Exploring the antibacterial effects of polymersomes embedded with silver nanoparticles and doxorubicin

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ABSTRACT

Antibiotic resistant bacteria have become a global concern. According to the CDC, approximately 2 million people acquire life threatening bacterial infections and about 23,000 people die from them each year. The purpose of this in vitro study was to explore the antibacterial effects of polymersomes embedded with silver nanoparticles and doxorubicin. Polymersomes have been studied extensively and they have proven to be a very effective drug-vehicle. The amphiphilic co-block polymer used in this study was mPEG-PDLLA, which demonstrates high potency as a nano-carrier. The silver nanoparticles (0.04 mg/mL) and doxorubicin (0.58 mg/mL) were encapsulated in the polymersomes using a syringe infusion pump method which aided the self-assembly to occur without any damage to their structure and morphology. Doxorubicin was encapsulated into the polymersomes using two different methods: an aqueous method and the organic method. The characterization tests showed that the organic method had a higher drug loading efficiency than the aqueous method (86.4% and 68.2%, respectively). Plain polymersomes, silver nanoparticle polymersomes and doxorubicin polymersomes were also synthesized for comparison. All of the polymersome samples were tested for anti-bacterial activity against S aureus, MRSA, E coli and MDR E coli. The results showed that the polymersomes made using the aqueous method were more effective on S aureus and MRSA (47.3% and 51.5% inhibition, respectively) and the polymersomes made using the organic method were more effective on E coli and MDR E coli (49.3% and 45.4% inhibition, respectively) after being treated with the samples for 4 hours.
Cytotoxicity tests with human dermal fibroblasts were also performed for all of the polymersomes made. The results showed that the polymersomes made using the aqueous method had a higher cell viability than the polymersomes made using the organic method after the cells were treated with the samples for 24 hours (83.7% and 58%, respectively). In summary, this study reports the antibacterial effects of polymersomes embedded with silver nanoparticles and doxorubicin and proved that the inhibition of bacteria can occur without harm to mammalian cells. Therefore, further studies should be performed to increase the effectiveness of the polymersomes against bacteria for a wide range of clinical applications.
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1.0 INTRODUCTION

1.1 MOTIVATION AND BACKGROUND

The Centers for Disease Control and Prevention (CDC) has claimed that antibiotic resistance is a worldwide problem.[1] Antibiotics, which were discovered around 1943, have transformed the field of medicine the way we know it and have saved millions of people because of their efficiency against infectious pathogens. However, with the rise of the antibiotic-resistant bacterial strains, the very drugs that were used to save millions of lives are under threat[2]–[4]. According to the CDC, antibiotic resistance has been called one of the world’s most pressing public health problems. Each year, in the United States alone, approximately 2 million people acquire life threatening bacterial infections that are resistive to commonly used antibiotics and as a direct result, approximately 23,000 people die each year[1]. In addition, these drug resistant bacteria also add significant costs to the US healthcare system. Infections caused by these pathogens directly lead to extended hospital stays and additional doctor visits, which ultimately results in more costs. The direct healthcare costs associated with antibiotic-resistant infections have been estimated to be as high as $20 billion. [1]. Recently, words such as “nightmare bacteria” and “catastrophic threat” have been used to describe the hazards and risks posed by these antibiotic resistant bacteria [2]. Therefore, the objective of this study was to develop a selective treatment against the different kinds of bacteria that can decrease the growing problem of antibiotic resistance.
1.2 OVERVIEW OF THE THESIS

Antibiotic resistant bacteria are a pressing and a global threat, and it can be reasonably concluded that alternatives to conventional antibiotics are urgently needed. This thesis focuses on the broad umbrella of using nanomedicine to combat the resistant strains of bacteria.

Nanomedicine can be generally defined as the application of nanotechnology to achieve certain goals in healthcare. It manipulates the properties of the material at the nanoscale to achieve either a physical, chemical or biological response [5]. Many of the mechanisms of the human body also occur at the nanoscale, thus, allowing the nanoparticles to potentially cross barriers that are usually not achieved though the use of existing drugs. This gives access to new sites of drug delivery, interactions with small proteins, blood, and organ and tissues on another level[5].

This thesis, in particular, explored the option of using a mixture of polymers, silver nanoparticles and doxorubicin to potentially create a drug that targets and combats bacterial infections.

Polymersomes, which are artificial amphiphilic vesicles made up of different chemical polymers, serve as a drug delivery vehicle for various purposes, like gene therapy and delivering cytotoxic drugs to tumor cells. [6] Another use of the polymersomes is to carry the drug to the targeted bacteria, which is what was done in this project. The different types of polymersomes, their functions and synthesis are examined in more detail in the next chapter.

Silver nanoparticles (AgNp) are attractive for their antibacterial applications. Nanoparticles in general have gained a lot of attention in the field of medicine as an alternative means to fight infection and cancer[7], [8]. AgNps in particular have attracted a lot of interest because of their unique physical, chemical and biological properties. They have very distinctive properties like high electrical and thermal conductivity and more. Studies have also shown that they exhibit
antibacterial and antifungal properties that makes them commercially applicable in a variety of consumer products like soaps and more. [9]

The last component of this project involved doxorubicin, which is an anti-cancer chemotherapy drug[10], [11]. Patients with cancer are at a higher risk for infection due to their immune system being compromised which is a direct result of their treatment with chemotherapy, radiation and/or surgery [1]. The development of antibiotic-resistant strains drastically reduces the ability of healthcare systems to combat and manage the infectious complications that cancer patients are highly vulnerable to doxorubicin, which is one of the most commonly used anthracycline drugs and is typically chosen for the treatment of a variety of cancers, including carcinomas, soft tissue sarcomas, and hematological malignancies[12][13]. Studies have shown that anthracyclines have an ability to inhibit bacteria growth through similar mechanisms to those that cause cytotoxicity in humans[10]. The mechanisms through which these drugs work will be described in more detail in the later chapters.

This thesis includes the following specific aims:

- To investigate the morphologies of the polymersomes embedded with silver nanoparticles and doxorubicin and characterize them in terms of size, zeta potential and loading efficiency of doxorubicin.
- To observe the antibacterial effects of different kinds of polymersomes on a variety of bacterial strains.
- To evaluate the cytotoxicity of the silver nanoparticle embedded polymersomes with doxorubicin and compare them with other polymersome samples.

Chapter 2 reviews the background on the use of polymersomes, specifically mPEG-PDLLA, silver nanoparticles, and doxorubicin separately as individual drugs and their health effect. This
chapter will also go into more detail about the rise of antibiotic-resistant bacteria and the different ways researchers are combated this right now. Chapter 3 describes the experimental equipment used for this work comprising dialysis tubes and stirred injection techniques. Experimental techniques used in this work include bacterial growth curves, bacterial cytotoxicity assays, and cell cytotoxicity assays, which will also be described. Chapters 4 describes the results of these investigations with a detailed discussion. Chapter 5 will conclude the project and Chapter 6 will talk about future recommendations for this field to grow.
2.0 CRITICAL LITERATURE REVIEW

2.1 INTRODUCTION

This critical literature review talks about the rationale behind the project and this chapter is divided into three sections:

1. Bacterial crisis: This section goes into detail about the existing crisis that is caused by antibiotic-resistant bacterial strains. It starts by introducing the different kinds of bacteria, goes into details behind the emergence of drug-resistant strains, and briefly touches upon existing research on finding novel ways to combat these infections.

2. Polymersomes: This section introduces the use of polymersomes and goes into more detail about their self-assembly property and establishes why mPEG-PDLLA is used in this project as the nano-carrier.

3. Silver nanoparticles (AgNp): This section evaluates the use of silver nanoparticles, discusses about the different ways they are made, their antimicrobial effects and touches upon their cytotoxicity.

4. Doxorubicin: This anticancer drug is introduced in this section and the mechanism with which it works against the tumor is examined in detail and finally concludes with the antimicrobial property of doxorubicin.
2.2 BACTERIAL CRISIS

Bacteria are single-celled organisms that are found inside and outside the human body. Bacteria can be broadly classified into two categories based on their cellular structures: gram-positive bacteria and gram-negative bacteria. The main difference between them is that gram-positive bacteria have a thick outer protective membrane while gram-negative bacteria have a thin membrane (Figure 2.2.1) [14], [15]. An example of a gram-positive bacteria is *Staphylococcus aureus*, and an example of a gram-negative bacteria is *Escherichia coli*. Most of the bacteria, in fact, are quite helpful. For example, the *Lactobacillus acidophilus* or *L. acidophilus* that resides in the intestines helps in the digestion of food. [16] However, there are some disease causing bacteria that can cause illnesses such as strep throat.

![Figure 2.2.1 Schematic showing the structural difference between gram-positive and gram negative bacteria](image)

Antimicrobial agents, or antibiotics, are drugs that were created to combat the diseases caused by bacteria. Antibiotics, specifically, are used against bacteria and they work by killing the bacteria
or by creating a hostile environment where the bacteria cannot multiply further[2]. Antimicrobial agents, like antibiotics and other similar drugs, have been used for the last 80 years to treat infectious diseases caused by bacteria. Although these antimicrobial agents have treated and saved millions of patients, the effectiveness of these drugs against bacteria has diminished drastically because of their overuse and misuse of these medications[1], [2]. Antibiotic resistance is the ability of the bacteria to resist the effects of the drug used which results in neither the destruction of the bacteria nor the stoppage of bacterial growth. Figure 2.2.2 shows the mechanism through which antibiotic resistance occurs.

Figure 2.2.2. An infographic from the CDC to briefly show the mechanism of antibiotic resistance.[1]

Scientifically, there are three fundamental mechanisms through which bacteria develop resistance: (1) enzymatic degradation of antibacterial drugs, (2) alteration of bacterial properties that are antimicrobial targets, and (3) changes in membrane permeability to antibiotics.[18]
Another major cause for the spread of antibiotic resistance is the spread of the resistant strains of the bacteria from person to person, or from other sources in the environment. In this way, antibiotic resistance has become a major problem in today’s world.

The major causes of the antibiotic resistance crisis are summarized in the bullet points below:

- Ironically, the major cause of the antibiotic resistance is the very use of antibiotics. Since the discovery of antibiotics, they have been the most commonly used drugs in human medicine. The revolution of antibiotic resistance is caused by the over use of antibiotics. Despite warnings regarding the overuse of antibiotics, they are over-prescribed all over the world. As seen in Fig 2.2.3, there are some areas where the number of antibiotic prescription exceeds the population of the state. Another cause for antibiotic resistance is inappropriate prescription. Studies have shown that up to 50% of the time, antibiotics are not optimally prescribed causing incorrect dosage and duration, thus giving conditions for the bacteria to develop resistance. The overuse and incorrect prescription of antibiotics promote resistance through genetic alterations, such as horizontal gene transfer (HGT) which allows resistance capability to be transferred between different species of bacteria, changes in gene expression...
Another major use of antibiotics is as a growth supplement for livestock, which is said to improve the overall health of the livestock, producing a higher yield and a better quality product. Studies have shown that approximately 80% of the antibiotics sold in the United States are used in animals.[2] The antibiotics used in animals kill non-resistive bacteria, thus, creating the ideal environment for antibiotic resistant bacteria to flourish, and these strains of bacteria are transmitted to humans when they consume the animals thus leading to infections in humans. [2]

Increasing antibiotic resistance can also be attributed to the lack of new antibiotics and regulatory barriers. Antibiotic development is no longer considered to be a good investment for the pharmaceutical industry mainly due to the short lifespan of any antibiotic (due to the development of antibiotic-resistant strains), and also because of the
relatively low cost of antibiotics. Another major cause for the lack of new antibiotic development is the increased regulatory approval (FDA) like bureaucracy, differences in clinical trial requirements across countries, and changes in regulatory and licensing rules that have made the development of antibiotics an uneconomical option. [2]

Antibiotic resistant infections are widespread across the globe and the United States. In 2013, the CDC declared that the world is now in a “post-antibiotic era” and in 2014, the World Health Organization (WHO) alerted the world of the alarming situation of antibiotic resistance [1], [20]. Among the gram-positive pathogens, the biggest threat is posed by the resistant strains of *S. aureus* and *Enterococcus*. Methicillin-resistant *S. aureus* (MRSA) causes more deaths than HIV/AIDS, Parkinson’s disease, emphysema and homicide combined[1]. Among the gram-negative pathogens, the emergence of multi-drug resistant gram-negative bacteria have directly affected the practice of medicine.[1] They have become more prevalent in the community and in healthcare settings causing a direct consequence. The CDC assessed antibiotic resistance bacterial infections and assigned a threat level to each of them based on seven factors: clinical impact, economic impact, incidence, 10-year projection of incidence, transmissibility, availability of effective antibiotics and barriers to prevention. The threat levels classification fall under the categories of “urgent”, “serious” or “concerning”. (Table 2.2.1)

### Table 2.2.1. Antibacterial Resistance Threats by CDC

<table>
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<th>Concerning Threats</th>
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<td>Vancomycin-resistant <em>Staphylococcus aureus</em> (VRSA)</td>
</tr>
<tr>
<td>Carbapenem-resistant <em>Enterobacteriaceae</em> (CRE)</td>
<td>Drug-resistant <em>Campylobacter</em></td>
<td>Erythromycin-resistant Group A <em>Streptococcus</em></td>
</tr>
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<td>Drug-resistant <em>Neisseria</em></td>
<td>Fluconazole-resistant <em>Candida</em> (a fungus)</td>
<td>Clindamycin-</td>
</tr>
<tr>
<td></td>
<td>Extended spectrum β-lactamase producing <em>Enterobacteriaceae</em> (ESBLs)</td>
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</table>


| gonorrhoae | • 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*  
| resistant Group B *Streptococcus* | 

Currently, there is ongoing research that are aimed at finding novel and alternatives to: traditional antibiotics including: (1) antimicrobial peptides, (2) therapeutic antibodies, (3) vaccines, (4) antibacterial biomaterials, and (5) artificial vesicles such as nano-carriers. [21]–[23]  

Antibiotic-resistant infections are a substantial health and economic burden to the United States healthcare system, as well as to the patients and the families. According to the CDC, the total economic burden placed by these infections on the U.S economy is estimated to be as high as $20 billion a year on direct health care costs and up to $35 billion a year in lost productivity. Costs per patients are estimated to range anywhere between $18,588 to $29,069, affecting not only the health care system in terms of longer hospital stays, more doctor visits, lengthier recuperations and chances of long-term disability, but it also affects the families and communities financially. [1]  

Antibiotic resistance bacteria are now a global threat and pose a substantial health and economic burden on the healthcare systems across the countries. Bacteria will always find ways of resisting the antibiotics developed by humans, which is why aggressive action is needed now to
keep new resistance from developing and to prevent the resistance that already exists from spreading.

2.3 POLYMERSOMES

Polymers are large molecules that are composed of many repeating subunits called monomers. Polymers have a large range of physical and chemical properties which makes them very useful in everyday applications. Familiar polymers include synthetic plastics (like polystyrene) and natural polymers (like DNA) and proteins that have biological properties and functions. Generally, natural polymers consist of several monomers while synthetic polymers have one kind of monomer. Polymers consisting of only one monomer are called homopolymers, and the polymers that are made with multiple types of monomers are called copolymers.

Polymersomes are artificial vesicles enclosing an aqueous cavity, resulting from the self-assembly of amphiphilic co-polymers [24], and their largest applications are as drug delivery vehicles or artificial organelles [25], [26]. Polymersomes are highly versatile and biologically stable because they act as a physical barrier between the material encapsulated and the materials outside. By applying various block copolymers that are biodegradable and stimuli responsive, the overall properties, drug encapsulation, and release capabilities of polymersomes can be easily manipulated, thus making them highly versatile systems. These advantages make polymersomes one of the most promising structures for potential applications in the delivery of drugs, genes and proteins in the field of nanomedicine. [6]

Block copolymers contain different adjacent blocks of chemically different monomers, different composition or different sequence distributions [27]. These block copolymers are used to make the polymersomes and they are amphiphilic in nature [28]. The amphiphilic nature is caused by a
block copolymer that contains both a hydrophobic and hydrophilic block and these amphiphilic block copolymers can self-assemble in an aqueous solution into various structures as required [27], [29], [30]. The structures obtained depend on many parameters like molecular weight, ratio, concentration and the geometry of the block co-polymers. The self-assembly is driven by van-der-Waals forces that are involved with the hydrophobic block. Their self-assembly is prompted by the fact that the aqueous phase prefers the hydrophilic block causing the hydrophobic part to avoid water contact.[31]

Another application in which self-assembling polymersomes offer many advantages is in chemotherapy. As documented, chemotherapy works by suppressing the cell growth, regardless of whether it is healthy or diseased which causes unwanted side effects for the body. Over the last few years, the effectiveness of chemotherapeutic agents has improved by encapsulating them in nanocarriers like polymersomes thus delivering them in a more targeted manner, without harming the healthy cells. Formulating them in nanocarriers improves the circulation time in the blood by preventing renal clearance and non-specific uptake [32]. Also, by enhancement via the permeability and the retention effect, an increased and targeted uptake into human tissue can be achieved. The enhanced permeability and retention (EPR) effect is phenomenon that is caused by the unique anatomical and pathophysical characteristics of a tumor[33]. As seen in Figure 2.3.1, normal blood vessels have a smooth muscle-cell layer that tighten the cell-cell junctions making the macromolecular agents difficult to extravasate and the tumor tissues, the blood vessels have loose cell-cell junctions through which the macromolecular agents can enter the tumor tissue.[32]
A di-block copolymer [a block copolymer containing two types of monomers] of methoxypoly(ethylene glycol)$_{5000}$ and poly(D-($L$)-lactic acid)$_{50000}$ (mPEG-PDLLA 5000:50 000 Da) was used in this project for the synthesis of polymersomes. In a study performed by Scott et al., it was proved that the mPEG block had the ability to display a “stealth” property to particles in vivo, which prevents premature clearance by the immune system. To go into more detail, the mPEG was covalently bonded with a red blood cell membrane to minimize transfusion reactions. The observations of this study included a profound decrease in the anti-blood group antibody binding. The reason behind the “camouflage” effect of the PEG was its physiochemical nature, which includes its size, large exclusion volume and extensive hydration. [34]

The racemic mixture of the PDLLA block helped create polymersomes that have a release rate with a positive thermal correlation, which means that there would be a low release of the encapsulated particles and drug at lower temperatures (for storage) and increased release in physiological temperatures. PDLLA is also a very stiff and brittle polymer block with a high
tensile strength.[35]–[37]. The impact strength of PDLLA by itself is extremely low, but after the addition of PEG, the impact strength is greatly enhanced proving that the combination of PEG and PDLLA effectively improves the overall strength and toughness. This particular di-block is also extremely biodegradable which means that it would not harm any of the human cells at all.

Figure 2.3.2 A schematic to show the self-assembly of the mPEG-PDLLA polymer block

As seen from Figure 2.3.2, the mPEG block is the hydrophilic block that encapsulates the aqueous core and interacts with the aqueous phase outside the polymersome. The PDLLA is the hydrophobic block that fills the space between the hydrophilic areas. Studies have shown that biodegradable polymersomes like mPEG-PDLLA make promising nanocarriers for anticancer drug delivery[26], [27]. In this particular study, paclitaxel (PTX) which is a crucial anticancer drug was loaded into mPEG PDLLA to obtain a high loading efficiency and a more controlled release concluding that mPEG-PDLLA makes an efficient nanocarrier in anticancer therapeutics. [26], [30], [38]
Therefore, for this particular study where both doxorubicin, an anti-cancer drug, and silver nanoparticles, an antimicrobial agent, are to be encapsulated in a nanocarrier, mPEG-PDLLA, makes an ideal candidate.

2.4 SILVER NANOPARTICLES

Silver nanoparticles (AgNp) have been gaining a large amount of interest in the field of nanomedicine because of their distinctive physio-chemical properties, which include high electrical and thermal conductivity, chemical stability, and enhanced surface Raman scattering [39]. Aside from their distinctive properties, AgNps display a wide range of bactericidal and fungicidal properties [40] which makes them applicable in a variety of products that range from plastics and textiles, to targeted drug delivery. Because of their versatile applications, they have attracted a large amount of interest in research and development.

AgNps can be used in various different forms and are hence synthesized in many different ways. The four main ways are chemical, physical, photochemical and biological and they are briefly discussed below.

The chemical methods for the synthesis of AgNps are the most commonly used methods for production. Different shapes such as silver nanocubes and spherical AgNps can be created using chemical synthesis. The chemical synthesis of AgNps uses three main components: (1) metal precursors, (2) reducing agents, and (3) stabilizing agents. The size and shape of the nanoparticles are largely dependent on the two stages of nucleation and subsequent growth, which involves the formation of the colloidal solutions from the reduction of the silver salts. Finally, in order to obtain a uniform size distribution for all the nanoparticles, certain reaction parameters (such as the pH and reaction temperature) can be adjusted.[41]–[43]
The physical methods for the synthesis of AgNps involve the utilization of a large amount of physical energies, such as thermal energy. This method allows for the production of AgNps with a very narrow size distribution and allows the production of a large amount of AgNps in a single process. Because this method utilizes such a large amount of physical energy, the primary production costs are more expensive than other methods. Another issue to consider is the large space and time requirement. [9]

The photochemical synthesis of AgNps can be divided into two categories: (1) photophysical or the top down approach and (2) photochemical or the bottom up approach. Because the photo induced processing is a clean process, some advantages like convenience of use and high spatial resolution can be enjoyed. This method also has great versatility in the sense that it enables the fabrication of the nanoparticles in various media like cells, polymer films, etc.[9], [44]

The biological synthesis of AgNps use live organisms as reducing or stabilizing compounds in the production of the nanoparticles. The live organisms used in this synthesis range from bacterial and fungi and algae and plants. This method of production gives a wide range of resources and is a low cost technique that is extremely environment friendly, however, this method of synthesis only allows for smaller batches of production. [9], [44]

The AgNps have been extensively studied and have proved to be an effective agent against a broad range of bacteria including both gram-negative and gram-positive bacteria. In studies performed by Sondi and Salopeck-Sondi [45]–[47], the results showed that the inhibition of both gram-positive bacteria and gram-negative bacteria was dependent on the concentration of the AgNp used to treat it, and it was also dependent on the degree of resistance acquired by the bacteria. Looking more in detail into the studies performed by Kim et al. [47], it was concluded that the AgNps had a larger degree of bactericidal effect on gram-negative bacterial than gram-
positive bacteria. Their studies showed that the minimum inhibitory concentration (MIC) needed for gram-negative bacteria (E. coli) was ten times less than that required for gram-positive bacteria (S. aureus) (3.3 nM and 33 nM respectively).\[47\] In studies performed by Kvitek et al. demonstrated that the antibacterial activity of the AgNps were also dependent on the surface modifications. The results of these studies showed that the MIC ranged between 1.69-13.5 µg/mL, depending on the bacterial strains and the surface modifications used.

After further studies were performed, it was noticed that the AgNps accumulated in the bacterial cell wall and penetrated it, thus damaging and destroying the cell membrane [45], [46]. The antibacterial activity of the AgNps against the gram-negative bacteria are divided into three steps (4):

1. AgNps of the size range 1-10nm accumulate in the bacterial cell wall and destroy its main functions like respiration and permeability
2. Once the bacterial cell wall is damaged, the AgNps then enter the bacteria and cause damage by interacting with sulfur and phosphorus containing compounds (like DNA)
3. These nanoparticles then release silver ions which causes an additional bactericidal effect due to reactive oxygen species generation

The reason why different methods of synthesis of AgNps were studied is because the shape and size of the nanoparticles also have an effect on the bacteria, and the different methods of synthesis directly affect the shape of the nanoparticles (Figure 2.4.1). There have been studies to show that the interaction between the AgNps and gram-negative bacteria also depends on the shape of the nanoparticles. It has been demonstrated that compared to ionic silver and rod-shaped/spherical nanoparticles, truncated triangular silver nanoplates with a \{1 1 1\} lattice plane as the basal place had the highest antibacterial effect. [48] The size of the nanoparticles ensures
that a significantly large surface area of the particle is in contact with the bacterial cells. Therefore, larger the surface area, greater the antibacterial effect.[47], [48] It has also been demonstrated that the reactivity of the silver is favored by high-atom-density facets such as \{1 1 1\}[47],[48]

![Figure 2.4.1 Shape dependent silver nanoparticles][49]

In terms of cytotoxicity of AgNp, many studies have been completed to understand the impact of these nanoparticles on human cells. Because of their small size, humans can be easily exposed to AgNPs through different ways like inhalation, ingestion, skin, etc. as described below.

1. For toxicity associated with inhalation, studies have shown that prolonged exposure makes lung a major target tissue. In studies performed by Ji et al., rats that were exposed to AgNps for 6 hours a day, 5 days a week for 4 weeks did not show any significant differences in hematology or blood chemical values. However, minimal pulmonary inflammation was found in the mice after 10 days. [50]

2. For toxicology associated with ingestion, studies performed by Kim et al showed that
there were no significant changes in the body weight relative to the doses of the silver nanoparticles. However, it was found that slight liver damage occurred when the doses exceeded 300 mg because there were dose-dependent changes in alkaline phosphatase and cholesterol values. [50]

3. When a dosage of 6.25-60 µg/ml of AgNps (7-20 nm) were exposed to human fibrosarcoma (HT-1080) and human skin/carcinoma cells (A431) for 24 hours, the results showed reduced cell viability, oxidative stress, DNA fragmentation and higher caspase-3 activity. [50]

Hence, it can be concluded that using just plain silver nanoparticles can be extremely toxic to human cells and can cause some adverse effects on the human body. Although AgNps are toxic, if they were to be encapsulated within a nano-carrier and were delivered in a targeted manner, it can be deduced that the toxicity to healthy cells can be drastically reduced. AgNps have shown a great effect on the inhibition of infectious pathogens and if used appropriately, can prevent microbial infections.

2.5 DOXORUBICIN

Doxorubicin (Figure 2.5.1), or adriamycin, from the class of anthracyline, is one of the most widely used drugs for chemotherapy[11], [13], [51]. It is a tetracyclic aglycone, water-insoluble compound that is red in color. This color is extracted from the cultures of Streptomyces peticetius var. caesius.
Doxorubicin is highly efficient against tumors, which is a direct result of its ability to cross-link with the DNA inducing topoisomerase II-mediated single- and double-strand breaks in the DNA.[13]

The picture above (Figure 2.5.2) shows the mechanism of doxorubicin intercalating with DNA. Intercalation is the mechanism through which molecules are inserted between the planar bases of DNA.
the DNA. Thus, by intercalating DNA, doxorubicin inhibits the progression of topoisomerase II, which is an enzyme that relaxes the supercoils in the DNA for gene expression. In this way, doxorubicin cross links with the DNA and deactivates it. While doxorubicin is extremely effective in tumor-ridden cells, it also harms the healthy cells causing some dangerous side effects. For this specific drug, the maximum cumulative dosage is between 550-700 mg/m². [13]

In a study conducted by Middleman et al., 67 patients who were administered doxorubicin intravenously showed rapid tumor regression. However, along with the rapid decrease in the tumors, there were also some toxic side effects observed. In detail, all of the patients that were administered doxorubicin displayed hair loss, 21% of the patients had nausea/vomiting and 6% had phlebitis[52]. Some of the more serious side effects include acute cardiac failure, which is a result of high dosage [52]. Some studies have shown that cardiac abnormalities induced by doxorubicin can occur within one year or more than ten years after chemotherapy. [13], [11], [53]

Patients who have received chemotherapy are also highly prone to infection; while the major use of doxorubicin is against cancer; studies have shown that it also has a high antibacterial effect. The mechanism with which it works against bacteria is very similar to the way doxorubicin works against tumor, that is, cross-linking with the DNA/RNA and causing damage. [10]

In a study performed by Peiris et al., doxorubicin was shown to slow down growth of *S. aureus* and *S. epidermis* in a concentrated dependent fashion. [54] The study also showed that doxorubicin was more effective against gram-positive bacteria than gram-negative bacteria. Doxorubicin, while effective against diseased cells and bacteria, is extremely toxic to the healthy cells which lead to unwanted side effects. However, if doxorubicin were to be encapsulated
successfully within a drug-delivery vehicle and delivered in a target manner, their toxicity for healthy cells could be decreased and they can be used specifically for their bactericidal effects.

### 2.6 SUMMARY

This critical literature review section was aimed at developing a rationale behind choosing the project. The major points are summarized as follows:

1. The development of safer and more targeted therapy against drug-resistant strains are necessary to combat the threat that is posed by these infectious pathogens.
2. From the literature, it can be inferred that polymersomes, specifically, mPEG-PDLLA would make an ideal candidate for the encapsulation of both AgNp and doxorubicin because of the camouflage effect displayed by the PEG block and the thermal sensitivity of the PDLLA block.
3. Silver nanoparticles (AgNps) display a wide range of antibacterial effects and its effects are more pronounced on gram-negative bacteria than gram-positive bacteria. The cytotoxicity of AgNps is relatively high as they inhibit cell growth in humans. However, encapsulating them in nano-carriers like polymersomes might reduce the cytotoxicity of the nanoparticles.
4. Doxorubicin, one of the most widely used chemotherapy agents, also displays antimicrobial effects but it is also extremely toxic to human cells. The mechanism of the antibacterial action is very similar to that of tumor cells, the doxorubicin cross links with the DNA and destroys it.
Therefore, the objectives of the current work were to encapsulate the AgNps and doxorubicin in mPEG-PDLLA successfully and test them against bacteria and mammalian cells. This involved the successful self-assembly of the polymers having a high loading efficiency of doxorubicin.
3.0 EXPERIMENTAL

3.1 INTRODUCTION

The following section of the project describes the materials and equipment used, analytical techniques used, and the experiments performed in order to get quantifiable data and draw conclusions.

3.2 MATERIALS USED

3.2.1 Synthesis of polymersomes

140 proof (70%) ethanol (Decon Labs, King of Prussia), deionized (DI) water (Water Purification System; EMD Millipore, Darmstadt, Germany), tetrahydrofuran (THF; Sigma-Aldrich, St.Louis, MO), dried dodecanethiol-stabilized silver nanoparticles (4nm, nanoComposix, San Diego, CA), doxorubicin hydrochloride, 0.01 M PBS (Sigma-Aldrich, St. Louis, MO), mPEG-PDLLA (methoxypoly(ethylyne glycol)5000 and poly(D)-(L)-lactic acid50 000 (mPEG-PDLLA 5000:50 000 Da) were used for this study.

3.2.2 Polymersome and nanoparticle characterization

1.5% uranyl acetate solution, Tetrahydrofuran (THF; Sigma-Aldrich, St. Louis, MO) and 0.01 M PBS (Sigma-Aldrich, St. Louis, MO) were used for the characterization studies.

3.2.3 Bacterial studies

*S aureus, E coli, MRSA, MDR E coli*, trypic soy broth (TSB), Agar, PBS, propidium iodide, and SYTO 9 dye (*BacLight Bacterial Viability Kit*; Thermo Fisher Scientific, Waltham, MA) were used for the bacterial studies.

3.2.4 Human cytotoxicity studies
Human dermal fibroblasts (HDF, Detroit 551 #CCL-110, American Type Culture Collection, Manasses, VA), Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (American Type Culture Collection, Masasses, VA) were used in the studies.

3.3 **EQUIPMENT USED**

3.3.1 **Synthesis of polymersomes**

Dialysis tubes (Spectra/Por Float-A-Lyzer G2; Spectrum Labs, Rancho Domínguez, CA), ultrasonication (Bransonic 2510R-DTH, Emerson Industrial Automation, Danbury, CT), glass vials (Wheaton, Millville, NJ), glass control syringe (1 cc, w/BD Luer-Lok tip; BD Cornwall, Radnor, PA), bevel tipped stainless steel needle (needle L x O.D. 115mm x 0.63 mm; SGE Analytical Science, Australia), analytical balance (Mettler Toledo Excellence XS; Mettler-Toledo, Columbus, OH), 15 mL borosilicate glass high strength round bottom centrifuge tubes (Kimble, Rockwood, TN), stir bars (8 mm x 3mm magnetic PTFE stir bars, BRAND, Wertheim, Germany), and a stirred injection pump were used in the studies.

3.3.2 **Polymersome and nanoparticle characterization**

JEOL TEM (Transmission Electron Microscopy), 300-mesh copper-coated carbon grids (Electron Microscopy Sciences, Hatfield, PA), DLS (90Plus Zeta, Brookhaven Instruments, Holtsville, NY), Freeze-dryer (FreeZone 2.5 Plus, LABCONCO, MO, USA), and a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA) were used in the studies.

3.3.3 **Bacterial Studies**
Conical tubes (Falcon propylene conical tube), 96 well plates, petri dishes, shaking incubator, static incubator, and a fluorescence microscope (ZEISS Axio Observer Z1) were used in the studies.

3.3.4 Human cytotoxicity studies

96 well plates, incubator enriched with 5% CO₂, and a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA) were used in the studies.

3.4 ANALYTICAL TECHNIQUES USED

All of the bacterial and cell studies were performed at least three times to indicate significance values (N=3). All of the data is displayed as the mean ± the standard deviation unless noted otherwise. The analysis of variance (ANOVA) was applied to evaluate the differences among the group averages of the different experiments, and a two-tailed student t-test was used to evaluate difference between means, with p<0.05 being considered as statistically significant.

The 24-hour bacterial growth curves were also fitted with the Gompertz model:

\[ y = A \cdot \exp \left( - \exp \left( \frac{\mu \cdot e}{A} (\lambda - t) + 1 \right) \right) \]

Where,

\( y \) = amount of bacteria (the corresponding optical density reading)

\( A \) = asymptote (maximum possible value of \( y \))

\( \mu \) = maximum growth rate (1/h)

\( \lambda \) = lag time (h)

\( A, \mu, \lambda \) were estimated using a GRG non-linear solver to the least-squares estimate and were fitted for comparison.
3.5 EXPERIMENTS PERFORMED

3.5.1 Synthesis of the polymersomes

In order to synthesize the polymersomes, a stirred injection method was used to enable the self-assembly of the polymersome nanocarriers which were embedded with the hydrophobic silver nanoparticles in the membrane bilayer and/or doxorubicin inside the aqueous core.

Before the synthesis, five dialysis tubes with a total volume of 5 mL each with a 50 kDa molecular weight cutoff were cleaned. The cleaning process included adding a mixture of 30 mL of ethanol and 170 mL of DI water in propylene graduated cylinders. The dialysis tubes were filled with this mixture of ethanol and DI water before being placed in the graduated cylinder. The dialysis tubes were left to stand in room temperature for 30 minutes. After 30 minutes, the dialysis tubes were washed with just DI water and left to stand in the graduated cylinders filled with only DI water for another 15 minutes.

Prior to the synthesis of the polymersomes, 2.5 mL of THF was added to 5 mg of the dried silver nanoparticles (2 mg/mL). This mixture was then sonicated. This made sure to prevent aggregation and promoted homogeneity of the mixture.

The polymersome synthesis was initiated with the preparation of organic phases. 0.1 mL of the silver nanoparticle and THF mixture was added to three 8 mL glass vials using a glass syringe and needle. Another 0.4 mL of THF was added to the glass vials to make a total volume 0.5 mL. Similarly, using a different needle and syringe setup, two different glass vials were filled with only THF (without the silver nanoparticles) to a total volume of 0.5 mL. Five quantities of the 5 mg of mPEG-PDLLA copolymer was measured and added to each of the five different glass vials. Two quantities of 2.9 mg of doxorubicin were measured and only one of them was added to one of the glass vials that contained the mixture of silver nanoparticles and THF. Each vial
was then sonicated at room temperature for a duration of five minutes to ensure that all of
copolymers and doxorubicin was dissolved.
Then, the aqueous phases were prepared in bulk using pure DI water and PBS. 5 mL of this
mixture was added to five different round bottom centrifuge tubes. 0.29 mg of doxorubicin was
added to one of the centrifuge tubes. Stir bars were added to these centrifuge tubes and the
contents were shaken on a magnetic stir plate at 1300 rpm. The organic contents were then
injected into the aqueous phase using an injection pump which injected the contents at the rate of
0.2 mL/minute. After the contents were injected, the solution was allowed to stir further for a
couple of minutes before transferring them to the dialysis tubes. The polymersomes were then
allowed to dialyze against PBS for 48 hours. The PBS solution was changed every 24 hours. The
dialysis ensured that excess nanoparticles, doxorubicin and THF were removed.
Using this method, five different polymersome solutions were synthesized:

1. 5 mg mPEG-PDLLA/5 mL DI water-PBS
2. 5 mg mPEG-PDLLA/0.2 mg silver nanoparticles/5 mL DI water-PBS
3. 5 mg mPEG-PDLLA/2.9 mg doxorubicin/5 mL DI water-PBS
4. 5 mg mPEG-PDLLA/2.9 mg doxorubicin/0.2 mg silver nanoparticles/5 mL DI water-PBS (aqueous method)

5. 5 mg mPEG-PDLLA/2.9 mg doxorubicin/0.2 mg silver nanoparticles/5 mL DI water-PBS (organic method)

3.5.2 Polymersome and nanoparticle characterization

The main microscopy mode to examine the morphology of the different polymersomes was the TEM. 5 µL of sample droplets were uniformly coated onto a 300 mesh copper coated carbon grid and was allowed to settle for approximately 5 minutes before they were negatively stained with a solution that contained 1.5% uranyl acetate solution. This grid was then plated in the TEM and the images were obtained.

For the DLS analysis, 1 mL of the sample solutions was placed in a 4.5 mL cuvette which was then loaded into the particle analyzer chamber for analysis. The DLS analysis was performed at 25 °C, a 90 ° scattering angle, and a wavelength of 659 nm. Each run lasted for one minute and 5 such runs were obtained for each of the samples. The zeta-potential was measured using the same particle analyzer but with a specialized software. The samples were loaded in the same manner as for the DLS analysis and the measurements were performed with an applied electric field of 13.89 V/com. Each run lasted for one minute and 5 such runs were obtained for each sample.

The amount of doxorubicin encapsulated in the polymersomes were characterized by obtaining a standard curve showing a linear correlation between different concentrations of doxorubicin in THF and the absorbance measured in the spectrometer. 1 mL of the doxorubicin containing polymersomes was freeze-dried overnight and then the dry powder was dissolved in THF after
which its absorbance was measured in the spectrometer. The concentration of doxorubicin in the sample was then obtained by correlating it to the standard curve that was acquired previously. The loading efficiency (%) was then calculated using the following equation:

\[
\text{Loading efficiency (\%)} = \frac{\text{Weight of the drug encapsulated}}{\text{Weight of the drug added}} \times 100\%
\]

3.5.3 Bacterial studies

Studies were performed to explore the bacterial inhibiting capacity of the five different polymersomes that were synthesized. The five different polymersomes that were analyzed were (1) Plain polymersomes, (2) Silver nanoparticle polymersomes, (3) Doxorubicin polymersomes, (4) Doxorubicin/silver nanoparticles polymersomes (aqueous method), and (5) Doxorubicin/silver nanoparticles polymersomes (organic method).

24-hour growth curves were performed to understand the effects of the different polymersomes. A single bacterial colony was allowed to grow overnight before it was used for the experiment. This was done by transferring the respective bacterial colony to a 15 mL conical tube that contained 4 mL of TSB, and was then was set in a shaking incubator (37ºC, 200 rpm) for approximately 14-16 hours. After this period of incubation, the bacteria were diluted to a bacterial density of \(10^6\) CFU/mL by adding 400\(\mu\)L of the bacterial solution to 800\(\mu\)L of TSB. Different concentrations of this dilution were then used to provide optical density (OD) measurements (562 nm) of 0.52 which corresponded to a bacterial density of \(10^9\) CFU/mL. 15\(\mu\)L of this bacterial dilution was mixed into 1.485 mL of TSB in order to obtain a final bacterial concentration of \(10^7\) CFU/mL. This mixture was stored until further use. The different polymersomes were then diluted by 50% (50 sample:50 TSB) and the control for this experiment was a mixture of 50% TSB and 50% PBS. 180 \(\mu\)L of the control and polymerosome samples were then reverse pipetted into the 96 well-plate. Each of the samples was replicated and a
background sample was also added. Finally, 20 µL of the bacteria solution that was prepared earlier was pipetted using a multi-channel pipette into the control and polymersome samples. For the background sample, 20 µL of TSB was added. The 96-well plate was then left to incubate at 37°C inside a spectrophotometer under static conditions. The absorption wavelength was set to 562 nm and the readings were taken every two minutes over a duration of 24 hours. The total number of readings yielded by one experiment was 721, which are an accurate representation of the interaction between the samples and the bacteria. Before any analysis, the readings were normalized by subtracting the background data (with only TSB) from the experimental data (with bacteria).

Another kind of bacterial study, colony-counting experiments, were designed and implemented in order to visually analyze the activity of the polymersomes against bacteria. Similar to the 24 hour-growth curve, a single bacterial colony was isolated and grown overnight in 4 mL of TSB. After the incubation period, the bacteria were diluted to a concentration of $10^7$ CFU/mL. The procedure of dilution was the same as the one followed for the 24-hour growth curve. Then, the samples were diluted by 50% (50 sample:50 TSB) and the control for this experiment was a mixture of 50% TSB and 50% PBS. 45 µL of the control and polymersome samples were then reverse pipetted into the 96 well-plate, with each sample being run in duplicate. Finally, 20 µL of the bacteria solution that was prepared earlier was pipetted using a multi-channel pipette into the sample wells. This 96-well plate was then incubated in a static incubator at a temperature of 37°C for 4 hours. After the incubation period, the 96-well plate was then taken out and serial dilutions were prepared. The dilutions depended on the kind of bacteria the samples were being tested against. 10 µL of each dilution were then added onto petri-dishes that were prepared with a mixture of Agar and TSB earlier. Each of the dilutions was spotted thrice in order to maintain the
significance of the data. These plates were then allowed to incubate in the static incubator for about 12-14 hours. After the incubation period, the bacterial colonies were manually counted, and then were further analyzed by multiplying the count by the corresponding dilution factor. Finally, a LIVE/DEAD assay was performed for further characterization. Like the previous experiments, the bacteria were diluted to the required number and were added to the samples in a 96-well plate. The 96-well plate was then allowed to incubate for 4 hours after which the plate was centrifuged for 10 minutes at 3000 rpm in order to pellet the bacteria. During the period of incubation, the staining solution was created. This was done by adding 3 µL of propidium iodide and 3 µL of SYTO 9 to 1 mL of 0.85% NaCl. The supernatant was removed and the staining solution was added. The bacteria were then allowed to incubate for 10 minutes after which they were observed under the fluorescence microscope.

3.5.4 Human cell toxicity studies

The effects of the polymersome samples against human dermal fibroblasts were determined using MTS cell proliferation assays. In addition to DMEM, the cells were cultured with 10% FBS and 1% penicillin-streptomycin and were allowed to grow. After sufficient cell growth, 5000 cells were added to each well of a tissue cultured 96 well-plate along with 100 µL of DMEM. The plate was then incubated for 24 hours in an environment that was enriched with 5% CO₂. After 24 hours, the media was aspirated out of each of the cells, leaving only the cells that were adhered to the bottom of the wells. To this, 100 µL of polymersomes samples that were diluted by 50% (50 sample : 50 DMEM) were added. For control, 100 µL of a DMEM/PBS mixture was used. The plate was then incubated for another 24 hours inside the CO₂ enriched incubator. After this period of incubation, the content of each well was aspirated and 100 µL of a mixture of 20% MTS and DMEM was added. The cells were then allowed to incubate with this
MTS mixture for another 4 hours. The addition of the MTS was done in the dark so the properties of the MTS were not compromised. After 4 hours of incubation, the absorbance of each well was measure using a spectrophotometer. The wavelength of absorbance for the reading was set at 490 nm. The readings were then procured and analyzed for further discussion.

3.6 SUMMARY

To summarize, the following polymersome samples were created using an injection pump method:

1. 5 mg mPEG-PDLLA/5 mL DI water-PBS
2. 5 mg mPEG-PDLLA/0.2 mg silver nanoparticles/5 mL DI water-PBS
3. 5 mg mPEG-PDLLA/2.9 mg doxorubicin/5 mL DI water-PBS
4. 5 mg mPEG-PDLLA/2.9 mg doxorubicin/0.2 mg silver nanoparticles/5 mL DI water-PBS (aqueous method)
5. 5 mg mPEG-PDLLA/2.9 mg doxorubicin/0.2 mg silver nanoparticles/5 mL DI water-PBS (organic method)

After the samples were created, a couple of characterization experiments were performed. TEM was used to look into the morphology of the sample made. Dynamic Light Scattering (DLS) was also done to look at the physiochemical size distributions and the zeta-potential of the sample. Experiments to understand the drug loading efficiency were also performed.

In order to study the effects of the samples on the bacteria, a 24 hour growth curve was implemented against different strains of bacteria. A colony-counting experiment was also designed and executed to understand the anti-bacterial effects further. Finally, a LIVE/DEAD assay was also performed for further characterization.
A MTS cell proliferation assay was performed to understand the cytotoxic effects of the polymersome samples against human dermal fibroblasts. All of the bacterial and cell studies were performed at least three times to indicate significance values (N=3).
4.0 RESULTS AND DISCUSSION

4.1 INTRODUCTION

This section describes the results of the experiments and studies performed and discusses them in detail to make reasonable conclusions. The first part discusses the characterization of the polymersomes made, which includes the zeta potential and the size of the particles. The second part of this section examines and explores the antibacterial activity of these particles and goes into more detail about the mechanisms. The last part of this study evaluates the cell viability studies and draws conclusions as well.

4.2 SYNTHESIS OF POLYMERSOMES

By using the syringe infusion pump method the nanoparticles were successfully encapsulated in the mPEG-PDLLA polymers to yield homogenous polymersomes that contained different combinations of particles. The polymersomes that were embedded with both silver nanoparticles and doxorubicin were synthesized in two different ways. The first way involved adding the doxorubicin to the aqueous phase initially and then injecting the organic phase with the silver nanoparticles with the pump. This method is called the aqueous Method. The second method involved the doxorubicin being added to the tetrahydrofuran with the silver nanoparticles and the polymers, after which the entire solution was then injected into the aqueous phase (only PBS). This method was called the organic Method.

It was observed that during the 48-hour dialysis period, the polymersomes that were made using the aqueous method released doxorubicin into the PBS solution causing the solution to go pink.
The polymersome that were made using the organic method did not show such a release (Fig 4.2.1).
Figure 4.2.1 (a) and (c) show the release of doxorubicin from the aqueous method for the preparation of polymersomes. (b) and (d) show no release of doxorubicin from the organic method of preparation of the polymersomes.

After the dialysis was completed and the polymersomes were transferred for storage, differences in the color of the particles were observed. The polymersomes made using the aqueous method showed a pink color and the polymersomes made using the organic method displayed a dark brown color. (Fig 4.2.2).

Figure 4.2.2 Polymersomes made from the organic method (left) and polymersomes made from the aqueous method (right)

The reason behind the color difference in both of the method of synthesis was believed to be because of the way the doxorubicin was encapsulated. The doxorubicin that was used for this study was in a hydrochloride form which makes it highly soluble in aqueous solutions (hydrophilic). When the solution of AgNp/polymers were injected into the aqueous solution
containing the doxorubicin and PBS, it is hypothesized that some of the doxorubicin remained in
the aqueous solution after the self-assembly of the polymersomes occurred. This would explain
the high release of doxorubicin during the dialysis period and also the pink color displayed by
the polymersomes. The polymersomes made using the organic method did not show any release
during the dialysis period because it is believed that most of the doxorubicin was encapsulated in
the aqueous core of the polymersome and the AgNps were embedded in the hydrophobic
membrane-bi-layer, which gives the polymersome solution a dark-brown color. [Figure 4.2.3]

![Figure 4.2.3 Schematic to show the polymersomes with AgNp and doxorubicin](image)

Thus, it was proposed that the loading efficiency of the doxorubicin was higher in the organic
method than the aqueous method. The other polymersomes made (plain, doxorubicin and silver
nanoparticles) seemed to be stable and did now show any abnormalities.
4.3 POLYMEROSOME AND NANOPARTICLE CHARACTERIZATION

After the polymersomes samples were synthesized, the first characterization study that was done was TEM imaging to ensure that the morphology of the particles did not change, which needed to be confirmed before any more studies could be performed. This is because if the morphology of the polymersomes were not maintained, different experimental methods of polymersome synthesis would have been pursued.

In DI water and PBS, the TEM images showed that the polymers and nanoparticles could self-assemble into polymersomes that encapsulated the silver nanoparticles. The doxorubicin was not observed under the microscope, and it was also observed that there was no difference between the particles created using the aqueous and organic methods. The diameter of the particles ranged from 69.9 nm to 93.8 nm, and this was later also confirmed by DLS studies (Fig 4.3.2). Hence, it could be concluded that the synthesis of the polymersomes using the injection-pump method was successful in maintaining the morphology of the particles.

(a)  (b)

Figure 4.3.1 TEM images of the AgNp/Doxorubicin polymersomes
The next studies that were performed were the DLS to elucidate the diameter of the particles.

The following graph shows the effective diameter (average) of all the samples made:

![Graph showing effective diameters](image)

**Figure 4.3.2 Results from the DLS studies**

The size of the AgNp/Doxorubicin polymersomes range from 64.5 nm to 72.2 nm which corresponds to the size of the particles that were viewed under the TEM.

Zeta-potential studies were also performed to understand the stability of the particles, by analyzing the magnitude of the zeta-potential, the degree of electrostatic repulsion can be understood. When the zeta-potential is small, the stability is low and the dispersion may break and cause coagulation and when the zeta-potential is high, it is considered to be extremely stable.

The range of zeta-potentials and their corresponding behavior is discussed below:[55], [56]

- 0 to ±5 indicates very low stability that causes rapid coagulation
- ±10 to ±30 indicates the beginning of a stable dispersion
- ±30 to ±40 indicates moderate stability
- ±40 to ±60 indicates good stability
- More than 61 indicates excellent stability

The zeta-potential measurements of the samples made are tabulated below:
Table 4.3.1 Zeta-potential studies

<table>
<thead>
<tr>
<th>Sample</th>
<th>ζ-potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain Ps</td>
<td>-40.07</td>
</tr>
<tr>
<td>AgNp Ps</td>
<td>-40.66</td>
</tr>
<tr>
<td>Doxorubicin Ps</td>
<td>-40.55</td>
</tr>
<tr>
<td>AgNp/doxorubicin Ps (Aq)</td>
<td>-40.7</td>
</tr>
<tr>
<td>AgNp/doxorubicin Ps(Org)</td>
<td>-40.54</td>
</tr>
</tbody>
</table>

As observed from the table above, the zeta potential of the sample varies between -40 to -40.7, indicating a good stability in aqueous solution. [49],[50]

The loading efficiency experiment was implemented to understand the amount of drug that was encapsulated by the polymersomes.

![Figure 4.3.3 DOXORUBICIN -THF Standard Curve](image)

From Figure 4.3.3, it can be observed that by using the standard curve between doxorubicin and THF, the amount of drug that was entrapped in the polymersomes could be analytically...
calculated. Using the equation of the slope and the equation to calculate the loading efficiency the following results were obtained:

**Table 4.3.2 Results from the loading efficiency experiment**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Loading Efficiency</th>
<th>Amount of drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNp/doxorubicin Ps (Org)</td>
<td>86.42%</td>
<td>0.50 mg/mL</td>
</tr>
<tr>
<td>AgNp/doxorubicin Ps (Aq)</td>
<td>68.18%</td>
<td>0.40 mg/mL</td>
</tr>
</tbody>
</table>

The amount of drug added originally for the synthesis was 0.58 mg/mL, and compared to that the the AgNp/doxorubicin polymersomes that were prepared in the organic method, had a larger amount of drug encapsulated (0.5 mg/mL) than the AgNp/doxorubicin polymersomes that were prepared in the aqueous method (0.40 mg/mL), which corresponds to a loading efficiency of 86.42% and 68.18%, respectively.

### 4.4 BACTERIAL STUDIES

Bacterial studies were performed on both gram-positive and gram-negative bacteria. The samples that were synthesized were tested on *Staphylococcus aureus* (*S. aureus*), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Escherichia coli* (*E. coli*) and *Multi-drug resistant Escherichia coli* (MDR E. coli).

The first set of studies that were performed were the 24-bacterial growth curve where the bacteria was allowed to grow with the samples for a duration of 24 hours and a reading was taken every 2 minutes, and then a colony counting assay was performed after which a LIVE/DEAD assay was also completed for further characterization.

It can be observed from the graphs above that the AgNp/doxorubicin Ps (Aqueous) had the highest inhibitory effect on *S. aureus* (51.5% viability). In the case of MRSA, both types of the
AgNp/Doxorubicin polymersomes show significant inhibitory effects, although the aqueous method (47.3% viability) was slightly more effective. This can also be correlated to the parameters obtained from the Gompertz model.

As seen from figures 4.4.3 (a) and 4.4.3 (b), the AgNp/doxorubicin Ps made using the aqueous method had a longer lag-time ($\lambda$) for both *S. aureus* and *MRSA*, and had a lower maximal bacteria growth rate ($\mu$). This confirms the conclusions and observations deduced from the other bacterial experiments.

It is very interesting to note that the polymersomes that had the lower loading efficiency were more effective than the higher one, and the reason behind this is hypothesized to be because of presence of doxorubicin in the aqueous solution in the polymersomes made using the aqueous method. Because the doxorubicin is present in the aqueous solution, it comes directly in contact with the bacterial strains and it is also known that doxorubicin, by itself, has an inhibitory effect on *S. aureus*. [54] It is also known that while AgNp does have antimicrobial properties against gram-positive bacteria, it is not as pronounced and requires a higher concentration than gram-negative bacteria. [40], [50], [57] This also explains why AgNp Ps and doxorubicin Ps both had a small bactericidal effect on both *S. aureus* and *MRSA* (Fig 4.4.1 (b) and Fig 4.4.2 (b)).
The following graph shows the interaction of *S. aureus* with the different samples.

(a)

(b)
Figure 4.4.1 (a) Growth curve of samples against *S aureus* (b) Colony counting assay of bacteria after being treated for four hours with different polymerosome samples, N=3, *p*<0.01 versus control, **p**<0.005 versus control (c) LIVE/DEAD assay (magnification x10): control (d)LIVE/DEAD assay (magnification x10): Polymersomes (aqueous method) (e) LIVE/DEAD assay (magnification x10): Polymersomes (organic method)

For *MRSA*:
Figure 4.4.2 (a) Growth curve of samples against MRSA (b) Colony counting assay of bacteria after being treated for four hours with different polymersome samples, N=3, *p<0.01 versus control, **p<0.005 versus control
Figure 4.4.3 (a) calculated lag-time for different samples against *S. aureus* and *MRSA* (b) calculated maximum bacterial growth rate for different samples
The effect of the polymerosome samples on *E. coli* told a different story. As seen from the following graphs and images, it can be observed that the AgNp/doxorubicin Ps (Organic) had a more significant effect on both *E. coli* (49.3% viability) and *MDR E. coli* (45.4% viability). This was also confirmed by the parameters obtained from the Gompertz model.

As the results show, the AgNp/doxorubicin polymersomes made using the organic method were more effective at reducing both *E. coli* and *MDR E. coli* growth (had a longer lag-time and smaller growth rate). As mentioned previously, the lag-time analysis and the maximal growth rate analyses both show that the effect is more prominent in *MDR E. coli* than in *E. coli*. Another comparison to be made is that the samples are definitely more effective on gram-negative than gram-positive bacteria.

From the literature review, it is established that the antibacterial activity of silver nanoparticles is more pronounced in gram-negative bacteria than in gram-positive bacteria.[45], [50], [57]. The interaction between silver nanoparticles and gram-negative bacteria is divided into three different steps:

1. The silver nanoparticles (1-10 nm) attach to the surface of the cell membrane and substantially effects its functions like permeability and respiration. [57]
2. Once they effect the functions of the cell membrane, they penetrate into the body of the bacterial cell and cause further damage by interacting with the sulfur and phosphorus containing compounds. [57]
3. Silver nanoparticles will release ions which will also impact damage to the cell membrane via reactive oxygen species. [57]

The reasons stated above also explain why just plain AgNp polymersomes also had a relatively significant bactericidal effect on *E. coli* (88.3% viability) and *MDR E. coli* (72% viability). With
the polymersomes made using the aqueous method, the doxorubicin which was already present in the aqueous solution came in contact with the bacterial cell and it did not have as prominent an effect as the polymersomes made using the organic method. The proposed mechanism for the polymersomes made using the organic method is that when they came in contact with the bacterial cell, the silver nanoparticles would be released leading to the disruption of the cell wall, and then the doxorubicin would be released from the aqueous core would work alongside to cross-link with the bacterial DNA to deactivate it. [10], [13]
(b)
Figure 4.4.4 (a) Growth curve of samples against *E coli* (b) Colony counting assay of bacteria after being treated for 4 hours with different polymosome samples, N=3, *p<0.01 versus control, **p<0.005 versus control (c) LIVE/DEAD assay (magnification x10): control (d)LIVE/DEAD assay (magnification x10): Polymersomes (aqueous method) (e) LIVE/DEAD assay (magnification x10): Polymersomes (organic method)

For *MDR E coli*:
Figure 4.4.5 (a) Growth curve of samples against MDR E.coli (b) Colony counting assay of bacteria after being treated for four hours with different polymersome samples, N=3, *p<0.01 versus control, **p<0.005 versus control
Figure 4.4.6 (a) calculated lag-time for different samples against *E coli* and *MDR E coli* (b) calculated maximum bacterial growth rate for different samples

### 4.5 HUMAN CYTOTOXICITY STUDIES

Lastly, the cytotoxicity of the different polymersome samples were investigated. The cell viability was compared to the control, which was assumed to be the highest viability that can be achieved. The plain polymersomes, AgNp polymersomes and doxorubicin polymersomes all showed higher cell viability than the control (Fig 4.5.1). This is a very interesting observation because it has already been established in the literature that AgNp (6.25-60 µg/mL)[50] and doxorubicin (550-700 mg/m²)[13] both are extremely toxic to human cells. However, when they were encapsulated in mPEG-PDLLA, they do not show any of their toxic characteristics.
The AgNp/doxorubicin polymersomes showed different results. Although both of them showed lower cell viability, the samples that were prepared using the aqueous method showed a higher viability (83.67%) than the samples prepared using the organic method (58.47%), which confirms the hypothesis that the organic samples had a higher loading efficiency of doxorubicin than the aqueous samples. It can also be concluded that when both AgNp and doxorubicin were loaded into the polymersomes, they had a synergistic effect that was relatively toxic to the cells.

![Figure 4.5.1 Cell cytotoxicity of polymersome samples made against human-dermal fibroblast after treatment for one day, N=3, *p<0.01 versus control, **p<0.005 versus control](image)

Figure 4.5.1 Cell cytotoxicity of polymersome samples made against human-dermal fibroblast after treatment for one day, N=3, *p<0.01 versus control, **p<0.005 versus control
4.6 SUMMARY

This section covers the results of all the experiments that were performed for this project and discusses the results in detail.

To summarize, five different samples were synthesized out of which the AgNp/doxorubicin polymersomes were synthesized two different ways, namely the aqueous method and the organic method. During the synthesis, the following observations were made:

1. It was observed that during the 48-hour dialysis period, the polymersomes that were created using the aqueous method showed a release of doxorubicin in the water leading to the conclusion that some of the drug was lost in the process which resulted in a lower rate of encapsulation.

2. After the dialysis period was over, the two different samples showed a different color. The sample which was prepared using the aqueous method showed a pink color and the one prepared using the organic method showed a dark-brown color, leading to hypothesize that in the aqueous method, some of the doxorubicin was left in the aqueous phase after the self-assembly of the polymersomes occurred.

After the synthesis, the polymersomes were characterized and the observations are noted as follows:

1. The TEM imaging showed that the morphology of the polymersomes remained intact after the self-assembly and the insertion of silver nanoparticles and doxorubicin orubin. There was also no difference in the morphology when the polymersomes were made in two different ways.

2. The DLS studies provided the diameter range of all the polymersomes made and they ranged from 64.5-150 nm which is considered as stable.
3. The zeta-potentials of the samples ranged from -40 to -40.7 indicating good stability in an aqueous solution.

4. Drug loading efficiency study proved the hypothesis that the AgNp/doxorubicin polymersomes (86.42%) made using the organic method encapsulated more doxorubicin than the AgNp/doxorubicin polymersomes (68.18%) made using the aqueous method.

After the characterization studies, the interaction between the samples and the bacteria were studied:

1. For gram-positive bacteria, the AgNp/doxorubicin polymersomes made using the aqueous method showed the most effect on *S. aureus* and for *MRSA*. AgNp Ps, doxorubicin Ps and the AgNp/doxorubicin Ps (organic method) did show bactericidal effects, but they were not as pronounced as the polymersomes made using the aqueous method. This was confirmed with the colony counting assay as well. The reason why the polymersomes made using the aqueous method is the most effective is believed to be because of the presence of the doxorubicin in the aqueous phase which readily interacts with the bacteria before the polymersomes do and the fact that doxorubicin has an antimicrobial effect that is more distinct in gram-positive bacteria.

2. For gram-negative bacteria, the AgNp/doxorubicin polymersomes made using the organic method showed the most effect on both strains. The Plain Ps and doxorubicin Ps did not show any significant effect against the bacteria, the AgNp Ps did show some bactericidal effect and this was attributed to the nature of the silver nanoparticles and their interaction with gram-negative bacteria. Finally, the AgNp/doxorubicin polymersomes made using the aqueous method did show significant inhibition of bacteria but it was not as pronounced as the one made using the organic method. This is believed
to be because of the nature of the interaction between the silver nanoparticles and the gram-negative bacteria; studies have shown that silver nanoparticles have a more pronounced effect on the gram-negative bacteria than gram-positive bacteria [9].

Lastly, cytotoxicity assays were performed to study the interaction between the sample and human dermal fibroblasts (HDF):

1. It was observed that the AgNp/doxorubicin Ps (aqueous method) had higher cell viability than the AgNp/doxorubicin Ps (organic method). This result correlates with the fact that the polymersomes made with the organic method had a higher amount of doxorubicin encapsulated and doxorubicin, as studies have shown, is very toxic to human cells. An interesting result was that the AgNp Ps and doxorubicin Ps showed a cell viability that was greater than 100%, studies have shown that both AgNp and doxorubicin are quite toxic to human cells, but this can be credited to the mPEG-PDLLA polymer used for encapsulating them. It is hypothesized that they minimized the interaction of the nanoparticles and doxorubicin with the healthy cells.
5.0 CONCLUSIONS

The objective of this in vitro study was to explore the antibacterial effects of polymersomes embedded with silver nanoparticles and doxorubicin. After all the studies performed and data obtained, it can be concluded that mPEG-PDLLA loaded with silver nanoparticles and doxorubicin show bacterial inhibition, and serve as a promising tool; however, further detailed studies are needed to explore ways to make it a very powerful tool against bacteria.

Some main takeaways from the studies conducted in this project are:

- mPEG-PDLLA serves as a very effective drug delivery vehicle and works very effectively in encapsulating both the silver nanoparticles and the doxorubicin. The morphology and the size of the polymersome is not affected and the self-assembly occurs in the very smooth manner. The mPEG-PDLLA also was also stable because there was no loss of drug or effectiveness of the sample after 3 weeks of storage.

- Silver nanoparticles are a very effective tool against both gram-positive and gram-negative bacteria. In this study, a concentration of 40µg/mL was used, and it showed significant bactericidal effect (roughly 50%) when used in combination with doxorubicin on the different strains used. The AgNp when encapsulated in Ps alone showed some bacterial inhibition but it was more noticeable in the gram-negative than gram-positive. This concentration of silver nanoparticles, when encapsulated in mPEG-PDLLA, also did not have very high cytotoxicity. However, according to several studies, a higher concentration of AgNp is required to show a greater inhibition on gram-positive bacterial strains (approximately 10 times more)[46].

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• Doxorubicin also has a very pronounced effect on bacterial strains, but they are extremely toxic to human cells. The concentration of doxorubicin used in this study was 0.58 mg/mL, and it displayed approximately 50% bacterial inhibition when used with AgNp and mPEG-PDLLA. Since the doxorubicin used in this study was hydrophilic, it was crucial to understand and implement a methodology that ensured a higher encapsulation rate. It has been established through studies that the mechanism of doxorubicin killing bacteria involves cross-linking with the DNA of the bacteria and thus deactivating it. While doxorubicin, in its purest form, is extremely bactericidal, it would not be a viable option because of its extremely high toxicity to healthy human cells. Hence, encapsulating it in a drug vehicle served as a very effective alternative to combat its toxicity because it was observed that the toxicity towards healthy cells was reduced drastically after the encapsulation.

In summary, the study demonstrated that mPEG-PDLLA could serve as a drug delivery vehicle for both silver nanoparticles and doxorubicin to inhibit the growth of bacterial strains. The combination of the drug and silver nanoparticles seemed to me more effective on gram-negative bacteria than on gram-positive bacteria. Further studies for enhancing the bactericidal effect, stability of doxorubicin and in-vivo studies are necessary.
6.0 RECOMMENDATIONS

Although this project provides promising evidence for the application of mPEG-PDLLA embedded with AgNp and doxorubicin against bacteria, additional studies are required. For example, it is necessary to understand the nature of the release of doxorubicin over time, and for this, a drug-release profile needs to be completed over a period of time to understand the sustained release of the drug. The encapsulation of silver nanoparticles also needs to be studied further in detail. It is helpful to know the exact amount of silver encapsulated in order to study the relationship between amount of AgNp and the bactericidal effects. For example, the TEM roughly showed the amount of silver nanoparticles encapsulated in each of the polymersome, but it is an inaccurate way to measure it. A better technique needs to be devised in order to accurately measure the amount of silver encapsulated in the polymersome. One possible technique would be to use the inductively coupled plasma mass spectrometry (ICMPS), which is an analytical technique for elemental analysis at very low concentrations.

Additional studies need to be performed by increasing the concentration of both the silver nanoparticles and doxorubicin. The concentration of AgNp (40µg/mL) and doxorubicin (0.58 mg/mL) used in this study displayed approximately 50% inhibition of bacteria (compared to the control). Studies have shown that a higher concentration of silver nanoparticles can be used for a greater inhibitory effect against bacteria which reduced toxicity to mammalian cells [34].

Increasing the concentration of doxorubicin is also a viable option. This study showed that by encapsulating doxorubicin in the mPEG-PDLLA polymersomes, the cytotoxicity was decreased
drastically. Hence, different ratios of AgNp and doxorubicin needs to be compared to see which one works most effectively against the bacteria and shows the least toxicity to mammalian cells. Another study that could be conducted is using different kinds of silver nanoparticles. There have been studies to show that the interaction between the AgNps and gram-negative bacteria also depends on the shape of the silver nanoparticles[50]. It has been demonstrated that compared to ionic silver and rod-shaped/spherical nanoparticles, truncated triangular silver nanoplates with a \{1 1 1\} lattice plane as the basal place had the highest antibacterial effect. [48] Using triangular silver nanoplates may increase the antibacterial effect of the drug. Another possible way to improve the overall efficiency of the drug would be to use to increase the overall charge of the drug. It is known that the cell walls of bacteria are negatively charged [18],[21], hence, having a positively charged drug would improve the interactions between the drug and the bacterial membranes. The final recommendation would be to perform in-vivo studies to understand the biodistribution of the polymersomes and its ability to deliver both the silver nanoparticles and doxorubicin in a targeted manner.
7.0 REFERENCES


