2018-01-01

Formulation Of Nanoparticle Systems For Drug Delivery Application In Biomedical Sciences

Victor Alfredo Rodriguez
University of Texas at El Paso, varodriguez2015@gmail.com

FORMULATION OF NANOPARTICLE SYSTEMS FOR DRUG DELIVERY APPLICATION IN BIOMEDICAL SCIENCES

VICTOR ALFREDO RODRIGUEZ

Master's Program in Biomedical Engineering

APPROVED:

Jwala Renukuntla, Ph.D., Chair

Rahul Kalhapure, Ph.D.

Thomas Boland, Ph.D.

Charles Ambler, Ph.D.
Dean of the Graduate School

Dedication

For my hardworking and immensely supportive family whom have always seen greatness in me and believed in my ability even during the days that seemed gloomy. My mother who speaks and guides me along my path. My father who has worked to provide and continuously approved of my endeavors. My grandfather who has supported, guided, mentored, spoke and taught me that integrity, respect and honesty are the qualities all should possess. This work is dedicated for those who have been there for me in every way.

FORMULATION OF NANOPARTICLE SYSTEMS FOR DRUG DELIVERY APPLICATION IN BIOMEDICAL SCIENCES

by

VICTOR ALFREDO RODRIGUEZ, B.S.

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Metallurgical, Materials, and Biomedical Engineering

THE UNIVERSITY OF TEXAS AT EL PASO

December 2018

Acknowledgements

Special thanks to Dr. Jwala Renukuntla for providing me the opportunity to study, learn and conduct research under her guidance. Dr. Rahul Kalhapure for the immense amount of knowledge and understanding to the field of formulation and drug delivery sciences. Dr. Boland, chair of the Biomedical Engineering program, for giving me the opportunity to study and conduct research with UTEP to advance my education, research skills, and curiosity for the field. Graduate students Pradeep Bolla and Stefani Perez, whom assisted and provided input to my experiments and experimental design process during my research journey. Much oblige to all undergraduate research assistants Carlos Meraz, Julian Franco, David Ramos, and Maya Ruiz.

Abstract

Antibiotic-resistant strains of bacteria may result in serious infections which are difficult to treat. In addition, the poor antibiotic pipeline has also contributed to the crisis. Recently, the complex of furosemide and silver (Ag-FSE) has been reported as a potential antibacterial agent. However, its poor aqueous solubility is limiting its activity as an effective antibacterial agent. The purpose of this study was to encapsulate Ag-FSE into chitosan nanoparticles (CSNPs) and evaluate antibacterial efficacy. Ag-FSE CSNPs were prepared using ionic gelation technique. The particle size, polydispersity index and zeta potential of Ag-FSE CSNPs were determined using dynamic light scattering (DLS) technique. Encapsulation efficiency and drug release studies were also performed and evaluated with the formulated Ag-FSE loaded CSNPs. To gain an insight into the formulation's effectiveness, *in vitro* antibacterial activity studies were also performed. Stability studies with the Ag-FSE loaded CSNPs were executed to understand the most optimal storage conditions for an extended storage life of the formulation to be appropriate. Based upon results and interpretations, it could be determined that the study is viable to continue forward for further studies.

Table of Contents

List of Tables

List of Figures

List of Equations

Chapter 1: Bacterial Infections & The Revolution of Medicine

The evolution of prokaryotic and eukaryotic cells has been at times dichotomous, and alternatively a collaborative relationship [1]. Within the human system, microbiomes of bacteria can be found involved with multitudes of processes, such as digestion, and nutrient absorption [2]. Alternatively, constant competition of survival for both forms of life exist, and thus "survival of the fittest" has led to the development of disease, infections, and uncontrolled plagues. This competitive nature of bacteria infecting and propagating along the human system brought the need for drug discovery, and formulation of medication. With efforts in producing antibiotics, diseases became eradicated, and infections became controlled & treatable. However, this same solution metamorphosized into the downfall of effective antibiotics, leading to new generations of bacteria, resistant to those once effective treatments.

SECTION 1.1. BACTERIAL INFECTIONS

The competitive nature between eukaryotic and prokaryotic organisms may lead to illness within a host. Bacteria, although simple single-celled entities with no nucleus or any other advanced features, prove to be burdensome when entering a system for competitive existence. Although many existing bacteria within the human system work symbiotically, some foreign bacterium act to dominate regions. When unchecked, the site of infection can propagate the genesis of serious complications.

Bacteria competing and overwhelming the immune system leads to the development of diseased states [3,4]. Easily contracted diseases could develop into plagues, shaping society and the ideals of modern medicine [5]. Specifically, into understanding the nature of bacteria and classifying types of bacteria found in common diseases and infections. Scientists observed that bacteria in common infections shared a common morphology [6–9].

Treatment of bacterial infections was possible with the determination that common infections were propagated due to a common bacterium. Common characteristics observed rooted in the form of morphology that the bacteria had. Synthesis of compounds to treat specific bacteria

for specific infections would lead to a medical revolution. A revolution in which eradication of bacterial infections caused by the discovery and production of antibiotics would be underway.

SECTION 1.2. ANTIBIOTIC EVOLUTION

Discovery and development of antibiotics became a pivotal moment, which eradicated diseases and increased the average life expectancy from 47 to over 70 years [10]. Yet, centuries before the discovery and use of antibiotics, individuals who contracted an infection primarily relied on holistic remedies with plant extracts or molds [11]. Ancient Egyptian and Sudanese practices indicated that knowledge of certain properties found in molds and other plant-based remedies would be used to treat infected wounds and other illnesses [11–13]. This gave rise to the notion that early forms of antibiotics had been used but with little understanding of the mechanisms of action.

Motivation to uncover solutions to infection and disease-causing bacterium initiated with early concepts of antibiotics during the late 19th and early 20th century. Early records of antibiotic development and discovery was recorded by Robert Emmerich and Oscar Löw [13]. Emmerich and Löw observed that a particular bacterium growing within the bandages of patients would inhibit the propagation of any other types of bacteria [13]. Although it had been determined an unfavorable solution with Emmerich and Löw's attempt, the door for new possibilities of treatment methods opened particularly for targeted treatment of bacterial infections.

The concept of specific and targeted treatment for bacterial infections with synthetic compounds was presented in the early 20th century by Paul Ehrlich [14]. Ehrlich's early concepts of dyes, such as methylene blue, was used to tag bacterium for specific treatment of infections. This method of staining was the basis for many early synthetic compounds in discovering the "magic bullet". The "magic bullet" is a concept which describes that a specific compound would target and destroy a specific bacterium based on chemical structure compatibility with no harm to uninfected host tissue [15,16].

These initiatives created the stepping stone for the first true antibiotics and revolution of pharmacological practice in treating diseases. Ehrlich's discovery of benzoporporine and a later derivative, Trypan red, allowed for the successful treatment of trypanosomal (malaria) infections [15]. Later, the discovery of the compound arsphenamine (Sulvarsan) was Ehlrich's 606th compound was used to treat syphilis [15,16]. Through trial and error of synthesis and molecular structuring of compounds with affinities to specific bacteria [15] were the first "antibiotics" discovered.

In a serendipitous discovery, the first true antibiotic was uncovered by Alexander Fleming [17]. Fleming's observation of a mold, *Penicillium notatum,* in a petri dish of *Staphylococcuss* bacteria transfecting itself to inhibit the bacterial propagation unveiled that through nature treatments for bacterial infections existed [11,18]. Penicillin's discovery sparked an advancement in antibiotic discovery which begun with Selman Waksman's work in discovering streptomycin to treat tuberculosis [19]. The golden era of antibiotics followed these initial breakthrough's with eradication of various diseases and infections which havocked society.

SECTION 1.3. DECLINE OF THE ANTIBIOTIC GOLDEN ERA

Early into the golden era of antibiotics, it was quickly observed that bacteria were acquiring resistance to the discovered antibiotics and to those remedies used beforehand with similar molds or bacteria [10,12,13,20]. Because of this early detection of resistance, generations of antibiotic derivatives were synthesized becoming the order of production for new antibiotics [13]. With focuses shifted from discovering novel antibiotics to producing derivatives of existing antibiotics, led to the gradual decline of the antibiotic pipeline. Other factors leading to the drying of the pipeline included the cost of drug discovery efforts and increasingly intensified FDA standards for clinical trials in protection of human subjects [20,21]. Along with focuses in medicine reshuffling to advance medical technology, caused a halt with antibiotic discovery [12,21].

Due to unsuccessful attempts and belief that all accessible scaffolds for new antibiotics had been uncovered, pharmaceutical focuses and investments for novel antibiotics seized leading to

the evolution of antibiotic resistant bacteria [21]. Infections that were once treatable, aggressively resurfaced with difficulty in implementing treatment methods. Combination therapies and increased dosages were utilized to treat infections with resistance bacteria. This improper use of prescribed drugs or low patient compliance would lead to the emergence of multiple-drug resistant bacteria [22–25]. This issue of bacterial resistance was forecasted to increase rates of mortality to treatable diseases and infections [26] unless revival of the current antibiotics were to occur.

SECTION 1.4. REVIVAL OF ANTIBIOTICS WITH NANOTECHNOLOGY

The threat of emerging drug resistant bacteria to send society back into a period of pre-antibiotics created the urgency to develop methodologies to revive the efficacy of existing antibiotics (Figure 1.1). Advancement of nanotechnology within the medicine, nanomedicine, had shown promise in overcoming the issue with drug resistant bacteria [27,28]. Nanomedicine could be utilized with a plethora of practices from wound healing promotion to infection prevention [25]. With high reproducibility, low cost effectiveness, high tunability, ease of modification, and target specificity, nanomedicine could be a favorable route of enhancing existing antibiotic effectiveness [23,25,27–29].

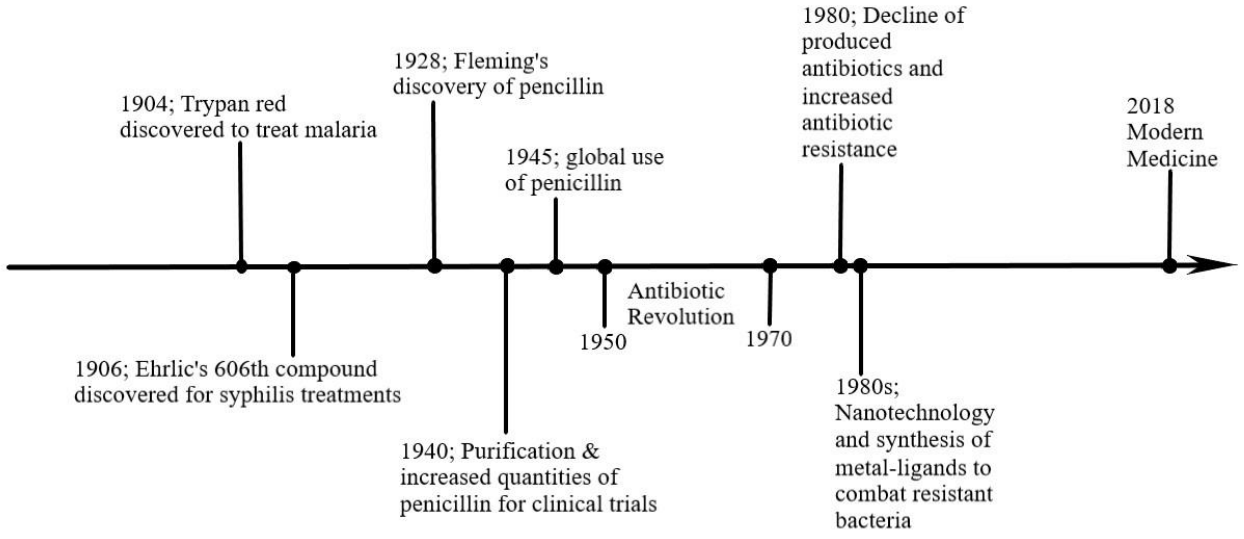

Figure 1.1: Timeline of the rise and fall of antibiotics.

Major drawbacks with existing antibiotics include low bioavailability within human physiological conditions, along with poor penetrability of the compound to the bacterial cell wall [22]. These drawbacks are also essential properties for antibiotics to hold susceptibility in treating bacterial infections and diseases, but when compromised are linked to the rise of resistant bacteria [22]. In response, research initiatives to develop nanotechnology platforms to improve properties of bioavailability, cell wall penetrability, as well as enhanced site specificity, prolonged therapeutic effects, biodegradability, biocompatibility for enhanced efficacy were of focus [24].

Formulating nanoparticles to combat antibiotic resistant bacteria aimed to enhance efficacy of existing antibiotics is one of many aspects with utilizing nanotechnology. Unique characteristics associated with nanoparticles is the ease of optimizing intrinsic physicochemical properties of the nanoparticles. Modification to nanoparticles for enhanced target site specificity, increased cellular interactions, improved drug encapsulation and release properties, while also improving solubility of poorly soluble drugs would be possible [24,25,30]. Nanoparticles used as drug delivery vehicles could vastly improve pharmacokinetic and pharmacodynamic properties to improve bioavailability for effective treatment methods against bacterial infections [22,27,30].

Nanoparticles with materials of either organic or inorganic nature also hold high influence with properties such as biodegradability, biocompatibility and antibiotic activity for enhanced efficacy [31]. Careful material selection could prove useful to provide synergistic effect the encapsulated drugs with the nanoparticle material to disrupt the formation of biofilms [23]. Biofilm is the assemblage of surface-associated microbial cells enclosed within extracellular polymeric substance matrices that provide enhanced structural and functional properties to bacteria and their cell walls [22,23,25,32]. Disruption of the biofilm could provide the edge required for antibiotics to regain efficacy and effectively treat bacterial infections [25,27,31]. Nanotechnology could provide the momentum to re-establish antibiotics as the gold standard of treatment against bacterial infections.

Chapter 2: Metal-Drug Complexes To Revive Antibiotics

Antibiotics were the epitome for revolutionizing modern medicine, yet became the downfall of medicine with the rise of drug resistant bacteria [31]. Poor initiatives in industry to synthesize and produce novel compounds led to the drying of the antibiotic pipeline [31]. Insights have turned to synthesizing compounds of currently existing drugs and complexing these drugs with a metal ion to create drug-metal complexes. Complexing of certain metals with drugs provide fresh initiatives with new mechanisms of action to combat bacterial infections is of high importance. Use of metal complexes along with nanotechnology could prove to be leading advantages in solving the issue with single-drug and multiple-drug resistant bacteria.

SECTION 2.1. SIGNIFICANCE OF METAL COMPLEXES AS ANTIBACTERIAL AGENTS

Synthesis of existing drugs complexed with metal ions to produce a drug-metal complex holds hopes of achieving three goals [33]. The first goal, would to create reversed mechanisms of microbial resistance. The second, promote development of novel compounds with action mechanisms unknown to bacteria. The third, to reduce toxicity of metal ions in form of complexes with ease of metabolic degradation [33]. Drug-metal complexes present a favorable avenue in solving the dilemma with synthesizing novel antibiotics for combating bacterial infections. Yet, understanding the mechanisms of action and biological interactions is crucial for play as efficient antibacterial agents.

Section 2.1.1. Common Metal Ions Within Biological Systems

Human biological systems are composed of 3% metal ions with various biological processes and functions [34,35]. One of the better known metals within the human system is iron (Fe), which is commonly used for cellular respiration and other biological processes and functions [36]. Other metals commonly found include sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), zinc (Zn), manganese (Mn), and cobalt (Co) (Figure 2.1) [37]. Each metal ion has various responsibilities in promoting cellular and subcellular functions. With

the help of bioinformatics, scientists determined that ~ 50% of all enzymes depend on metal ions for catalysis indicating the importance of metal ions with the promotion of life [37].

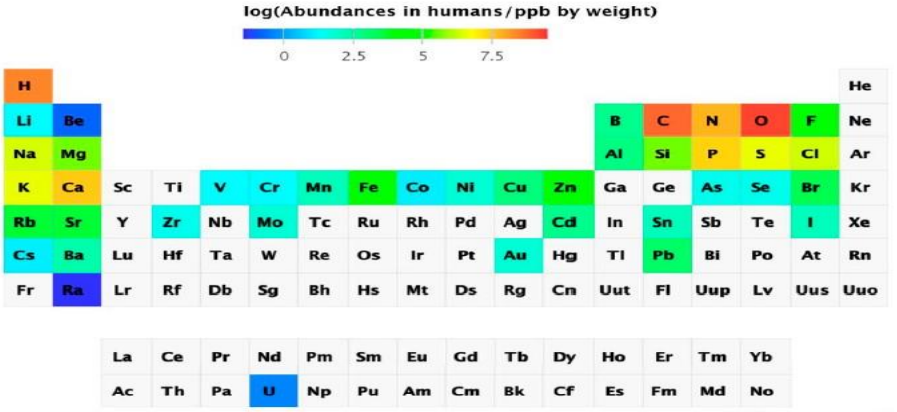

Figure 2.1: Abundance of elements found in the human biological system [37].

While the discovery of metal ions within biological systems is recent. Knowledge of metals to combat and prevent bacterial infections has been understood for centuries. Ancient Egyptians understood the use of Cu as a water sterilizer, along with Chinese and Arabic practices incorporating gold as remedies against infections [35]. Silver (Ag), is one of the earliest recorded metals to exhibit antibacterial activity [38]. Although these metals have been used for centuries to prevent or treat bacterial infections, the threat of promoting gene transferring processes resulting in metal ion resistance bacteria still exists [39].

Section 2.1.2. Drug-Metal Complexes Reviving Antibiotics

Research initiatives in recent years has increased to sought out possible solutions to the rising issue of resistant bacteria. Interestingly, metal ions ligated with drugs to yield a metal-drug complex show promise as an avenue to combat the threat of resistant bacteria [34,35,40,41]. Particularly metals with natural occurrence within biological systems, biometals, [37] due to the inherent antibacterial activity. Other metals that do not normally occur in biological systems but possessed antibacterial properties could also be investigated for activity against resistant bacteria.

Biometals complexed with various antibiotics had shown increased antibacterial activity against resistant bacteria strains [34,41,42]. Those biometals included Cu, Zn, Ni, Fe, and Co ions when complexed with various antibiotic ligands [33,34,42]. As these biometals were complexed,

enhancement of antibacterial activity for some combinations were observed. Foreign metals were also investigated as potential candidates for effective metal-drug complex combinations [41,43]. Ag exhibited the highest antibacterial activity even at low concentrations and against the other foreign metals continued to exhibit enhanced antibacterial activity even against biometals [34,43]. Because of Ag's historical background as an antibacterial agent, favored economical standards, and current applications with topical treatment was Ag determined as a viable candidate for further exploration [34,38,40,44].

SECTION 2.2. SILVER COMPLEXES AS AN ANTIBACTERIAL AGENT

For over a millennia, Ag has been utilized as an antibacterial agent as early as 4000 BC [38,40]. In 1884, silver nitrate ($AgNO_3$) was introduced and utilized for the prevention of ophthalmia neonatorum [38]. Mechanisms of action which Ag utilizes to combat bacteria occurred via various pathways. These pathways included ionic interactions inhibiting DNA replication, deactivating enzymatic activity, disruption to electron transport systems, poisoned respiratory enzymes by reactive oxygen species (ROS) (Figure 2.2) [25,31,34]. More efficient dosage forms were required, primarily to act as an antibacterial agent in treating infections and secondly to prevent local toxicity to tissues and depletion of required electrolytes [38].

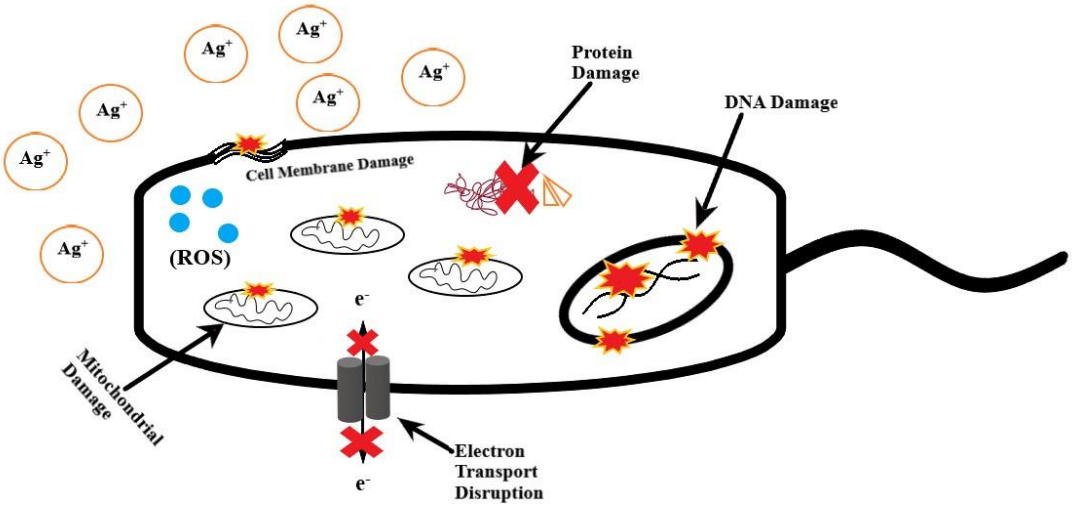

Figure 2.2: Antibacterial actions of silver (Ag) ions.

The first Ag-complexed system was introduced in 1965, specifically silver sulfadiazine (AgSD) to replace $AgNO_3$ as the standard for treating *P. aeruginosa* infections [38,40,44]. AgSD remained as the standard treatment for topical infections, but was associated with limitations. Low stability due to Ag ions complexing with other available counter ions, precipitation of the AgSD complex, and low solubility properties were some limitations [40]. Consequentially, overuse of the complex led to the evolution of AgSD resistant bacteria [44]. With AgSD compromised, this called for the investigation of novel silver complexes to combat resistant bacteria.

Research incentives focused on synthesizing various Ag-complexes with promise in becoming gold standard treatment. Most intuitively, research has focused on the synthesis of Ag complexed with ligands exhibiting synergistic effects to achieve primary goals of creating new mechanisms of inhibition against resistant bacteria. Ag had been observed to strongly bind to ligands with phosphorus and sulfur donors along with nitrogen and oxygen which could yield stable complexes [38]. With strongly stable complexes, enhanced antibacterial activities could be observed due to synergistic actions from both constituents. Although effective, this feat sacrificed solubility for synergy and stability.

With the growing threat of resistant bacteria, it has become imperative that research surrounding metal-drug complex synthesis not only aims to produce complexes but extends to achieve all properties required for viable antibacterial agents. This would include improved solubility, complex stability, synergistic activity, thermodynamic stability, and enhanced de-/ligation kinetics. Acquiring all said requirements could produce a viable metal-drug complex with enhanced antibacterial efficacy to eradicate the threat of resistant bacteria.

SECTION 2.3. SILVER-FUROSEMIDE COMPLEX AS NOVEL ANTIBACTERIAL

Recently, a novel Ag-complex with potential to act as an antibacterial agent was synthesized. Particularly, the ligand of choice for complexation was not a former antibiotic agent. Rather, the drug Furosemide (FSE) was chosen for complexing with Ag to form Ag-FSE [45]. In normal practices, FSE is a sulfonylamide compound that belongs to a class of diuretics [46].

Although FSE would not normally be used for applications of bacterial treatment, it was observed that FSE exhibited slight antibiofilm activity [45]. FSE's antibiofilm activity is a vitality with potential to cause a paradigm shift in the fight against resistant bacteria. Particularly, the antibiofilm activity with FSE could be an advantageous property as FSE is not conventionally utilized for antibacterial treatment. Thus, complexing FSE with a known antibacterial agent, Ag, for synergistic effects could effectively treat bacterial infections of both resistant and non-resistant in nature.

When complexing the Ag to FSE, it was observed that a hydrogen donation from the carboxylic group of FSE occurred [45]. The conjugated Ag ion promoted a carbon shift to an oxygen which resulted in a geometric spacing in the yielded product to be thermodynamically stable resulting in high complex stability [45]. The increased complex stability arose due to reinforced coordination of FSE to Ag via the carboxylate group [45] which allowed for thermodynamically favorable bonding. Yet, this high stability also resulted with poor solubility properties of the complex.

The Ag-FSE complex was observed to be insoluble in water and other organic solvents, but remained highly soluble in dimethyl sulfoxide (DMSO) [45]. Ag-FSE's insolubility ultimately translated into poor permeability properties to effectively inhibit bacterial cell propagation [47]. The poor solubility properties of the Ag-FSE complex could also potentially cause hinderance on the deligation kinetics of Ag to appropriately act on the bacterial cells [45,47]. Herein, nanotechnology initiatives could prove advantageous with encapsulation of the Ag-FSE complex into a drug delivery vehicle such as a nanoparticle. Encapsulation of Ag-FSE could result with enhanced intrinsic complex properties leading to improved antibacterial efficacy with enhanced solubility, controlled/sustained drug release, and appropriate nanoparticle characterization.

Chapter 3: Encapsulation of Silver-Furosemide Complex with Formulated Chitosan Nanoparticles

With increasing challenges in antibiotic therapy, scientists are exploring novel combinations of antibiotics, excipients and drug delivery carriers. These novel antibiotic drug delivery systems are expected to be less susceptible to bacterial resistance. In addition to novel drug delivery systems targeting bacterial infections, another approach is the complexation of antimicrobials with silver (Ag), an antibacterial metal. These antibacterial metal complexes can be further developed into nanoparticulate formulation for sustained and targeted release. Silver (Ag) exhibits antibacterial activity via multiple mechanism of actions making it difficult for bacteria to develop resistance [48]. Previously known complexes include silver sulfadiazine and clotrimazole silver complex [38,49]. A complex of silver and furosemide (Ag-FSE) has been developed as an antibacterial agent [45]. The poor solubility problem associated with Ag-FSE can be resolved using nano drug delivery systems such as nanosuspensions, nano micelles, liposomes and nanoparticles [50,51]. In recent years, chitosan has been widely used in the areas of bioengineering, biopharmaceutical, and nanomedicine fields for the development of efficient drug delivery systems [52]. CS is a synthetically produced biopolymer through a deacetylation process of a chitin, a naturally occurring biopolymer in exoskeletons of insects, crustaceans, and fungi [53]. CS is biocompatible, biodegradable and tunable for drug encapsulation and controlled drug release. Considering the advantages with CS, we hypothesize that chitosan nanoparticles loaded with Ag-FSE can improve solubility and antibacterial activity. The formulated of Ag-FSE-CSNPs were evaluated for size, zeta potential (ZP), polydispersity index (PDI), encapsulation efficiency (EE%), antibacterial activity and stability.

SECTION 3.1. MATERIALS

Furosemide was purchased from Acros Organics (New Jersey, USA). Chitosan, 85% deacetylated powder was procured from Alfa Aesar (Ward Hill, MA). Silver nitrate ($AgNO_3$), potassium hydroxide (KOH), sodium triphosphate (TPP), dimethyl sulfoxide (DMSO), buffer

salts, acetic acid and methanol (ACS grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water used in experiments was obtained from Milli-Q® IQ 7000 Ultrapure Water System (Millipore, Bedford, MA, USA). Dialysis tubing with MWCO of 14,400 was procured from Sigma-Aldrich (St. Louis, MO, USA). Mueller-Hinton broth (CM0405) was obtained from Oxoid Microbiology Products (USA), bacterial strains were purchased from the American Type Culture Collection (ATCC), and sterile microtiter plates were procured from Fisher Scientific (Fair Lawn, NJ, USA).

SECTION 3.2. METHODS

Section 3.2.1. Synthesis of Ag-FSE complex

Synthesis of the Ag-FSE complex was performed as reported by Lustri *et al.* 2017 with minor modifications to the procedure (Figure 3.1) [45]. Briefly, an aqueous solution (2 ml) of $AgNO_3$ (2.13 mmol, 0.362 g) was added dropwise into a methanolic solution (20 ml) of furosemide (0.704 g, 2.13 mmol) and KOH (0.119 g, 2.13 mmol) with stirring. This reaction mixture was then subjected to continuous stirring at room temperature for 2h and a final product of white precipitate was collected by filtration. The white precipitate of Ag-FSE Complex was then washed with cold water three times and dried in a desiccator overnight under negative pressure for 24 h.

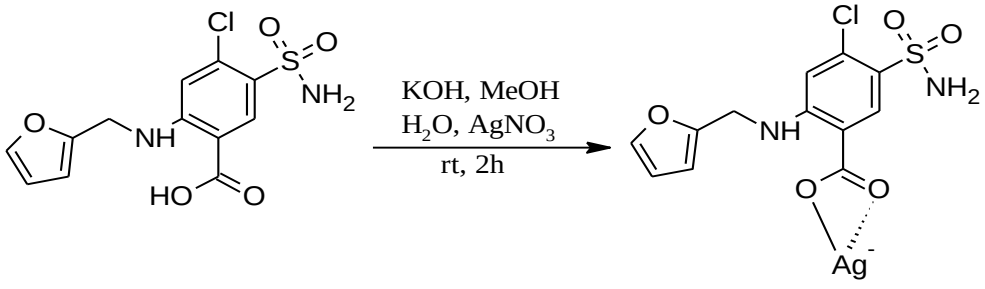

Figure 3.1. Synthesis of Ag-FSE complex.

Section 3.2.2. Preparation of CSNPs

CSNPs were formulated by ionic gelation technique as described by Kalhapure *et al.* 2017 with slight modifications, for both blank and Ag-FSE loaded nanoparticles [54]. For the preparation of blank CSNPs, CS solution (10 ml) (1% w/v in 1% acetic acid) was kept under stirring at 1000 rpm for 10 minutes followed by dropwise addition of 0.1% w/v TPP solution (20 ml) under continuous stirring. The resultant solution was kept under stirring for 5 minutes after the addition of all TPP solution. The pH of the final solution was adjusted to 5.5 with 0.5 M NaOH. Following this, the formulation was sonicated at 30% amplitude using probe sonicator (Fisher scientific^{TM} Model 505 Sonic Dismembrator) in an ice bath. Thereafter, the formulation was stirred at 1000 rpm for 20 minutes in the dark. Ag-FSE loaded CSNPs were prepared using the same method as blank CSNPs. A weighed amount of the Ag-FSE complex was added into the TPP solution (20 ml) and then subjected to probe sonication at 30% amplitude. During sonication the solution was kept in an ice bath to maintain room temperature. The homogenous Ag-FSE-TPP solution was then added dropwise into CS solution (10 ml) and processed further as the blank CSNPs to obtain Ag-FSE loaded CSNPs.

Section 3.2.3. Determination of size, PDI, and ZP

The size, PDI, and ZP analysis of the CSNPs was performed using dynamic light scattering (DLS) technique. CSNPs (100 µl) were dispersed in deionized water (10 ml) and measurements were performed with a Zetasizer Nano ZS90 (Malvern Zetasizer Nano ZS90, Malvern Instrument Ltd., UK) at 25℃ and 90 ^{o} scattering angle.

Section 3.2.4. Morphology Studies

Single drop of dilute dispersion of Ag-FSE CSNPs were placed on gold grids, with excess of liquid dispersion on gold grid been wicked using a piece of filter paper. The grid was kept for drying in a sterile fume hood for two days. The sample grid was then transferred into the STEM (Hitachi HD-2300A) where images were recorded in transmission electron microscopy (TEM) mode at 200kV.

Section 3.2.5. Drug encapsulation efficiency (EE%)

Drug loaded Ag-FSE CSNP encapsulation efficiency was determined using a modified ultrafiltration technique [55]. Ag-FSE loaded CSNPs (500 µl) were pipetted into Ultracel® centrifugal filter units with a MWCO of 10 kDa. These samples were then centrifuged at 10,000 rpm (accuSpin Micro 17R, Fisher Scientific, USA) for 30 minutes at 20 °C allowing for the separation of unentrapped drug from encapsulated drug. After centrifugation, filtrate was collected and 300 µl was diluted to 3 ml with milli-Q water and analyzed using UV-spectrophotometer (UV-vis) (Shimadzu UV-1800, Japan) at λmax = 277 nm. The regression equation used for calculations was $y = 0.0486x + 0.0135$ with $R^2 = 0.9995$. EE% of the CSNPs was determined using Equation 3.1.

$$EE\% = (\text{Total drug} - \text{Free drug})/ (\text{Total drug}) \times 100$$

Equation 3.1. EE% of Ag-FSE complex with CSNPs.

Section 3.2.6. X-ray diffraction (XRD)

XRD analysis of the Ag-FSE CSNPs was performed using PANanlytical's X-ray diffractometer (PANanlytical's X'pert Pro Tokyo, Japan) equipped with X'Celerator high speed detector. XRD studies were performed for CS, TPP, Ag-FSE and Ag-FSE CSNPs. Powdered samples were placed on an aluminum sample holder and uniformly packed using glass slide. Radiation source was CuKα operated at 45 kV and 40 mA. All the measurements were recorded with continuous scanning mode over a 2θ range of 5^o to 70^o.

Section 3.2.7. Differential scanning calorimetry (DSC)

DSC analysis for Ag-FSE CSNPs and CS was performed using DSC Q20 instrument with TA universal analysis software to obtain scans. Samples (5 -10 mg) were placed in aluminum pans and sealed. DSC analysis was performed at a heating rate of 10 ^{o}C/min over a range of 40 ^{o}C to 300 ^{o}C with a nitrogen flow of 20 ml/min.

Section 3.2.8. In vitro drug release studies

Drug release behavior from the CSNPs and free drug (Ag-FSE) in phosphate buffer saline (PBS) (pH 7.4, 6.5 and 5.5) was evaluated using the dialysis bag method (MW 8000 – 14,400 Da) [56,57]. Briefly, 3 ml of Blank CSNPs, Ag-FSE and Ag-FSE CSNPs in PBS were transferred into separate dialysis bags. These dialysis bags were placed in conical tubes (50 ml capacity) containing 30 ml of PBS as the release medium (pH 7.4, 6.5 and 5.5) maintained at 37 ± 0.5 ^{o}C in a shaking water bath (100 rpm). At a pre-determined time-intervals, 3 ml of the samples were collected from the conical tubes and replaced with 3 ml of fresh PBS to maintain sink conditions. Pre-determined time intervals were 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24h. The amount of drug released from the formulations at different timepoints was quantified using a UV-visible spectrophotometer (Shimadzu UV 1800, Japan) at λmax = 277 nm. All the experiments were performed in triplicate.

Section 3.2.9. In vitro antibacterial activity

Ag-FSE loaded CSNPs and free Ag-FSE were evaluated for antibacterial activity against *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) using a broth microdilution method. Colonies of each bacteria were grown overnight in Muller-Hinton broth (MHB) in an incubator at 37 °C. Single bacterial culture colonies of each bacteria were suspended in MHB and further diluted with MHB until a cell density of 0.5 Mcfarland for each bacterium was attained. Diluted bacterial suspensions (100µL) were transferred into 96-well plates. Upon seeding with designated bacteria, serial dilutions of 100 µL of Ag-FSE CSNPs, blank CSNPs, free Ag-FSE in 10% w/v DMSO, MHB, and plain 10% DMSO were performed. Plates were incubated overnight at 37 °C and minimum inhibitory concentration (MIC) was assessed as the concentration at which no bacterial growth was observed. All experiments were completed in triplicate.

Section 3.2.10. Stability studies

Three batches of Ag-FSE CSNPs were prepared under optimum conditions and their size, PDI and ZP were analyzed. Thereafter, the samples were stored at 25 °C and 4 °C conditions.

Stability was assessed using DLS analysis for four months (120 days). The parameters monitored to assess the stability were physical appearance, size, PDI and ZP.

Section 3.2.11. Statistical analysis

Statistical analysis was performed using t-test followed by Bonferroni's multiple comparison test using GraphPad Prism® (Graph Pad Software Inc., Version 5.0, San Diego, CA). A p-value < 0.05 was considered statistically significant.

SECTION 3.3. RESULTS & DISCUSSION

Section 3.3.1. CSNP Characterization

Section 3.3.1.1. Effect of sonication time on blank and Ag-FSE CSNPs

Blank CSNPs and Ag-FSE CSNPs were formulated by ionic gelation technique. Effect of sonication time on the size, PDI and ZP of CSNPs was studied. Table 3.1 and Figure 3.2 summarizes the effect of sonication time (3, 5, 10 and 20 minutes at 30% amplitude) on size, PDI and ZP on blank CSNPs. Size, PDI and ZP of CSNPs ranged from 204.8 ± 4.428 to 472.1 ± 42.81, 0.195 ± 0.023 to 0.517 ± 0.107, and 36.0 ± 2.54 to 44.1 ± 2.21 respectively. It was observed that with increasing sonication time there was a significant decrease in particle size from 3 to 10 minutes, however, at 10 minutes and 20 minutes there was no significant difference (p > 0.05) in particle size. Thus, 10 minutes of sonication was considered as optimum sonication time for preparing CSNPs with < 300 nm size, low PDI and high ZP. Low PDI and high ZP of CSNPs suggests that the system was monodispersed and electrostatically stable. Similar results were observed by Tang et.al where increase in sonication time caused cleaving free unpolymerized chitosan, reducing particle size and enhancing monodispersity of the system [58].

Table 3.1: Effects of sonication time on size, PDI, and ZP of CSNPs.

Time (minutes)	Size (nm)	PDI	ZP (mV)
3	344.6 ± 98.96	0.517 ± 0.107	44.1 ± 2.21
5	472.1 ± 42.81	0.507 ± 0.139	42.9 ± 3.86
10	261.3 ± 12.23	0.195 ± 0.023	42.8 ± 1.31

20	204.8 ± 4.428	0.205 ± 0.019	36.0 ± 2.54

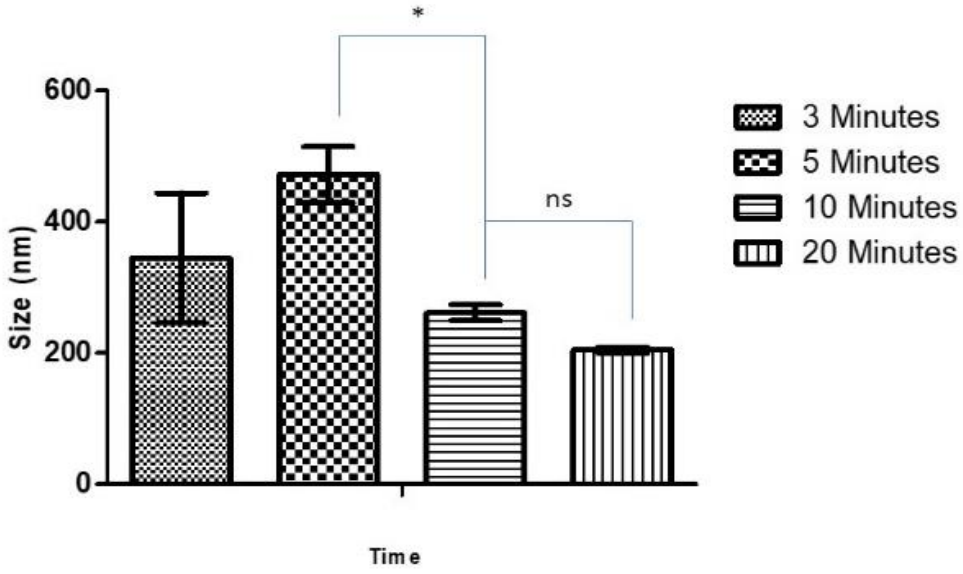

Figure 3.2: Effect of sonication time on size of blank CSNP; *significant difference with CSNP size at 5- and 10-minute sonication ($p < 0.05$); ns, non-significant.

After optimizing the sonication time for blank CSNPs, the effect of sonication time on the size of Ag-FSE CSNPs was studied. Initially, 5 mg Ag-FSE was loaded into the CSNPs and analyzed for differences in size, PDI, and ZP with respect to 5 and 10-minute sonication times. Effect of sonication time on the size, PDI and ZP of Ag-FSE CSNP is provided in Table 3.2 and Figure 3.3. Results confirmed that there was a significant difference with respect to size between 5 minutes and 10 minutes sonication ($p < 0.05$). Therefore, 10 minutes was considered as the optimum sonication time both blank and Ag-FSE CSNPs.

Table 3.2: Effects of sonication time on CSNPs loaded with 5 mg of Ag-FSE.

Time (minutes)	**Size (nm)**	**PDI**	**ZP (mV)**
5	481.4 ± 28.95	0.520 ± 0.100	42.2 ± 2.44
10	290.3 ± 162.6	0.395 ± 0.222	35.5 ± 1.52

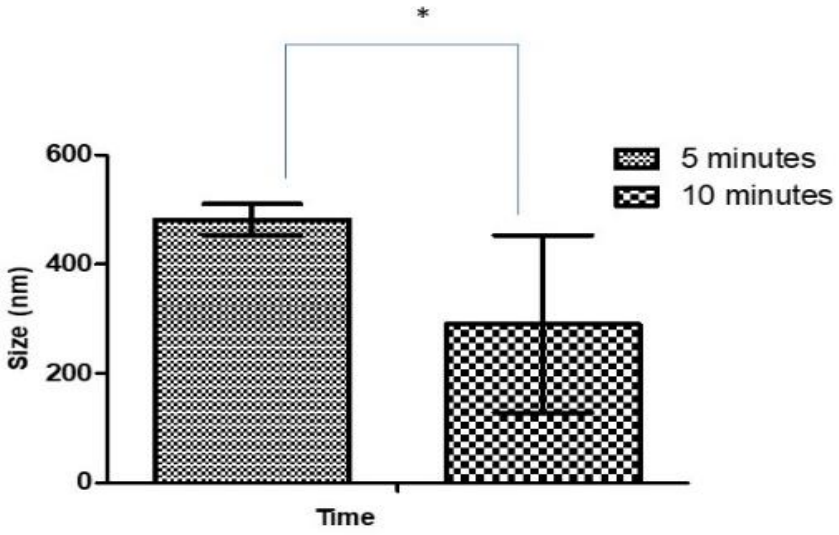

Figure 3.3: Effect of sonication time on size of Ag-FSE CSNPs (5mg). *significant difference with CSNP sizes ($p < 0.05$).

Section 3.3.1.2. Effect of drug loading on size, PDI, and ZP of Ag-FSE CSNPs

After the preliminary studies, 10 minutes was considered the optimum sonication time for the formulation of Ag-FSE CSNPs. Effect of increased drug loading (10 mg) on the size, PDI and ZP of Ag-FSE was studied and summarized in Table 3.3 and Figure 3.4. Interestingly, it was found that increase in Ag-FSE loading from 5 mg to 10 mg resulted in CSNPs with significantly smaller particle size ($p < 0.05$). Size, PDI and ZP of 10 mg loaded Ag-FSE CSNPs was 216.6 ± 26.80 nm 0.194 ± 0.021 and 25.8 ± 3.05 mV respectively. The low PDI values and high ZP indicate that under 10-minute sonication with 10 mg loaded into the CSNPs continued to exhibit monodispersity and electrostatic stability. With higher dosage loading the size of the CSNPs will remain optimal with < 300 nm sizes as no significant differences ($p > 0.05$) was observed with size being affected from higher dosage.

Table 3.3: Effects of drug loading on size, PDI, and ZP of CSNPs.

Ag-FSE loading (mg)	Size (nm)	PDI	ZP (mV)
5	290.3 ± 162.6	0.395 ± 0.222	35.5 ± 1.52
10	216.6 ± 26.80	0.194 ± 0.021	25.8 ± 3.05

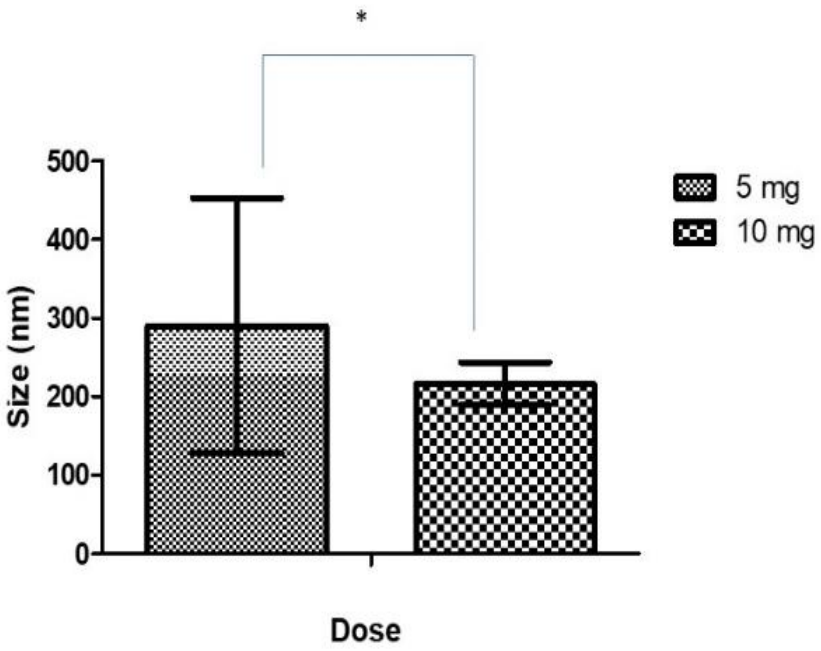

Figure 3.4: Effect of drug loading on size of CSNPs. *significant difference between 5 mg and 10 mg loading ($p < 0.05$).

Section 3.3.2. Morphology

TEM images of the Ag-FSE CSNPs indicated that the nanoparticles were globular in shape (Figure 3.5). Analysis of the Ag-FSE CSNP morphology indicated that a decrease in particle size occurred when compared to DLS results due to dehydration of the particles when undergoing drying on the gold grids for preparation of TEM analysis [59].

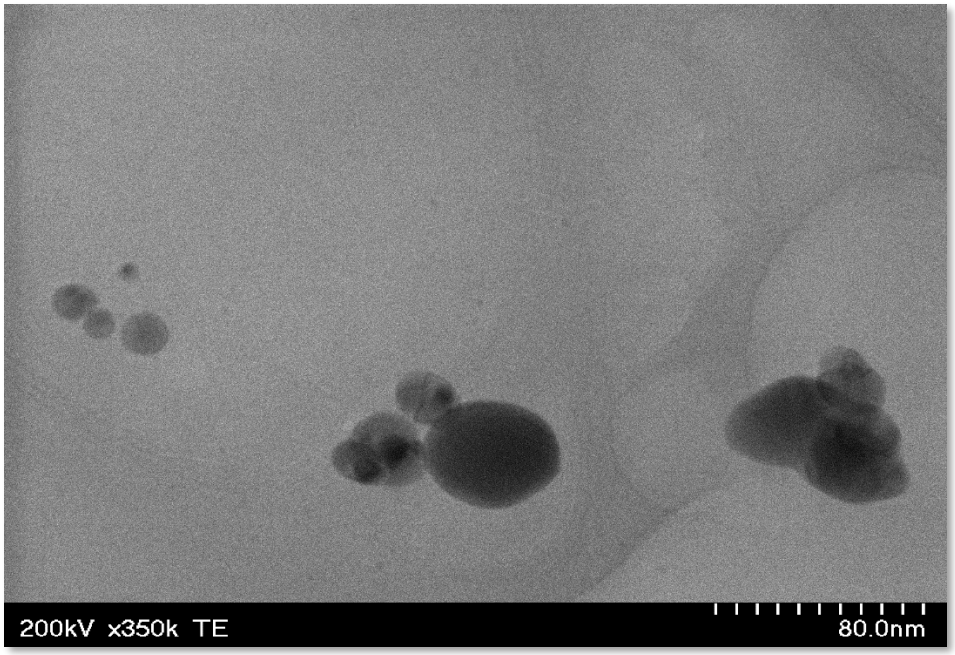

Figure 3.5: TEM images of Ag-FSE loaded CSNPs, nanoparticles appeared globular in shape.

Section 3.3.3. Drug encapsulation efficiency (EE%)

Ag-FSE CSNPs showed high encapsulation with EE% of 66.72 ± 4.14 %. The high EE% for Ag-FSE could be attributed to the electrostatic nature of the two constituents. CS being cationic while Ag-FSE complex holds an anionic nature, allowing the polymerization of CS and formulation of the nanoparticles with loaded drug [60].

Section 3.3.4. XRD

As silver complexes inherently reported to have poor aqueous solubility [38], it is crucial to study the crystalline nature of the formulation. Crystalline nature of the drug can affect the solubility, stability and bioavailability. XRD was performed for Ag-FSE, CS, and Ag-FSE CSNPs to confirm the crystalline nature. Results showed that Ag-FSE exhibited crystalline nature with characteristic peaks at 6.18^{o}, 10.28^{o}, 18.48^{o}, 25.92^{o}, and 32.8^{o}. However, when Ag-FSE was encapsulated into CSNPs, it was observed that Ag-FSE had undergone phase transition from crystalline to amorphous state (Figure 3.6). The transition can be confirmed by absence of the

characteristic peaks of Ag-FSE in the XRD of Ag-FSE CSNP (Figure 3.6c). Similar findings were observed with other CSNP formulations [61].

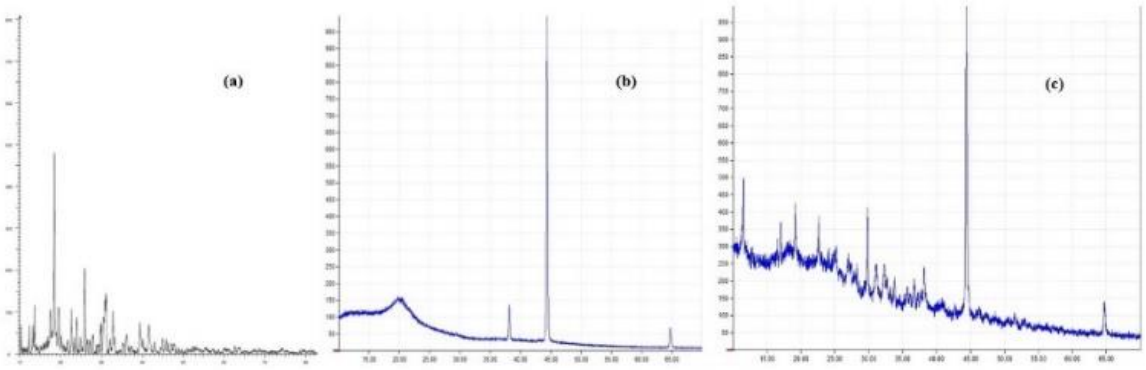

Figure 3.6: XRD results for (a) plain Ag-FSE, (b) plain CS and (c) Ag-FSE CSNPs.

Section 3.3.5. DSC

DSC evaluates the melting and crystalline behavior of drugs. Phase transition of Ag-FSE from crystalline to amorphous form was further confirmed by DSC. Results of Ag-FSE, CS and Ag-FSE CSNPs are provided in Figure 3.7. Thermograms showed that Ag-FSE had a sharp exothermic peak at 211.69 °C. However, the characteristic exothermic peak of Ag-FSE was absent in DSC of Ag-FSE CSNPs confirming the encapsulation of Ag-FSE into CSNPs.

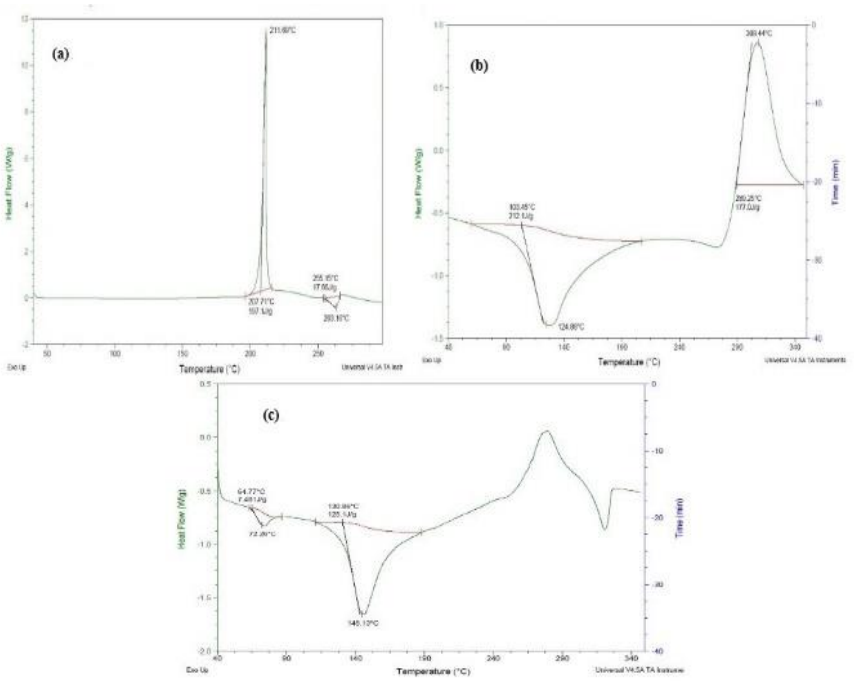

Figure 3.7: DSC results of (a) Ag-FSE, (b) CS and (c) Ag-FSE CSNPs.

Section 3.3.6. In vitro drug release studies

Ag-FSE encapsulated CSNPs showed sustained release with cumulative release of ~75% for over a period of 24 hours. In vitro drug release profiles of Ag-FSE CSNPs and free Ag-FSE are provided in Figure 3.8. As bacterial infections are associated with varying pH at the infection site, release profiles at physiological pH (7.4) and acidic pH (6.5 and 5.5) were also studied (Figure 3.9). Ag-FSE CSNPs showed sustained and controlled release over a period of 24 hours at all pH's. At pH 5.5 and 6.5, the CSNPs had an initial burst like release during the first 4 hours with a cumulative release ranging from 41.78 % – 70.57 %. This initial burst could be due to unencapsulated Ag-FSE molecules that remained along the surface of the CSNPs [62]. It was observed that at pH 5.5 and 6.5 conditions ~ 42 – 44% of Ag-FSE was released within 0.5 hours whereas, at pH 7.4 only ~ 29% release was observed in first 0.5 hours. Similar release profile was observed with CS under acidic conditions [56]. Whereas with the free Ag-FSE complex, the release was greater at all time points compared to nanoparticles (Figure 3.8). A significant

difference between encapsulated Ag-FSE with CSNPs versus free Ag-FSE drug release was observed ($p < 0.0001$).

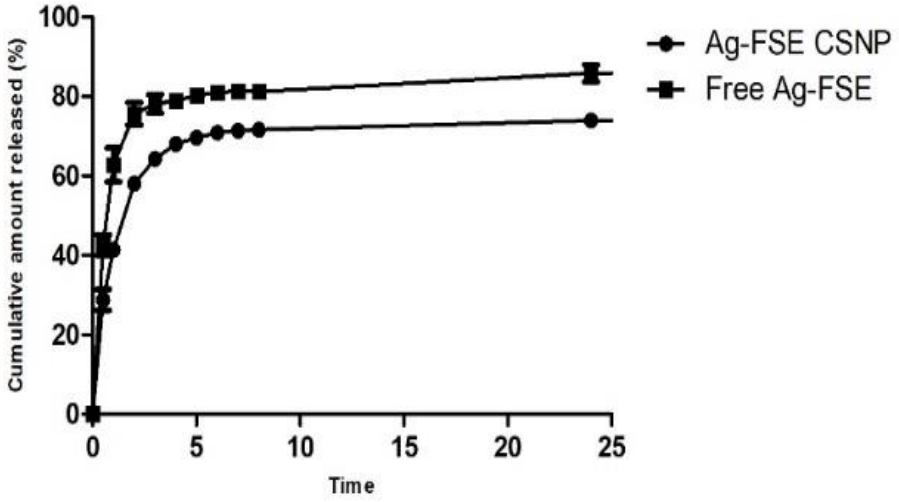

Figure 3.8: In vitro release profile of Ag-FSE in PBS and Ag-FSE CSNPs. Data is expressed in mean ± SD (n = 3).

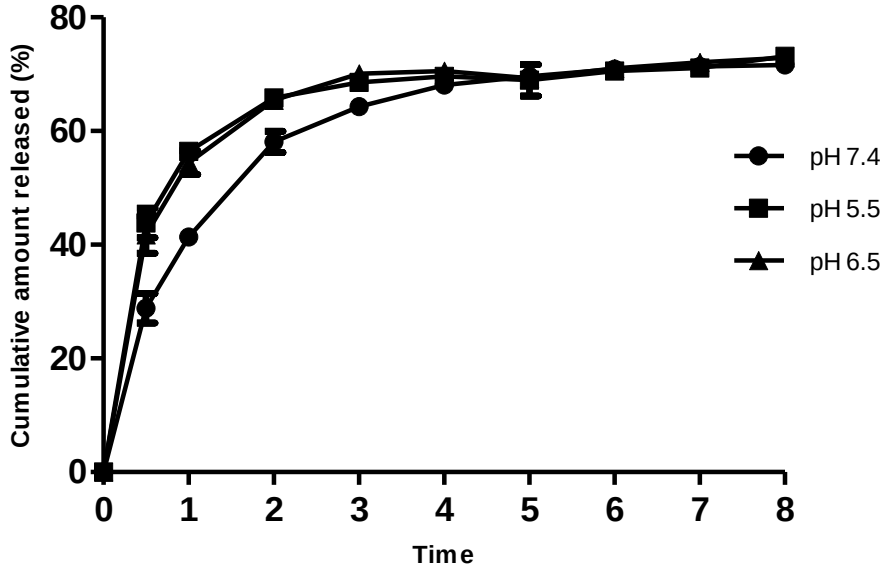

Figure 3.9: In vitro release profile of Ag-FSE CSNPs at pH 7.4, 5.5 and 6.5. Data is expressed in mean ± SD (n = 3).

Section 3.3.7. In vitro antibacterial activity

In vitro antibacterial activity was assessed by broth microdilution method. Results showed that Ag-FSE CSNPs exhibited significantly greater inhibitory activity against *E. Coli* and *S. aureus* compared to Ag-FSE. Against *E. coli*, MIC of Ag-FSE was 166.5 µg/ml whereas value for plain Ag-FSE was 500 µg/ml. There was a 6-fold enhancement in activity against *S. aureus* (Table 3.4). The MIC values suggested that the Ag-FSE loaded CSNP formulation had notably greater antibacterial activity compared to unencapsulated drug. These synergistic characteristics may be due to CS being a bioactive polymer with antibiofilm and antimicrobial properties [63]. Results confirmed that encapsulation of Ag-FSE in to CSNPs enhanced the antibacterial efficiency of Ag-FSE.

Table 3.4: Minimum Inhibition Concentration (MIC) for Blank CSNP, Ag-FSE CSNP, Ag-FSE in 10% DMSO and 10% DMSO against *E. coli* and *S. aureus* (n = 3).

Formulation	**MIC (µg/ml)**	
	E. coli	***S. aureus***
Blank CSNPs	NA	NA
Ag-FSE CSNPs	166.5	41.63
Ag-FSE in 10% DMSO	500	250
10% DMSO	NA	NA

Section 3.3.8. Stability Studies

Stability studies were performed on both 5 mg and 10 mg Ag-FSE loaded CSNPs over 120 days. Size, PDI and ZP were investigated to confirm the physical stability of CSNPs. Formulations were stored at 4 °C and 25 °C, and analyzed over the course of time (day 0, 30, 60 and 120). Stability study results for 5 mg and 10 mg Ag-FSE loaded CSNPs are provided in Table 3.5 and Table 3.6 respectively. Results showed that there was a steady decrease in the size of the 5 mg Ag-FSE loaded CSNPs from days 30 to 60. The decreased size may be due to the cationic and anionic interactions of CS and Ag causing the particle size to decrease [64]. Also, 10 mg Ag-FSE loaded

CSNPs stored at 25 °C showed slight increase in size for 4 °C. Day 120 analysis indicated an increase in particle size for both 5 mg and 10 mg Ag-FSE loaded CSNPs. The increased size may be due to particle aggregation or swelling caused by changes in environmental conditions (temperature, pH, ionic strength) [65]. The overall difference in size, PDI and ZP of all the samples at different time points was not significant ($p > 0.05$). Therefore, both the formulations can be considered stable at 25 °C and 4 °C for 120 days.

Table 3.5: Effect of storage on particle size, PDI and ZP of 5 mg loaded CSNPs for 30, 60 and 120 days at 4 °C and 25 °C.

Storage Time	Size (nm)		PDI		ZP (mV)	
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
Day 0	290.3 ± 162.6	290.3 ± 162.6	0.395 ± 0.222	0.395 ± 0.222	35.5 ± 1.52	35.5 ± 1.52
Day 30	239.8 ± 9.877	241.5 ± 5.568	0.224 ± 0.023	0.191 ± 0.021	33.7 ± 1.70	35.6 ± 1.08
Day 60	229.7 ± 3.167	232.0 ± 11.19	0.217 ± 0.008	0.242 ± 0.018	39.0 ± 2.09	35.5 ± 8.90
Day 120	296.8 ± 2.769	331.8 ± 16.11	0.236 ± 0.014	0.288 ± 0.038	29.8 ± 2.03	30.7 ± 0.66

Table 3.6: Effect of storage on particle size, PDI and ZP of 10 mg loaded CSNPs for 30, 60 and 120 days at 4 °C and 25 °C.

Storage Time	Size (nm)		PDI		ZP (mV)	
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
Day 0	216.6 ± 26.80	216.6 ± 26.80	0.194 ± 0.021	0.194 ± 0.021	25.8 ± 3.05	25.8 ± 3.05
Day 30	224.6 ± 13.78	176.5 ± 10.49	0.217 ± 0.025	0.272 ± 0.030	30.5 ± 11.1	30.3 ± 5.45
Day 60	217.4 ± 5.276	207.3 ± 9.907	0.232 ± 0.017	0.248 ± 0.018	37.3 ± 3.79	36.2 ± 6.61
Day 120	324.8 ± 50.74	321.7 ± 33.53	0.587 ± 0.162	0.407 ± 0.021	39.7 ± 1.92	37.3 ± 2.40

Conclusions

In the present study we successfully encapsulated a novel antibacterial complex, Ag-FSE, into CSNPs for enhancement in its antibacterial efficacy. The size of formulated Ag-FSE CSNPs was < 300 nm with high %EE of ~ 66 %. Ag-FSE CSNPs were globular in shape with smooth surfaces. XRD and DSC confirmed the transition of Ag-FSE from crystalline to amorphous form. Ag-FSE CSNPs showed significant antibacterial activity against *E. Coli* (Gram negative) and *S. aureus* (Gram positive). Stability studies indicated that the Ag-FSE CSNPs at 4 °C and 25 °C conditions remained stable over 120 days. Further exploration of developed CSNPs with in depth in vivo studies could lead to its further transition to clinical trials.

References

[1] Cooper GM, The Origin and Evolution of Cells, in: Cell A Mol. Approach, 2nd ed., Sunderland (MA): Sinauer Associates, 2000. https://www.ncbi.nlm.nih.gov/books/NBK9841/.

[2] J.R. Sistrunk, K.P. Nickerson, R.B. Chanin, D.A. Rasko, C.S. Faherty, Survival of the fittest: How bacterial pathogens utilize bile to enhance infection, Clin. Microbiol. Rev. 29 (2016) 819–836. doi:10.1128/CMR.00031-16.

[3] Bacteria and Infections, (n.d.). https://www.reactgroup.org/toolbox/understand/bacteria/bacteria-and-disease/ (accessed September 3, 2018).

[4] M. Clinic, Infectious Diseases, (n.d.). https://www.mayoclinic.org/diseases-conditions/infectious-diseases/symptoms-causes/syc-20351173 (accessed September 1, 2018).

[5] C. for D.C. and Prevention, History of the Plague, (n.d.). https://www.cdc.gov/plague/history/index.html (accessed September 5, 2018).

[6] B. Waggoner, Antony van Leeuwenhoek (1632-1723), (n.d.). http://www.ucmp.berkeley.edu/history/leeuwenhoek.html (accessed September 7, 2018).

[7] R. Hajar, History of Medicine Timeline, Hear. Views. (2015) 43–45. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4379645/ (accessed September 5, 2018).

[8] V.S. Governments, Infections- bacterial and viral, (2014). https://www.betterhealth.vic.gov.au/health/conditionsandtreatments/infections-bacterial-and-viral (accessed September 9, 2018).

[9] Common Causes of Infectious Diseases, (2017). https://www.atsu.edu/faculty/chamberlain/Website/common.htm (accessed September 9, 2018).

[10] W.A. Adedeji, the Treasure Called Antibiotics., Ann. Ibadan Postgrad. Med. 14 (2016) 56–57. http://www.ncbi.nlm.nih.gov/pubmed/28337088%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5354621.

[11] J. Parascandola, The History of antibiotics: a symposium, (1980) 136. http://books.google.co.uk/books/about/The_History_of_antibiotics.html?id=_NRsAAAAMAAJ&pgis=1.

[12] R.I. Aminov, A brief history of the antibiotic era: Lessons learned and challenges for the future, Front. Microbiol. 1 (2010) 1–7. doi:10.3389/fmicb.2010.00134.

[13] K. Gould, Antibiotics: From prehistory to the present day, J. Antimicrob. Chemother. 71

(2016) 572–575. doi:10.1093/jac/dkv484.

[14] F. Bosch, L. Rosich, The contributions of paul ehrlich to pharmacology: A tribute on the occasion of the centenary of his nobel prize, Pharmacology. 82 (2008) 171–179. doi:10.1159/000149583.

[15] G.F. Gensini, A.A. Conti, D. Lippi, The contributions of Paul Ehrlich to infectious disease, J. Infect. 54 (2007) 221–224. doi:10.1016/j.jinf.2004.05.022.

[16] Case Studies in Public Health_Robert Koch and Paul Ehrlich_2018, (n.d.).

[17] A. Ciechanover, The future of medicine, Tedx Talks. (2017). https://www.youtube.com/watch?v=ZYAgNpVZLRc (accessed September 10, 2018).

[18] S.Y. Tan, Y. Tatsumura, Alexander Fleming (1881–1955): Discoverer of penicillin, Singapore Med. J. 56 (2015) 366–367. doi:10.11622/smedj.2015105.

[19] H.B. Woodruff, Selman A. Waksman, winner of the 1952 nobel prize for physiology or medicine, Appl. Environ. Microbiol. 80 (2014) 2–8. doi:10.1128/AEM.01143-13.

[20] J. Davies, Where have all the antibiotics gone?, Can. J. Infect. Dis. Med. Microbiol. 17 (2006) 287–290. doi:10.1155/2006/707296.

[21] J. Daves, Antibiotic discovery: Then and now, Microbiol. Today. 39 (2012) 200–203. doi:10.1007/978-1-4614-1400-1.

[22] M.H. Xiong, Y. Bao, X.Z. Yang, Y.H. Zhu, J. Wang, Delivery of antibiotics with polymeric particles, Adv. Drug Deliv. Rev. 78 (2014) 63–76. doi:10.1016/j.addr.2014.02.002.

[23] R.Y. Pelgrift, A.J. Friedman, Nanotechnology as a therapeutic tool to combat microbial resistance, Adv. Drug Deliv. Rev. 65 (2013) 1803–1815. doi:10.1016/j.addr.2013.07.011.

[24] R.S. Kalhapure, N. Suleman, C. Mocktar, N. Seedat, T. Govender, Nanoengineered drug delivery systems for enhancing antibiotic therapy, J. Pharm. Sci. 104 (2015) 872–905. doi:10.1002/jps.24298.

[25] L. Wang, C. Hu, L. Shao, The antimicrobial activity of nanoparticles: Present situation and prospects for the future, Int. J. Nanomedicine. 12 (2017) 1227–1249. doi:10.2147/IJN.S121956.

[26] C. for D.C. and Prevention, Antibiotic/Antimicrobial Resistance, (n.d.). https://www.cdc.gov/drugresistance/index.html (accessed July 19, 2018).

[27] A.J. Huh, Y.J. Kwon, "Nanoantibiotics": A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era, J. Control. Release. 156 (2011) 128–145. doi:10.1016/j.jconrel.2011.07.002.

[28] X. Zhu, A.F. Radovic-Moreno, J. Wu, R. Langer, J. Shi, Nanomedicine in the management of microbial infection - Overview and perspectives, Nano Today. 9 (2014) 479–498. doi:10.1016/j.nantod.2014.06.003.

[29] S.T. Khan, A.A. Al-Khedhairy, Metals and Metal Oxides: Important Nanomaterials With Antimicrobial Activity, Elsevier Inc., 2017. doi:10.1016/B978-0-323-52733-0.00008-2.

[30] S.J. Lam, E.H.H. Wong, C. Boyer, G.G. Qiao, Antimicrobial polymeric nanoparticles, Prog. Polym. Sci. 76 (2018) 40–64. doi:10.1016/j.progpolymsci.2017.07.007.

[31] B. Das, S. Patra, Antimicrobials: Meeting the Challenges of Antibiotic Resistance Through Nanotechnology, Elsevier Inc., 2017. doi:10.1016/B978-0-323-46152-8.00001-9.

[32] R.M. Donlan, Biofilms: Microbial life on surfaces, Emerg. Infect. Dis. 8 (2002) 881–890. doi:10.3201/eid0809.020063.

[33] A.F. Santos, D.F. Brotto, L.R.V. Favarin, N.A. Cabeza, G.R. Andrade, M. Batistote, A.A. Cavalheiro, A. Neves, D.C.M. Rodrigues, A. dos Anjos, Study of the antimicrobial activity of metal complexes and their ligands through bioassays applied to plant extracts, Brazilian J. Pharmacogn. 24 (2014) 309–315. doi:10.1016/j.bjp.2014.07.008.

[34] M. Rizzotto, Metal Complexes as Antimicrobial Agents, A Search Antibact. Agents. (2012). doi:10.5772/45651.

[35] J.A. Obaleye, A.C. Tella, M.O. Bamigboye, Metal Complexes as Prospective Antibacterial Agents, Intech Open. 2 (2012) 64. doi:10.5772/32009.

[36] M.R. Laret, Hemoglobin and Functions of Iron, Univ. Calif. San Fr. Med. Cent. (2016) 1. https://www.ucsfhealth.org/education/hemoglobin_and_functions_of_iron/.

[37] W. Maret, The metals in the biological periodic system of the elements: Concepts and conjectures, Int. J. Mol. Sci. 17 (2016) 1–8. doi:10.3390/ijms17010066.

[38] J.L. Clement, P.S. Jarrett, Antibacterial Silver, (1869) 467–482. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2364932/pdf/MBD-01-467.pdf.

[39] K. Poole, At the Nexus of Antibiotics and Metals: The Impact of Cu and Zn on Antibiotic Activity and Resistance, Trends Microbiol. 25 (2017) 820–832. doi:10.1016/j.tim.2017.04.010.

[40] X. Liang, S. Luan, Z. Yin, M. He, C. He, L. Yin, Y. Zou, Z. Yuan, L. Li, X. Song, C. Lv, W. Zhang, Recent advances in the medical use of silver complex, Eur. J. Med. Chem. 157 (2018) 62–80. doi:10.1016/j.ejmech.2018.07.057.

[41] V. Uivarosi, Metal complexes of quinolone antibiotics and their applications: An update, Molecules. 18 (2013) 11153–11197. doi:10.3390/molecules180911153.

[42] M. Selvaganapathy, N. Raman, Pharmacological Activity of a Few Transition Metal

Complexes: A Short Review, J. Chem. Biol. Ther. 01 (2016) 1–17. doi:10.4172/2572-0406.1000108.

[43] J.S. Möhler, T. Kolmar, K. Synnatschke, M. Hergert, L.A. Wilson, S. Ramu, A.G. Elliott, M.A.T. Blaskovich, H.E. Sidjabat, D.L. Paterson, G. Schenk, M.A. Cooper, Z.M. Ziora, Enhancement of antibiotic-activity through complexation with metal ions - Combined ITC, NMR, enzymatic and biological studies, J. Inorg. Biochem. 167 (2017) 134–141. doi:10.1016/j.jinorgbio.2016.11.028.

[44] L. Balogh, D.R. Swanson, D.A. Tomalia, G.L. Hagnauer, A.T. McManus, Dendrimer-Silver Complexes and Nanocomposites as Antimicrobial Agents, Nano Lett. 1 (2001) 18–21. doi:10.1021/nl005502p.

[45] B.C. Lustri, A.C. Massabni, M.A.C. Silva, F.A. Resende Nogueira, R. Aquino, A.C. Amaral, A.C. Massabni, H. da Silva Barud, Spectroscopic characterization and biological studies in vitro of a new silver complex with furosemide: Prospective of application as an antimicrobial agent, J. Mol. Struct. 1134 (2017) 386–394. doi:10.1016/j.molstruc.2016.12.056.

[46] American Society of Health-System Pharmacists, Furosemide: MedlinePlus Drug Information, (2017). https://medlineplus.gov/druginfo/meds/a682858.html (accessed September 25, 2018).

[47] J. Renukuntla, FSE–Ag complex NS: preparation and evaluation of antibacterial activity, IET Nanobiotechnology. (2018) 1–5. doi:10.1049/iet-nbt.2017.0284.

[48] A.A. Keller, A.S. Adeleye, J.R. Conway, K.L. Garner, L. Zhao, G.N. Cherr, J. Hong, J.L. Gardea-Torresdey, H.A. Godwin, S. Hanna, Z. Ji, C. Kaweeteerawat, S. Lin, H.S. Lenihan, R.J. Miller, A.E. Nel, J.R. Peralta-Videa, S.L. Walker, A.A. Taylor, C. Torres-Duarte, J.I. Zink, N. Zuverza-Mena, Comparative environmental fate and toxicity of copper nanomaterials, NanoImpact. 7 (2017) 28–40. doi:10.1016/J.IMPACT.2017.05.003.

[49] R.S. Kalhapure, S.J. Sonawane, D.R. Sikwal, M. Jadhav, S. Rambharose, C. Mocktar, T. Govender, Solid lipid nanoparticles of clotrimazole silver complex: An efficient nano antibacterial against Staphylococcus aureus and MRSA, Colloids Surfaces B Biointerfaces. 136 (2015) 651–658. doi:10.1016/j.colsurfb.2015.10.003.

[50] B.Z. Shakhashiri, G.E. Dirreen, F. Juergens, Color, solubility, and complex ion equilibria of nickel(II) species in aqueous solution, J. Chem. Educ. 57 (1980) 900. doi:10.1021/ed057p900.

[51] S. Kalepu, V. Nekkanti, Insoluble drug delivery strategies: Review of recent advances and business prospects, Acta Pharm. Sin. B. 5 (2015) 442–453. doi:10.1016/j.apsb.2015.07.003.

[52] A. Ghadi, S. Mahjoub, F. Tabandeh, F. Talebnia, Synthesis and optimization of chitosan nanoparticles: Potential applications in nanomedicine and biomedical engineering, Casp. J. Intern. Med. 5 (2014) 156–161.

[53] M. Dash, F. Chiellini, R.M. Ottenbrite, E. Chiellini, Chitosan - A versatile semi-synthetic polymer in biomedical applications, Prog. Polym. Sci. 36 (2011) 981–1014. doi:10.1016/j.progpolymsci.2011.02.001.

[54] R.S. Kalhapure, M. Jadhav, S. Rambharose, C. Mocktar, S. Singh, J. Renukuntla, T. Govender, pH-responsive chitosan nanoparticles from a novel twin-chain anionic amphiphile for controlled and targeted delivery of vancomycin, Colloids Surfaces B Biointerfaces. 158 (2017) 650–657. doi:10.1016/j.colsurfb.2017.07.049.

[55] A. Abdelkader, M.A. El-Mokhtar, O. Abdelkader, M.A. Hamad, M. Elsabahy, O.N. El-Gazayerly, Ultrahigh antibacterial efficacy of meropenem-loaded chitosan nanoparticles in a septic animal model, Carbohydr. Polym. 174 (2017) 1041–1050. doi:10.1016/j.carbpol.2017.07.030.

[56] Y. Pan, Y.J. Li, H.Y. Zhao, J.M. Zheng, H. Xu, G. Wei, J.S. Hao, F. De Cui, Bioadhesive polysaccharide in protein delivery system: Chitosan nanoparticles improve the intestinal absorption of insulin in vivo, Int. J. Pharm. 249 (2002) 139–147. doi:10.1016/S0378-5173(02)00486-6.

[57] H. Liu, J. He, Simultaneous release of hydrophilic and hydrophobic drugs from modified chitosan nanoparticles, Mater. Lett. 161 (2015) 415–418. doi:10.1016/j.matlet.2015.09.006.

[58] E.S.K. Tang, M. Huang, L.Y. Lim, Ultrasonication of chitosan and chitosan nanoparticles, Int. J. Pharm. 265 (2003) 103–114. doi:10.1016/S0378-5173(03)00408-3.

[59] C.G. Zhang, Q.L. Zhu, Y. Zhou, Y. Liu, W.L. Chen, Z.Q. Yuan, S. Di Yang, X.F. Zhou, A.J. Zhu, X.N. Zhang, Y. Jin, N-succinyl-chitosan nanoparticles coupled with low-density lipoprotein for targeted osthole-loaded delivery to low-density lipoprotein receptor-rich tumors, Int. J. Nanomedicine. 9 (2014) 2919–2932. doi:10.2147/IJN.S59799.

[60] E. Guibal, Interactions of metal ions with chitosan-based sorbents: A review, Sep. Purif. Technol. 38 (2004) 43–74. doi:10.1016/j.seppur.2003.10.004.

[61] L. Qi, Z. Xu, Lead sorption from aqueous solutions on chitosan nanoparticles, Colloids Surfaces A Physicochem. Eng. Asp. 251 (2004) 183–190. doi:10.1016/j.colsurfa.2004.10.010.

[62] L. Sun, Y. Chen, Y. Zhou, D. Guo, Y. Fan, F. Guo, Y. Zheng, W. Chen, Preparation of 5-fluorouracil-loaded chitosan nanoparticles and study of the sustained release in vitro and in vivo, Asian J. Pharm. Sci. 12 (2017) 418–423. doi:10.1016/j.ajps.2017.04.002.

[63] E.M. Costa, S. Silva, S. Vicente, C. Neto, P.M. Castro, M. Veiga, R. Madureira, F. Tavaria, M.M. Pintado, Chitosan nanoparticles as alternative anti-staphylococci agents: Bactericidal, antibiofilm and antiadhesive effects, Mater. Sci. Eng. C. 79 (2017) 221–226. doi:10.1016/j.msec.2017.05.047.

[64] J. Wang, C. Chen, Chitosan-based biosorbents: Modification and application for

biosorption of heavy metals and radionuclides, Bioresour. Technol. 160 (2014) 129–141. doi:10.1016/j.biortech.2013.12.110.

[65] T. López-León, E.L.S. Carvalho, B. Seijo, J.L. Ortega-Vinuesa, D. Bastos-González, Physicochemical characterization of chitosan nanoparticles: Electrokinetic and stability behavior, J. Colloid Interface Sci. 283 (2005) 344–351. doi:10.1016/j.jcis.2004.08.186.

Appendix

Micro & Nano Letters manuscript submission contact information:

editorialoffice_1@iet-review.rivervalleytechnologies.com

Vita

Victor A. Rodriguez is a student of the field of bioengineering. Mr. Rodriguez attained his bachelors of science from the University of California, Merced in bioengineering. From then, Mr. Rodriguez continued his studies in the field of biomedical engineering at the University of Texas at El Paso in order to attain a masters of science. Within the realm of biomedical engineering research, Mr. Rodriguez became interested in understanding the properties and concepts surrounding drug delivery sciences. Particularly with implementing and formulating polymeric nanoparticles composed of materials with biocompatible and biodegradable properties to enhance efficacy of currently existing drugs.

Delving into the realm of drug delivery research, Mr. Rodriguez begun to understand the concepts and principles of enhancing drug efficacy of currently existing agents. But also ventured into synthesizing novel metal complex compounds for enhancing antibacterial agents in order to revive the effects antibacterial drugs against resistant bacteria. Improving bioavailability, solubility, drug release characteristics, therapeutic effects, target site specificity and shelf life of the formulated nanoparticle systems. Along with improved drug encapsulation properties with the formulated nanoparticle systems.

Since then Mr. Rodriguez has published one review article and has presented three research posters at the 2018 Drug Resistance Gordon Research Conference. He currently has one research article under review for acceptance. Mr. Rodriguez also has accepted abstracts at various research conferences such as the BioMedical Engineering Society (BMES) and American Association of Pharmaceutical Scientists (AAPS) 2018 conferences. Mr. Rodriguez continues to strive to expand his understanding of the mysteries surround biological systems and the engineering of those systems in order to improve the efficacy of medical practices.

Contact Information: varodriguez11@miners.utep.edu