2014

Polymer-based delivery systems for support and delivery of bacteriophages

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Polymer-based delivery systems for support and delivery of bacteriophages

by

Alyssa Marie Brown

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Materials Science and Engineering

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Ames, Iowa
2014
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LIST OF ABBREVIATIONS

Alg: Alginate
AM: Alveolar macrophage
APC: Antigen-presenting cell
CENPE: Centromere associated protein E
Chi: Chitosan
Col: Collagen
CPH: 1,6-bis-(p-carboxyphenoxy)hexane
CPTEG: 1,8-bis-(p-carboxyphenoxy)-3,5-dioxaoctane
Da: Dalton
DNA: Deoxyribonucleic acid
G-block: Guluronic acid block
Gel: Gelatin
LPS: Lipopolysaccharide
MraY: Phosphor-MurNAc-pentapeptide translocase
OmpC: Outer membrane protein C
OmpF: Outer membrane protein F
LB: Lysogeny broth
M-block: Mannuronic acid block
PAMP: Pathogen-associated molecular pattern
PEG: Poly(ethylene glycol)
PFU: Plaque-forming units
PLGA: Poly(lactic-co-glycolic acid)
PRR: Pattern recognition receptor
RNA: Ribonucleic acid
SA: Sebacic acid
TEM: Transmission electron microscopy
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor α
v/v%: Volume per volume percent
w/v%: Weight per volume percent
ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Rebecca Cademartiri for her guidance and encouragement throughout my graduate education. Special thanks to Dr. Kaitlin Bratlie and Dr. Ian Schneider for their time and counsel. I would also like to extend my thanks to Dr. Balaji Narasimhan and Dr. Tim Brenza for their collaboration and for providing direction during this project. A special thanks also to Dr. Santosh Pandey and Zach Njus for their contributions. Finally, I thank my friends and family for their support and encouragement.
ABSTRACT

One of the most urgent problems in the fields of medicine and agriculture is the decreasing effectiveness of antibiotics. Once a miracle drug, antibiotics have recently become associated with the creation of antibiotic-resistant bacteria. The main limitations of these treatments include lack of both adaptability and specificity. To overcome these shortcomings of current antibiotic treatments, there has been a renewed interest in bacteriophage research.

Bacteriophages are naturally-occurring viruses that lyse bacteria. They are highly specific, with each bacteriophage type lysing a narrow range of bacteria strains. Bacteriophages are also ubiquitous biological entities, populating environments where bacterial growth is supported. Just as humans are exposed to bacteria in their daily lives, we are exposed to bacteriophages as well.

To use bacteriophages in practical applications, they must be delivered to the site of an infection in a controlled-release system. Two systems were studied to observe their support of bacteriophage lytic activity, as well as investigate the possibility of controlling bacteriophage release rates. First, hydrogels were studied, using crosslinking and blending techniques to achieve a range of release profiles. Second, polyanhydride microparticles were studied, evaluating release rates as a function of monomer chemistries.
CHAPTER 1: INTRODUCTION

1.1 Bacteriophage Overview

In our environment, there exists an ubiquitous antibiotic entity that works silently to maintain microbial equilibrium: the bacteriophage. A bacteriophage is a type of virus that infects a specific bacterium through its membrane and transfers viral DNA into the cell, either into or outside of the cell’s genome. Eventually the infected bacterium will replicate hundreds of phages and undergo membrane rupture.

These viruses exist in soil, seawater, and any other environment that supports bacterial growth. In fact, bacteriophages are the most abundant and diverse biological entity on earth [1]. They are also remarkably effective: for nearly every known strain of bacteria, there exists a corresponding bacteriophage that can infect it [2]. Bacteriophages are thought to be responsible for between 10-80% of bacteria deaths in aquatic ecosystems [3]. As each environment varies in temperature, chemical composition, and microbiota, so too does the concentration of bacteria and its impact on the ecosystem [4].
The general structure of a bacteriophage is similar to that of a common virus, with genetic material encased in an icosahedral capsid. Bacteriophages are made up almost entirely of protein and nucleic acid material, though the structures of different types of bacteriophages vary. In these experiments, we mainly used the well-studied T4 and T7 bacteriophages, shown in Figures 1.1 and 1.2 [2], and these phages will be the focus of structure comparison. We also briefly considered the phage φX174, which will be used for comparison when appropriate.

Relatively complex bacteriophages, such as T4, feature a tail collar, tail core and sheath, base plate (or tail plate), tail pins, and six tail fibers. The T4 phage, due to its long tail and many auxiliary structures, has a total length of 200 nanometers: a 100 nanometers capsid length and 100 nanometers total tail length [2]. In contrast, the T7 phage is much simpler, with six tail fibers as its only auxiliary structure. The T7 phage has a length of 50 nanometers and total tail length of 20 nanometers [5]. Despite the differences between phages T4 and T7, both are specific to Escherichia coli (E.coli) bacteria and undergo the infection process through similar means. The phage φX174 contains no tail, and is also icosahedral in shape with a length of 25

Figure 1.2
Size and structure of bacteriophages T4, on the left; and T7, on the right.
nanometers [6]. Its infection process is different from that of T4 and T7 and will be used to illustrate the multiple possible infection mechanisms.

1.2 Bacteriophage Biology

Bacteriophages are remarkable in that each bacteriophage type may only infect a specific bacterial strain. Due to co-evolution of bacteriophages and bacteria, the viruses have adapted to the biochemical markers in the membrane of the target cell. The process of co-evolution has given rise to both structures to facilitate infection (such as the tail) and alternative virus-membrane binding requirements [1].

To infect a bacterium, the bacteriophage must first adsorb onto the surface of the cell. In phages with tail fibers, the fibers identify a high concentration of receptors on the membrane surface of the target cell, where the tail fibers act as a “ligand” for the receptors [7]. The interaction between the phage and bacterial membrane is often compared to a chemical reaction and is shown to occur in two steps: reversible adsorption, followed by irreversible fixation. The reversible adsorption process follows first-order adsorption kinetics characterized by a phage adsorption constant [4], requiring physical contact between three of the six tail fibers and membrane receptor [2].

After adsorption, the phage binds irreversibly to a second receptor membrane, which has been identified as the heptose residue of the lipopolysaccharide, or LPS, inner core [1]. In phage T4, this irreversible binding occurs via a conformational change in the baseplate [2]. Phage T7, which does not contain a baseplate, contains tail-related proteins inside the capsid that serve as a core to guide DNA transfer. The viral DNA from phage T7 is transferred more slowly than that of T4, and the transfer process is dependent on the host cell RNA polymerase to act as a molecular motor [1]. The bacteriophage tail guides the viral nucleic acid material across the
bacterial membrane into the cell. Phages with contractile sheaths further ensure the transfer of viral nucleic acid material by shortening the distance between the viral head and cell interior.

Once the viral DNA has been introduced to the cell interior, transcription begins upon contact with the host RNA polymerase. These first-phase genes are known as immediate early genes. Transcription of these genes results in molecules that serve to protect the viral DNA and further tailor the host cell’s biology toward phage survival by degrading host DNA or destroying enzymes and other proteins that impede the production of new bacteriophages [1]. The next phase of viral DNA is known as the middle genes, which codes for new viral DNA. Lastly, the final phase of viral DNA is the late genes. These genes, when transcribed, produce the proteins necessary for production of new phage components to encase and transport the newly-made viral DNA [1]. After phage production is complete, the viral DNA will initiate a rupture of the cellular membrane.

Depending on the type of phage, membrane rupture can occur via the lytic cycle or the lysogenic cycle. The phages T4 and T7, as well as φX174, undergo the lytic cycle, as illustrated in Figure 1.3 [1]. However, the process differs between phages with double-stranded DNA, such as T4 and T7, and those with single-stranded DNA, such as φX174. In double-stranded DNA phages, the viral genetic code produces the enzyme endolysin. To trigger membrane rupture, endolysin must interact with the membrane protein holin, which is also encoded in the viral DNA.

The holin production is the limiting factor in the lytic process, and the timing of its production can be controlled via production of holin inhibitors coded in viral genes. The delay in holin production allows time for the immediate early, middle, and late phase viral DNA to be transcribed. Holin production can also depend on growth conditions of the new viruses [1].
When between 100 and 300 daughter viruses are produced, holin proteins are allowed to accumulate and lysis can begin. The holin proteins then solubilize the bacterial membrane to such a degree that the endolysin enzyme is able to break down the bacterial cell walls[8].

To compare, the lytic process of a single-stranded DNA bacteriophage is characterized by transcription of viral DNA to produce centromere-associated protein E, or CENPE. This protein then inhibits the membrane enzyme phosphor-MurNAc-pentapeptide translocase, or MraY. The MraY enzymes catalyze the synthesis or murein, which makes up a crosslinked mesh outside of the bacterial membrane. After murein inhibition, the bacterial membrane is weakened, and CENPE proteins oligomerize into a trans-membrane tunnel [9]. After sufficient membrane breakdown, daughter bacteriophages escape the cell. The phage φX174, a single-stranded DNA phage, was briefly considered for study, but was ruled out due to its instability in organic solvents.

The term “virulent” is generally applied to bacteriophages that undergo the lytic cycle, as compared to “temperate” for lysogenic bacteriophages. Since the lytic cycle is more rapid than lysogeny, a virulent phage population is expected to have a more immediate effect on its target bacterial population. It is important to note that the rate of bacteriophage population change is independent of peak phage population, which is the determining factor for total elimination of
the target bacteria strain [4]. This is to say, virulent bacteriophages are not intrinsically more effective than temperate bacteriophages, but they do lyse bacteria more quickly.

1.3 History of Bacteriophage Use

Before bacteriophages were formally identified, their bacteriocidal effects had been observed in microbiology work. One of the first references to bacteriophages is thought to be a work by Ernest Hankin in 1896. While studying cholera microbes in the Ganges and Jumma rivers, he observed a bacteriocidal property in filtered river water [10]. After the turn of the century, two independent researchers identified bacteriophages almost simultaneously. Frederick Twort of the United Kingdom and Felix d’Herelle of France, in 1915 and 1917 respectively, demonstrated the lytic activity of bacteriophages. By 1921, d’Herelle had found success in treating chickens with bacteriophages against avian typhosis. He expanded his research to field work in rural France, treating flocks of chickens in areas where avian typhosis was an epidemic. He found that flocks that had received bacteriophage had fared much better: fewer chickens died, the duration of the illness was shortened, and the possibility of secondary infection was minimized [1].

D’Herelle went on to successfully immunize himself, his coworkers, and his family against dysentery by administering injections of an anti-Shinga bacteriophage suspension. In 1925, d’Herelle treated four bubonic plague patients by injecting anti-plague bacteriophage into the infected lymph nodes, or buboes, of the patients. As d’Herelle’s successes became more well-known, he traveled to the Haffkine Institute in Bombay, India to develop a treatment for cholera epidemics. He developed bacteriophage therapy regimens both as a preventative measure as well as a treatment for those already suffering with the disease [1]. By the late-1920s, bacteriophage therapy was a popular area for research. In addition to d’Herelle’s
treatments, typhoid fever, *Bacillus anthracis* and *Staphylococcus* had been successfully treated with bacteriophage therapy [10]. Therapy trials were administered using both oral and injected doses, to great success.

In the same time frame, bacteriocidal activity of mold and fungi was also being observed, eventually leading to Alexander Fleming’s discovery of penicillin in 1928. After his discovery, bacteriophage research became stagnant in North America and Western Europe due to the presence of both positive and negative results seen in literature regarding bacteriophage therapy. However, Eastern Europe established a research institute for bacteriophage study in 1923 in Tbilisi, Georgia which continued its bacteriophage work. The findings were published in Russian scientific journals, stimulating interest in bacteriophage therapy in Russia and Poland [11].

In the 1940s bacteriophages regained popularity, when Max Delbrück spearheaded a study in bacteriophage genetics, specifically the phages lambda and T4, to investigate the lysis mechanism of the viruses. In the course of his research, he demonstrated that a bacterium was lysed by one virus type, highlighting the specificity of bacteriophages. To simplify his research, Delbrück focused on T-series phages and their lysis of the B strain of *E.coli*. His research led to T-series, which includes bacteriophages T1 through T7, being the most well-studied bacteriophages [2]. Bacteriophages that are classified as T-even (T2, T4, and T6) were studied first because the viral DNA of these entities contain a novel base: 5-hydroxymethylcytosine, which clearly differentiated viral DNA from host cell DNA in early experiments [1]. Unfortunately, the renewed interest in bacteriophages was short-lived due to World War II, at which time antibiotics saw a surge in popularity. Such levels of interest in bacteriophages did
not return until 1967, when lambda phages were used to isolate lambda repressor, a DNA-binding protein [11].

1.4 Bacteriophages in Biological Systems

With the recent rise of environmental research, the ubiquity of phages has become clear: bacteriophages “are by far the most numerous biological entity on our planet, [10]” with concentrated populations in bacteria-rich environments such as soil, oceans, and wastewater. It is estimated that bacteriophages outnumber cellular organisms ten to one. While it is commonly known that the human body’s cells are outnumbered by colonizing bacteria, those bacteria are in turn outnumbered 100 to one by colonizing bacteriophages [10].

Humans and animals are exposed to bacteriophages throughout life, so bacteriophage treatments are generally harmless when proper bacteriophage preparations are administered [1]. Healthy humans have low levels of bacteriophages present in the digestive tract, which are thought to have little impact on the intestinal microbiota. In patients with internal illness, fecal phage titers increase, as do the diversity of phages present. The magnitude of phage titers is also observed to have a positive correlation with the severity of the disease [3]. Similarly, in canine populations, fecal bacteriophage presence is dependent on living conditions. Dogs that are kept in a clean environment as house pets show very low levels of bacteriophage, while dogs living in kennels show higher bacteriophage presence [3]. The presence of bacteriophages does not cause the disease, but rather the increased presence of bacteria (i.e. severity of the disease) causes an increase in bacteriophage lysis and multiplication.

1.4.1 Bacteriophages in Medicine

In the 1960s through the 1980s, large-scale bacteriophage studies were conducted in Eastern Europe to evaluate effects of bacteriophage therapy on antibiotic-resistant bacteria
infections. Soviet scientists tended to focus on prophylactic bacteriophage therapy, while Polish scientists tended to apply bacteriophages to patients with pre-existing infections.

One of the most prominent bacteriophage studies conducted in the Soviet republic of Georgia was to use bacteriophages as prophylaxis against *Shigella*, or dysentery. The study was conducted on over 30,769 children, where 17,044 received a weekly oral dose of bacteriophages, and 13,725 received placebo treatment as a negative control. The children received weekly visits from scientists, who administered phage and performed a health evaluation. After a 109-day period, fecal samples were tested for presence of *Shigella* bacteria. Clinical diagnosis based on weekly health evaluation found that dysentery rates were 6.7 and 1.76 cases per 1,000 children, for placebo and bacteriophage-treated groups respectively. Diagnoses based on fecal bacteria culture indicated that dysentery rates were 1.82 and 0.7 cases per 1,000 children, for placebo and bacteriophage-treated groups respectively. The bacterial culture also tested for unspecified diarrhea-causing bacterial infections, which yielded similar results: presence of diarrhea-causing bacteria showed a 2.3-fold reduction between the placebo and bacteriophage-treated group [12].

In Poland, scientists studied several pathogens (*Staphylococcus*, *Pseudomonas*, *Escherichia*, *Klebsiella*, and *Salmonella*) in order to find a potent corresponding bacteriophage. They then applied this knowledge to a large study with 518 patients with cases of antibiotic-resistant infection. The patients received bacteriophage therapy one of three ways: orally (with a baking soda to neutralize stomach pH), locally (with wound dressings or application in the pleural or peritoneal cavities) or via drops applied to the nasal mucosa, eye, or middle ear. Throughout the study, the bacterial infection was monitored for bacterial resistance against bacteriophage infection. If resistance was detected, the patients received treatment with a new
type of bacteriophage. The treatment was found to completely eradicate the infection in 94% of patients [12].

Other successful studies conducted in the Soviet Union included treatments for staphylococcal infections in the lung, eye infections, neonatal sepsis, urinary tract infections, and surgical wound infection. In Poland, successful applications of bacteriophage therapy included treatments for cerebrospinal meningitis in a newborn, various chronic bacterial infections, and stabilization of tumor necrosis factor alpha (TNF-α) levels in blood serum. However, many of these studies do not hold up to the rigor of today’s scientific standards: many studies lack negative controls, while some studies do not report information on the number of subjects receiving each type of treatment [12].

1.4.2 Bacteriophages in Agriculture

Bacteriophages have great potential in agricultural applications in addition to medicine. As antibiotics fall out of favor as a prophylactic treatment for livestock, bacteriophage therapy has shown great potential to reduce infections in both animals and plants, resulting in better protection against foodborne contamination [1].

As discussed above, Felix d’Herelle successfully used bacteriophages to treat avian typhosis in chicken flocks. Subsequent experiments performed by other scientists expanded the scope of study to other animals, which showed positive outcomes as well as negative outcomes. This can be attributed to variation in animal model, target bacteria, and bacteriophage potency. In a study of salmonella treatment, chicken and mouse animal models were used with bacteriophages did not show lytic activity in vitro. In this case, the experiment failed to show protection against salmonella, leading to the conclusion that failed in vitro tests were a good predictor of failed phage performance in vivo [1].
Recent veterinary experiments have shown that bacteriophage treatments in animals with doses as low as $10^2$ PFU have the capacity to prevent infection when administered simultaneously with the infecting bacteria. However, a low dosage is effective primarily as a prophylactic measure, as a $10^2$ PFU dosage is less effective when administered after the infection has been introduced [3].

Based on these initial findings, there has been interest in administering bacteriophages to pigs for the treatment of \textit{Salmonella typhimurium}, which is the bacteria that causes salmonellosis in humans. Effective treatment and containment of \textit{Salmonella} would decrease risk of foodborne illnesses greatly. Due to livestock living conditions, \textit{Salmonella} was found to disseminate rapidly among pigs, with an increase in infection rates between farm and lairage, and again between lairage to slaughterhouse. In one phage study, a group of pigs were experimentally infected with \textit{Salmonella}, then treated with bacteriophage against \textit{Salmonella} three hours later. It was found that the pigs’ tonsil and cecum tissues harbored the most \textit{Salmonella} cells, resulting in the bacteriophage treatment being targeted to these areas. The treatment did not eliminate the \textit{Salmonella} infection in its entirety, but it did reduce the number of bacterial cells in the target area and significantly decreased the risk of spreading the disease. However, the intestinal contents of the pigs still did show a considerable amount of \textit{Salmonella} cells, which indicates that the porcine intestine may reduce phage propagation and activity [13].

1.4.3 Bacteriophage as an Alternative to Antibiotics

Bacterial infections in humans, animals, and plants are currently controlled with antibiotics. Antibiotics are produced most commonly by soil bacteria and fungi, though the antibiotic molecules’ function in nature is still in dispute [11]. Based on the antibiotic synthesis processes found in nature, pharmaceutical labs have implemented multistep procedures to
produce synthetic antibiotics. Most of the antibiotics produced today are based on either a natural compound, or a by-product of an attempt to synthesize a natural compound. However, in the past 25 years, there has been little impetus for pharmaceutical labs to seek out new antibiotic compounds from natural sources because of the success of the current formulations. In recent years, antibiotic resistance has increased the demand for new forms of antibiotics, changes in current formulations to overcome resistance mechanisms in bacteria, as well as alternative treatments altogether [14]. Unfortunately, this process may take several years [12]. This is because antibiotic formulations have varying degrees of effectiveness, and relatively few formulations are safe for humans, as compared to the vast amount of antibiotic chemicals that exist in nature. Furthermore, approval of new antibiotic drugs adds additional years between discovery and general use.

When treating infections with bacteriophage therapy, it is important to note the expected differences as compared to commonly-used antibiotic treatment. Firstly, antibiotic treatment can target a broad range of bacteria whereas bacteriophages are highly specific. A bacteriophage treatment would be best suited to an infection in tissue inhabited by both healthy and pathogenic bacteria, where the widespread destructive power of antibiotic therapy may produce undesirable complications, such as in the digestive tract. A bacteriophage treatment would be more difficult in the case of infection by several different strains of bacteria [1]. In this case, the target bacteria would need to be identified quickly and the patient would receive a cocktail of bacteriophages.

In human studies, treatments of certain infections using lytic phages, such as *Staphylococcus* bacteria, have been shown to be more effective than their antibiotic counterparts. In a study of staphylococcus infection in the lung, intravenous bacteriophage, oral bacteriophage, and oral antibiotic treatments were compared. The intravenous bacteriophage treatment
eradicated the infection in 95% of patients, and oral bacteriophage treatment showed complete recovery in 82% of patients, as compared with 64% of patients in the antibiotic group [12]. In animal studies, a bacteriophage preparation that has been carefully selected for the target bacteria has also been shown to be as effective as an antibiotic regimen, if not more so [1].

Early Eastern European studies have shown that bacteriophages are generally a safer treatment than antibiotics. Antibiotic treatments often eradicate bacteria nonspecifically, and such treatments are liable to cause intestinal disorders as a result of disruption of digestive microbiota, allergies, and secondary (e.g. yeast) infections [12]. In contrast, no studies reported serious side effects of bacteriophage treatments, likely because of the vast amount of bacteriophages that patients encounter through normal daily activity. To ensure that bacteriophages do not mount an immune response, Polish scientist Stefan Slopek analyzed antibody titers during a study of treatment for staphylococcal bacteria. Of the 57 patients participating in the study, 44 had no measurable antibodies, while 8 patients had a titer under 1:40, and two patients had titer of 1:320-1280. Slopek concluded that bacteriophage immunogenicity did not interfere with antibacterial processes [15]. Minor side effects of bacteriophage treatment are thought to be caused by endotoxins released by the lysed target bacterium, though the free endotoxins are present in any antibiotic treatment.

Along the same vein, the pharmacology of bacteriophages differ from antibiotic treatment. Since antibiotics are non-living molecules, they must either be targeted directly at the infection site (which is often an internal organ), or must be administered via a systemic, or body-wide, oral or injected dose. Bioavailability of the antibiotic is therefore dependent on absorption, metabolism, and degradation. To overcome these barriers, a relatively high dose of antibiotic is necessary to maintain minimum effectiveness [16].
Bacteriophages also face the same barriers. However, because of the body’s familiarity with bacteriophages, a relatively high dose of bacteriophages is not harmful to digestion or other bodily functions. Once bacteriophages reach the site of infection, the viruses proliferate upon lysis of target bacteria. In this way, the virulent effect increases exponentially and reduces the need for frequent doses [12]. In a mouse study on the bioavailability of bacteriophage, the bacteriophage was removed from the mouse’s bloodstream after 48 hours, but a high titer remained in the mouse’s spleen [15].

As mentioned above, antibiotic molecules are static formulations. Engineering new, effective antibiotics can be a time-consuming process. In contrast, bacteriophages have a history of co-evolution with bacteria, a process that can be replicated in lab by exposing phage-resistant bacteria to other types of phages. When exposed to a high number of diverse phages, there is a high chance of finding a new treatment, whether by phage tail fiber adaptation or a separate infecting phage altogether.

1.5 Difficulties with Bacteriophage Treatment

Bacteriophages are always found in environments that support growth of bacteria. As a result, bacteriophages and bacteria have co-evolved with one another. Bacterial mutations may improve immunity to infections by altering certain membrane receptors, or removing them altogether. This immunity is generally temporary, as the mutated cell becomes susceptible to other bacteriophages that target different types of membrane receptors.

In the event of receptor alteration, bacteriophages can often adapt tail fibers to compensate for the change and still complete the adsorption process. For example, the T4 phage is able to bind to a lipopolysaccharide specific to the B strain of *E.coli*, as well as the OmpC membrane protein found in the K strain, and the OmpF protein. Interestingly, mutation in cells
occurs more easily than counter-mutation in viruses. In this case, the bacteriophages rely on their sheer numbers in order to maintain microbial equilibrium [1]. However, anti-bacteriophage mutations are not always beneficial: deletion of membrane proteins may protect from bacteriophage infection, but may also prove detrimental to the cell’s survival. The receptors targeted by bacteriophages are often necessary for the cell’s survival. Loss of these proteins may lead to a weakened physiological state or loss of important functions in the cell [1].

Due to the ubiquity of both bacteria and bacteriophages, regional and individually-hosted bacteriophages may affect research efforts. It is possible that co-evolution can favor the bacteria rather than the bacteriophage, due to a higher degree of adaptability in a particular cell than the corresponding virus. For example, a 2009 cholera outbreak in Zimbabwe spurred research into bacteriophages against the cholera microbe, also called vibriophages. However, bacteriophage treatment was hindered by the fact that the vibriophage populations of Zimbabwe had affected evolution of the cholera bacteria by significantly altering serotype prevalences found on the cell membrane [17]. Bacteria and bacteriophages exist in a balance in nature, and if relatively few new phages are added to an environment, the bacteria may resist bacteriophage lysis over time. However, intervention of new bacteriophages to a bacteria population can counteract this effect, targeting alternative membrane proteins in the bacteria.

Lastly, bacteriophage therapy suffers from the perception of a lack of credibility because of negative results reported by several early studies. Critics of bacteriophage therapy cite the narrow bacterial host range as a drawback because early studies did not use bacteriophage cocktails to compensate for anti-bacteriophage mutations. In addition, many early bacteriophage treatments were ineffective or caused various side effects. Scientists would often treat infections with bacteriophages intermixed with lysates (including endotoxins) from recently-burst host
bacteria, which can be responsible for side effects. Very early scientists also did not distinguish between lytic and lysogenic viruses when developing treatments, and some conducted studies using the slower, less-effective lysogenic class of bacteriophages, producing negative results [12].

Commercially-available bacteriophage stocks only reinforced the lack of credibility. Early bacteriophage treatments also contained phenol and merthioloate for sterility and stability, as was standard practice for vaccines, which resulted in inactivation of bacteriophages [18]. Many pharmaceutical companies advertised stocks containing over 100 separate types of bacteriophages to treat many different infections, but these did not hold up to scrutiny by scientist Max Delbrück. He found that the manufacturers did not grow phage types separately, but rather combined them before exposing the phage cocktail to a cycle of infection and phage growth. Over many such passages, he found that the final phage stock contained only one type: T7 phage, which is specific to certain strains of *E.coli* [18]. By the time scientists learned more about bacteriophages, antibiotics had risen in popularity and bacteriophage therapy was no longer a research priority.

**1.6 Bacteriophage Delivery**

In order to maximize effectiveness of bacteriophage treatment, polymers can be used as a vehicle to reach the site of infection in either humans or livestock animals. Currently, drug treatments have been successfully delivered both orally and nasally. In this study, the aforementioned vectors will be applied to bacteriophage delivery and support.

**1.6.1 Oral Delivery**

Oral drug delivery systems are popular because of their ease of use for both medical professionals and patients. Furthermore, controlled-release oral delivery provides a consistent
drug delivery over time, reducing the frequency of administration. In controlled release systems, drug is released under certain conditions, including osmosis, pH, and other physiological factors [19].

In the digestive tract, drugs are absorbed through the intestinal lumen. Absorption of drugs can be hindered by intestinal pH and enzymatic activity in the lumen, as well as structural barriers like the mucous layer. The epithelial layer, which is folded into the villi, consists of strongly-bound cells, such only very small, polar drug molecules can pass through the tight junction. Therefore, most drugs must be absorbed by the epithelial cells in order to reach the bloodstream [20]. Large, polar drugs rely on the concentration gradient to aid diffusion into the epithelial cells. Drugs with poor water solubility require surfactants, prodrugs, or other additives for absorption [21].

Interestingly, highly water-soluble drugs can also pose difficulties in controlled-release systems. Such drugs demonstrated first-order release kinetics, characterized by a large initial burst of drug with subsequent low release levels. Since water-soluble drugs are easily absorbed by epithelial cells, first-order kinetics can cause a very high amount of drug to enter the bloodstream in a short amount of time. Ideal drugs, with average water solubility, show release closer to the desired zero-order release kinetics, which demonstrates consistent release over time [19].

Hydrogels, which consist of a stable network of crosslinked polymers, are often used in drug delivery applications. Water-soluble drugs can easily be distributed throughout the gel, which generally consists of about 95% water. While hydrogels are initially stable in water, they are prone to swelling and eventual degradation, which causes release of drug. The chemistry of
hydrogels can be altered to reflect a desired rate of degradation, however by-products must be assessed to determine risk of inflammatory response [22].

1.6.2 Nasal Delivery

Drug delivery via nasal sprays and particles originally began as a treatment for infections and allergies in the nasal pathway. More recently, there has been success with introducing systemic drugs via nasal delivery, especially when an immediate effect is necessary. Systemic drug circulation is found to be effective for drugs that are difficult to inject or are poorly absorbed orally [23] using molecules with low molecular weight, including proteins, that would otherwise be difficult to use in injected treatments, as they are prone to metabolism by the host [24].

The absorption of nasal drugs depends on the permeability of the nasal mucosa, which depends on the polarity of the drug. Nonpolar drugs have been shown to absorb particularly well via nasal delivery, showing bioavailability near 80%. This is because nonpolar drugs are absorbed in a transcellular process through passive diffusion or active transport through channels across the membrane. Polar drugs, by contrast, show a much lower permeability and less bioavailability, near 10%. The molecular weight of polar drugs can affect absorption as well. Drugs with low molecular weights (below 1,000 Da) are able to follow a paracellular route through the tight junctions between cells. This route is not efficient for transport of drugs with high molecular weights, which must be transported in small amounts via an endocytotic transport process, wherein the drug is engulfed by the cell membrane into a vesicle. If a drug is not absorbed within 15 to 20 minutes, the excess will be removed by mucociliary clearance [24].

To promote absorption of a polar drug, chemicals like surfactants or bile salts can be used as enhancer agents. The enhancer agents work through one of several methods. Some alter the
phospholipid bilayer of epithelial cells, and others strip off the outer mucosa layer entirely. Others alter the tight junctions between surface cells, which may include acting as an enzyme inhibitor [24]. While many of these additives showed success in animal studies by greatly increasing drug bioavailability, absorption by animal mucosal tissues was positively correlated with damage from the additives [23]. Similarly, the success is less pronounced in human studies than animal because of the irritation of the mucosa layer. Some gentler additives, like cyclodextrins and chitosan, enhanced absorption enough to justify their mucosal irritation [24].

Bacteriophages against respiratory-pathway diseases are good candidates for nasal delivery. In a patient with a pre-existing infection in the lung, a nasal bacteriophage dose would act as an immediate treatment because it would not need to travel systemically [24]. However, bacteriophages can be described as a protein-based particle of high molecular weight. In these experiments, polyanhydride microparticles are used as a nonpolar encapsulation method for bacteriophages, promoting the transport of bacteriophages across the nasal mucosa.

1.7 References


CHAPTER 2: VISUALIZING BACTERIOPHAGE ACTIVITY

2.1 Introduction

Bacteriophage activity is most commonly evaluated using the agar overlay method, wherein a layer of top agar and indicator bacteria are spread over a layer of base agar. The bacteriophages to be tested are introduced on top of the agar, at which time they lyse indicator bacteria during an incubation period. The purpose of the base agar is to provide nutrients for the bacteria. The top agar, because of its lower volume, contains fewer nutrients. Instead, its purpose is to create an even bacteria lawn. The mobility of the bacteriophages and the subsequent cleared area is limited by the concentration in the top agar, so the 0.5% agar powder is held constant. This method is used in our experiments, and is described in more detail in the Experimental section of this chapter.

While the agar overlay method is most commonly used, it is not the only method of measuring bacteriophage activity. For scientists who wish to determine the host range of a bacterium, a spot test method is used. In this case, the indicator bacteria is replaced by a suspension of multiple host bacteria. The bacteriophages are plated ("spotted") on the top agar, then incubated [1]. The host range can be visualized by the size of the plaque, though complete clearing within the drop area is not automatically a positive result. In some cases, very high bacteriophage concentrations can cause “lysis-from-without,” wherein the sheer number of bacteriophages overwhelm a cell’s ability to synthesize proteins, resulting in cell death [2]. To prevent false positive results, the bacteriophage suspension is plated in serial dilutions. An example of serial dilutions atop a bacteria-agar overlay is shown in Figure 2.1.
In this experiment, we used the agar overlay method to evaluate the activity of T4 bacteriophage against a K12 *E.coli* lawn. The T4 bacteriophage infects K12 *E.coli* by binding to the lipopolysaccharide, or LPS, a receptor protein found in the outer cell membrane. The interaction is a two-part phenomenon, as the LPS requires an interaction with protein Ib before acting as a receptor for T4 [7].

![Figure 2.1 (Dilution of T4 bacteriophage plated using the overlay method on a lawn of K12 *E.coli*. The darker area indicates clearing of bacteria. The scale bar indicates 1.3 cm.)](image)

Bacteriophages may also be evaluated by microscopy. Transmission electron microscopy (TEM) is an option for obtaining an image of the viruses. The size and shape of the capsid is apparent under TEM imaging, as well as the presence of tails and tail fibers. Historically, electron microscopy has been used to image viruses since the 1940s when the German scientist Helmut Ruska observed the bacteriophage-induced lysis of enterobacteria and enterococci. Since then, the TEM has been useful in classifying novel bacteriophages using existing bacteriophage families as a comparison [3]. An example of TEM imaging of bacteriophage activity is seen in Figure 2.2 [4].
As biological entities primarily made up of proteins, bacteriophages may also be detected by fluorescent microscopy. The capsid contains the small outer capsid protein, which is used as an attachment site for a marker protein such as green fluorescent protein. This is a useful technique for detecting encapsulated bacteriophages, or detecting the presence of the corresponding target bacteria under a fluorescence microscope [5]. An example of fluorescent imaging of bacteriophages is shown in Figure 2.3 [6].

![Figure 2.3](image1)

A plaque created by λ bacteriophage is shown in epifluorescence illumination. The scale bar denotes 150 μm.

![Figure 2.2](image2)

TEM imaging of T4 bacteriophages. The image on the left shows a bacteriophage attached to the cell membrane, with a densely-packed capsid. The image on the right shows a bacteriophage attached to the cell membrane with a hollow core after DNA injection through the membrane.

2.2 Experimental

In this experiment, we visualized the effect of T4 bacteriophage on the K12 strain of *E.coli*. The T4 bacteriophages were purchased from ATCC in freeze-dried form. The K12 *E.coli* were also purchased from ATCC as a freeze-dried pellet.

Our experiments use K12 *E.coli* as the indicator bacteria. *E.coli* is the most thoroughly-studied bacterium and has been historically used in microbiology and genetics work. The
bacteria require as few as 8 hours to grow the full lawn required for bacteriophage indication. While the K12 strain is nonpathogenic, it can be used as a model for *E. coli* O157:H7, which is responsible for foodborne illness.

### 2.2.1 Method for Bacteriophage and Bacteria Stocks

Preparation of stocks and other subsequent measurements were performed on Miller LB agar plates. The base agar was prepared by mixing Miller LB media with 1.5% agar powder. The solution was autoclaved and 20 ml of liquid agar was measured into each plate. The agar was allowed to set, after which the plates were stored at 4°C for up to two months. The top agar was prepared by mixing Miller LB media with 0.5% agar powder. The liquid agar was aliquotted into small bottles and autoclaved. After autoclaving, one bottle was kept in a 50°C water bath and the others were stored at 4°C and solidified. To prepare the solidified agar for use as top agar, the bottle was boiled in a hot water bath until the agar melted. The agar was melted thoroughly to prevent microcrystals, which appear as lumps when the top agar is poured and can affect bacteriophage activity [1]. The melted top agar was cooled to 50°C before plating so that the indicator bacteria would not undergo heat shock.

The K12 *E. coli* master stock, stored at -80°C, was used to spread a smear plate on Miller LB base agar (1.5% agar). The plate was then inverted and incubated at 37°C overnight. After the growth cycle, colonies of K12 *E. coli* were evident. Each colony was assumed to be genetically uniform. A general-use stock of *E. coli* was made by brushing up one colony with a pipette tip, then suspending the bacteria in 5 ml of Miller LB media. The stock was incubated under shaking at 37°C overnight. All subsequent stocks were grown with one colony from the smear plate. Each grown stock produced full bacteria lawns for seven days, after which the stock
began to produce lawns with spots of clearing. The smear plate was replaced after approximately two months.

To make a T4 stock, the freeze-dried bacteriophages were suspended in Miller LB media, then serial dilutions were made with Miller LB media as the diluent. We first allowed Miller LB agar plates to come to room temperature. To these, we poured Miller LB top agar (0.5% w/v agar) mixed with 2% v/v K12 \textit{E.coli} stock on top of the base agar. The top agar was allowed to set. The diluted bacteriophage solution was plated by dropping 5μl aliquots on the agar. The solution drops were allowed to dry. The plate was then inverted and placed into a 37°C incubator for 18 hours. After incubation, bacteriophage clearings called “plaques” were present on the bacteria lawn.

Each bacteriophage plaque is considered to be genetically uniform, so one plaque was selected for amplification. The plaque was brushed up by a sterile pipette tip, and stirred into a microcentrifuge tube containing 200 μl lambda buffer. The bacteriophage-buffer solution was incubated at room temperature for 2 hours, after which it was applied to a K12 \textit{E.coli} Petri dish as described above. The dish was incubated overnight, after which a large percentage of the plate showed clearing of bacteria.

We then added 10 ml of lambda buffer to the agar plate and allowed it to sit for 5 minutes, which loosened the top agar. The top agar was scraped off with a sterile L-spreader and placed into a 50 ml centrifuge tube. We added 10 ml of chloroform to the mixture and mixed vigorously. The mixture was centrifuged down, leaving separate chloroform, agar gel, and aqueous phases. The aqueous phase, which contained bacteriophages, was skimmed off and run
through a 0.45 μm-pore filter to remove all remaining agar. The final solution was then diluted to calculate concentration.

2.2.2 Bacteriophage Concentration and Activity

To find the concentration of bacteriophage suspended in a prepared stock, the stock was serially diluted by one tenth into lambda buffer. The solutions were plated by 5 μl aliquots onto a bacteria lawn as described above. The plate was then inverted and incubated at 37°C overnight. After incubation, a countable dilution was found. To be countable, the dilution had to show between three and 30 small, distinct plaques. The formula for determining concentration is as follows:

\[
\text{Concentration} \left[ \frac{PFU}{ml} \right] = (\text{Plaque count}) \times (\text{Dilution factor})^{-1} \times \left( \frac{1000 \mu l}{\text{volume plated}} \right)
\]

Using this formula, a non-diluted 5 μl drop is countable at a minimum concentration of approximately 1,000 PFU/ml. A concentration of \(10^3\) PFU/ml would therefore be unacceptable as a starting phage stock in experiments, as a controlled release of bacteriophage would be under detection limits much of the time. When the bacteriophage has a stock of \(10^5\) PFU/ml or more, a 5 μl drop will appear as one large clearing due to the individual plaques merging together.

Bacteriophage activity can also be qualitatively visualized by observing the diameter of the clearing, especially as a result of a delivery material containing bacteriophage solution. We would expect a high bacteriophage concentration to produce a clearing with a large diameter. Because of the number of variables in this method, it is difficult to quantify the number of PFUs from the diameter of the clearing.
2.3 Results

2.3.1 Concentration of Bacteriophage Stocks

When we followed the protocol as described above, the resulting bacteriophage stocks showed very high activity. Generally, the concentration of a new stock was found to be $10^{10}$. The maximum concentration that we observed in a new stock was $10^{14}$, while the minimum we observed was $10^8$. The bacteriophage stocks were stored at 4°C without stabilizers. The bacteriophage stocks maintained the original concentration for at least two months.

2.3.2 Growth of Bacteriophage over Time

We were able to create a visual representation of bacteriophage activity over time using a scanner in a temperature-controlled 37°C chamber. The experiments were carried out for 24 hours, with scans taken every 5 minutes.

To measure the radial spread of bacteriophage activity, we placed 15 μl of bacteriophage suspension on a sterilized disc of 703 blotting paper obtained from VWR. The blotting paper absorbed the liquid completely, such that the entire volume of liquid applied to the paper was transferred onto the plate.

In early experiments, we plated the bacteriophage-loaded discs onto agar without pre-growing the bacteria. We found that early clearing was difficult to visualize as there was no contrast between bacteriophage activity and the early stages of lawn growth. Since bacteriophages are known to be less effective on fully pre-grown lawns, we instead plated the paper discs on lawns that had been incubated for 2 hours.
As seen in Figure 2.4, we loaded the paper discs with one of the following concentrations: $10^7$, $10^8$, $10^9$, or $10^{10}$ PFU/ml. In those plates, all of them showed some contamination (denoted by the dark gray area). Two of the plates (not shown) indicated clearing independent of the bacteriophage-loaded discs due to condensation on the lid falling onto the agar and impeding bacterial growth. However, the images do show that lower concentrations yield smaller clearings. The images shown were taken after 7 hours of incubation.

We measured the distance of bacteriophage clearing on the above images, though the radii should be considered an approximation due to the clearing from the condensation, as well as the interference from the contamination. We took 4 radius samples per paper disc, and the radii were measured in ImageJ. The results are recorded in Table 2.1 and graphed in Figure 2.5.
Table 2.1

<table>
<thead>
<tr>
<th>Bacteriophage Concentration, PFU/ml</th>
<th>$10^7$</th>
<th>$10^8$</th>
<th>$10^9$</th>
<th>$10^{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottom</td>
<td>5.48</td>
<td>5.39</td>
<td>6.88</td>
<td>7.06</td>
</tr>
<tr>
<td>Left</td>
<td>5.35</td>
<td>5.75</td>
<td>6.43</td>
<td>7.06</td>
</tr>
<tr>
<td>Top</td>
<td>4.85</td>
<td>6.43</td>
<td>7.01</td>
<td>6.88</td>
</tr>
<tr>
<td>Right</td>
<td>5.48</td>
<td>5.98</td>
<td>6.65</td>
<td>5.80</td>
</tr>
<tr>
<td>Average</td>
<td>5.29</td>
<td>5.89</td>
<td>6.74</td>
<td>6.76</td>
</tr>
</tbody>
</table>

Using the scanner, we also obtained images every 5 minutes for a span of 24 hours. In Figure 2.6, discs of blotting paper were loaded with 15 μl of bacteriophages, of either $10^8$ or $10^{10}$ PFU/ml.
The radius of bacteriophage activity was measured in ImageJ and was taken at four points: top and bottom of the disc, as well as to the left and right. The measurements are given in Table 2.2 and graphed in Figure 2.7. The bacteriophages showed rapid growth until hour 12, after which the cleared area remained approximately the same size.

*Figure 2.6*
Scanner images of bacteriophage clearing over time. Discs on left contain $10^{10}$ PFU/ml concentration, discs on right contain $10^{8}$ PFU/ml concentration. The black circles indicate the papers. The gray area is the lawn of *E.coli* growth, while the white area indicates cleared areas. Images taken at A. Time 0; B. 6 hours; C. 12 hours; D. 24 hours
Table 2.2

<table>
<thead>
<tr>
<th>Time 0</th>
<th>Hour 6</th>
<th>Hour 12</th>
<th>Hour 24</th>
<th>Time 0</th>
<th>Hour 6</th>
<th>Hour 12</th>
<th>Hour 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottom</td>
<td>0.00</td>
<td>1.16</td>
<td>2.02</td>
<td>2.02</td>
<td>Bottom</td>
<td>0.00</td>
<td>2.38</td>
</tr>
<tr>
<td>Left</td>
<td>0.00</td>
<td>1.30</td>
<td>2.31</td>
<td>2.31</td>
<td>Left</td>
<td>0.00</td>
<td>2.53</td>
</tr>
<tr>
<td>Top</td>
<td>0.00</td>
<td>1.16</td>
<td>2.24</td>
<td>2.24</td>
<td>Top</td>
<td>0.00</td>
<td>2.31</td>
</tr>
<tr>
<td>Right</td>
<td>0.00</td>
<td>2.02</td>
<td>4.04</td>
<td>4.26</td>
<td>Right</td>
<td>0.00</td>
<td>2.60</td>
</tr>
<tr>
<td>Average</td>
<td>0.00</td>
<td>1.41</td>
<td>2.65</td>
<td>2.71</td>
<td>Average</td>
<td>0.00</td>
<td>2.46</td>
</tr>
</tbody>
</table>

Figure 2.7

Graphical representation of bacteriophage activity, as measured by the distance from the edge of the paper disc to the edge of the cleared area, over time.

2.4 Conclusions

The bacteriophage stocks that we made from the incubation of single plaques were more successful than those grown from older stocks. Similarly, bacteria stocks grown from colonies produced by the frozen master stock were more stable and less prone to mutation than those grown from colonies produced by refrigerated stock. Multiple iterations of using existing stock
to make new stock eventually resulted in spotty bacteria lawns and weak plaque formation. This is likely to be a result of a mutation in the bacteria than the bacteriophage, which do not adapt as easily.

Bacteriophage stocks were evaluated for concentration after they were made, resulting in very high concentrations of $10^8$-$10^{14}$ PFU/ml. At these concentrations, the bacteriophage stocks are very stable when stored at 4°C.

When bacteriophage solution is applied to blotting paper, the resulting clearing is a function of the volume of solution as well as the concentration. A blotting paper disc with a relatively high amount of bacteriophage solution is less dry upon plating, which helps to support bacteriophage activity.

2.5 Acknowledgments

The authors would like to thank Dr. Santosh Pandey of the Iowa State University Department of Electrical Engineering for the use of his laboratory’s incubated scanner. Special thanks to Zach Njus for performing the overnight scans of our bacteriophage plates and obtaining video files of growth over time.

2.6 References


CHAPTER 3: DELIVERY VIA POLYMER HYDROGEL

3.1 Introduction

The principles of bacteriophage delivery are similar in many ways to those of drug delivery. Biomaterials used for drug delivery, especially degradable materials, must be compatible with the body’s systems. When treating inflammation or infections, the delivery material must be able to withstand both normal body chemistry, as well as the chemistry of the mounted immune response. At the site of infections, the microenvironment experiences a rise in temperature and a decrease in pH [1].

Hydrogels are a class of material that has been successful in drug delivery applications. Due to their wide range of polymer compositions and preparation methods, hydrogels are often used for controlled release, especially for local delivery [2]. Many hydrogels are made from naturally-derived polymers, which are the focus of this study. We chose naturally-derived polymers – in the forms of polysaccharides and proteins – because they show a high degree of biocompatibility, both in hydrogel form and after degradation [3].

The main polymer that we used in this experiment was alginate. A widely used biomaterial for wound healing and tissue engineering, alginate is a polysaccharide that is extracted from brown algae. Alginate is a copolymer, consisting of (1,4)-linked β-D-mannuronic acid monomers (abbreviated as M) and α-L-guluronic acid (abbreviated as G). The polysaccharide is made up of three distinct blocks: M-blocks (i.e. MMMMMM), G-blocks (i.e. GGGGGGG), or alternating blocks (i.e. GMGMGM) [4]. As shown in Figure 3.1, the structure of the G-blocks is more angular and provides stiffness, while the M-blocks are more linear and provide flexibility [5].
Alginate hydrogel transitions from a viscous liquid into a gel via ion exchange. The sodium ions present in the alginate solution are exchanged for the divalent ions in the ion crosslinker solution. The divalent ions form bridges between monomers of the G-blocks. The cross-linked regions form a three-dimensional helix shape, also called an “egg-box,” as modeled in Figure 3.2 [6]. When placed in lambda buffer, the calcium ions eventually exchange with the monovalent sodium ions in the buffer, which breaks these linkages. When the crosslinks break, the bead takes in water and swells. Eventually, the degree of crosslinking falls enough for the beads to dissolve completely [6].

![Chemical structures of alginate M-blocks, G-blocks, and alternating blocks](image)

In this experiment we compared the release rates of pure alginate beads with alginate-blend beads. Firstly, gelatin is one of the blend polymers that we chose. Unlike alginate, gelatin is a protein. In medicine, gelatin is used as a film, in oral delivery, or in optical applications. Its mechanical properties can be modified by crosslinking with small molecules like genipin [7], though gelatin also exhibits temperature-sensitive solidification.
The second polymer we chose is collagen. Like gelatin, collagen is a protein and can be modified by crosslinking either by exposure to ultraviolet light or with small molecules. Collagen is considered to be more biocompatible than gelatin, which is most often restricted to the digestive system. In comparison, collagen may be used in nanoparticles, wound dressings, and tissue engineering. In drug delivery, collagen may be tailored to the target area of the body. Corneal infections are often treated with collagen film, while bone formation may be stimulated with a collagen-bone morphogenetic protein composite. When treating wet tissue, collagen is often in hydrogel form [8].

Lastly, we studied chitosan hydrogel. Like alginate, chitosan is a polysaccharide. It is known for quickly responding to changes in its environment. Chitosan polymer is especially sensitive to pH, as it dissolves in an acidic environment. Like the previous polymers, it is often modified by grafting or crosslinking to improve its toughness and stability [9]. Chitosan has been successfully used in wound healing, tissue engineering, and drug delivery applications [10], making it a candidate for bacteriophage delivery.
3.2 Experimental

3.2.1 Materials

3.2.1a Alginate Hydrogel

The alginate hydrogel was made from low-viscosity alginic acid sodium salt from Alfa Aesar. The alginic acid powder was dissolved in nanopure water to form a 5% solution, then autoclaved. The alginate gel was left to thicken at room temperature overnight before use. Ion crosslinker solutions were made from calcium chloride and barium chloride powders from J. T. Baker. The salts were dissolved in a nanopure water to a concentration of 100 mMol.

3.2.1b Gelatin Hydrogel

For some of the experiments, alginate hydrogel was blended with one of the following: gelatin, collagen, or chitosan. The gelatin was obtained from Amresco. Gelatin powder was dissolved in nanopure water to form a 10% solution under gentle heat and stirring, then autoclaved. The gelatin gel was stored in a 50°C water bath to prevent solidification.

3.2.1c Chitosan Hydrogel

The chitosan powder was obtained from TCI. To prepare the hydrogel, we dissolved the chitosan in 0.5% acetic acid under gentle heat and stirring to form a 1% solution. The solution was then autoclaved. After autoclaving, genipin from Cayman Chemical Company was suspended in lambda buffer at a 5% concentration. A 0.5 ml aliquot of genipin suspension was introduced to the chitosan solution and was gently shaken at 37°C for 2.5 hours to allow for crosslinking. The crosslinking reaction produces a change in color, and the final solution turned a light blue color. The crosslinked chitosan yielded a viscous liquid, which was combined with alginate and incubated in calcium ion solution.
3.2.1d Collagen Hydrogel

A bovine achilles tendon collagen powder was obtained from Alfa Aesar. The collagen powder was dissolved in 0.5% acetic acid under gentle heat and stirring to form a 1% solution. The solution was then autoclaved. After autoclaving, a crosslinking agent was added to the hydrogel solution. In the first trial, approximately 0.4 mg transglutaminase powder was added and the mixture was incubated at 37°C under shaking. In the second trial, 0.5 ml of a 5% genipin suspension was added and the mixture was incubated at 37°C under shaking. The resulting solution was mixed with alginate and incubated in calcium ion solution.

The prepared gels were placed in lambda buffer to measure release kinetics. The lambda buffer contains 0.58% sodium chloride from BDH, 0.02% magnesium sulfate from Fisher Chemical, 5% Tris buffer (1M, pH 7.5) from Alfa Aesar and 0.01% reagent-grade gelatin from Amresco.

3.2.2 Experimental Design

Hydrogels are known for their variable degradation rates, depending on processing conditions. In this experiment, we aim to compare release kinetics of pure alginate with alginate blends. We chose alginate as a base polymer because of its ability to be shaped into millimeter-scale beads, comparable in size to readily-available gel-cap medications. The polymers were prepared with nanopure water (or acetic acid diluted with nanopure water, as appropriate) and autoclaved to reduce contamination. The gels were prepared with water instead of bacteriophage-supporting lambda buffer to reduce the number of variables affecting the ion crosslinks.

The alginate or alginate blend gels were mixed with bacteriophage solution with a vortex mixer. The gel was then drawn up by a pipet or micropipette and dropped into the ion
crosslinker solution under gentle stirring. The gel beads incubated in the ion solution for 10 minutes. Early experiments showed that the degradation time of alginate beads was the same for 10- and 15-minute incubations.

After incubation, the ion solution was drained and the excess was removed with a sterile micropipette. We used sterile tweezers to transfer the beads into either well plates or microcentrifuge tubes containing 1 ml of lambda buffer each. The beads were placed in a shaker at 37°C. The beads were kept at 37°C to mimic human body temperature. We placed the beads under shaking to distribute the released bacteriophage throughout the buffer. Although we aim to mimic human physiology through temperature and movement, we used lambda buffer in favor of a PBS solution because the lambda buffer is more well-known for supporting bacteriophage activity, which is necessary for the release solution.

At each time point, we saved the release solution from a set number of beads, and then plated those beads on an *E.coli* lawn. The release solutions were serially diluted and measured for concentration. The beads that were not plated were kept for subsequent time points, and their release solutions were discarded and replaced with fresh lambda buffer. The new lambda buffer mimicked bacteriophage moving away from the hydrogel and being absorbed into the bloodstream. The concentration gradient of bacteriophage was reset, increasing the driving force of bacteriophage to leave the hydrogel. In addition, the new lambda buffer introduced new ions to the environment, which interact with the alginate’s ion crosslinkers. The presence of these new ions promotes degradation of the polymer network, further stimulating bacteriophage release.
3.3 Results

3.3.1 Effect of Crosslinker Ion

Calcium is commonly used as a crosslinking ion for alginate, and was used as a baseline for all other comparisons. We found that calcium-crosslinked alginate beads swelled over the first 48 hours of incubation in buffer, after which they began to break down. The beads then dissolved completely after 1 to 5 days, as shown in Figure 3.3. The calcium-alginate beads held their spherical shape for most of that period of time. The water influx that causes swelling also widens the bead’s pores. It is expected that the moderate rate of swelling would also correlate with delayed bacteriophage release, rather than an initial burst of release. We found that calcium-alginate beads delayed the release of bacteriophage by 8 to 12 hours. The release of calcium-crosslinked beads is shown in Figure 3.6. The releases shown compare two dispensation methods, as well as a comparison of solution storage times: 1 month storage at 4°C and no storage.

Barium is another common crosslinker for alginate. Like calcium, barium is a divalent ion belonging to the alkaline earth period. Barium-crosslinked alginate beads have a cloudy exterior, whereas calcium-crosslinked alginate is transparent. Barium-alginate began to swell immediately, and lost its spherical shape after 6 hours of incubation in buffer. However, the polymer was still in a gel state and had not dissolved into the liquid. The barium-alginate also showed an initial burst of bacteriophage release. Due to the early loss of spherical shape, the bacteriophages suspended in the interior of the bead were quickly exposed to the buffer environment. The barium-crosslinked beads dissolved after 2 to 3 days of incubation, as shown in Figure 3.3. The release of barium-crosslinked beads is shown in Figure 3.6. Like the
calcium-crosslinked beads, this figure compares dispensation methods as well as release solution storage.

We also considered magnesium as a possible crosslinker. Magnesium is a divalent ion that shares a period with calcium and barium. We incubated alginate in a 100 mMol solution of magnesium sulfate, which is a salt consisting of a divalent cation and divalent anion. The alginate did not form spheres.

A subsequent literature search found that magnesium does not bind with alginate as effectively as calcium, and requires a five-fold increase in the concentration of the ion solution [11]. Additionally, sulfate salts are not commonly used in alginate crosslinking. Monovalent chloride anions appear be preferable to divalent sulfate ions in binding the exchanged sodium ions, which indicates that ion exchange between the sodium ions and magnesium ions is not entropically favorable in this particular case. This leads us to believe that a high concentration of magnesium chloride (MgCl$_2$) may crosslink the alginate into spheres. This hypothesis will be tested in future experiments.

3.3.2 Effect of Bead Dispensation Method

Two methods of dispensing hydrogel into ion crosslinker solution were compared. In the first, we tried cutting a 1-ml micropipette tip at the 250 μl mark, resulting in a diameter of 6 mm. Using this system, beads of approximately 100 μl in volume were dispensed. However, the scissors used to cut the tip were thought to be a potential source of contamination, despite pre-sterilizing the blades. We also attempted dispensing beads via a 15-ml pipet tip. The 15-ml pipet was individually sealed in a sterile container, with a tip diameter of 3 mm. Despite the difference in diameter size, the 15-ml pipet dispensed beads of a similar size – approximately
83.3 μl – because of the hydrogel’s cohesive force when forming a drop. In Figure 3.5, the two dispensation methods are compared.

We did not see a reduction in contamination, however. In both cases, the beads did not show contamination after 21 hours of incubation, but did show a small amount of contamination around the bead after 24 hours.

3.3.3 Effect of Incubation Containment

Early alginate experiments were incubated in 1.5 ml microcentrifuge tubes. The opening of the microcentrifuge tube is approximately 1 centimeter, and is tapered at the base. We found that the alginate bead initially settled at the bottom of the tube, with only the upper surface area in contact with the free buffer. As the bead swelled, it floated to the midsection of the microcentrifuge tube and was surrounded more evenly by the buffer. The swelling of the barium-crosslinked beads spanned the area of the midsection, while the calcium-crosslinked beads gained less volume. The barium-crosslinked beads were more obstructive to attempts to draw up release solution, and were often destroyed by the micropipette tip in the process.

Subsequent experiments were performed in a 24-well plate, which is made up of cylindrical wells without tapering. Each well was approximately 1.5 centimeters in diameter. In this environment, the alginate beads are able to float freely in buffer. The swelling of the alginate beads did not reach the diameter of the wells. Dissolution happened more naturally in this environment, without a narrow boundary to limit burst, and reduced disturbance by micropipette tips.

3.3.4 Sterilization Technique

Early experiments were performed with 2% pure alginate, which was sterilized via filter sterilization. However, this was found to be an inefficient technique due to the viscosity of the
hydrogel. Multiple filters were required in order to sterilize small amounts of alginate. After sterilization, we found that the filtered hydrogel was easily contaminated.

We then attempted to autoclave the 2% alginate solution, but the solution lost a significant amount of viscosity. The autoclaved 2% solution was thin enough that it did not form beads in calcium ion solution. A subsequent literature search confirmed this phenomenon, showing that a 1% alginate solution decreased in viscosity by 78%, and a 3% alginate solution decreased in viscosity by 86%. The loss in viscosity is caused by the depolymerization of alginate that occurs at temperatures above 100°C. Subsequent experiments would instead begin with a higher concentration and thus a higher viscosity to compensate for solution thinning. A 5% alginate solution was autoclaved, then left to stand overnight. The solution appeared to regain a small amount of viscosity after storage at room temperature. The autoclaved alginate was sterilized in small amounts to prevent excessive transfer between containers, and was found to harbor less contamination than the filter sterilized solution.

3.3.5 Effect of Blending with Gelatin

The alginate:gelatin blend was made with a 5% alginate solution and 10% gelatin solution. Several alginate-gelatin blends were considered. In the first trial, we studied 25:75, 50:50, and 75:25 alginate:gelatin blends. The 25:75 alginate:gelatin blend dissolved upon incubation in the calcium ion solution, and was taken off-study. The 50:50 and 75:25 alginate:gelatin blends successfully formed into beads, though their shape was an ellipsoid rather than a sphere. The dissolution times of the beads increased with increasing amounts of alginate, and are shown in Figure 3.4. The release profiles for calcium-crosslinked and barium-crosslinked alginate are shown in Figures 3.6 and 3.7, respectively.
In the second trial, we studied 33:67, 60:40, and 90:10 alginate:gelatin blends. Each blend formed a bead upon incubation in the calcium ion solution. The 33:67 blend dissolved very quickly, while the 60:40 alginate:gelatin blend stayed in gel form longer. The 90:10 alginate:gelatin blend showed a dissolution time similar to that of pure alginate. The dissolution times are shown in Figure 3.4, and the release profile is shown in Figure 3.9.

3.3.6 Effect of Blending with Collagen

The alginate:collagen beads were made with a 1% collagen solution, which introduces more water into the hydrogel mixture than the 10% gelatin hydrogel. The dry collagen was obtained from bovine achilles tendon, and was a stringy and airy network rather than a powder. When dissolved in 0.5% acetic acid, the collagen solution appeared white and cloudy. After autoclaving the solution, we attempted two different crosslinking agents: transglutaminase and genipin. Following a protocol by Janine M. Orban [12], we allowed the transglutaminase powder to incubate in the collagen solution for 2 hours at 37°C. However, this method did not crosslink the collagen, and it remained a liquid. Subsequent mixing with alginate and exposure to calcium ions did not produce beads. Further investigation into crosslinking by transglutaminase was not pursued for budgetary reasons.

In our second trial, genipin was used to crosslink collagen. Based on a protocol by Shunji Yunoki [13], we incubated 0.5 ml of 5% genipin in lambda buffer and 1% collagen solution at 37°C under shaking, while observing periodically for a change in color. The collagen was crosslinked and in a pourable state after 19 hours of incubation. The collagen was mixed with alginate in a 33:67, 60:40, and 90:10 ratio of alginate:collagen. Upon incubation, we found that the 33:67 and 60:40 alginate:collagen blends did not form beads, likely because of the high water content introduced by the collagen solution. We continued the experiment with the beads
formed from 90:10 alginate:collagen. The release of the alginate:collagen blend is found in Figure 3.9.

3.3.7 Effect of Blending with Chitosan

A chitosan solution was made by dissolving chitosan powder into 0.5% acetic acid, to form a 1% solution. Using the protocol described above, we incubated the chitosan solution with 0.5 ml of 5% genipin in lambda buffer. The crosslinking reaction reached a crosslinked, pourable state after 2 hours. The chitosan was mixed with alginate quickly, as the chitosan tended to become sticky and immiscible if left at room temperature for more than 1 hour. Like the collagen hydrogel, the chitosan was mixed with alginate in 33:67, 60:40, and 90:10 alginate:chitosan ratios. Again, the 33:67 and 60:40 alginate:chitosan ratios did not form beads. The 90:10 alginate:chitosan beads were used in this experiment. The release from these beads is shown in Figure 3.9.

3.3.8 Dissolution and Release

![Figure 3.3](image)

Dissolution times observed in 3 iterations of alginate bead experiments. Two iterations tested beads dispensed with a cut 1-ml pipette tip, while one iteration tested beads dispensed with a 15-ml pipet. All other processing conditions were equal. Error bars denote standard deviation.
The dissolution times, seen in Figures 3.3, 3.4, and 3.5 were recorded for all hydrogel blends. The calcium-crosslinked alginate was found to dissolve slowest, followed by the 90:10 and 75:25 alginate:gelatin blends. The 33:67 alginate:gelatin and 90:10 alginate:collagen blends...
blends dissolved most quickly. It is hypothesized that allowing the collagen to crosslink for a longer period of time may increase the time before dissolution. The barium:alginate blend had the most consistent time before dissolution.

The hydrogel release graphs show a burst of release from the quick dissolving blends, such as 90:10 alginate:collagen and 33:67 alginate:gelatin. The slower-dissolving blends, such as calcium-crosslinked alginate and 90:10 alginate:gelatin, show a more sustained release of bacteriophages over time, which resembles zero-order release kinetics. In practice, the different release patterns are both valuable, however, depending on the infection site and severity. A burst-release would be beneficial for a severe infection in the stomach, while a slower release may be able to stay gelled long enough to reach the intestine.

![Figure 3.6](image_url)

Cumulative bacteriophage release profile of calcium-crosslinked alginate beads. The beads show a variable dissolution time, and a relatively consistent release. Error bars indicate standard deviation.
Figure 3.7
Cumulative bacteriophage release profile of barium-crosslinked alginate. Release begins earlier than calcium-crosslinked beads, and release is relatively consistent until dissolution after a small initial burst. Error bars indicate standard deviation.

Figure 3.8
Cumulative bacteriophage release profile of alginate:gelatin blends. Blends with higher gelatin content dissolved more quickly with a high burst of release. Error bars indicate standard deviation.
3.3.9 Swelling Calculations

The rate of bacteriophage release appears to be dependent on the degree of swelling, as the increased volume fraction of water in a gel widens the polymer pores. Hydrogel swelling can be calculated by comparing the beginning volume with the final volume. The volumes were estimated by imaging the beads with a scanner, measuring the bead diameters with ImageJ, and using the results to calculate volume. The swollen volume was measured using the timepoint before the bead lost its shape. After calculation, we found the extension ratio (Figure 3.10).

\[ \lambda_x = \lambda_y = \lambda_z = \left( \frac{V}{V_0} \right)^{\frac{1}{3}} \]

The term \( \lambda \) denotes the extension ratio, which can be applied to any of the Cartesian directions. In this case, the hydrogel is non-directional and we can assume that the extension...
ratio is equal in all directions. The initial and final volumes can also be used to find the volume fraction of polymer in the swollen hydrogel, indicated as $\varphi$ (Figure 3.11).

$$\frac{V}{V_0} = \frac{1}{\varphi}$$

Polymers with a high extension ratio and low volume fraction of polymer in a swollen state experienced the most swelling. Thus, these polymers tend to release bacteriophages in a quick burst, as the polymer strands that retain the bacteriophages form wider pores. Polymers that had a low extension ratio tended to release bacteriophages more steadily.

![Figure 3.10](image)

Figure 3.10

Extension ratio of polymers tested. The stars indicate polymer blends that demonstrated the highest burst release. Error bars indicate standard deviation.
The high error values of the polymer volume fraction can be attributed to the different burst points of the hydrogel beads, as well as the fact that different beads were measured at each timepoint. The relatively low values of the extension ratio are a result of the cube root of the volume ratio, which reduced the magnitude of error.

### 3.3.10 Other Hydrogels Considered

There were two other hydrogels considered for this study. The first is hyaluronic acid, which is a naturally-derived polysaccharide similar to alginate. Both hyaluronic acid and alginate are copolymers containing guluronic acid monomers, with different chirality. Current
applications of hyaluronic acid include drug delivery, wound dressing, and tissue engineering (Yi).

We attempted to crosslink hyaluronic acid hydrogel by precipitating sodium hyaluronate in methacrylic anhydride, adjusting the pH to 8 using sodium hydroxide. The amount of sodium hydroxide to be added was calculated, but the amount expected to adjust the pH was found to be insufficient to change the color of the Neutral Red indicator. We later found that the indicator used was faulty, and the hyaluronic acid hydrogel was no longer considered.

The second hydrogel considered was sodium polyacrylate, which is a self-crosslinking synthetic polymer. Sodium polyacrylate gels upon exposure to water under stirring. A 100 ml sample of 5% sodium polyacrylate solution gelled within 5 minutes of stirring. The gel was not able to be shaped, and incubation of sodium polyacrylate gel in lambda buffer did not produce a release solution. In fact, the sodium polyacrylate gel had absorbed the lambda buffer. The sodium polyacrylate hydrogel was abandoned because we hypothesized that it would not release bacteriophages into a solution efficiently.

3.3.11 Future Work

These experiments demonstrated release into lambda buffer, which is a supportive environment for bacteriophages. Future experiments performed in an acidic solution would better predict the outcome of bacteriophage release and survival. While bacteriophages are not stable in acidic environments, early bacteriophage experiments showed activity when administered orally alongside a sodium bicarbonate solution to neutralize acidity in the stomach. A future experiment can take these findings into account by testing bacteriophage release into a neutralized simulated gastric fluid solution.
Additional experiments with alginate blended with collagen or chitosan would also be beneficial, to further explore varying degrees of crosslinking and its effect on bacteriophage release. In theory, a collagen or chitosan hydrogel that has been loaded with bacteriophage and tightly crosslinked could be mixed with an alginate-bacteriophage solution and exposed to calcium chloride. A two-phase release could be visualized upon the early breakdown of alginate, followed by a secondary dissolution of collagen or chitosan. In addition, magnesium can be explored as a possible crosslinking ion for alginate hydrogel.

### 3.4 Conclusions

Alginate-based hydrogels show great promise as delivery systems for bacteriophages. Controlling the rate of bacteriophage release can determine where the bacteriophages will be released in the digestive tract. This can be done by blending alginate with other hydrogels in varying ratios.

### 3.5 References


CHAPTER 4: DELIVERY VIA POLYANHYDRIDE MICROPARTICLES

4.1 Introduction

Besides oral delivery, bacteriophages may also be introduced to the body through the nasal mucosa. Nasal treatments are a non-invasive way to target the respiratory system, or distribute the drug systemically through the bloodstream. However, effectiveness of nasal delivery depends on the type of drug selected. As mentioned in section 1.6.2 of Chapter 1, bacteriophages can be considered to be large protein-based entities, ranging in length from 100-200 nm. Polar particles of high molecular weight have been shown to be the most difficult to cross the mucosal membrane [1], therefore a delivery vehicle must be used in conjunction with the bacteriophages.

For peptide and protein-based drugs, bioavailability after nasal application is often less than 1%. Since many of these drugs are not readily absorbed through the mucosal membrane, the excess is either degraded enzymatically or eliminated by the mucociliary clearance process within approximately 20 minutes. To improve the absorption of high-molecular-weight, polar drugs, there are approaches to consider: chemical modification, application of enzyme inhibitors, inclusion of absorption promoters like surfactants and bile salts, and encapsulation in a polymer microparticle [2]. In the case of bacteriophage therapy, chemical modification is not an option. As mentioned in 1.6.2 of Chapter 1, enzyme inhibitors and absorption promoters tend to damage the mucosal membrane [1]. Polymer encapsulation is the best choice for effective bacteriophage delivery.

Unlike the hydrogels of the previous chapter, polymer microparticles are a dry microenvironment, which can adversely affect the stability of bacteriophages. A study of bacteriophages against *Staphylococcus aureus* encapsulated in poly(lactic-co-glycolic acid), or
PLGA, found that bacteriophage activity tapered off after one week in dry storage. This result was seen at storage temperatures of both 4°C and 22°C. However, bacteriophage activity can be retained in dry conditions with the addition of stabilizers. The most common, non-immunogenic stabilizers include trehalose, sucrose, and polyethylene glycol (PEG) [3].

In this study, the stabilizing effects of trehalose were considered. Trehalose is an osmolyte, and preserves the activity of bacteriophages (and proteins) by stabilizing labile proteins in a dry state, as well as acting as a water substitute through hydrogen bonding. Despite the weak interaction with the proteins that make up the bacteriophage, trehalose is found to be inert towards the bacteriophage. The stabilizing effects of trehalose are also seen in high-pressure environments, like microparticle formation, in which the bacteriophages’ protein capsids and tails are susceptible to pressure denaturation [4].

Effective absorption of bacteriophages not only depends on survival in a dry state, but also promotion of particle internalization. The bacteriophage treatment will ideally be absorbed in the same manner as the infectious agent, while avoiding an immune response. In this case, polyanhydride microparticles are an ideal delivery vector due to their biocompatibility, degradability, and hydrophobic nature [5].

The polyanhydride microparticle is able to mimic a pathogen by activating the body’s innate immune system. The immune response is mounted when a pathogen-associated molecular pattern (PAMP) is detected by the pattern recognition receptors (PRRs) on an antigen-presenting cell (APC), located among epithelial cells [6]. An example of a PAMP is the lipopolysaccharide, or LPS, found on E.coli cells [5]. Specifically, LPS interacts with Toll-like receptors (TLRs), among other PRRs. In the case of polyanhydride microparticles, the hydrophobic nature of the polymer mimics PAMPs, which are often hydrophobic as well. Upon interaction with PRRs, the
microparticle is internalized by the dendritic cell, either by phagocytosis or endocytosis, depending on polymer chemistry. The polyanhydride then degrades, releasing bacteriophages. One possible application for bacteriophage-loaded microparticles is in the treatment of tuberculosis. The disease is caused by the presence of \textit{Mycobacterium tuberculosis} in the alveolar macrophages, or AMs, which is a type of APC. This type of infection is known as delayed type hypersensitivity. Upon internalization by the AM, bacteriophages released from the cell would target the \textit{M. tuberculosis} bacterium immediately. A nasal bacteriophage treatment would target the lungs directly, as opposed to systemic oral drug delivery treatments used currently. Systemic treatments rely on high doses of drug to reach the alveoli, which can cause several side effects in various physiological systems [7]. Nasal bacteriophage delivery, on the other hand, is a targeted method of delivery using a highly specific biological entity, which is a promising combination for fast, effective recovery.

4.2 Experimental

4.2.1 Materials

The polyanhydride microparticles tested are copolymers with varying ratios, consisting of two of the following monomers: sebacic acid (SA), 1,6-bis(\textit{p}-carboxyphenoxy)hexane (CPH) and 1,8-bis(\textit{p}-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). The structures of these monomers

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure4.1.png}
\caption{Polyanhydride monomer chemical structures. Clockwise from top left: SA, CPH, and CPTEG.}
\end{figure}
are shown in Figure 4.1. The polymers used for microparticle formation were synthesized in Balaji Narasimhan’s lab using methods outlined in literature [8].

Degradation rates of polyanhydrides can be controlled by copolymerizing monomers of separate chemistries. For example, monomers of aliphatic chemistries (such as SA) degrade relatively quickly, because water molecules are able to access and hydrolyze the anhydride bonds. In contrast, aromatic monomers (such as CPH) have less accessible anhydride bonds due to the adjacent aromatic ring. These polymers require much more time to degrade [9]. The CPTEG monomers degrade through a different mechanism than that of SA and CPH. As seen in Figure 4.1, CPTEG contains a region made up of ethylene glycol that is more accessible than the anhydride bonds. Water molecules are able to hydrolyze the ethylene glycol region quickly, precluding the necessity of hydrolyzing the anhydride bonds [8].

Hydrolysis of anhydride bonds produces dicarboxylic acid by-products [9]. The degradation products from SA, CPH, and CPTEG have been found to be non-toxic and non-immunogenic. However, dicarboxylic acids tend to lower the pH of the surrounding microenvironment. The most acidic polyanhydride used in the microparticles was SA, which upon degradation lowers the pH to 4.2. The by-products of CPH lower the microenvironmental pH to 5.5. To compare, the by-products of the commonly-used poly(lactic acid-co-glycolic acid), or PLGA, lower the microenvironmental pH to 2 [8]. Since bacteriophages lose activity in highly acidic environments, the polyanhydrides SA, CPH, and CPTEG are therefore better choices in this application.
4.2.2 Experimental Design

4.2.2a Microparticle Formation

There are three main methods of microparticle formation: precipitation, double emulsion in water/oil/water, and spray-drying. We attempted precipitation of 20:80 CPTEG:CPH by dissolving the polymer in chloroform and precipitating the particles in a 1:80 excess of pentane at -20°C. We found that the size of the resulting microparticles was less than 1 μm in diameter, which is below our target size of 1-5 μm in diameter. Due to the failure of precipitation to form the desired microparticle size, we opted to investigate spray-drying rather than emulsion, as spray-drying tends to produce larger particles. We verified that spray-dried 20:80 CPTEG:CPH microparticles were the correct size through SEM. All subsequent microparticles were produced by spray-drying.

The spray-dryer used is the model Buchi B-290 mini spray-dryer, as shown in Figure 4.2 [10]. We began by spray-drying a control of bacteriophage suspension without polymer in order to obtain a control of bacteriophage activity retention without polymer. The bacteriophages

![Figure 4.2](image)

Schematic of Buchi B-290 spray-dryer. Our phage-polymer solution was mixed on a stir plate before being heated and introduced to the drying chamber. The gas used in the drying chamber was argon.
were suspended in lambda buffer. The aqueous nature of the solvent required the spray-dryer to run at 80-85°C to ensure appropriate evaporation. The bacteriophages saw a drop in activity of 5 orders of magnitude, i.e. from $10^{10}$ PFU/ml before spray-drying to $10^5$ PFU/ml after spray-drying and reconstitution.

To prepare microparticles of polymer and bacteriophage, the hydrophobic raw polymer was first dissolved in chloroform, after which the bacteriophage was added. With this solution, the spray-dryer was run at 40°C to reflect the lower liquid-gas transition temperature of chloroform. All other settings remained the same. A white powder was produced, which adhered to the cyclon and holding vial. Due to the low glass-transition temperature of CPTEG-rich polymers, the microparticles were removed from the cyclon and the vial in a walk-in refrigerator held at 4°C.

4.2.2b Bacteriophage preparation

To produce spray-dried microparticles, the encapsulating polymer was dissolved in chloroform as it is unstable in water. The bacteriophages are stored in lambda buffer, but are stable in chloroform for a short amount of time. To evenly distribute bacteriophage in the polymer solution, two methods were investigated: lyophilization (freeze-drying) or use of a surfactant.

We found that lyophilization reduced the bacteriophage activity by 4 orders of magnitude, i.e. a sample with an activity of $10^{10}$ PFU/ml initially showed activity of $10^6$ PFU/ml after lyophilization and reconstitution. These results were found with bacteriophages originally in lambda buffer and reconstituted in sterilized water, as the lambda buffer salts were assumed to be present in the resulting freeze-dried pellet after lyophilization.
To prevent an initial drop in bacteriophage activity before spray-drying, surfactants were also considered. The surfactant Tween-20 was mixed with bacteriophage solution, after which we evaluated bacteriophage activity. There was no reduction in bacteriophage activity found in the bacteriophage-Tween-20 mixture. In subsequent experiments, we chose to introduce an aqueous surfactant-bacteriophage solution to the polymer-chloroform solution before spray drying.

Early experiments were performed with both T4 and T7 bacteriophages. Since spray-drying subjects the bacteriophages to high shear stresses, we noted that T7, the smaller bacteriophage, may fare better than a longer bacteriophage, T4. We hypothesized that T4 may become inactivated during spray-drying due to damage to its thin tail. In later experiments, we performed trials on batches of T7 bacteriophages only, with the intention of expanding the scope to T4 bacteriophages if successful. The use of trehalose as a stabilizer was also considered. As mentioned in section 4.1, trehalose shows a stabilizing effect in dry environments. We hypothesized that the trehalose may lessen the chance of bacteriophage inactivation by pressure denaturation.

4.3 Results

Three formulations of polymer were tested for bacteriophage release: 20:80 CPTEG:CPH, 15:85 CPTEG:CPH, and 20:80 CPTEG:SA. With the 15:85 CPTEG:CPH formulation, we tested the effectiveness of 1% w/v trehalose in the bacteriophage stock.

4.3.1 20:80 CPTEG:CPH

Microparticles of 20:80 CPTEG:CPH were spray-dried in three batches. The first batch was a blank control made with sterilized water instead of bacteriophage solution. The second batch was made with a $10^{12}$ PFU/ml T4 bacteriophage stock diluted to $10^{11}$ with sterilized water.
The third batch was made with a $10^{11}$ T7 bacteriophage stock diluted to $10^{10}$ with sterilized water. The aqueous bacteriophage phase was mixed with Tween-20 surfactant, then stirred into the polymer-chloroform phase. The mixture was kept under stirring during the spray-dry process.

The total amount of bacteriophages in the pre-spray-dried solution was calculated as follows:

$$\frac{PFU}{sample} = \left( \frac{weight \ of \ sample}{weight \ of \ microparticle \ yield} \right) \times \left( \frac{PFU}{ml \ of \ bacteriophage \ stock}{Volume \ of \ stock \ in \ polymer \ solution} \right)$$

After the microparticles were obtained from the spray-dryer, we divided each batch into two groups: a slow-release group and a dry storage group. The slow release group was kept in lambda buffer at 37°C under shaking. At various timepoints, the lambda buffer was removed to count the amount of bacteriophages released, and the microparticles were resuspended in new buffer. The dry storage group was kept at 4°C in the refrigerator. At various timepoints, a sample was dissolved in chloroform, which breaks down the polyanhydride to release the bacteriophage. A sample of chloroform was then mixed with buffer to encourage the movement of bacteriophages into the aqueous phase to be counted. The microparticles were also imaged with scanning electron microscopy (SEM). The SEM images are seen in Figure 4.3.
Our first slow release sample was taken 2 hours after spray-drying. We found that the T7 bacteriophages had a higher initial activity than T4, as seen in Figure 4.4. The dry storage samples showed activity at the initial timepoint, as well as after 2 and 5 days of storage. When plated, the storage samples from day 2 showed contamination that was not eradicated upon chloroform exposure. On day 5, the T4 storage sample showed an activity of $10^4$ PFU/ml, while the T7 storage sample showed an activity of $10^3$ PFU/ml. From 6 days onward, the storage samples showed no activity. The study was concluded after 28 days.

Figure 4.3

SEM images at 5,000x magnification of 20:80 CPTEG:CPH microparticles containing. A: blank control. B: T4-containing C: T7-containing.
The 20:80 CPTEG:CPH experiment was conducted with one slow-release sample. Future experiments with multiple samples will be performed for more robust release data. Additional error was introduced in the bacteriophage stocks themselves, which were grown from existing stock rather than an individual plaque. The result of this improper growth technique is irregularly-sized plaques that tended to be larger than expected. This problem was rectified in later experiments.

4.3.2 15:85 CPTEG:CPH

The first iteration of 15:85 CPTEG:CPH microparticles were created using the same method as described above. Instead of separating slow release and dry storage samples, the microparticles were divided into 16 microcentrifuge tubes for bacteriophage-containing microparticles and 8 control tubes for slow release experiments. The microparticles were again imaged under SEM, as seen in Figure 4.5. In this experiment, only T7 bacteriophages were used.
At each timepoint, the release solution was removed and the microparticles were washed with lambda buffer. New lambda buffer was then added for incubation. One of the samples was diluted to a countable level, and was assumed to reflect the release of all microparticle samples. At two timepoints per week, two T7-containing samples and one control sample were dissolved in chloroform to evaluate the amount of bacteriophage remaining in the microparticle sample. This test was known as a quick release.

At the initial timepoint, about 30 minutes after microparticle formation, all samples showed bacteriophage activity, although the quick release performed after the removal of lambda buffer showed no activity. The counted sample was measured to have a concentration of approximately $10^5$ PFU/ml. At the next timepoint 2 hours later, 5 of the 14 bacteriophage-containing samples showed activity of approximately $10^3$ PFU/ml. No activity was seen in the slow release or quick release samples until the timepoint on day 5.

When a timepoint was taken 5 days after microparticle formation, a contaminant was found in the control samples, which showed strong bactericidal activity. The contaminant did not show bacterial growth, and appeared to be either bacteriophage presence or a bactericidal chemical. Of the bacteriophage-containing microparticles, 9 of the 10 samples showed a

![SEM images of 15:85 CPTEG:CPH at 5,000x magnification. A: blank control. B: T7-containing microparticle.](image)

*Figure 4.5* SEM images of 15:85 CPTEG:CPH at 5,000x magnification. A: blank control. B: T7-containing microparticle.
countable level of activity. We hypothesize that a smaller amount of contaminant was in the bacteriophage-containing samples than the blank samples. In the day 6 sample, we found that the activity in control samples persisted while the bacteriophage-containing samples again showed countable activity of approximately $10^3$ PFU/ml. We filter-sterilized the subsequent samples and found that the control-sample contaminant was still present at day 7, absent at day 10, and present again at day 14. The bacteriophage-containing microparticles showed activity of $10^3$ PFU/ml in 2 of the 8 remaining samples. The quick release timepoints measured within these time frames showed no activity. All further samples, taken until day 28, were found to have no activity.

A second iteration of 15:85 CPTEG:CPH was tested using a bacteriophage solution with 1% w/v trehalose as a stabilizer. This experiment was set up much like the previous iteration, although 8 tubes of T7-microparticles were used instead of 16 due to limitations in the amount of raw polymer available.

An initial timepoint was performed at about 30 minutes after microparticle formation. The release into lambda buffer showed activity in all bacteriophage-containing samples of approximately $10^3$ PFU/ml. The quick release, performed after the lambda buffer exposure, showed no activity. All subsequent timepoints, taken until day 28, showed no activity.

**4.3.3 20:80 CPH:SA**
Early experiments with 20:80 CPH:SA showed that it does not support bacteriophage activity well due to the acidity of the SA by-products. In this study, we prepared microparticles with bacteriophage only, without stabilizers. The microparticles were prepared in three batches, similar to the 20:80 CPTEG:CPH study: a blank control, T4-containing, and T7-containing microparticles. The resulting powder was divided into two microcentrifuge tubes per bacteriophage type. Some of the microparticles were imaged under SEM, shown in Figure 4.6.

![SEM images of 20:80 CPH:SA](image)

**Figure 4.6**

SEM images of 20:80 CPH:SA. A: blank control. B: T4-containing microparticles. C: T7-containing microparticles. The blank control polymer did not form into microparticles, unlike the T4- and T7-containing polymer mixtures. We hypothesize that this may be because of the bacteriophages acting as nucleation points during microparticle formation.

The first timepoint was taken 2 hours after microparticle formation. Both T4 samples showed no activity into lambda buffer. The T7 samples showed activities of $10^2$ and $10^3$ PFU/ml in each respective tube. The control did not show activity. Subsequent timepoints over 7 days showed no activity. After 8 days, we amplified the lambda buffer release solutions for a qualitative assessment of bacteriophage activity. To amplify bacteriophage activity, we placed 10 µl of the release solution in 90 µl of bacteria solution. The mixture was incubated for 12 hours at 37°C. The samples were then centrifuged to separate the bacteria from the bacteriophages. The bacteriophage-containing samples were then plated. Bacteriophage activity was present at 2 hours, 3 days in the T4-containing samples, 4 days in one T7-containing
samples, and 5 days in one T4-containing sample. These results were not able to be quantified, but rather were used as a qualitative analysis.

4.3.4 Future Work

The CPTEG:CPH blends appear to be the best polymer type for supporting bacteriophage activity. The 20:80 CPTEG:CPH blends show the longest-term support for bacteriophage activity, at 5 days. A wider-scale study with more samples will be performed to test the repeatability of these findings. In addition, a trehalose stabilizer will be added to the 20:80 CPTEG:CPH blends to determine whether bacteriophage activity can be retained during spray-drying. In addition, all subsequent studies will be performed with a chloroform quick release on a sample of freshly-made microparticles, without prior exposure to lambda buffer, for a better estimate of bacteriophage activity retained after encapsulation.

4.4 Conclusions

While the 20:80 CPTEG:CPH experiment appeared to be the most promising of the chemistries studied, additional iterations of the experiment must be conducted. The 15:85 CPTEG:CPH formulation showed initial bacteriophage release both with and without the trehalose addition. The 20:80 CPH:SA microparticles released bacteriophage in the smallest amounts, likely because of the acidity of the microenvironment that results from SA degradation.

4.5 Acknowledgments

The authors would like to thank the laboratory of Balaji Narasimhan for graciously allowing us access to their spray-dryer. The authors gratefully thank Tim Brenza for the synthesis of raw polymer for use in this study, as well as guidance in microparticle synthesis.

4.6 References


CHAPTER 5: SUMMARY AND CONCLUSIONS

Historically, bacteriophages have been shown to be effective in treating bacterial infections as well as acting as prophylactic measures. Recently, antibiotics have fallen out of favor due to an increasing prevalence of antibiotic-resistant bacteria, as well as difficulty in developing new formulations of antibiotics. Bacteriophages are a remarkable alternative to antibiotics due to their ubiquity, specificity, and variability in mechanisms for bacterial lysis.

Effective delivery of bacteriophages involves maintaining lytic activity, as well as tailoring a release rate appropriate to a particular situation. Delivery methods may be engineered to release bacteriophages in a burst for rapid treatment of a local infection, or may be released at a low, steady level for prophylactic effect. For these uses, we look to biodegradable polymers for controlled release of bacteriophages.

Hydrogels are a class of biomaterial commonly used in controlled release studies. They are easily modified for stability in various environments through crosslinking. In this study, we have shown that hydrogels are able to support bacteriophage activity, and are able to release bacteriophages into a buffer solution both in a burst or steady output, depending on the method of blending and crosslinking.

Polymer microparticles are another possible vector for bacteriophage delivery. The microparticles are commonly applied to the nasal mucosa, either for systemic release or to directly treat pulmonary infections. The polyanhydride microparticles, specifically the CPTEG:CPH copolymers, have been shown to support bacteriophages for a short amount of time, releasing them in an initial burst. Future experiments will be performed to investigate the possibility of longer-term release.