish; clear</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>200 ppm</td>
<td>Brown; turbid</td>
<td>Pink; clear</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>100 ppm</td>
<td>Brown; clear</td>
<td>Light pink; clear</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>50 ppm</td>
<td>Brown; clear</td>
<td>Transparent; clear</td>
</tr>
</tbody>
</table>

**Table 6:** A list showing the color and UV observation of all the different combinations of ceftazidime and gold tried for the synthesis of Cef-GNPs
3.2. Characterization of Ceftazidime Gold Nanoparticles (Cef-GNPs):

UV-Vis spectroscopy remains one of the most quick and reliable tools for identifying the formation of GNPs. This is due to the fact that GNPs possess an inherent property called surface plasmon resonance (SPR), showing an intense peak absorption in the visible region, sensitive to size, shape, aggregation, and nature of solvent. UV-Vis spectra of resulting Cef-GNPs suspension showed peak absorption at 548 nm, characteristic for spherical GNPs (Figure 5(a)). The peak was sharp with no formation of humps, indicating the Cef-GNPs to be monodispersed and non-aggregated.

To validate the UV-Vis data, samples were observed under high resolution TEM for morphological characterization. The image captured at 100 kX magnification showed well-dispersed spherical particles in the size range of 25 ± 5 nm (Figure 5(b)). The images clearly support our hypothesis as proposed in the synthesis mechanism. TEM images of other concentrations were also observed which showed signs of low yield, large size and aggregation. With the TEM data, the concentration chosen for the synthesis yielded the best nanoparticles among all the other concentrations. (see Appendix E for additional TEM image)

To further corroborate the results, dynamic light scattering (DLS) was performed on the dilute suspension of Cef-GNPs at a scattering angle of 90°. DLS is another widely used characterization technique for nanoparticles where a light emitted by a laser source is passed through the nanoparticles which are in constant motion in solution. Intensity of scattered light from nanoparticles is plotted as a function of time and the hydrodynamic size of particles is determined. DLS is highly sensitive and the size of nanoparticle is influenced by the substances (ligand) adsorbed on the surface of nanoparticle unlike the
case in microscopic techniques which measures only the metallic core of nanoparticle. As a result, the resulting size from DLS generally differs marginally from the TEM or UV data. DLS analysis of Cef-GNPs showed a sharp peak with an average size distribution of 45 ± 15 nm (Figure 5(c)).
Figure 5: Representative images showing morphological characterization of Cef-GNPs. (a) UV-Vis spectra of Cef-GNPs showing a strong absorption peak at 548 nm which is characteristic of spherical GNPs. The figure in the inset represents color change from light brownish to pink indicating formation of GNPs. (b) TEM photograph showing formation of well-dispersed spherical GNPs in the size range of $25 \pm 5$ nm by reducing KAuCl$_4$ using an cephalosporin antibiotic, ceftazidime. (c) Plot showing average particle size distribution obtained using dynamic light scattering (DLS) at a scattering angle of 90°.
FTIR\textsuperscript{67} was also used as a qualitative tool to determine the changes in the peaks of functional groups of ceftazidime upon its capping onto GNP surface. For the analysis, IR spectrums of pure ceftazidime and Cef-GNPs were recorded and the spectrums were overlapped as shown in Figure 6. Spectrum of Cef-GNPs had similar peaks as observed in pure ceftazidime, thereby confirming it’s capping on GNP surface. The peaks in the IR spectrum were as follows: 1680-1630 cm\textsuperscript{-1}: C=O axial deformation of amide group, 1350-1300 cm\textsuperscript{-1}: axial deformation from C-N, 1600-1475 cm\textsuperscript{-1}: aromatic ring C=C deformation, 1750-1725 cm\textsuperscript{-1}: C=O stretching of carboxylic functional group, 3660-3250 cm\textsuperscript{-1}: N-H axial deformation respectively. A closer look on the peaks of Cef-GNPs, shows the peak indicating N-H deformation were gone or not significant, indicating their involvement in the reduction and capping of ceftazidime with nanoparticles. Minor stretching’s for carbonyl were also observed indicating their role in the synthesis of Cef-GNPs. The peaks responsible for the β-lactam ring were undisturbed, thereby preserving the inherent bactericidal action of the antibiotic.
**Figure 6:** An overlay IR spectrum of Cef-GNPs and pure ceftazidime. The IR peak of Cef-GNPs closely resembled with that of pure ceftazidime. The N-H stretching in the region of 3660-3250 cm\(^{-1}\) as evident in pure ceftazidime was absent or insignificant in Cef-GNPs, indicating their involvement in the reduction of ionic gold. Minor stretching was also observed for carbonyl group in the region 1750-1725 cm\(^{-1}\). The figure in the inset represents the molecular structure of ceftazidime with major functional groups which has strong IR peaks. The peaks responsible for the \(\beta\)-lactam ring (highlighted section) were undisturbed, thereby preserving the inherent bactericidal action of antibiotic.
SEM analysis of Cef-GNPs revealed the formation of spherical GNPs (Figure 7(a) inset). Quantitative analysis of the surface elemental composition was determined from the SEM preparation using EDX spectroscopy. The EDX spectrum of Cef-GNPs synthesized using ceftazidime is shown in Figure 7(a). Vertical axis represents the number of x-ray counts while the horizontal axis shows the energy (keV). Peaks at binding energy of 0.285, 0.395, 0.535 and 2.135 keV corresponded to carbon (C), nitrogen (N), oxygen (O) and gold (Au) respectively. EDX analysis showed the presence of ~87% of reduced gold and ~5% of carbon, ~2% of nitrogen and ~3% of oxygen respectively. Gold peak in the EDX analysis was from the GNPs while the carbon, oxygen and nitrogen peak were attributed to the organic molecule, ceftazidime present on the surface of GNPs. SEM-EDX results confirmed the presence and capping of ceftazidime on the surface of GNP.

Quantitative determination of the weight content of the organic ceftazidime was determined using thermogravimetric analysis (TGA) as shown in Figure 7(b). As a control, free ceftazidime was also subjected to TGA analysis. From the thermogram, two major weight loss steps were observed for both Cef-GNPs and free ceftazidime. The first weight loss was due to the moisture loss till ~150 °C and the second weight loss from 200 °C to 850 °C was attributed to the decomposition of organic ligand (ceftazidime). For the Cef-GNPs, the peak became horizontal after a weight loss of 35.61% representing the amount of ceftazidime bonded on GNP surface. In comparison, a total weight loss of 88.54% was observed for the free ceftazidime. Total decomposition of pure ceftazidime wasn’t observed due the presence of 10% of sodium carbonate (Na₂CO₃) in the ceftazidime drug as stabilizer. Sodium carbonate being an ionic compound has a high
melting point which is beyond 850 °C. As a result, only 90 % weight loss was observed which represented the ceftazidime present in the drug. From the TGA study, a high percentage of organic ceftazidime was found on the GNP surface which confirms all the above observations obtained from TEM, SEM, UV-Vis, DLS and FTIR data (see Appendix F for TGA data of Cef-GNPs taken under air flow).
Figure 7: Represents qualitative and quantitative analysis of organic ligand (ceftazidime) on GNP surface. (a) Energy dispersive spectroscopy (EDS) spectra of Cef-GNPs showing the presence of elemental peaks for carbon (C), nitrogen (N), oxygen (O) and gold (Au) constituting ~ 5%, 2 %, 3 % and ~85% respectively. Figure in the inset represents scanning electron microscope (SEM) image of Cef-GNPs suspension layered on an aluminum stub obtained at an accelerating voltage of 20 keV with 5kx magnification. SEM-EDX results confirmed the presence and capping of ceftazidime on the surface of GNP. (b) Thermogravimetric analysis (TGA) thermogram showing weight loss of organic matter for ceftazidime (——) and Cef-GNPs (- - -) respectively. The samples were heated from room temperature to 650 °C at a rate of 10 °Cmin⁻¹ under nitrogen flow followed by heating till 850 °C under air. A total weight loss of 35.61 ± 0.25 % in Cef-GNPs indicated the amount of ceftazidime bounded on GNP surface. Total
decomposition of pure ceftazidime was not observed due to the presence of 10 % of ionic sodium carbonate which has high melting point.

Change in the UV-Vis spectrum of Cef-GNPs was monitored to determine its storage stability. After recording the UV spectrum of two samples of freshly synthesized Cef-GNPs, one sample was kept at room-temperature while the other was kept in refrigerator. UV-Vis spectra of both the samples were recorded after 30 days and 60 days respectively. An overlay plot of UV-Vis spectra’s of two samples taken at 0, 30 and 60 days is shown in (Figure 8 (a and b)). The peak absorption was found to be 548 ± 2 nm (refrigerator sample) and 549 ± 2 nm (room-temperature sample) respectively. No significant change in the peak intensity or SPR wavelength for both the samples suggested the storage stability of Cef-GNPs. A slight decrease in the intensity could be attributed to the handling error while performing the analysis.

In addition to UV, TGA analysis was also performed to determine the change in the percentage weight loss upon storage. Two samples, one at room temperature and other in refrigerator were subjected for TGA after 60 days of storage. An overlay plot of thermogram for both samples showed a weight loss of 36.22 ± 0.2 which was near identical to the weight loss obtained for the fresh Cef-GNP’s sample (Figure 8(c)). The TGA data shows no leaching of ceftazidime from GNP surface upon its storage.
Figure 8: Illustrates assessment of stability of Cef-GNPs. (a) An overlay of UV-Vis spectrum of 0, 30 and 60 days old sample of Cef-GNPs suspension stored at room temperature showing no major shift in the intensity and peak absorption value. The average peak absorption value was found to be 549 ± 2 nm. (b) An overlay of UV-Vis spectrum of 0, 30 and 60 days old sample of Cef-GNPs suspension stored in refrigerator showing no major shift in the intensity and peak absorption value. The average peak absorption value was found to be 548 ± 2 nm. From the UV results, no signs of leaching or aggregation were observed. (c) TGA curves comparing the change in the percentage of organic material for 60-days old Cef-GNPs kept in refrigerator and room temperature. The % weight loss for both samples was found similar (36.22 ± 0.85 %). The weight loss was almost equal to the weight loss of fresh Cef-GNPs.
3.3. Evaluation of *In-vitro* Antibacterial Activity:

3.3.1. Cef-GNPs against Gram-negative bacteria:

The overriding purpose of the study was to determine the efficiency of synthesized Cef-GNPs in combating bacteria as compared to the pure ceftazidime. The primary focus was the activity against Gram-negative bacteria (*P. aeruginosa* and *E. aerogenes*) due to the wide use of ceftazidime in the treatment of such bacterial infections. Conventional antibacterial testing methods such as bacterial growth assay and spread plate assay were used with minor modifications. Growths of fresh cultures of respective bacterial strain were monitored periodically for 12 hours in presence of different concentrations (0.06, 0.012, 0.025, 0.05, 0.10 and 0.20 mg mL\(^{-1}\)) of Cef-GNPs by measuring the optical density (OD) at 600 nm, which shows minimal absorption from nutrient media and GNPs. The lowest concentration of Cef-GNPs which showed 90% inhibition of bacterial growth was taken as the minimum inhibition concentration (MIC). From the results of bacterial growth assay, dose-dependent bacteriostatic action of Cef-GNPs was observed against both Gram-negative bacterial strains. The MIC of Cef-GNPs was found to be 0.05 mg mL\(^{-1}\) against *P. aeruginosa* and *E. aerogenes*. Furthermore antibacterial testing was performed by counting the viable bacterial cells grown on a solid agar plate. Overnight cultures of bacteria grown in presence of different concentration of Cef-GNPs were diluted and spread onto solid agar plate. The petri plates were incubated at 37 °C for 14-18 hours. Samples of bacteria grown in absence of nanoparticles were taken as control. From the spread plate results, gradual decrease in the number of viable bacterial cells with increasing nanoparticle concentration were observed confirming the bactericidal activity of Cef-GNPs. The concentration that yielded an insignificant number
of viable bacterial colonies was taken as the MIC. The MIC results from spread plate assay against Gram-negative bacteria were in compliance with the growth assay. The number of viable cells of *P. aeruginosa* and *E. aerogenes* at the MIC of Cef-GNPs were less than 5% as compared to the control. To check the precision of results, a separate colorimetric assay was also performed using a detecting agent XTT, which shows a change in color from yellowish to orange (formazan product) in presence of living bacterial cells. 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 results of XTT against *P. aeruginosa* and *E. aerogenes* were in compliance with the previous assays. The results of all antibacterial assays against *P. aeruginosa* and *E. aerogenes* are shown in Figure 9 and Figure 10 respectively. To determine the aggregation effect of Cef-GNPs with nutrient media, various concentration of Cef-GNPs were incubated with L.B. and T.S broth individually and absorbance was checked at 600 nm periodically (see Appendix G for graph)
Figure 9: Illustrates dose dependent inhibition by Cef-GNPs against Gram-negative, *P. aeruginosa*. (a) Monitoring the growth of *P. aeruginosa* in presence of increasing concentrations of Cef-GNPs by measuring the OD at 600 nm every 3 hours for a period of 12 hours. MIC of Cef-GNPs was found to be 0.1 mg mL⁻¹. (b) Visualizing the growth of *P. aeruginosa* on a solid agar plate in presence of MIC of Cef-GNPs obtained from the growth assay. Untreated sample of bacteria was used as control. (c) *P. aeruginosa* susceptibility testing against varying concentrations of Cef-GNPs by colorimetric assay which involves reduction of a yellow tetrazolium salt (XTT) to orange formazan product by metabolic active bacterial cells. The MIC (0.1 mg mL⁻¹) was similar to the MIC obtained using spread plate and growth assay. (d) A plot of corresponding XTT assay obtained by measuring OD of wells at 492 nm, which shows peak absorption for orange formazan derivative. Wells with 90 % reduction in OD_{492 nm} compared to positive control or no orange color formation was taken as MIC.
**Figure 10:** Illustrates dose dependent inhibition by Cef-GNPs against Gram-negative, *E. aerogenes*. (a) Monitoring the growth of *E. aerogenes* in presence of increasing concentrations of Cef-GNPs by measuring the OD at 600 nm every 3 hours for a period of 12 hours. MIC of Cef-GNPs was found to be 0.1 mg mL⁻¹. (b) Visualizing the growth of *E. aerogenes* on a solid agar plate in presence of MIC of Cef-GNPs obtained from the growth assay. Untreated sample of bacteria was used as control. (c) *E. aerogenes* susceptibility testing against varying concentrations of Cef-GNPs by colorimetric assay which involves reduction of a yellow tetrazolium salt (XTT) to orange formazan product by metabolic active bacterial cells. The MIC (0.1 mg mL⁻¹) was similar to the MIC obtained using spread plate and growth assay. (d) A plot of corresponding XTT assay obtained by measuring OD of wells at 492 nm, which shows peak absorption for orange formazan derivative. Wells with 90% reduction in OD₄₉₂ nm compared to positive control or no orange color formation was taken as MIC.
3.3.2. **Cef-GNPs against Gram-positive bacteria:**

For evaluating the broad-spectrum activity of synthesized Cef-GNPs, additional antibacterial testing was performed against Gram-positive bacteria (*S. bovis* and *E. durans*). Growths of fresh cultures of respective bacterial strain were monitored periodically for 12 hours in presence of different concentrations (0.125, 0.250, 0.50, 1 and 1.2 mg mL\(^{-1}\)) of Cef-GNPs by measuring the optical density (OD) at 600 nm, which shows minimal absorption from nutrient media and GNPs. From the results, the Cef-GNPs showed a dose-dependent inhibition of bacterial growth but the concentration required was significantly high. The MIC was found to be 1.2 mg mL\(^{-1}\) against *S. bovis* and *E. durans*. Further antibacterial assays were not performed as the concentration obtained from growth assay was too high. The results of growth assay against Gram-positive bacteria are shown in **Figure 11**. In general, third generation cephalosporin are found to be more potent against Gram-negative as compared to Gram-positive microorganisms. This could be the reason for high MIC of Cef-GNPs against *S. bovis* and *E. durans*. Nevertheless, Cef-GNPs were found to have broad-spectrum activity with a higher potential against Gram-negative bacteria.
Figure 11: Illustrates dose dependent inhibition by Cef-GNPs against Gram-positive bacteria. (a) Monitoring the growth of *S. bovis* in presence of increasing concentrations of Cef-GNPs by measuring the OD at 600 nm every 3 hours for a period of 12 hours. MIC of Cef-GNPs was found to be 1.2 mg mL$^{-1}$. (b) Monitoring the growth of *E. durans* in presence of increasing concentrations of Cef-GNPs by measuring the OD at 600 nm every 3 hours for a period of 12 hours. MIC of Cef-GNPs was found to be 1.2 mg mL$^{-1}$. From the above results, Cef-GNPs were proved to have broad-spectrum antibacterial activity.
In order to evaluate the efficacy of Cef-GNPs over pure ceftazidime, bacterial growth assays were carried out against all the above bacterial strains in presence of pure ceftazidime. The MIC of pure ceftazidime for Gram-negative (P. aeruginosa and E. aerogenes) was found to be 0.35 mg mL\(^{-1}\) while that for Gram-positive (S. bovis and E. durans) was found to be 1.0 mg mL\(^{-1}\). A plot of MIC comparison for Cef-GNPs and ceftazidime by itself against all the bacterial strains under test is shown in Figure 12. From the above results, it was evident that Cef-GNPs were more potent than ceftazidime by itself against Gram-negative bacteria.
Figure 12: Illustrates a plot of MIC comparison for pure ceftazidime and bound ceftazidime (Cef-GNPs) against multiple strains of Gram-positive and Gram-negative bacteria. The MIC of Cef-GNPs (0.1 mg mL\(^{-1}\)) was found to be significantly lower than the free ceftazidime (0.35 mg mL\(^{-1}\)) against Gram-negative bacteria which proves its in-vitro efficiency as a potent antibacterial agent. As far as Gram-positive bacteria, the Cef-GNPs activity was almost similar to that of pure ceftazidime. If the actual dose of ceftazidime present on GNP surface is taken into consideration, the MIC values are much lower for Cef-GNPs as compared to ceftazidime by itself.
3.4. Visualizing Bacterial Permeability of Cef-GNPs:

To confirm the bacterial membrane permeability of Cef-GNPs, propidium iodide (PI) assay was used to determine the ratio of viable/dead cells. The assay was performed against one Gram-negative (*P. aeruginosa*) and one Gram-positive (*E. durans*) bacteria. For the assay, overnight samples of bacteria grown in presence of Cef-GNPs were incubated with PI and observed under Leica fluorescence microscope. PI shows red fluorescence after binding with the nucleic acids. Untreated samples of bacteria with nanoparticles were taken as control. The results showed ~90% permeability of PI into bacterial cells against both *P. aeruginosa* and *E. durans* at a Cef-GNP concentration of 0.1 mg mL\(^{-1}\) and 1.2 mg mL\(^{-1}\) respectively (*Figure 13*). Permeability of PI suggests formation of perforations in presence of Cef-GNPs. In contrast, the control sample didn’t show any significant fluorescence as the bacterial cells remained intact, restricting the PI influx into the cytoplasm. The above data confirms the bactericidal action of Cef-GNPs as evident from the disruption of cell membrane which resulted in leakage of cell contents including nucleic acids.
Figure 13: Illustrates fluorescence images of Cef-GNPs induced cell membrane permeability using propidium iodide (PI) dye which has strong binding affinity towards nucleic acids. Upper panel represents Gram-negative, *P. aeruginosa* and lower panel represents Gram-positive, *E. durans*. (a) For each image, the left half represents differential interference contrast mode, while the right half represents the corresponding fluorescence image. Untreated samples of respective bacteria with Cef-GNPs were taken as control. (b) Represents a plot showing percentage permeability of *P. aeruginosa* and *E. durans* bacterial cells in presence (MIC) and absence (control) of Cef-GNPs.
4. CONCLUSION

A novel and efficient antibacterial strategy which involves the use of the commercial antibiotic ceftazidime capped onto the surface of gold nanoparticle was prepared in a single-step process without using any toxic chemical agents. The overall objective of the study was to develop a novel antibacterial formulation to fight against bacterial strains. Monodisperse, spherical GNPs in the size range of $25 \pm 5$ nm were formed which showed great storage stability as conferred from UV and TGA analysis. According to the FTIR data, the interaction of ceftazidime with GNP surface was attributed to the amine and carbonyl groups. A high percentage of ceftazidime was found to be capped on GNP surface. Antibacterial testing showed dose-dependent bactericidal activity against both Gram-negative and Gram-positive bacteria. The MIC of Cef-GNPs against Gram-negative bacteria ($P. aeruginosa$ and $E. aerogenes$) was found to be 0.1 mg mL$^{-1}$ while that for Gram-positive ($S. bovis$ and $E. durans$) was found to be 1.2 mg mL$^{-1}$. In comparison, the MIC of pure ceftazidime against Gram-negative was 0.35 mg mL$^{-1}$ while for Gram-positive was 1.0 mg mL$^{-1}$. A significant decrease in the MIC of Cef-GNPs as compared to ceftazidime by itself proved its greater efficiency in combating Gram-negative bacteria. Cef-GNPs were found to have high bacterial permeability thereby disrupting cellular environment which causes death of bacterial cell. In conclusion, we report a simple bio-friendly process using combined reducing and capping ability of antibiotic to make stable and efficient GNPs which holds a promising future for its clinical use as antibacterial agent. Future work is necessary to elucidate the $in$-vivo antibacterial activity of Cef-GNPs against resistant bacterial strains using animal models and also to assess the cytotoxicity of GNPs which could facilitate its biomedical use.
5. REFERENCES

(3) Bacteria in food production http://www.effca.org/content/bacteria-food-production (accessed Jul 2, 2014).
(4) Industrial Microbiology - Industrial Application of Microbes.
(10) Goldman, A. S.; Prabhakar, B. S. Immunology Overview. In *Medical Microbiology*; Baron, S., Ed.; University of Texas Medical Branch at Galveston: Galveston (TX), 1996.

**ANTIBIOTIC RESISTANCE: AN ECOLOGICAL PERSPECTIVE ON AN OLD PROBLEM**; Colloquium Reports; The American Academy of Microbiology, 2009.

(20) WHO | Antimicrobial resistance

Schwartz, E. Who’s Trying to Fix the Pipeline Problem? – Hunting the Nightmare Bacteria


FDA Data: Slight Uptick in Animal Antibiotic Use, Resistance Remains Issue in Meat


Banned Antibiotics Found in Poultry | The Scientist Magazine®


Antibiotic resistance “threat to UK”

Activist. Activist Post: The “Return of Our Old Enemies in an Untreatable Form.”

Threat Report 2013 | Antimicrobial Resistance | CDC


(47) There’s Plenty of Room at the Bottom. Wikipedia, the free encyclopedia, 2014.


6. APPENDIX

6.1. Appendix A: Product Specification of Ceftazidime Hydrate:

![Figure 14: Molecular Structure of Ceftazidime hydrate](image)

<table>
<thead>
<tr>
<th>Property</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Name:</td>
<td>Ceftazidime hydrate – 90.0-105.0 %</td>
</tr>
<tr>
<td>Formula:</td>
<td>C_{22}H_{22}N_{6}O_{7}S_{2} \cdot xH_{2}O</td>
</tr>
<tr>
<td>Formula Weight:</td>
<td>546.58 g/mol</td>
</tr>
<tr>
<td>Storage Temperature:</td>
<td>2 - 8 °C</td>
</tr>
<tr>
<td>Appearance (Color):</td>
<td>White to off-White</td>
</tr>
<tr>
<td>Appearance (Form):</td>
<td>Powder</td>
</tr>
<tr>
<td>Solubility (Color):</td>
<td>Colorless to faint Yellow</td>
</tr>
<tr>
<td>Solubility (Turbidity):</td>
<td>Clear</td>
</tr>
<tr>
<td>Moisture content:</td>
<td>≤ 15.0 %</td>
</tr>
<tr>
<td>Sodium carbonate content:</td>
<td>9 – 11 %</td>
</tr>
<tr>
<td>Assay (Dry Basis):</td>
<td>90.0 – 105.0 %</td>
</tr>
</tbody>
</table>
6.2. Appendix B: Step-wise process involved in TGA analysis of Cef-GNPs:

**Figure 15:** A detailed step-wise process involved in the TGA analysis of Cef-GNPs. Method A refers to the analysis in presence of nitrogen and air while Method B refers to analysis under total air flow.
6.3. Appendix C: Petri Plates Images for Calculating Bacterial CFU/mL:

Gram-negative bacteria:

**Figure 16:** (a) Images of petri plates showing viable bacterial cells after respective dilution of the preculture. The dilution of $10^{-7}$ resulted in countable colonies in the range of 300-400 colonies. (b) Shows the dilution factor required for the antibacterial assays against Gram-negative bacteria to yield $1 \times 10^6$ CFU/mL in the resulting solution.
Gram-positive bacteria:

Figure 17: (a) Images of petri plates showing viable bacterial cells after respective dilution of the preculture. The dilution of $10^{-7}$ resulted in countable colonies in the range of 300-400 colonies. (b) Shows the dilution factor required for the antibacterial assays against Gram-positive bacteria to yield $1 \times 10^6$ CFU/mL in the resulting solution.
6.4. Appendix D: Microtiter plate design for bacterial growth assay and XTT assay:

![Diagram of microtiter plate design](image)

**Figure 18:** Design of microtiter plate for antibacterial assays. The perimeter wells are filled with water to avoid sample evaporation. Sample wells are B, C and D from 2 to 7 which contain 100 µL of drug + 150 µL of bacterial culture (1X 10^6 CFU/mL). The highest concentration of drug is added in B2 and is serially diluted in the succeeding wells. Blank wells are F, G from 2 to 7 which contains same concentration of drug as in sample wells with 150 µL of nutrient media instead of bacteria. This is used to remove background absorbance from drug and media. Control well is D and E 10 which contains bacteria, used to monitor the growth of bacteria.
6.5. Appendix E: Additional Transmission Electron Microscopy (TEM) image of Cef-GNPs:

**Figure 19:** TEM photograph showing formation of well-dispersed spherical GNPs in the size range of 25 ± 5 nm by reducing KAuCl4 using a cephalosporin antibiotic, ceftazidime.
6.6. Appendix F: Thermogravimetric analysis (TGA) of Cef-GNPs under air flow:

![Thermogravimetric analysis (TGA) thermogram](image)

**Figure 20:** Thermogravimetric analysis (TGA) thermogram showing weight loss of organic matter for ceftazidime (---) and Cef-GNPs (- - -) respectively. The samples were heated from room temperature to 850 °C at a rate of 10 °C/min under air flow. A total weight loss of 35.38 ± 0.35 % in Cef-GNPs indicated the amount of ceftazidime bounded on GNP surface. Total decomposition of pure ceftazidime was not observed due to presence of 10 % of ionic sodium carbonate which has high melting point. The data was similar to the one obtained using nitrogen as gas.
6.7. Appendix G: Interaction of nutrient media with Cef-GNPs:

**Figure 21:** (a) Interaction of different concentration of Cef-GNPs with Luria Broth media which was the nutrient media used for Gram-negative bacteria. (b) Interaction of different concentration of Cef-GNPs with Tryptic Soy media which was the nutrient media used for Gram-positive bacteria.
7. ABBREVIATIONS

°C  Degree Celsius
μL  Microliter
CDC  Centers for Disease Control and Prevention
Cef-GNPs  Ceftazidime-Gold Nanoparticles
CFU  Colony Forming Unit
DLS  Dynamic Light Scattering
EDS  Energy Dispersive X-Ray Spectroscopy
FDA  Food and Drugs Administration
FTIR  Fourier Transform Infrared Spectrometer
g  Gram
GNP  Gold Nanoparticles
hr  Hours
IR  Infrared
L.B  Luria Broth
MDR  Multi-Drug Resistant
Mg  Milligram
MIC  Minimum Inhibition Concentration
mL  Milliliter
mM  Millimolar
nm  Nanometer
OD  Optical Density
QIDP  Qualified Infectious Drug Product
SEM  Scanning Electron Microscope
SPR  Surface Plasmon Resonance
T.S  Tryptic Soy
TEM  Transmission Electron Microscope
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA</td>
<td>Thermogravimetric Analysis</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</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>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>Peak Absorption</td>
</tr>
</tbody>
</table>