

ULTRASONICALLY CONTROLLED ANTIBIOTIC RELEASE FROM
HYDROGEL COATINGS FOR BIOFILM PREVENTION

by

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ABSTRACT

Medical devices are routinely employed in healthcare settings since they provide clinicians with a means of administering nutrients, drawing blood samples and drug delivery. However, local and systemic infections are frequently associated with the use of medical devices and implants. In fact, implanted devices often provide a highly suitable surface for bacterial adhesion and colonization resulting in the formation of complex, differentiated and structured communities known as biofilms. Once a biofilm infection is established, conventional treatments frequently fail as bacteria in the form of biofilms are much more resistant to antibiotics than their planktonic counterparts. A variety of implantable drug-delivery systems have been developed to combat biofilm related infections.

The main goal of this research was to investigate the effectiveness of a drug-delivery method using polymer hydrogels. The University of Washington Engineered Biomaterials (UWEB) group has developed a novel drug-delivery polymer matrix consisting of a poly 2-hydroxyethyl methacrylate hydrogel coated with ordered methylene chains forming an ultrasound-responsive coating. The polymer hydrogel was loaded with ciprofloxacin, an antibiotic well known for its action against gram-negative bacteria. This system was able to retain the drug inside the polymer in the absence of ultrasound but showed a significant drug release when low intensity ultrasound was applied.

A consistent experimental program has been developed to determine the effectiveness of the UWEB hydrogels against *Pseudomonas aeruginosa* biofilms. Biofilms were grown on hydrogel surfaces in flowcells. Ultrasound was applied for twenty minutes every twenty four hours for three days using a 43 kHz ultrasonic bath. Confocal images were taken both before and after ultrasound application. The confocal data was then analyzed quantitatively using the biofilm analysis software package, COMSTAT. In addition, a numerical model was developed to demonstrate and characterize drug-delivery from hydrogel surfaces.

Experimental results showed that biofilm accumulation on ciprofloxacin loaded hydrogels with ultrasound induced drug-delivery was significantly reduced compared to biofilms grown in control experiments. The results of these studies may ultimately facilitate future development of medical devices sensitive to external ultrasonic impulses, capable of treating or preventing biofilm growth via “on demand” drug release.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The frequency of device related and medical implant infections in human patients has spurred a rapidly growing field of research directed at controlling or eliminating these infections. Patients who are host to nosocomial infections often suffer systemically due to the pathogenic nature of the infecting organisms and the host cell response to both the infection and the implanted device. Various device related infections have been well documented on vascular catheters, prosthetic hips and knees and other orthopaedic implants [Gristina et al., 1993; Passerini et al., 1992; Strachan, 1995]. Among the more common organisms causing the infections are *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* .

Research in the area of bacteria habitation on surfaces (biofilm bacteria), has led scientists and medical professionals to implicate biofilms in many medical device related infections. The sustained survival of biofilms on implanted devices has raised questions on how to effectively treat or prevent biofilm infections. Infections of this nature often show high resistance to traditional systemic antibiotic treatments. Once a biofilm has been established, it becomes increasingly difficult to eradicate as the infection matures. In extreme cases when a biofilm infection has established itself, patients must undergo another series of revision surgeries to mechanically remove the infection and in some cases removal of the initial implant is necessary. This process

further increases the vulnerability of the patient to further nosocomial infections. For this reason, scientists and engineers have focussed attention on the prevention of biofilm infections due to the implantation of medical devices.

The effort to prevent biofilm formation on implant surfaces has proven to be an arduous and delicate task. The materials of which implants are manufactured must not promote an overly adverse response by the host cells. The development and manufacturing of such materials has been classified as Biomaterial Science. In recent years Biomaterial Scientists have also devoted efforts to creating materials that prevent or minimize biofilm infection, promoting further research into the causes, formation and survival of biofilms. The task of developing biomaterials that are compatible with a human host environment (biocompatible) while actively combating biofilm formation has demanded engineering innovation from the research and medical communities.

Many of the materials that have been developed incorporate drug delivery into the system. The idea is to be able to deliver antibiotics or other drugs to the site at which biofilm formation is likely to occur. Earlier models for these materials included an antibiotic, known to be active against certain bacteria, loaded into a polymer matrix of a biomaterial. Although somewhat effective, the release of the antibiotic out of the polymer matrix into a surrounding bulk fluid was only passively controlled or uncontrolled. The uncontrolled release of an antibiotic out of a polymer delivery system is known as leaching. At later stages, scientists began to develop actively

controlled drug delivery systems. Several delivery systems have been designed that are controlled chemically, thermally or mechanically.

The University of Washington Engineered Biomaterials (UWEB) Lab in Seattle Washington has taken part in the development of novel biomaterials specifically designed to prevent and maintain biofilm infections on medical implants. In 2000, UWEB developed a biocompatible coating that responds actively to ultrasonic energy [Kwok et al., 2000, 2001]. A poly 2-hydroxyethyl methacrylate (pHEMA) polymer matrix was embedded with ciprofloxacin or insulin. The polymer was molded into a thin film (0.015”) and was then coated with ordered methylene chains which created a self-assembled molecular structure that served as a barrier membrane. These polymers have come to be known as hydrogels. When hydrated, these hydrogels showed minimal release characteristics or leaching. However, when ultrasonic pressure waves at specific frequencies and power densities were introduced, drug release was initiated. When ultrasonic energy was removed from the system, the hydrogel again showed minimal drug release. This process was repeated several times and could conceivably be continued until the loaded drug was exhausted. The concept of using ultrasound to induce controlled drug delivery on the surface of a hydrogel is attractive to the medical community because ultrasound at low power levels is non-invasive and has been used in other areas of medical treatment and diagnosis. With this controlled drug delivery system, drug therapy could be initiated externally to the patient without the use of invasive surgeries.

Although the release of certain drugs from the UWEB coated pHEMA hydrogels have been documented, it has remained unclear if these hydrogels would be effective against biofilm formation and growth. The purpose of this research was to investigate the effectiveness ciprofloxacin loaded UWEB coated pHEMA hydrogels against the formation and growth of *Pseudomonas aeruginosa* biofilms.

Bacterial Biofilms

Bacterial behavior has traditionally been studied using cells that have been cultured in an aqueous solution. These cells are said to be “planktonic” or suspended in a liquid medium [Wilson, 2001]. However, it has become clear that in natural environments planktonic cells do not dominate the bacterial community. Bacteria exists in much higher quantities in complex aggregations called biofilms [Costerton et al., 1999]. The complexities of biofilms vary by organism and environment and, hence, require a complex definition. However, in general terms, biofilms have been defined as a group of bacteria (or other micro-organisms) attached to a surface [Wilson, 2001; Costerton et al., 1999; Stoodley et al., 2002; Wimpenny et al., 2000; Watnick and Kolter, 2000].

Typically, biofilms tend to cause many serious problems. Specifically they have been recognized as a source of fouling industrial equipment, dental surfaces, household products, pharmaceutical products and medical implants [Characklis and Marshall, 1990; LeChevallier et al., 1987; Bryers, 2000; Sternberg et al., 1999]. These problems

have motivated recent research efforts aiming at an enhanced understanding of biofilm behavior and properties.

Biofilm Formation

Biofilm formation is a complex process. A list of five components of biofilm formation are provided by Stoodley et al. [2002] and identified in Figure 1. Other authors have determined a more expanded list of components involved in the formation of biofilms [Characklis and Marshall, 1990; Bryers, 2000]. The first step in the formation process is characterized by the recognition and initial attachment of planktonic cells to a surface. In *Pseudomonas aeruginosa* biofilms, flagellar motility is reported by O'Toole and Kolter [1998] as being essential in initial attachment. Colloid chemistry also plays a role in initial attachment [Eginton et al., 1995]. As a colloid (cell) approaches a substratum (surface), the repulsion forces and attraction forces balance. When a colloid is in a state of minimum energy, the colloid no longer exhibits Brownian motion.

The second stage of biofilm formation is the production of extracellular polymeric substances (EPS). The EPS is a product of microbial cells and is an organic polymer gel [Characklis and Marshall, 1990]. Davies and Geesey [1995] have shown that for *Pseudomonas aeruginosa* biofilms in continuous culture, the EPS production is the result of specific gene functions that have been upregulated in biofilm cells.

The third and fourth components of biofilm formation, represented in Figure 1, are the stages of biofilm growth. Step three is the initial growth of the biofilm,

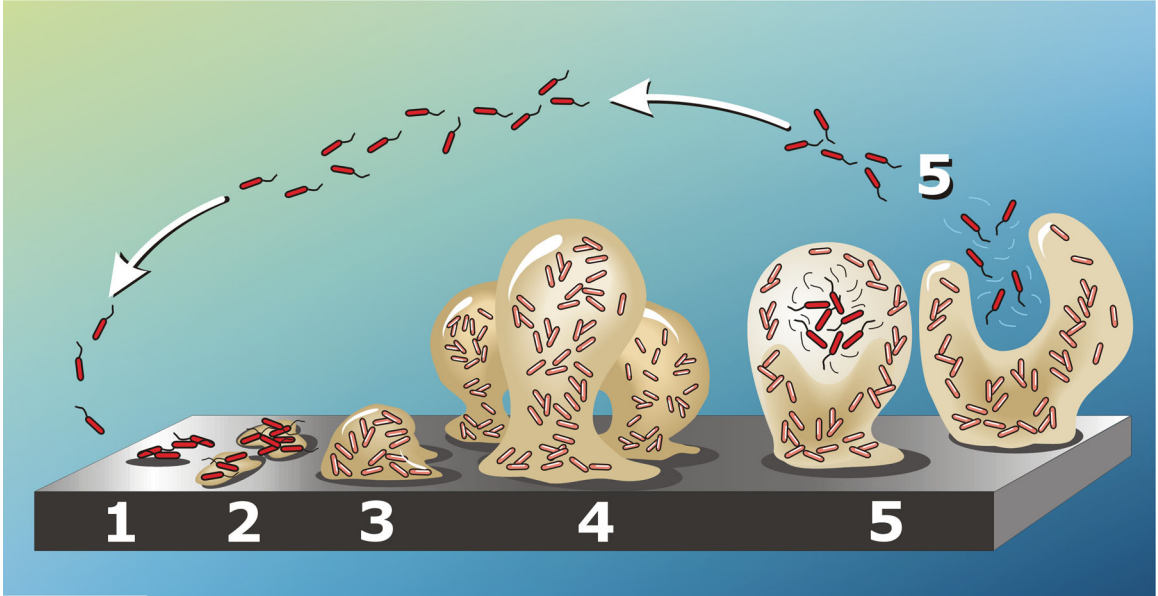


Figure 1: Biofilm formation: 1) Initial cellular attachment to the substratum. 2) Production of EPS. 3) Initial biofilm growth. 4) Formation of complex structures. 5) Detachment of cells back into the bulk fluid. Image used by permission [Stoodley et al., 2002].

and step four is the formation of complex structures. In step three, the biofilm begins to grow outward away from the substratum. Many of the pioneering biofilm researchers viewed biofilms as flat slab entities growing outward normal to a surface [Characklis and Marshall, 1990]. Modern microscopy has allowed scientists to view mature biofilms as complex structures with voids and channels, towers and mushroom shaped structures [Bryers, 2000; Zhang and Bishop, 1994; Wilson, 2001; Heydorn et al., 2000; Costerton et al., 1995; Stewart et al., 1995]. The formation of complex structure has been primarily described in terms of mass transport and growth rates that are highly dependant on nutrient supply [Wimpenny et al., 2000; Wasche et al., 2002; de Beer et al., 1994].

The fifth stage of biofilm development is detachment. Detachment is a mechanism by which viable cells and other biofilm components transfer back into the surrounding bulk fluid. Initially, biofilm detachment mechanisms were characterized in terms of interfacial shear stress which provide a mechanism for the biofilm to reach a steady state thickness [Bryers, 2000]. Recent mathematical models have allowed detachment terms to be functions of biofilm growth, which coincides more closely to laboratory observations and the dynamic nature of growing biofilms [Stewart, 1993; Peyton and Characklis, 1992].

Biofilm Resistance to Antibiotics

The minimum inhibitory concentration (MIC) is the concentration of a particular antimicrobial or antibiotic that prevents a particular organism from growing. It is noted that the MIC of biofilm cells are at least 500 - 5000 times greater than identical organisms existing in the planktonic state [Costerton et al., 1995; Khoury et al., 1992]. This observation has motivated research of biofilm resistance. As a result, several mechanisms of biofilm resistance have been identified.

The first of these mechanisms is the mass transport of antimicrobial agents [Costerton et al., 1995]. As a biofilm grows, it produces EPS which acts like a glue to hold a biofilm colony together. In addition, the EPS also acts as a diffusive barrier to mass particles, particularly antimicrobials. However, an opinion has been expressed that limited diffusion of antimicrobial agents in biofilms is insufficient to account for

reduced biofilm susceptibility to antimicrobial agents [Stewart, 1996]. Diffusion in biofilms does occur, but at a slower rate.

The second possible mechanism of the antimicrobial resistance of biofilms is nutrient limitation and the reduction in metabolic activity. This mechanism combines both mass transport limitation and the stress response of the organisms contained in a biofilm [Mah and O'Toole, 2001; Costerton et al., 1995]. In this case the mass transport limitation is that of nutrients instead of antimicrobials. It is noted that the same mathematical principles that govern the transport of antimicrobials through a biofilm, also apply to the nutrients needed by the bacteria for survival. When a cell is nutrient limited, its metabolic activity and growth rate decrease substantially. It was suggested by Brown et al. [1988] that the lower growth rates in biofilms are a mechanism of decreased antibiotic susceptibility.

The third suggested mechanism of biofilm resistance is that bacteria within microbial communities employ distinct biofilm specific mechanisms to resist antimicrobial agents [Mah et al., 2003]. Research in this area is led by the idea of a biofilm specific phenotype that is different than planktonic bacteria [Mah and O'Toole, 2001; Costerton et al., 1999]. This is the most complicated and, at this point, somewhat speculative mechanism of biofilm resistance. Multi-cellular interactions have been studied in conjunction with the idea of a biofilm phenotype [Stoodley et al., 2002; Stewart and Costerton, 2001]. Current studies include cell-cell communication, gene transfer and antimicrobial inactivation.

It is becoming apparent that there is not a simple answer to the antimicrobial resistance of biofilms. Despite some progress in this research area, there is a need for further studies in order to understand this phenomena. In the case of medical biofilm infections, the goal of preventing infection altogether has emerged as the primary objective. It is clear that the more mature an infection, the harder it is to eradicate using antibiotics.

Biofilms in Medicine

Medical Biofilm Infections

The relationship between human disease and biofilm presence is not definitive, rather, it is suggested that the presence of biofilm bacteria is often coincidental with some human diseases. Some of the diseases in which biofilms have been implicated are endocarditis, otitis media (middle ear infection), prostatitis and cystic fibrosis [Donlan and Costerton, 2002]. These diseases are often chronic ailments that progress cyclically over time. The conundrum of these human diseases is that the biofilms that have been implicated are often composed of common environmental pathogens [Costerton et al., 2003]. In cystic fibrosis (CF) patients, for example, *Pseudomonas aeruginosa* have been determined to be the major constituent organism in the patients lungs. However, when biofilm infection occurs as the result of a surgery or appears on the surface of an implanted device, the link between the biofilm and the overall health of the patient is more conceivable [Donlan, 2001].

Implant Infections

When a medical implant, whether it be metal, polymer or ceramic, is inserted into a human host, it is instantaneously a prime candidate for biofilm formation. Implanted devices such as heart valves, artificial joints or other orthopaedic devices provide inert surfaces that are not well protected by host defense, hence, they provide opportunity for infecting pathogens [Reid, 1999]. Upon implantation, body fluids surround the “foreign body” and proteins begin to coat the surface to provide a conditioning film. This conditioning film is intended to assist in tissue integration and provides active sites for cell adhesion. Therefore, once the conditioning film is in place, host cells and pathogenic or nonpathogenic bacteria compete to occupy the surface. If bacteria begin to colonize and form biofilms on the surface of the implant before it can be integrated into the surrounding tissue, the host cells have difficulty recovering and often respond with inflammation [Gristina, 1987; Habash and Reid, 1999]. In orthopaedic joint implants, these types of infections are the second leading cause of joint failure that ultimately results in revision surgery or implant removal [Neut et al., 2003].

De Lucas-Villarrubia et al. [2004] report that in 2000, nearly 10% of all joint reconstructions in their hospital were followed by infection. Strategies to prevent such infections include shorter operating times, strict sanitation requirements, plastic surgical gowns and drapes and clean air requirements [Widmer, 2001; Vogely et al., 2000]. However, these precautionary measures do not totally eliminate cases of acute

or chronic infection. Biomaterial scientists are now developing new ways of combating biofilms where they are likely to occur, on the surfaces of implanted devices.

Biomaterials and Biocompatibility

The persistence of device related infections has brought forth a need to design and develop special materials to be used in the fabrication of medical implants. The goal of biomaterials is two fold, firstly to promote the acceptance of the implant by host cells and, secondly, to prevent the formation of infectious bacteria [Gristina et al., 1993]. Among the more popular methods of achieving these goals are surface treatments and antimicrobial loading for controlled release.

In order to select a material for medical applications it must first be classified as biocompatible. Biocompatibility encompasses an array of properties which include protein absorption, cell adhesion, cytotoxicity, tissue compatibility and hemocompatibility (blood compatibility). Because of the scope of biomaterials, it has been difficult to design a standard biocompatibility test to which all biomaterials must comply. For this reason cytotoxicity, hemocompatibility and over all functionality are the biomaterial properties that dominate the screening and classification of biocompatibility [Vienken et al., 1995; Kirkpatrick et al., 1998; Hendricks et al., 2000]. More recent investigations into hemocompatibility suggest that design of biomaterials should incorporate hydrophilic surfaces since they absorb less proteins than hydrophobic surfaces [Wang et al., 2004].

Surface Treatments

Surface treatments on various polymers and metals have proven effective in cases where tissue integration is not desired. For this reason, most surface treatments are intended to prevent bacterial attachment, although some are designed to promote host cell attachment. Portoles et al. [1994] report up to 99% inhibition of *P. aeruginosa* and other organisms on hydrophilic contact lenses treated with poloxamer 407, a bacterial abhesive. Francois et al. [1996] report that poly(vinyl pyrrolidone) (PVP) surface treatments on central venous catheters provide a smoother surface, reducing the protein absorption associated with the conditioning layer and therefore the adhesion of bacteria. Although some surface treatments do provide some defense against biofilm formation initially, conditioning films may eventually form and render the surface treatments ineffective [Habash and Reid, 1999].

Controlled Release of Antibiotics

Materials loaded with antimicrobial agents have been shown to reduce infection. The loading of antibiotics and other drugs into polymer based biomaterials was initially intended to deliver drugs to a specific site and override the need for systemic antibiotic therapy, with the overall goal of maintaining drug concentrations in the therapeutic range. Commercially available controlled release systems achieve this goal with a variety of mechanisms including: chemically controlled, solvent activated and diffusion controlled systems [Lohmann, 1995; Kost and Langer, 1984].

Chemically controlled systems are sometimes referred to as bioerodible systems or biodegradable systems. In chemically controlled systems, drugs are either covalently bound to a bioerodible polymer backbone or a drug core is encapsulated by a bioerodible polymer membrane [Robinson and Lee, 1987]. The polymers used for bioerodible drug delivery systems are generally non-diffusive and the release rate of the incorporated drug is mediated by the erosion rate of the polymer. The advantage of using a bioerodible system is that the drug delivery system is eventually totally absorbed by the host and does not need to be surgically removed [Kost and Langer, 1984]. Bioerodible polymer drug delivery systems have been studied *in vitro* as coatings for orthopaedic implants with gentamicin as the delivery drug. Price et al. [1996] report significant reduction of implant colonization over 24 days when biodegradable polymers with gentamicin were used compared to controls.

Solvent activated delivery systems are generally either reservoir systems or matrix systems. Reservoir systems incorporate a core of solid drug encased by a semipermeable membrane that is non-diffusive to the internal drug. Environmental solvents (e.g. water) are passed through the exterior membrane due to osmosis at a controlled rate. The solvent then dissolves the drug and begins to deliver it through a single orifice [Lohmann, 1995; Kost and Langer, 1984]. Matrix solvent activated systems are also non-diffusive and incorporate a delivery drug into the matrix of the polymer. As solvents penetrate the polymer, it begins to swell and release the encased drug [Lohmann, 1995; Kost and Langer, 1984]. Some of the advantages of solvent activated

systems included to delivery of large macromolecules and high constant delivery rates [Robinson and Lee, 1987].

Similar to chemically controlled delivery systems and solvent activated systems, there are two basic types of diffusion controlled systems [Lohmann, 1995; Kost and Langer, 1984]. The first are reservoir systems in which a reservoir of drug is encapsulated by a diffusive membrane. The thickness, porosity and diffusive properties of the membrane mediate the passage of the drug from the internal reservoir to the outer bulk fluid. The second type of diffusion controlled systems are monolithic matrix systems in which antibiotics or other drugs are incorporated into a polymer matrix. In the case of monolithic matrix systems, the diffusion of the drug is mediated by the diffusive properties of the polymer, the size of the molecule being diffused and the solubility of the drug in the surrounding bulk fluid. One of the classical examples of a monolithic delivery system is orthopaedic bone cement loaded with various antibiotics. However, long term exposure to low doses of antibiotics being released from bone cement has raised questions about their relationship to the emerging problem of antibiotic resistant strains [van de Belt et al., 2001].

Polymer Systems for Controlled Release

Polymers that are typically used as coatings for implants or used in soft tissue reconstruction are often loaded with antibiotics and are classified as monolithic diffusion controlled delivery systems. The polymers that are used are biocompatible and are generally commercially available and their properties have been well

characterized. These polymers are often referred to as “hydrogels”. They are generally hydrophilic with varying degrees of porosity [Lowman and Dziubla, 2003]. Among the more common polymers classified as hydrogels are poly(ethylene glycol) (PEG), poly(hydroxyethylmethacrylate) (pHEMA), poly(acrylamide) (PAA) and poly(N-vinyl-2-pyrrolidone) (PVP) [Wang et al., 2004]. Bacterial adhesion and biofilm formation has been studied and characterized on pHEMA hydrogels because of their applications pertaining to contact lenses [Cook et al., 1993]. Scientists and engineers are now investigating polymer hydrogels for controlled release purposes in a variety of applications.

Numerous antimicrobial loaded polymers (monolithic hydrogel systems) have been developed for the purpose of inhibiting bacterial accumulation on their surfaces [Ackart et al., 1975; Golomb and Shpigelman, 1991; Kwok et al., 1999; Hendricks et al., 2000]. These hydrogels were loaded with antibiotics that have known action against gram positive bacteria, gram negative bacteria or both. These hydrogels are loaded with a finite concentration of antibiotics and therefore have a finite delivery time. Due to the high solubility of some antibiotics such as ciprofloxacin, diffusion of the antibiotic out of the hydrogel is initially rapid and then slows as the drug is depleted from the matrix of the polymer. For this reason, antimicrobials with low solubility are being investigated for use in polymer delivery systems. Francolini et al. [2004] used usnic acid, which is classified as a secondary lichen metabolite, in a polymer matrix to show the inhibition of *Staphylococcus aureus* biofilm formation on the

surface of polymer discs under laminar flow. Usnic acid is a promising active agent against gram positive bacteria because of its low solubility in water, and gradual release kinetics from the polymer.

Smart Polymers

Efforts to actively control drug release have resulted in the development of a class of biomaterials known as “smart polymers”. These materials are classified as either open-loop systems or closed-loop systems [Kost and Lapidot, 2003]. Open-loop drug delivery polymer systems deliver antibiotics or other drugs by means of an externally controlled trigger mechanism such as ultrasound [Kost et al., 1989], magnetic fields or externally applied heat. Closed-loop systems are self-regulated. Their functions are controlled internally, usually by environmental feedback parameters like pH or temperature [Hoffman et al., 2002; Galaev and Mattiasson, 1999]. In general, active control of drug delivery is advantageous because it eliminates some of the concerns that are introduced by passive delivery systems such as uncontrolled leaching and less than desirable drug residuals that can lead to antibiotic resistance. Both open-loop and closed-loop smart polymer delivery systems are still in developmental stages and need to be explored further to characterize their effectiveness against biofilm formation and infection.

UWEB pHEMA Hydrogels

The University of Washington Engineered Biomaterials (UWEB) group at the University of Washington in Seattle has developed a novel ultrasonically responsive membrane that can be coated onto pHEMA hydrogels for the purpose of actively controlling drug release [Kwok et al., 2001]. Self-assembled monolayers (SAMs) of ordered methylene chains were successfully formed on the pHEMA polymer by binding C12 isocyanate to the hydroxyl groups on the surface of the hydrogel (Figure 2). The effectiveness of surface coatings and coverage has been confirmed using various surface analysis techniques including X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (TOF-SIMS). The self-assembled monolayer coating initially served as a barrier membrane to minimize the passive diffusion of the loaded drug (Insulin or ciprofloxacin) out of the pHEMA matrix into the surrounding bulk fluid. When a 1.1 MHz ultrasonic frequency was applied to the loaded hydrogels, release rates spiked significantly until the ultrasound application was arrested at which point the hydrogels returned to a state of exhibiting minimal leaching [Kwok et al., 2000]. This process could be repeated as needed until the drug content in the polymer matrix was exhausted.

The described open-loop smart polymer hydrogels have an array of possible clinical applications. Among the proposed applications is an antimicrobial coating for orthopaedic implants by which drug delivery could be initiated and controlled externally after the implant has been surgically inserted in the patients body. The use

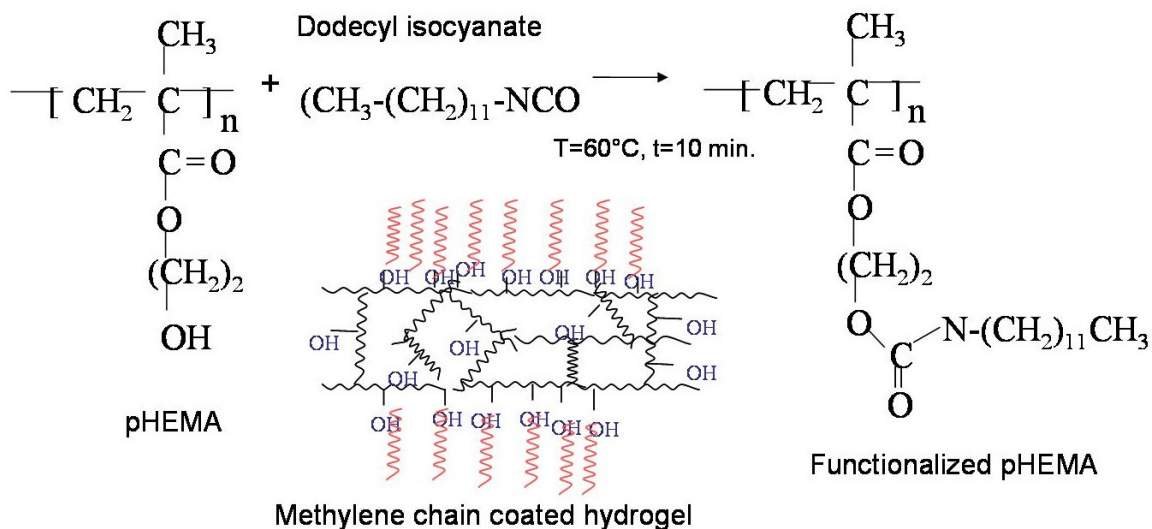


Figure 2: pHEMA polymer with functional hydroxyl groups on the surface are used to create an ultrasonically responsive membrane of methylene chains.

of ultrasound as a trigger of controlled drug release on the surface of a foreign body such as an implant is attractive, because ultrasound has been used widely for various medical applications. The biological effects of ultrasonic energy is well understood [Mourad, 1999]. The purpose of this research was to determine the effectiveness of the UWEB coated pHEMA hydrogels against biofilm formation. The hydrogels used in this study were loaded with the antibiotic ciprofloxacin. *P. aeruginosa* biofilms were grown on the surface of the pHEMA hydrogels and ultrasound was applied at specified intervals. The effectiveness of the treatment is reported along with discussion of the results.

Ciprofloxacin

Ciprofloxacin is a member of a group of antibiotics classified as fluoroquinolones. The exact mechanism by which fluoroquinolones act against bacteria is uncertain, but it is proposed that in the case of gram negative bacteria, fluoroquinolones interfere with activities of the DNA gyrase, which regulates DNA transcription and therefore the ability of bacteria to reproduce [Wolfson and Hooper, 1989; Blondeau, 1999]. Ciprofloxacin is a broad spectrum antibiotic that has known action against aerobic gram negative and aerobic gram positive bacteria, but is not known to be effective against anaerobic bacteria [Wolfson and Hooper, 1989]. Ciprofloxacin and other fluoroquinolones have been used extensively to treat acute urinary tract infections and urinary catheter related infections involving *P. aeruginosa* bacteria [Goto et al., 1999; Soboh et al., 1995; Reid et al., 1993, 1994]. The use of fluoroquinolones in combination with other drugs has also been used effectively to treat osteomyelitis and orthopaedic device infections due to *Staphylococcus aureus* and *Pseudomonas aeruginosa* [Lew and Waldvogel, 1999]. Ciprofloxacin was used as the model antibiotic loaded into the UWEB pHEMA hydrogels because of its proven effectiveness against *P. aeruginosa* bacteria.

Bioacoustic Effect

Since biofilms have shown to be extremely resistant to antibiotic treatment, several techniques have been developed to assist the delivery of antibiotics to biofilm

bacteria. Among these techniques is the bioacoustic effect. The bioacoustic effect was defined as the synergistic effect of ultrasound and antibiotics on the efficacy of biofilm bacteria [Qian et al., 1997], and have shown up to $2.26 \log_{10}$ reduction of live cells of *Pseudomonas aeruginosa* biofilms after exposure to $12 \mu\text{g/ml}$ gentamicin sulfate and low intensity ultrasound (10 mW/cm^2) at various frequencies ranging from 70 kHz to 10 MHz. Controls of antibiotic only and ultrasound only produced 0.9 and 0.1 $\log_{10}$ reductions respectively under the same testing conditions. The bioacoustic effect has also been shown to be effective in the eradication of *Escherichia coli* biofilms using gentamicin [Rediske et al., 1999; Miller and Quddus, 2000].

In order to show that the bioacoustic effect is a real phenomena, and not just a result of ultrasonic wave perturbations, Qian et al. [1996] observed a biofilm of *Pseudomonas aeruginosa* using time lapse Confocal Scanning Laser Microscopy (CSLM). A flowcell was constructed to facilitate both the microscope and the ultrasonic source (Panametrics V318, 500 kHz transducer). Time lapse images showed very little difference between the biofilm before ultrasound application and the biofilm after ultrasound had been applied continuously for 35 min without antibiotic treatment. This experiment shows that the reduction of viable cells after sonication is not due to a significant change in biofilm structure in the presence of low intensity ultrasonic pressure waves.

CHAPTER 2

GOAL AND OBJECTIVES

Goal

The overall goal of the research reported here was to evaluate the effectiveness of UWEB pHEMA ultrasonically responsive smart polymer hydrogels loaded with ciprofloxacin on the efficacy and overall health of *Pseudomonas aeruginosa* biofilms grown under flow conditions.

Objectives

1. Determine the role of the bioacoustic effect in the killing of Pseudomonas aeruginosa biofilm cells in the presence of various concentrations of ciprofloxacin with various ultrasound sources. The bioacoustic effect has been well documented using various ultrasound sources and antibiotics against specific bacteria. Although certain studies have shown bioacoustic effects present using *P. aeruginosa* biofilms and various antibiotics, it is unclear whether there would be any bioacoustic effect related killing using *P. aeruginosa* biofilms and ciprofloxacin with the ultrasound sources used in other parts of this research, specifically the 43 kHz ultrasonic bath that is used to initiate controlled release of ciprofloxacin from the surface of UWEB pHEMA coated hydrogels.

2. Demonstrate the controlled release of ciprofloxacin from UWEB pHEMA coated hydrogels in the presence of ultrasound. The controlled release of ciprofloxacin from UWEB pHEMA hydrogels has been shown by the UWEB manufacturer. However, it was desired to demonstrate this concept under the specific flow and geometry conditions that were used to determine the effectiveness of the hydrogels and controlled release against biofilm bacteria. For this reason, controlled release was demonstrated in a biofilm flowcell reactor in the absence of biofilm bacteria.

3. Determine the effectiveness of UWEB pHEMA coated hydrogels and controlled release of ciprofloxacin against the formation and efficacy of *Pseudomonas aeruginosa* biofilms grown for three days under flow conditions. The UWEB pHEMA coated hydrogels have a proposed use as a coating for orthopaedic implants and other medical devices. It is proposed that their use in this capacity would eliminate or reduce the formation and virulence of medical biofilm infections at the site of implant. For this reason *in vitro* studies must first be conducted to validate a proof of concept. After the hydrogel effectiveness has been established, the link between the results from the lab and clinical applications will be more clear.

Summary of Experimental Procedures

In order to determine the effects of the pHEMA hydrogels coated with methylene chains along with any combined effects of low intensity ultrasound on the growth and efficacy of *P. aeruginosa* biofilms, it was necessary to develop several sets of experiments. The first of these experiments involved only the effects of low intensity ultrasound. These experiments were designed to determine if there was any significant bioacoustic effect involved in the application of low intensity ultrasound systems. The second set of experiments was intended to observe the release kinetics of ciprofloxacin into a bulk fluid flowing in a flowcell. The third and final set of experiments consisted of *P. aeruginosa* biofilms grown on the UWEB ultrasonically responsive smart hydrogels. These experiments were made up of a subset of four experiments that were designed to determine if the structure of the *P. aeruginosa* biofilm colonies were significantly altered due to the release of ciprofloxacin when ultrasound was applied to coated hydrogels that were loaded with the antibiotic. The results from the combination of these three sets of experiments provided the first step in ultimately determining whether or not these smart hydrogels will have clinical applications against biofilm infections.

CHAPTER 3

MATERIALS AND METHODS

Basic Experimental ProceduresBacterial Cultures

Bacterial cultures for all experiments requiring biofilm bacteria were prepared in the same way. The strain of *Pseudomonas aeruginosa* that was cultured was pMF-230. This strain of *P. aeruginosa* includes a plasmid that produces a green fluorescent protein (GFP) along with an antibiotic resistance to carbenicillin [Nivens et al., 2001]. Batch cultures were made from pure cultures of frozen stock. The batch culture consisted of 9 ml of full strength Luria Bertani (LB) broth, 1 ml of 3000 $\mu\text{g}/\text{ml}$ carbenicillin solution in purified nanopure water, and 100 μl of frozen stock. The solution was then incubated at 37°C. The batch culture was then ready to be used for inoculation.

Ciprofloxacin Calibration Curve

Ciprofloxacin concentrations were calibrated using total absorbance measurements from a Spectronic Genesys 5 Spectrophotometer with a 339 nm wavelength [Hendricks, 1998]. An initial concentration of 500 $\mu\text{g}/\text{ml}$ was suspended in 10 ml of 1/10 strength LB broth. Dilutions ranging from 50 $\mu\text{g}/\text{ml}$ to 0.05 $\mu\text{g}/\text{ml}$ were

prepared in the same broth, and the total absorbance of each dilution was measured and recorded. A “blank” solution was also prepared which consisted of a 1/10 LB solution without any ciprofloxacin. The absorbance of the blank was recorded and subtracted from all ciprofloxacin concentration absorbance readings. A calibration curve was generated and used to determine ciprofloxacin concentrations in hydrogel release studies.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of *P. aeruginosa* pMF-230 was determined using AB Biodisk ciprofloxacin loaded Etest strips. The Etest strips were 50 mm long and contained a gradient of ciprofloxacin ranging from 32 $\mu\text{g}/\text{ml}$ to 0.002 $\mu\text{g}/\text{ml}$. A full strength LB agar plate was prepared, inoculated with 100 μl of a batch culture and spread for full plate coverage. The plate was allowed to dry and two Etest strips were affixed to the surface of the agar. The plate was incubated at 37 °C for 24 hours and observed. The MIC of ciprofloxacin against pMF-230 was determined to be 0.125 $\mu\text{g}/\text{ml}$.

Bioacoustic Effect

Bioacoustic effects were observed for two different systems. The first of these systems involved the use of low intensity ultrasound generated by an ultrasonic transducer. This transducer was calibrated before it was used to apply ultrasound to

biofilm bacteria (Methods and results from these experiments are reported in Appendix A). The second system consisted of stationary phase planktonic 48 hour batch culture in conjunction with a low intensity ultrasonic cleaning bath.

Bioacoustic Effect: Ultrasonic bath Experimental Setup

The ultrasound source used for these experiments was a Branson 200 43 kHz ultrasonic cleaning bath. Eight 48 hour batch cultures were grown at 37°C. This incubation time insured that the batch cultures were in stationary phase, simulating the metabolic activity of biofilm bacteria. Two of the cultures were used as controls, two were treated with a specified concentration of ciprofloxacin, two underwent 20 minutes of ultrasound treatment in the ultrasonic bath and the final two were treated with ciprofloxacin and underwent ultrasound treatment. All cultures were allowed to incubate for four hours before plating to allow the antibiotic time to act against the cells. The cultures were plated out to five ten-fold reduction dilutions on full strength LB agar plates. The plates were incubated overnight at 37°C before counting.

UWEB pHEMA Hydrogels: Antibiotic Release

Controlled Release

In order to demonstrate the controlled release of the UWEB pHEMA ultrasonically responsive hydrogels loaded with ciprofloxacin, experiments were designed to capture the effluent of a flowcell containing these hydrogels when ultrasound was applied. A 1 ml/min flow rate of purified water was imposed and effluent was captured

in 5 ml fractions. During the collection process, ultrasonic energy was delivered to the hydrogel at specific time increments for a specified duration. The intent of these experiments was to demonstrate the on/off effect on the ciprofloxacin concentrations being released from the hydrogel in the presence of ultrasound.

Flowcells and Hydrogels.

The hydrogels used in these experiments were manufactured by UWEB in support of this research. The hydrogels were loaded with 11.1 mg/ml of ciprofloxacin. The hydrogels were then cut with the dimensions of a standard microscope slide before the surface was functionalized with methylene chains.

Hydrogels were incorporated into a Biosurface Technologies, Inc BST-FC81 flowcell. These flowcells were comprised of a polycarbonate base with a 2.5 mm thick lumen between a standard microscope slide and a standard number two microscope coverslip. The flowcell reactor was clamped together using two clamping plates, held together by ten clamping screws. The hydrogels were manufactured with the same dimensions as a standard microscope slide. All hydrogels were hydrated for 24 hours before incorporating them into the flowcell systems. Hydrogels were inserted into the system by overlaying them over the microscope slide and assembling the flowcell as shown in Figure 3. The force provided by the two clamping plates was sufficient to keep the hydrogel flat against the microscope slide and prevent flowcell leakage. The

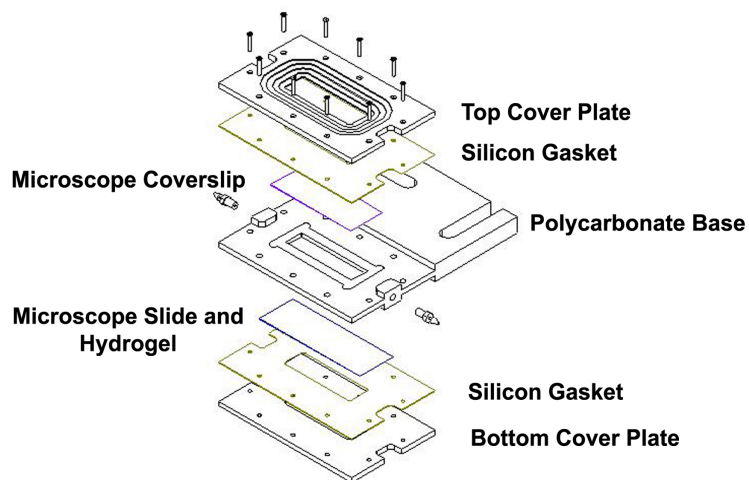


Figure 3: Standard flowcell configuration used for UWEB pHEMA hydrogel controlled release and UWEB pHEMA hydrogel biofilm control experiments.

hydrodynamic properties of the flowcell lumen are outlined in Table 1. The hydrodynamic properties are calculated assuming fully developed and steady state flow with a flow rate of 1 ml/min.

Flowcell Effluent Collection System.

A Cole Palmer 7553-80 peristaltic pump was used to pump nanopure purified water through the flowcell at a flow rate of 1 ml/min. The flowcell containing the hydrogel was immersed in a Branson 200 43 kHz ultrasonic cleaning bath. The effluent of the flowcell was collected using a Spectra/Chrom CF-1 fraction collector, programmed to collect effluent in five minute intervals. Ultrasound was applied to the flowcell system for 15 minutes in intervals of one hour. Total absorbance readings were measured and recorded for each of the fractions. The absorbance readings were then

Table 1: FC81 flowcell hydrodynamic parameters

Parameter	Definition	Value	Units
h	lumen depth	0.2032	cm
L	lumen length	5.08	cm
w	lumen width	1.27	cm
Q	flow rate	1.0	ml/min
dp/dx	driving pressure	-1.784×10^{-2}	Pa/cm
v_{max}	maximum velocity	0.10	cm/s
$\langle v \rangle$	average velocity	6.460×10^{-2}	cm/s
D_h	hydraulic diameter	0.3503	cm
ρ	density of water at 25°C	996.95	kg/m ³
μ	dynamic viscosity at 25°C	8.889×10^{-4}	Pa*s
Re	Reynolds number($D_h * \langle v \rangle * \rho / \mu$)	2.507	
V	System volume: taken from inoculation port	1.80	ml
RT	Residence Time (V/Q)	1.80	min
τ_{xy}	Shear stress at the hydrogel surface at the midplane with respect to width	1.813×10^{-3}	Pa

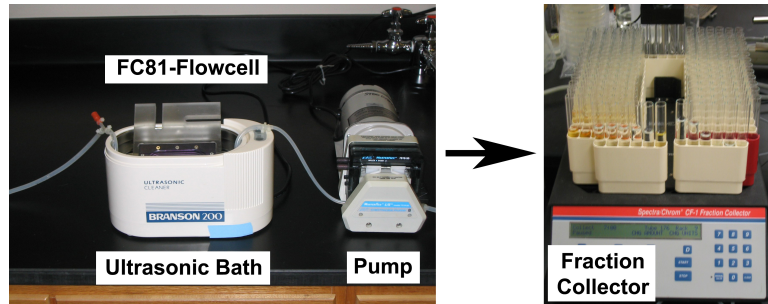


Figure 4: System used to collect effluent from a flowcell system. This system was designed to demonstrate the controlled release of ciprofloxacin from a UWEB pHEMA hydrogel when ultrasound was applied at specified time increments.

compared to the ciprofloxacin calibration curve to give ciprofloxacin concentrations in each of the fractions (Figure 4).

Background Release

In addition to examining the controlled release properties of the hydrogels with ultrasound application, the release of ciprofloxacin from hydrogel surfaces was observed without ultrasound. Hydrogels that were loaded with ciprofloxacin were cut into circular discs with a diameter of 1.27 cm. Some of these hydrogels were coated with methylene chains and some were not coated. The hydrogels were immersed in 250 ml of buffer solution and absorbance readings using 339 nm were recorded every 24 hours. After each absorbance reading, the 250 ml of buffer fluid was exchanged for clean buffer solution. Release rates for both coated and non-coated hydrogels were recorded between each 24 time increment for seven days.

UWEB pHEMA Hydrogels: Biofilm Control

P. aeruginosa biofilms were grown on UWEB pHEMA ultrasonically responsive hydrogels for three days. Four sets of independent experiments were conducted to determine if the methylene coated polymers, in conjunction with low intensity ultrasonic energy had significant effects on the biofilm structure over a 72 hour time period. These four sets of experiments were designed to separate the effects of the applied ultrasound and the release of ciprofloxacin into the bulk fluid (Figure 5). Two of these experiments included the application of ultrasound, one with a ciprofloxacin loaded hydrogel and the other with a hydrogel that was not loaded with ciprofloxacin. The other two experiments did not include the application of ultrasound, again, one with

a ciprofloxacin loaded hydrogel and the other with a hydrogel that was not loaded. Hydrogels were incorporated into a flowcell system and inoculated with 2 ml of a 16 hour batch culture. The flowcell was allowed to incubate for 30 minutes before a continuous flow of 1/10 strength LB broth at 1 ml/min was induced. In 24 hour increments, the biofilm in the flowcell was observed and recorded using a Leica TCS-NT confocal scanning laser microscope (CSLM). A total of ten image stacks were recorded at each time increment and later processed quantitatively using the biofilm image analysis software package COMSTAT. Four statistical biofilm parameters were analyzed including maximum biofilm thickness, average biofilm thickness, biomass and the roughness coefficient. These parameters were then analyzed to determine if there was any statistical differences in the four hydrogel system configurations. It was determined that the doubling time (DT) of exponential phase *P. aeruginosa* bacteria was on the order of 120 minutes. By comparing the doubling time to the flowcell residence time (RT=1.8 min, Table 1), it was determined that $DT \gg RT$, indicating that there was complete washout of planktonic bacteria in the flowcell.

Flowcell Biofilm Reactor

Identical flowcell configurations were used for each of the four hydrogel system configurations. Hydrogels were incorporated into Biosurface Technologies, Inc BST-FC81 flowcells as described in the previous section. One Cole Palmer 7553-80 peristaltic pump provided influent to a bubble trap at a flow rate of slightly higher than 1 ml/min. Another Cole Palmer 7553-80 peristaltic pump on the effluent side of the

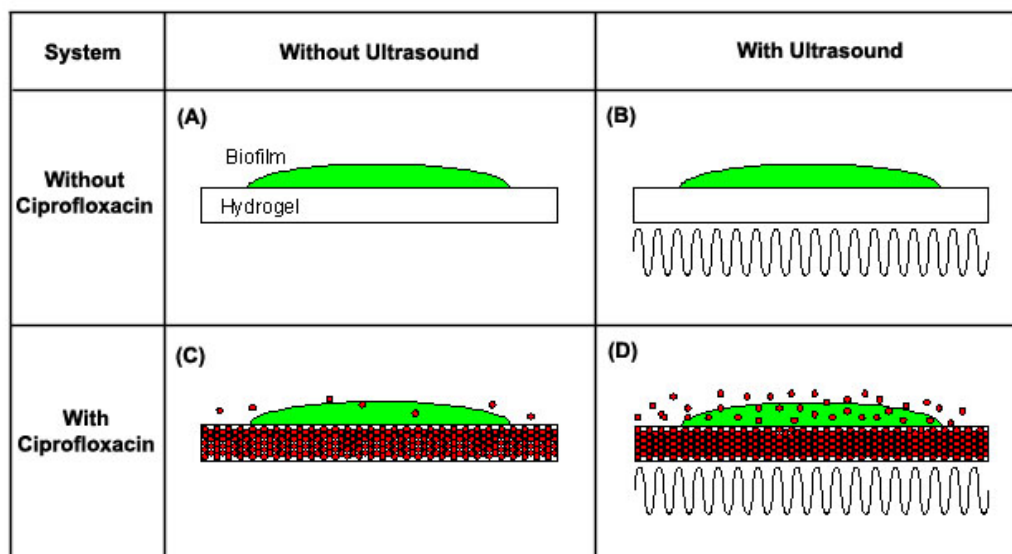


Figure 5: Four hydrogel configurations used to evaluate the effects of controlled release on the health and structure of *P. aeruginosa* biofilms grown for three days under flow conditions.

flowcell, pumped media out of the bubble trap, through the flowcell and into a waste carboy at a flow rate of 1 ml/min. The bubble trap also had an overflow effluent port and a port where air was continuously pumped into the media with a Elite 801 fish tank style air pump (Figure 6).

Hydrogel System Configurations

The four hydrogel system configurations used for these experiments are described above (Figure 5). The first of these configurations included a coated hydrogel that was initially loaded with 11.1 mg/ml of ciprofloxacin. It was determined by UWEB that there was negligible loss of drug during the manufacturing process. Ultrasonic energy was introduced to this hydrogel by immersing the flowcell system into a Branson 200

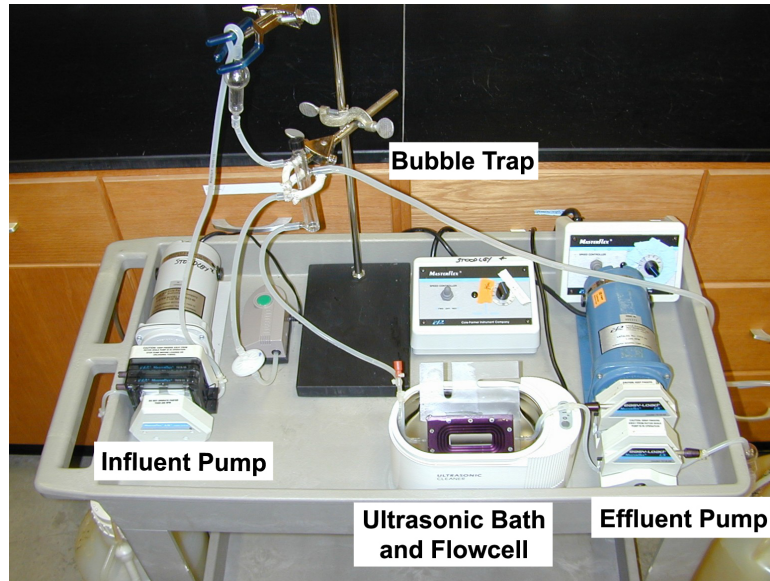


Figure 6: System used to grow and observe biofilm incorporation a flowcell and ultrasonic bath.

43 kHz ultrasonic bath for 20 minutes every 24 hours. The second of the four systems also included a loaded hydrogel, loaded with the same ciprofloxacin concentration as the previous hydrogel system configuration. However, this system did not include any ultrasound treatment during the 72 hour observation period. The third flowcell configuration included a coated hydrogel that was not loaded with antibiotics. This system also underwent ultrasound treatment for 20 minutes at the same 24 hour time intervals. The final hydrogel system also included a coated hydrogel that was not loaded with antibiotic. This final system configuration did not include any ultrasound treatment.

Image Acquisition

A Leica TCS-NT confocal scanning laser microscope was used to collect images of colony biofilms growing on the hydrogels. The BST-FC81 flowcell was designed in such a way to be mounted securely onto a microscope stage and allow image acquisition through the coverslip window. A Leica 40x long working distance microscope objective was used to collect all image stacks. Images were acquired at either 512x512 pixels or 1024x1024 pixels. A Leica 488 nm laser was used to excite the GFP produced by the *P. aeruginosa* pMF-230 biofilm cells, which emits light at a wavelength of 488 nm excitation wavelength. For the hydrogel systems not exposed to ultrasound, ten biofilm colonies growing on the surface of the hydrogels were imaged every 24 hours for 72 hours. For the hydrogel systems that did include ultrasound, five biofilm colonies were imaged before ultrasound application and another five biofilm colonies were imaged after ultrasound application.

Effluent Collection

The effluent from the two hydrogel systems that required ultrasound application was collected over the application time period of 20 minutes. The flow rate in the flowcell was 1 ml/min, therefore, 20 ml of effluent was collected. Effluent turbidity was then recorded by observing 1 ml of the effluent in a Spectronic Genesys 5 spectrophotometer. Total viable cells were recorded by diluting and plating.

Biofilm analysis using COMSTAT

The image analysis software package COMSTAT was developed at The Technical University of Denmark, Lyngby, Denmark [Heydorn et al., 2000], specifically for analyzing confocal image stacks of biofilm. The software interfaces with Matlab, and utilizes Matlab's image analysis software toolbox. COMSTAT first requires the user to convert 8-bit images into binary images, and then reads white pixels as area occupied by biofilm and black pixels as open space. The software then computes the location of occupied pixels in each of the images in a given confocal image stack. COMSTAT offers an array of functions and is capable of generating up to ten different statistical parameters for the purpose of quantifying three dimensional biofilm structure. For purposes of this research, four of the parameters were utilized in determining differences in the biofilms grown with each of the hydrogel system configurations. These parameters were: maximum biofilm thickness, average biofilm thickness, roughness coefficient and biomass. The maximum biofilm thickness was the maximum distance from the substratum that the biofilm colony reaches. The average thickness was the average biofilm height taken over the entire field of view. For the 40x microscope objective used, the field of view was 250 μm wide by 250 μm long. The total area of the field of view was therefore, 62,500 μm^2 . The roughness coefficient was calculated according to Heydorn et al. [2000] and is given by the equation:

$$R_a = \frac{1}{N} \sum_{i=1}^N \frac{|L_{fi} - \bar{L}|}{\bar{L}} \quad (3.1)$$

where L_{fi} was the thickness measurement of the i th pixel and overbar L is the mean thickness over the entire field of view. The roughness coefficient was a measure of the structural heterogeneity within a given field of view. A roughness coefficient approaching zero would represent a flat slab type biofilm structure. The higher the roughness coefficient, the more heterogeneous the structure in the biofilm being analyzed. The biomass parameter was perhaps the most significant. Biomass was calculated by dividing the volume of the biofilm by the surface area of the field of view, giving the biomass parameter a unit of length. The biofilm volume is calculated by COMSTAT using a complex volume filtration and interpolation algorithm to determine the volume of biofilm between images in the confocal image stack and then summed to give the total volume of the biofilm. The biomass parameter represents the amount of biofilm present in a given confocal image stack.

Statistical Significance using ANOVA

Analysis of variance (ANOVA) is commonly employed to determine statistical differences in given populations. The commercially available software package Minitab 13 was used to perform all ANOVA comparisons. Minitab 13 requires the user to input sets of data in a spreadsheet format. Various statistical functions, including ANOVA, can then be performed by the user comparing multiple sets of data. All ANOVA operations were performed on the Biomass biofilm parameter generated by the COMSTAT software package. The p value generated by an ANOVA comparison is an indication of the confidence of the statistical differences between two populations.

Values less than 0.05 indicate 95% confidence that the populations were considered statistically significantly different.

The first set of biomass comparisons that were made were between the before ultrasound biofilms and the after ultrasound biofilms. An ANOVA was set up and performed on each set of before and after ultrasound biofilms for each experiment for each observation day. These ANOVA tests were performed to validate grouping the before and after ultrasound biomass parameters into one population.

After the before and after ultrasound biomass parameters were grouped into one population for each day for each experiment, another set of ANOVA tests were performed to determine if there was any statistical differences in the biomass parameter in the biofilms grown on each of the different hydrogel system configurations. The hydrogel system configuration without ultrasound application, without ciprofloxacin was used as the baseline configuration to which all other system configurations were compared. Comparisons for these ANOVA tests were performed on a per observation day basis.

CHAPTER 4

RESULTS

Basic Experimental ProceduresCiprofloxacin Calibration Curve

Ciprofloxacin concentration was linear with absorbance at a wavelength of 339 nm for an absorbance range of 0.003 to 0.110 and for ciprofloxacin concentrations between 0.15 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ (Figure 7). The data points on this curve represent the results of five experiments with the error bars indicating standard deviation. This ciprofloxacin calibration curve was used to determine ciprofloxacin concentrations in the effluent in the controlled release experiments.

Bioacoustic Effect: Ultrasonic bath

There were no bioacoustic effects observed using the Branson 200 ultrasonic bath with *P. aeruginosa* PMF-230 and ciprofloxacin. None of the experiments showed a significant $\log_{10}$ reduction in CFU/ml (Colony Forming Units/ml) from the control when ultrasound was applied for 20 minutes ($p=0.896$). For experiments using 5 $\mu\text{g/ml}$ of ciprofloxacin, there was approximately a 1.5 $\log_{10}$ reduction in CFU/ml when the ciprofloxacin was introduced without the application of ultrasound ($p<0.001$). There were no further reductions in CFU/ml in samples with both ciprofloxacin and

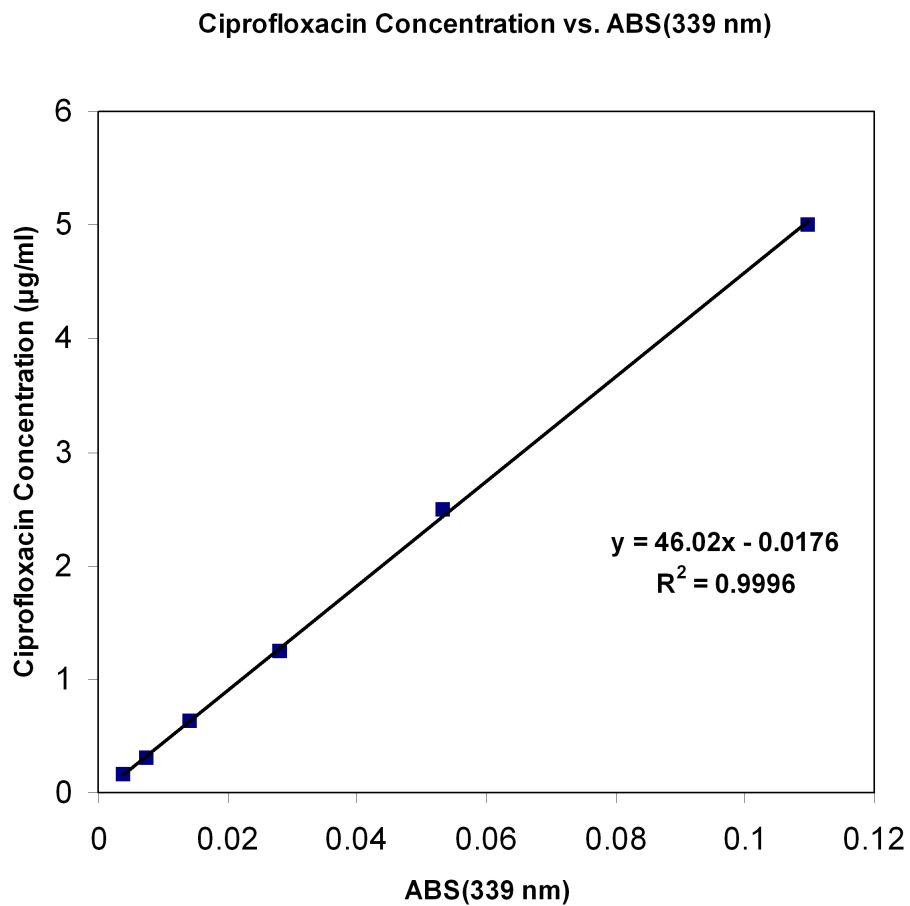


Figure 7: Ciprofloxacin calibration curve used to determine total ciprofloxacin concentration in a given sample. Calibration was for absorbance values between 0.003 and 0.110 and ciprofloxacin concentrations between 0.15 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$. Error bars indicate standard deviation (contained in the data points).

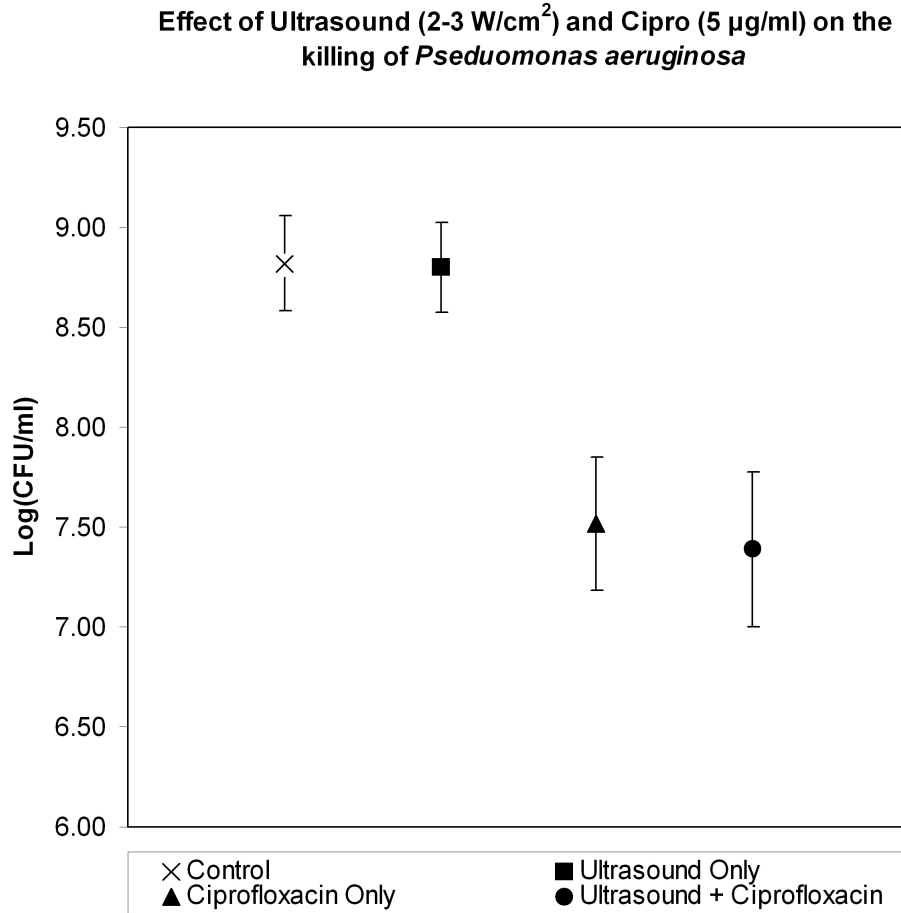


Figure 8: The effects of ultrasound generated by a Branson 200 43 kHz ultrasonic bath in the presence of 5 μ g/ml of ciprofloxacin on planktonic *P. aeruginosa* cells grown to stationary phase in a batch culture. The plot shown represents the average values over five experiments. There was no bioacoustic effect observed under these conditions. Error bars indicate standard deviation.

ultrasound application ($p=0.604$)(Figure 8). Each of the data points in Figure 8 represents the mean of five independent experiments, with the error bars indicating standard deviation. Similar results were found using 0.5 μ g/ml and 50 μ g/ml of ciprofloxacin (Data for these experiments are shown in Appendix B).

UWEB pHEMA Hydrogels: Antibiotic Release

Controlled Release

Controlled release of ciprofloxacin from ultrasonically responsive UWEB pHEMA hydrogels was demonstrated in these experiments. An initial ciprofloxacin concentration was observed when flow was induced over the hydrated hydrogel (Figure 9). This initial concentration decayed gradually over time as purified water was pumped over the surface of the hydrogel. When ultrasound was applied, an instantaneous spike in ciprofloxacin concentration was observed and collected in the fraction collector. When ultrasound application was arrested, the ciprofloxacin was gradually washed out of the flowcell and also showed a decaying trend over a short period of time. The three ultrasound treatments administered to the hydrogel all showed a spike in effluent concentration when ultrasound was applied with a rapid decay of ciprofloxacin when ultrasound was turned off. The initial decay in ciprofloxacin was in the first 75 minutes decaying from approximately $0.25\text{ }\mu\text{g/ml}$ to $0.125\text{ }\mu\text{g/ml}$ (MIC). When ultrasound was initiated, the concentration spikes to approximately $0.6\text{ }\mu\text{g/ml}$. The second and third ultrasound treatments show a spike to approximately $0.5\text{ }\mu\text{g/ml}$. The release of ciprofloxacin from these smart polymers was clearly demonstrated in these experiments, and was compared mathematically to a Heaviside function, where release of the antibiotic was initiated in conjunction with the application of ultrasound.

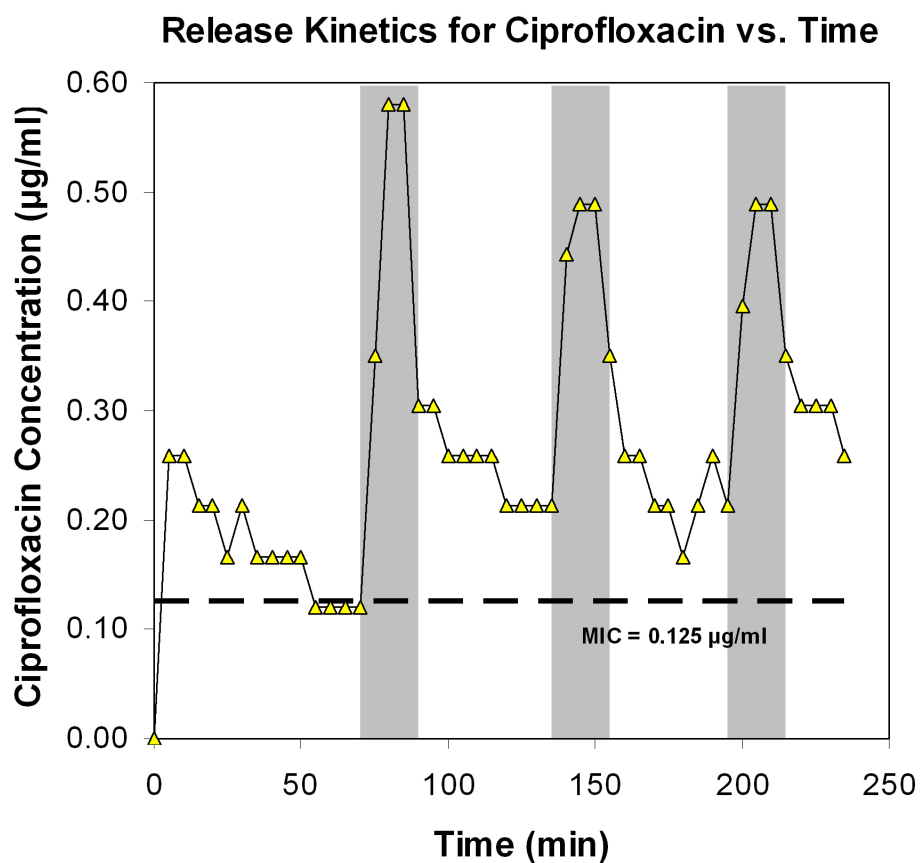


Figure 9: Controlled release of ciprofloxacin from coated pHEMA hydrogels when ultrasound was applied. Ultrasound supplied by a Branson 200 43 kHz ultrasonic bath for 15 minutes in intervals of one hour. Curve shows average ciprofloxacin concentration (triangles) collected in 5 ml fractions. Shaded area indicates ultrasound application.

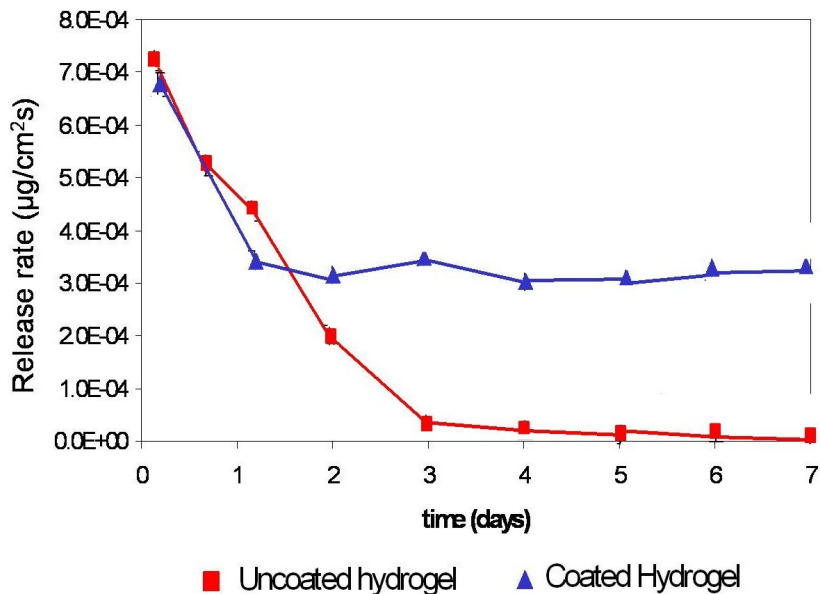


Figure 10: Background release rate of ciprofloxacin from coated and non-coated UWEEB pHEMA hydrogel discs over seven day observation period.

Background Release

Background release rates were measured for seven days for both coated and non-coated hydrogels. The release rate of ciprofloxacin decays exponentially for hydrogels that were not coated with methylene chains (Figure 10). The decay continued until the third day when the release rate approached zero and the ciprofloxacin inside the hydrogel was depleted. For coated hydrogels, the release rate curve flattened out after only one day which represents a zero-order kinetics mass release. The zero-order constant release rate was approximately $3.5 \times 10^{-4} \mu\text{g}/\text{cm}^2\text{s}$.

UWEB pHEMA Hydrogels: Biofilm Control

The controlled release of ciprofloxacin from the UWEB pHEMA ultrasonically responsive hydrogels was demonstrated clearly in the hydrogel release studies. It was also shown that there was no significant bioacoustic effect using the 43kHz Branson 200 ultrasonic cleaning bath using stationary phase pMF-230 cells in the presence of multiple concentrations of ciprofloxacin. The stationary phase cells used in the bioacoustic effect studies were assumed to have similar metabolic response to the antibiotic as biofilm cells. Therefore, any killing of biofilm cells or alterations in biofilm structure was accounted only to the presence of a ciprofloxacin residual in the bulk fluid during ultrasound application.

Effluent Collection

The total absorbance at 660 nm and the plated colonies from the effluent of the two hydrogel configurations requiring ultrasound are reported in Table 2. The total absorbance was similar in all instances on the first observation day. The total absorbance on the second and third observation days increased for hydrogel configurations without ciprofloxacin loading, but remained similar to the first observation day for hydrogel configurations with ciprofloxacin loading. The CFU increased over the three day observation period for hydrogel configurations without ciprofloxacin. The effluent of hydrogel configurations with ciprofloxacin did not yield any detectable CFU for any of the experiments on any of the observation days.

Biofilm analysis using COMSTAT

P. aeruginosa biofilms that were grown on ciprofloxacin loaded hydrogels showed significant structural differences when ultrasound was applied for 20 minutes every 24 hours for three days (Figure 11). Each of the data points in Figure 11 represents the average parameter value generated by COMSTAT over three experiments. Each experiment consisted of ten confocal image stacks per day. For experiments that included ultrasound, the confocal image stacks for before ultrasound application and

Table 2: Absorbance and CFU values reported for effluent collected during ultrasound application for biofilm grown on hydrogels.

Hydrogel Configuration	Experiment	Day	abs(660 nm)	log ₁₀ CFU
Without Ciprofloxacin	1	1	0.049	1.95
		2	0.077	5.39
		3	0.078	5.27
Without Ciprofloxacin	2	1	0.042	2.28
		2	0.054	3.11
		3	0.055	3.29
Without Ciprofloxacin	3	1	0.048	2.25
		2	0.069	4.39
		3	0.078	5.19
With Ciprofloxacin	1	1	0.045	ND
		2	0.042	ND
		3	0.040	ND
With Ciprofloxacin	2	1	0.042	ND
		2	0.044	ND
		3	0.042	ND
With Ciprofloxacin	3	1	0.044	ND
		2	0.043	ND
		3	0.040	ND

ND - Not Detectable

after ultrasound application were grouped together in one population. The statistical justification for this grouping is discussed below. Biomass, maximum biofilm thickness and average biofilm thickness all increased over time for biofilms grown on hydrogels without ciprofloxacin loading, both with and without ultrasound application. The same was true for biofilms grown on hydrogels with ciprofloxacin loading when ultrasound was not applied. Biomass, maximum biofilm thickness and average biofilm thickness all decreased over time for biofilms grown on ciprofloxacin loaded hydrogels when ultrasound was applied for 20 minutes every 24 hours. The roughness coefficients displayed opposite trends for all hydrogel system configurations. The data compiled using COMSTAT can be found in Appendix C.

The images shown in Figure 12 represent the average biofilm grown on the respective hydrogel configuration on the given observation day. Images were selected by summing the error of the four COMSTAT parameters for each image stack based on the average parameter value for the respective hydrogel configuration and observation day, and then selecting the image stack that generated the least amount of total error. The biofilms grown on hydrogels loaded with ciprofloxacin, with applied ultrasound have decreasing colony sizes over the three day observation period (Figure 12). Biofilms grown on all other hydrogel system configurations have increasing colony sizes over the three day observation period.

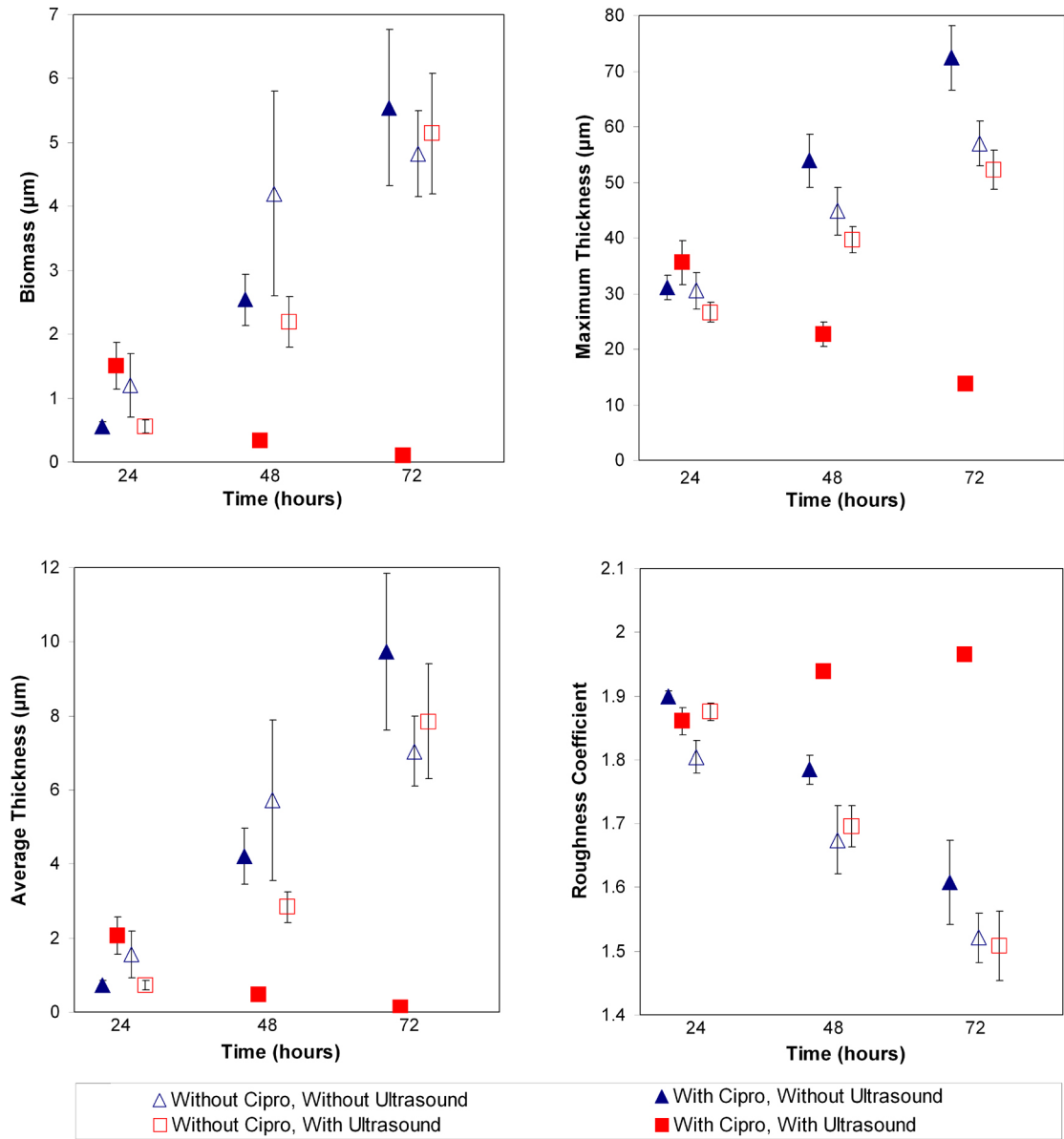


Figure 11: Biofilm parameters observed over three day time period. Each data point represents the average of the respective parameter over three experiments calculated by COMSTAT from 10 confocal image stacks per experiment. Hydrogels without ciprofloxacin, with ultrasound application (empty squares). Hydrogels with ciprofloxacin, without ultrasound application (filled triangles). Hydrogels with ciprofloxacin, with ultrasound application (filled squares). Error bars indicate standard error.

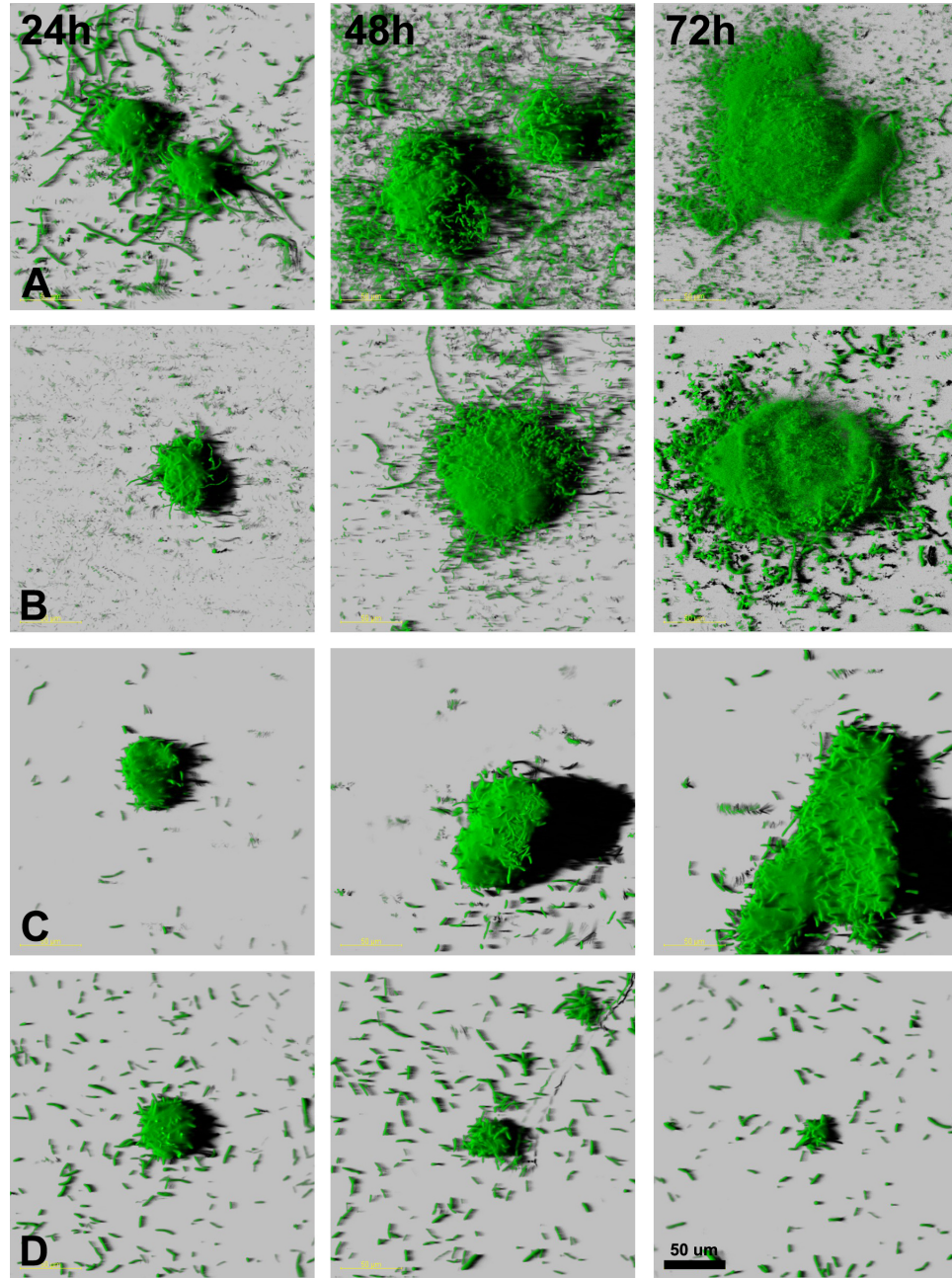


Figure 12: Biofilm images from UWEB pHEMA hydrogel biofilm control experiments. Images shown for each observation time period for each system configuration. (A) Biofilms grown on hydrogels without ciprofloxacin, without ultrasound. (B) Biofilms grown on hydrogels without ciprofloxacin, with ultrasound application. (C) Biofilms grown on hydrogels with ciprofloxacin, without ultrasound. (D) Biofilms grown on hydrogels with ciprofloxacin, with ultrasound application.

Statistical Significance using ANOVA

Analysis of variance techniques were used to qualify the results displayed in Figure 11 and Figure 12. The biomass parameter was taken to be the most clinically significant biofilm parameter and was the only parameter used in the analysis of variance study. Table 3 shows the p values output by the software package, Minitab 13, comparing biomass data from before ultrasound application and after ultrasound application. These tests were performed on each individual experiment, for each individual day. There were a total of three experiments conducted on the hydrogel systems with ultrasound application, without ciprofloxacin loaded hydrogels and three experiments conducted with ultrasound application, with ciprofloxacin loaded hydrogels. Using the p value as an indication of confidence, 16 out of the 18 tests generated p values greater than 0.05, indicating that there were no statistical differences between the confocal image stacks acquired before and after ultrasound application. When the three experiments were grouped together and ANOVA tests were performed on a per day basis, there was no statistical differences in any of the tests with the smallest p value being 0.232 (Table 4). Based on these results, the populations of before ultrasound application and after ultrasound application were grouped into one population for further ANOVA studies on the statistical differences in biomass between each of the hydrogel system configurations.

Biofilms grown on hydrogels without ciprofloxacin and without ultrasound application were compared to the other hydrogel system configurations at each observation

Table 3: ANOVA p values for before and after ultrasound application. Analysis performed on the biomass parameter for biofilms grown on hydrogels with the application of ultrasound. Results shown for each experiment.

Hydrogel Configuration	Day	Experiment	p
Without Ciprofloxacin	1	1	0.928
Without Ciprofloxacin		2	0.272
Without Ciprofloxacin		3	0.391
Without Ciprofloxacin	2	1	0.061
Without Ciprofloxacin		2	0.554
Without Ciprofloxacin		3	0.044
Without Ciprofloxacin	3	1	0.476
Without Ciprofloxacin		2	0.743
Without Ciprofloxacin		3	0.270
With Ciprofloxacin	1	1	0.224
With Ciprofloxacin		2	0.463
With Ciprofloxacin		3	0.509
With Ciprofloxacin	2	1	0.328
With Ciprofloxacin		2	0.838
With Ciprofloxacin		3	0.749
With Ciprofloxacin	3	1	0.032
With Ciprofloxacin		2	0.603
With Ciprofloxacin		3	0.056

day. Table 5 shows p values for each hydrogel configuration compared to hydrogels without ciprofloxacin and without ultrasound application. There were no significant differences in biomass populations after one day. On day two and day three, however, the biomass populations for the hydrogels with ciprofloxacin and with ultrasound application were significantly different with p values less than 0.05. There was not a significant difference in the day two and day three biomass populations for hydrogels with ciprofloxacin, without ultrasound application and hydrogels without ciprofloxacin, with ultrasound application.

Table 4: ANOVA p values for before and after ultrasound application. Analysis performed on the biomass parameter for biofilms grown on hydrogels with the application of ultrasound. Results shown for grouped before and after ultrasound populations.

Hydrogel Configuration	Day 1	Day 2	Day 3
Without Ciprofloxacin	0.447	0.838	0.764
With Ciprofloxacin	0.633	0.400	0.232

Table 5: ANOVA p values for all hydrogel system configurations. Analysis performed on the biomass parameter for biofilm grown on all system configurations. The system configuration without ciprofloxacin, without ultrasound application was used as the base configuration.

Hydrogel Configuration	Day 1	Day 2	Day 3
Without ultrasound, With Ciprofloxacin	0.219	0.332	0.602
With ultrasound, Without Ciprofloxacin	0.222	0.232	0.803
With ultrasound, With Ciprofloxacin	0.627	0.021	<0.001

CHAPTER 5

MODEL OF ANTIBIOTIC RELEASE

There has not been a mathematical model developed to characterize the controlled release of ciprofloxacin from UWEB hydrogel surfaces. The uniqueness of the UWEB hydrogels is that there is a change in surface structure when ultrasound is introduced into the system. This instantaneous effect causes the drug concentration on the surface of the hydrogel to spike. A two dimensional numerical model was developed in order to understand and demonstrate the dynamic nature of the UWEB pHEMA coated hydrogels loaded with ciprofloxacin. The domain of the model was created in space between parallel plates. Flow was driven between the plates by a pressure differential in the axial flow direction, with no slip boundary conditions enforced on both the upper and lower boundaries of the domain. The flow between the plates was considered fully developed. Mass was introduced into the system as a constant concentration boundary on the lower plate (hydrogel surface) when ultrasound was turned on, and allowed to diffuse into the surrounding fluid. When ultrasound was off, the lower boundary assumed a no flux condition allowing the mass in the domain to be driven out by the velocity profile. The boundary condition with respect to mass on the upper plate was also a no flux boundary (Figure 13). Table 6 gives a complete list of the model input parameters and important calculated parameters.

The purpose of this model was to demonstrate the effects of ultrasound induced release of ciprofloxacin from a UWEB hydrogel surface. The lower boundary was initially set to a C_0 concentration for a specified amount of time, in which a ciprofloxacin concentration profile developed between the two parallel plates. At the end of that time period, the lower boundary changed to a no mass flux condition to simulate the condition when ultrasound was arrested and the ciprofloxacin concentration profile was washed downstream of the domain. For purposes of these simulations, the C_0 concentration was considered to have a value of one, and therefore all results are on a scale of zero to one and can be considered a percentage of the actual C_0 concentration. This model is intended to assist in further development of the hydrogels and serve as a building block to future modelling efforts. The model was solved in MATLAB; the code for this model is contained in Appendix D.

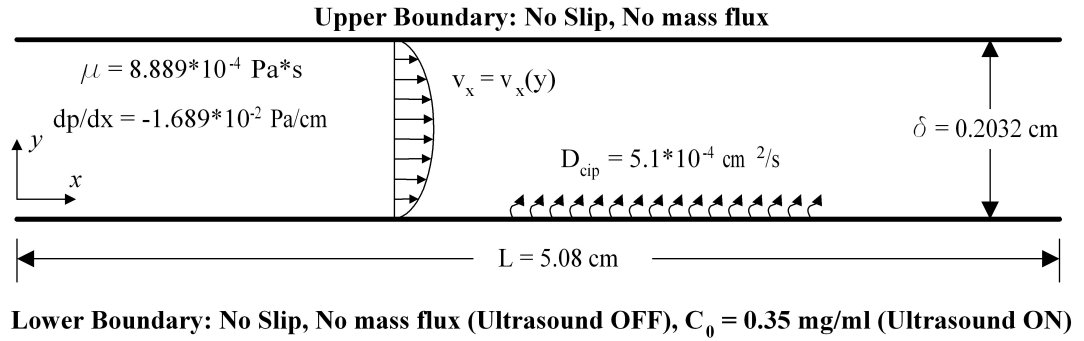


Figure 13: Two dimensional domain between parallel plates used for numerical analysis.

Table 6: Numerical Model Parameters

Parameter	Definition	Value	Units
x	variable horizontal direction		
y	variable vertical direction		
δ	depth of the gap between plates	0.2032	cm
L	domain length	5.08	cm
Q	flow rate	1.0	ml/min
dp/dx	driving pressure	-1.881*10 ⁻²	Pa/cm
v_{max}	maximum velocity	0.108	cm/s
$\langle v \rangle$	average velocity	7.193*10 ⁻²	cm/s
C_{Loaded}	loaded ciprofloxacin concentration	11.1	mg/ml
C_0	solubility of ciprofloxacin in water	0.35	mg/ml
D_{cip}	diffusion coefficient	5.1*10 ⁻⁶	cm ² /s
ρ	density of water at 25°C	996.95	kg/m ³
μ	dynamic viscosity at 25°C	8.889*10 ⁻⁴	Pa*s
Re	Reynolds number($\delta \langle v \rangle \rho / \mu$)	1.619	
Pe	Péclet number($L \langle v \rangle / D_{cip}$)	7.164*10 ⁴	

The dimensional parameters used for this model were taken from the controlled release experiments discussed in Chapter 3 when they were available. The loaded ciprofloxacin concentration was assumed to be the loaded concentration as described by the UWEB manufacturer. However, the C_0 boundary condition was limited by the solubility of ciprofloxacin hydrochloride in water. The gap between the plates (δ) was the same as the lumen depth between the hydrogel surface and the microscope coverslip in the BST FC81 flowcell. The length of the domain (L) was taken from the length of the exposed hydrogel surface in the FC81 flowcell. The pressure differential was determined by assuming a 1 ml/min flow rate between the plates with the width of the FC81 flowcell. The viscosity of the fluid between the plates was assumed to be that of water at 25°C [Munson et al., 1998]. The parameter values of dp/dx and μ

with the specified dimensions of the domain, produce a maximum velocity between the plates of 0.108 cm/s. The diffusion coefficient for ciprofloxacin in water at 25°C was taken from recent literature [Stewart et al., 2000].

Governing Equations

There are several phenomenon governing the ciprofloxacin distribution between the parallel plates. The first phenomena that was considered was the flow induced in the axial direction by the pressure differential. The convention used throughout this exercise was that the axial direction was the x direction and the perpendicular direction was the y direction with the origin located in the lower left hand corner of the domain. As with any flowing system, the momentum in the system must be balanced. Beginning with the continuity equation (Equation 5.1), the x-momentum equation (Equation 5.2) and the y-momentum equation (Equation 5.3), the steady state velocity distribution was determined to be in the x direction and only a function of y ($v_x = v_x(y)$).

$$\frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y} = 0 \quad (5.1)$$

$$\rho \left(\frac{\partial v_x}{\partial t} + v_x \frac{\partial v_x}{\partial x} + v_y \frac{\partial v_x}{\partial y} \right) = -\frac{\partial p}{\partial x} + \mu \left[\frac{\partial^2 v_x}{\partial x^2} + \frac{\partial^2 v_x}{\partial y^2} \right] + \rho g_x \quad (5.2)$$

$$\rho \left(\frac{\partial v_y}{\partial t} + v_x \frac{\partial v_y}{\partial x} + v_y \frac{\partial v_y}{\partial y} \right) = -\frac{\partial p}{\partial y} + \mu \left[\frac{\partial^2 v_y}{\partial x^2} + \frac{\partial^2 v_y}{\partial y^2} \right] + \rho g_y \quad (5.3)$$

The first assumption made to reduce the above equations was that the flow between the plates was laminar and therefore there was only fluid velocity in the axial direction. This assumption was validated by the computation of the Reynolds number after the average velocity in the system was determined. Therefore, all velocities in the y direction were zero, and all derivatives of velocities in the y direction were zero. Based on this assumption, Equation 5.3 was reduced to give only a hydrostatic pressure distribution in the y direction. The continuity equation reduces to the first derivative of velocity in the x direction with respect to x equal to zero. This led to fact that x velocities between the plates are constant in the x direction and are therefore only a function of y . The x-momentum equation, Equation 5.2, then reduced to

$$\frac{d^2 v_x}{dy^2} = \frac{1}{\mu} \frac{dp}{dx} \quad (5.4)$$

When Equation 5.4 was integrated twice and the no slip boundary conditions were applied at $y=0$ and $y=\delta$, the resulting equation was

$$v_x = -\frac{1}{2\mu} \frac{dp}{dx} y^2 \left[\frac{\delta}{y} - 1 \right] \quad (5.5)$$

where dp/dx was considered a constant and the maximum velocity occurs at $y=\delta/2$.

Using the parameters from Table 6, and calculating the Reynolds number by

$$\text{Re} = \frac{\rho \langle v \rangle \delta}{\mu} \quad (5.6)$$

the Reynolds number for this system was $Re=1.619$, which is well within the range of laminar flow and therefore validates the laminar flow assumption made earlier.

With the velocity distribution defined, the ciprofloxacin transport in the system was then evaluated. The generalized governing differential equation in two dimensional space for the transport of mass was given as

$$\rho \left(\frac{\partial \omega_A}{\partial t} + v_x \frac{\partial \omega_A}{\partial x} + v_y \frac{\partial \omega_A}{\partial y} \right) = \rho D_{AB} \left[\frac{\partial^2 \omega_A}{\partial x^2} + \frac{\partial^2 \omega_A}{\partial y^2} \right] + r_A \quad (5.7)$$

where

$$\begin{aligned} \rho &= \text{fluid mass density (kg/cm}^3\text{)} \\ \omega_A &= \text{dimensionless mass fraction of species A} \\ D_{AB} &= \text{Diffusion coefficient of species A into bulk fluid (cm}^2\text{/s)} \\ r_A &= \text{Reaction of species A} \end{aligned}$$

with time dependence and convection terms on the left hand side of the equation and diffusion terms and reaction terms on the right hand side of the equation. With only one species in the system being considered (ciprofloxacin) and no reaction, Equation 5.7 reduces to

$$\frac{\partial c}{\partial t} = -v_i \frac{\partial c}{\partial x} + D_{cip} \frac{\partial^2 c}{\partial y^2} \quad (5.8)$$

which is the well-known advection diffusion equation. Equation 5.8 indicates that diffusion in the x direction was neglected. This assumption was made by evaluating the magnitude of the Péclet number (Table 6). Péclet numbers much greater than one indicate that convection dominates diffusion in a given coordinate direction.

Numerical Solution

Numerical methods were employed to solve Equation 5.8 in two dimensional space and time. An alternating direction implicit (ADI) scheme was used to solve for the concentrations at specified node points in the domain [Chapra and Canale, 2002]. Finite difference equations were used to discretize the time derivative and the two spatial derivatives that appear in the advection-diffusion equation. Due to the nature of the equation, two boundary conditions with respect to mass transport are needed in the y direction and one in the x direction. The initial concentration profile was also needed in order to completely specify the problem. The two boundary conditions in the y direction were very easily understood. When ultrasound was not being applied to the system, the two boundaries were no mass flux conditions simulated by the parallel plates. When ultrasound was being applied to the system, the lower plate assumed a constant concentration boundary with the concentration being the solubility of ciprofloxacin in water. The boundary condition imposed on the x direction was not as intuitive. Because the velocities in the system are always positive, and there was no diffusion in the x direction ($Pe \gg 1$), there was no mechanism for mass to move upstream against the direction of flow. For this reason, fluid space that resides on the left hand side of the domain considered would never be greater than zero. The boundary condition imposed in the x direction was therefore on the left hand boundary requiring that ciprofloxacin concentrations be zero in that space.

Because there was no boundary condition imposed on the right hand side of the domain, and mass must be allowed to pass through this boundary, backward difference equations were used to discretize the concentration derivative with respect to the x direction. Central difference equations were used to discretize the second order concentration derivative with respect to the y direction and forward difference equations were used to discretize the time derivative. Equations 5.9- 5.11 show how each of the derivatives that appear in the advection-diffusion equation were discretized.

$$\frac{\partial c}{\partial x} \cong \frac{c_{i,j} - c_{i,j-1}}{\Delta x} \quad (5.9)$$

$$\frac{\partial^2 c}{\partial y^2} \cong \frac{c_{i-1,j} - 2c_{i,j} + c_{i+1,j}}{\Delta y^2} \quad (5.10)$$

$$\frac{\partial c}{\partial t} \cong \frac{c_{i,j}^{l+1} - c_{i,j}^l}{\Delta t} \quad (5.11)$$

The ADI scheme used solves for each time step in two half steps, where each half step solves a system of linear algebraic equations in one direction. Figure 14 shows how the nodal values are solved at time step $t+1/2$ in the x direction, which are then used to solve for the nodal values at time step $t+1$ in the y direction. Discretizing the advection-diffusion equation for time step $t+1/2$ in the x direction gives

$$\frac{c_{i,j}^{l+\frac{1}{2}} - c_{i,j}^l}{\Delta t/2} = -v_i \left(\frac{c_{i,j}^{l+\frac{1}{2}} - c_{i,j-1}^{l+\frac{1}{2}}}{\Delta x} \right) + D_{cip} \left(\frac{c_{i-1,j}^l - 2c_{i,j}^l + c_{i+1,j}^l}{\Delta y^2} \right) \quad (5.12)$$

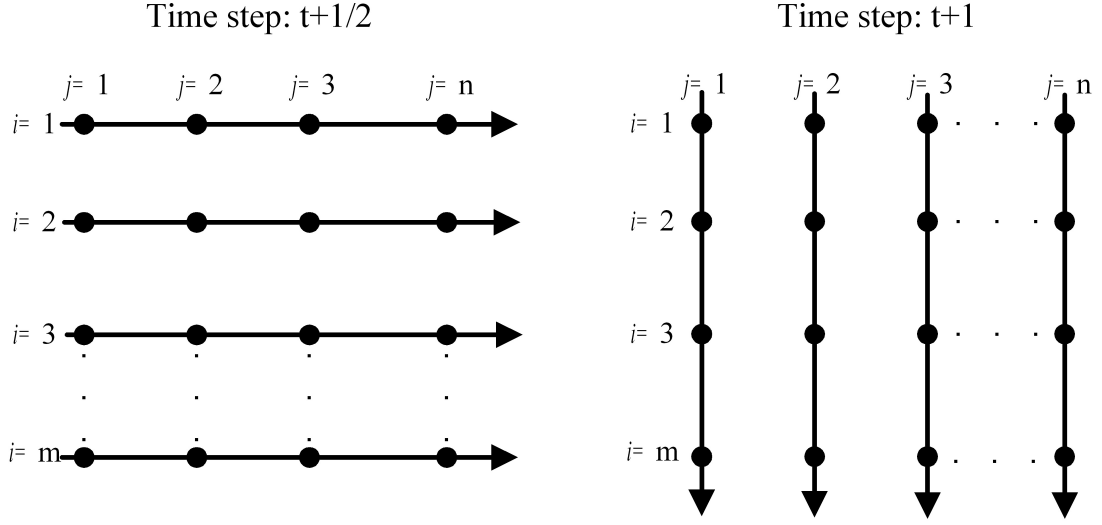


Figure 14: Two dimensional domain between parallel plates with indexed nodes. The ADI scheme solves for nodal values line by line in the horizontal direction at a time step of $t+1/2$, and then solves for nodal values line by line in the vertical direction for each incremental time step.

where l is the time variable. Rearranging Equation 5.12 gives

$$\left(-\frac{v_i}{\Delta x}\right) c_{i,j-1}^{l+\frac{1}{2}} + \left(\frac{2}{\Delta t} + \frac{v_i}{\Delta x}\right) c_{i,j}^{l+\frac{1}{2}} = \left(\frac{D_{cip}}{\Delta y^2}\right) c_{i-1,j}^l + \left(\frac{2}{\Delta t} - \frac{2D_{cip}}{\Delta y^2}\right) c_{i,j}^l + \left(\frac{D_{cip}}{\Delta y^2}\right) c_{i+1,j}^l \quad (5.13)$$

which gives a linear set of equations that are used to solve for the concentrations of each node at time step $t+1/2$. The advection-diffusion equation was then discretized again in the y direction to give the equation

$$\frac{c_{i,j}^{l+1} - c_{i,j}^{l+\frac{1}{2}}}{\Delta t/2} = -v_i \left(\frac{c_{i,j}^{l+\frac{1}{2}} - c_{i,j-1}^{l+\frac{1}{2}}}{\Delta x} \right) + D_{cip} \left(\frac{c_{i-1,j}^{l+1} - 2c_{i,j}^{l+1} + c_{i+1,j}^{l+1}}{\Delta y^2} \right) \quad (5.14)$$

which was rearranged to give

$$\left(-\frac{D_{cip}}{\Delta y^2}\right) c_{i-1,j}^{l+1} + \left(\frac{2}{\Delta t} + \frac{2D_{cip}}{\Delta y^2}\right) c_{i,j}^{l+1} - \left(\frac{D_{cip}}{\Delta y^2}\right) c_{i+1,j}^{l+1} = \left(\frac{v_i}{\Delta x}\right) c_{i,j-1}^{l+\frac{1}{2}} + \left(\frac{2}{\Delta t} - \frac{v_i}{\Delta x}\right) c_{i,j}^{l+\frac{1}{2}} \quad (5.15)$$

This equation also produces a set of linear algebraic equations that can be solved to give the concentration of each node at time step $t+1$. This process was then repeated for each time step for the desired length of time.

Special Cases

In order to validate the solutions given by this model, several special cases were examined. The first of these special cases occurred when the velocities between the plates were set to zero. The reduced form of Equation 5.8 for this special case was then

$$\frac{\partial c}{\partial t} = D_{cip} \frac{\partial^2 c}{\partial y^2} \quad (5.16)$$

which is Fick's second law of diffusion. The resulting effect was one dimensional diffusion with a constant concentration specified on one boundary and a no mass flux condition imposed on the other boundary. A closed form series solution was given for a one dimensional diffusion equation with identical boundary conditions by Stewart et al. [2000].

$$\frac{C}{C_0} = 1 - 2 \sum_{n=0}^{\infty} \frac{(-1)^n}{\left(n + \frac{1}{2}\right) \pi} \exp \left[- \left(n + \frac{1}{2}\right)^2 \pi^2 \frac{t D_{cip}}{\delta^2} \right] \cos \left[\left(n + \frac{1}{2}\right) \frac{\pi y}{\delta} \right] \quad (5.17)$$

A numerical solution was generated for this problem using the ADI scheme described above with 21 vertical nodes. Solutions at time steps of 20, 200, 500 and 900 seconds were compared to solutions given by Equation 5.17. Figure 15 shows plots of the concentration profiles over the thickness of the gap between the plates ($\delta=0.2032$ cm) for both the analytical and the numerical solutions. It was noted that the coefficient of determination was nearly one for all time points observed ($R^2 > 0.99$). For the limiting case of one dimensional diffusion, the ADI numerical solution produced accurate curve fits with confidence.

The other special case that was evaluated for the advection diffusion equation was that of no diffusion. In this case, there was no mass transport in the y direction, therefore, mass was only transported along streamlines in the x direction. Since velocity was constant along each streamline, it was considered a constant at each position along the y axis. The reduced form of Equation 5.8 for this special case was then

$$\frac{\partial c}{\partial t} = -v \frac{\partial c}{\partial x} \quad (5.18)$$

This equation can be solved using Laplace Transformation method. A complete solution to Equation 5.18 can be found in Farlow [1993]. For purposes of this exercise, the domain was initially set to contain a completely mixed solution with concentration C_0 . The resulting analytical solution was then given by

$$c(x, t) = \begin{cases} C_0 & t < x/v \\ 0 & t \geq x/v \end{cases} \quad (5.19)$$

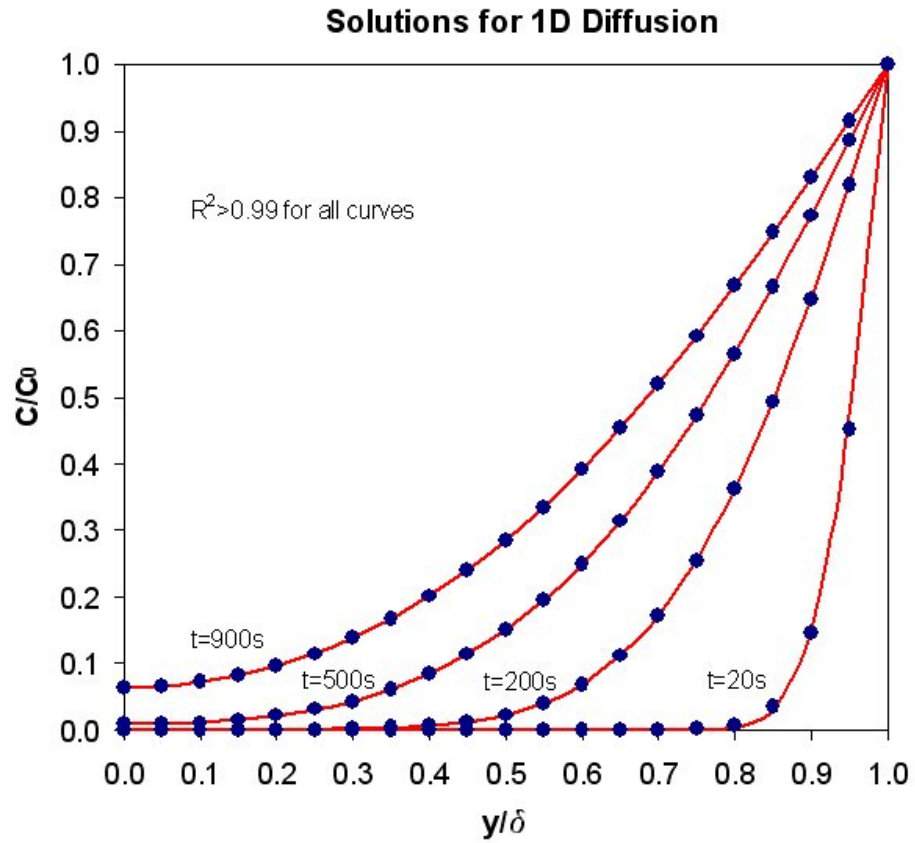


Figure 15: One dimensional diffusion between parallel plates where the right hand side of the graph represents a constant concentration boundary and the left hand side of the graph represents a no mass flux boundary. Solid curves represent the analytical solution at the specified time, and the data points overlayed on the curves represent values from the numerical model.

This equation produced a concentration front where the concentrations in the domain have a value of zero on the left hand side of the front and a value of C_0 on the right hand side of the front. The concentration front moves with the velocity profile v through the domain. As a result, the concentration front has a parabolic shape and spreads axially as time progresses. This effect is known as convective dispersion [Bird et al., 2002]. Figure 16 shows solutions to Equation 5.18 for various time points. It should be noted the scaling of the x and y axes are not the same, and are displayed this way for ease of viewing. Contour plots are shown for the numerical solution using the described ADI scheme and the line with filled circles shows the concentration front defined by the analytical solution as described by Equation 5.19. The numerical solution uses 21 nodes in the y direction and 100 nodes in the x direction over the height and length dimensions of the domain. Although the solution given by the ADI numerical scheme was considered adequate for this analysis, it should be noted that there was a steep concentration gradient produced around the concentration front given by the analytical solution in Figure 16. This concentration gradient is an artifact of the nature of the numerical solution and cannot be completely eliminated. The numerics simply do not have a mechanism for handling a discontinuous concentration front.

Controlled Release

For controlled release simulations, the concentration in the domain was initially set to zero. Equation 5.8 was used as the governing differential equation and the ADI

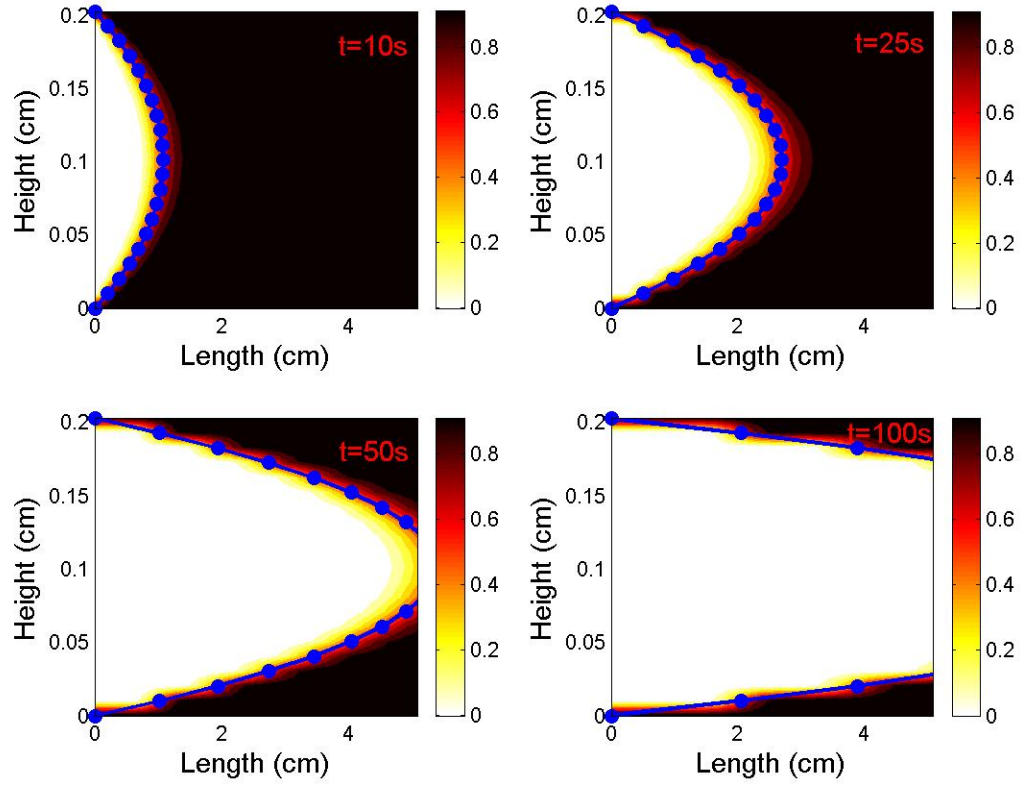


Figure 16: One dimensional mass convection between parallel plates shown for various times. The analytical solution of the concentration front is represented by the curve with filled circles. The analytical solutions are overlaid onto the numerical domain solution at the respective time. The domain was initially filled with concentration C_0 and then washed down stream by the driving velocity profile.

scheme developed above was utilized in its entirety. Ultrasound was then simulated for 15 minutes allowing the concentration on the lower boundary to have a constant value of C_0 . At the end of 15 minutes, the ultrasound simulation was turned off and the ciprofloxacin concentration profile between the plates was washed out by convective transport. A time step of 1 second was used and therefore, there are 900 domain solutions while ultrasound was simulated on, and an additional 900 domain solutions with ultrasound simulated off. In order to determine if the system reached a steady state solution in the first 900 seconds, the sum of all nodal values in the domain was determined for each time point and compared to the sum of the nodal values from the previous time point. When the difference between adjacent sums became less than 0.001, the system was assumed to have reached a steady state solution. Figure 17 shows that the system reaches steady state after 162 seconds. This process was again repeated for the last 900 time points when the ultrasound was simulated off. When ultrasound was off, the steady state configuration did not include any ciprofloxacin concentration within the limits of the domain. Figure 17 showed that this occurred at a time of 1290 seconds (390 seconds after ultrasound was discontinued).

Figure 18 shows contour plots of the released ciprofloxacin concentrations in the domain at various time points in the first 900 seconds. It is noted again that the scaling of the x and y axes are not the same. It was also noted that the system reached steady state fairly rapidly compared to the overall simulation time and that the concentration profiles for 163 seconds through 900 seconds were identical to the

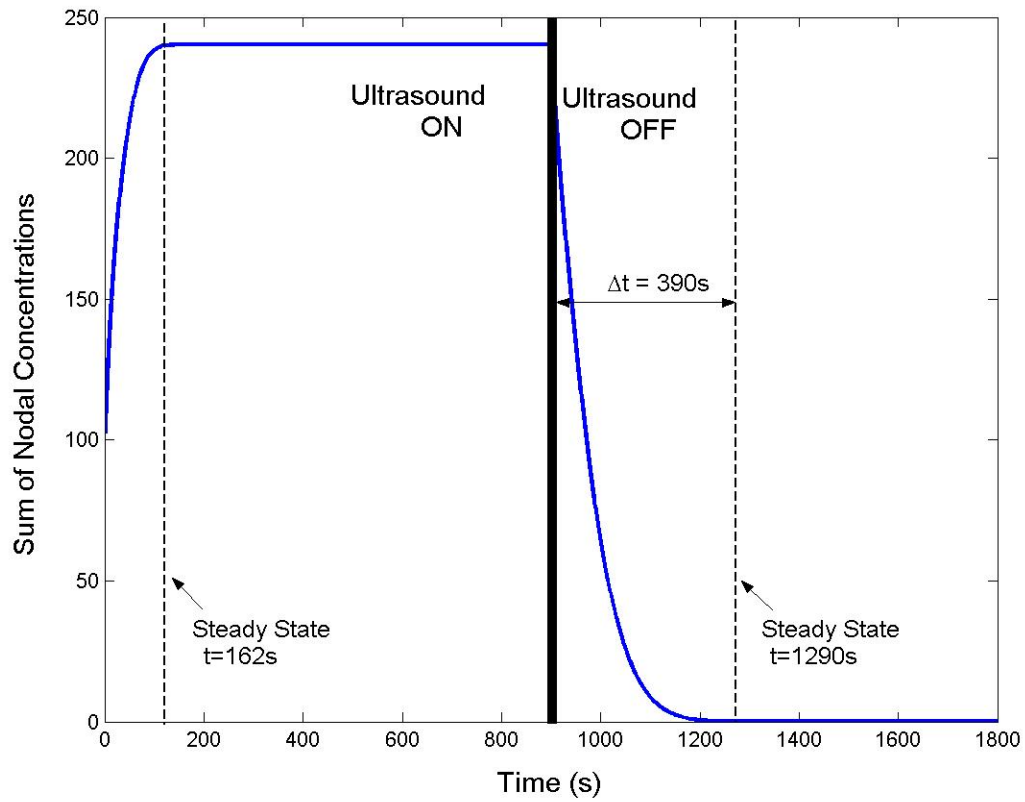


Figure 17: Steady state solutions in the domain were determined for the first 900 seconds when ultrasound was simulated on, and the last 900 seconds when ultrasound was simulated off. The system reached steady state after 162 seconds of ultrasound application and again at 390 seconds after ultrasound was simulated off.

contour plot displayed for 162 seconds. Dashed lines in Figure 18 represent the depth in the domain at which the average ciprofloxacin concentration is 10 times the MIC concentration reported in Chapter 3 ($\text{MIC} = 0.125 \mu\text{g/ml}$).

Figure 19 shows contour plots of the ciprofloxacin concentration profiles in the second 900 seconds, after ultrasound had been turned off and the lower boundary assumes a no mass flux condition. After the ultrasound was turned off, the ciprofloxacin in the system was washed down stream beyond the right hand boundary of the domain.

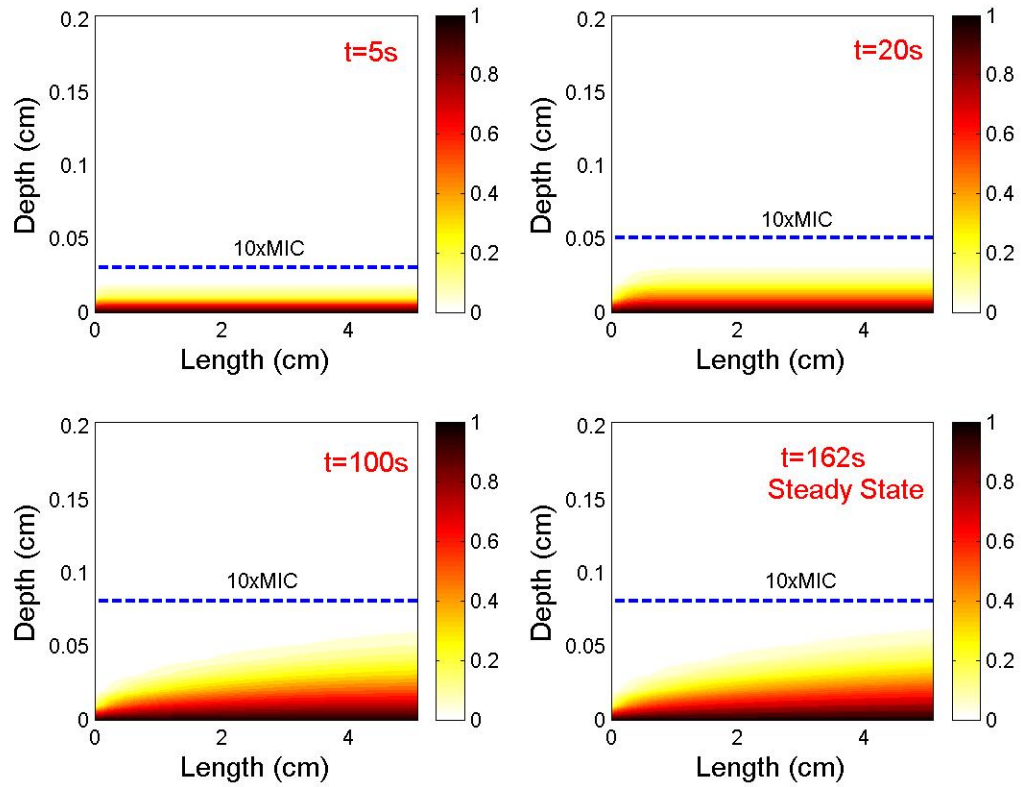


Figure 18: Various time solutions of the domain with ultrasound simulated on. For time values of 162 seconds to 900 seconds, the domain has a steady state solution. Dashed lines represent 10 times the MIC value ($\text{MIC}=0.125 \mu\text{g/ml}$).

At time 1290 seconds, the system again reached steady state, which in this case represented the complete washout of ciprofloxacin in the domain. Dashed lines in Figure 19 again represent ciprofloxacin concentration levels of 10 times the MIC value.

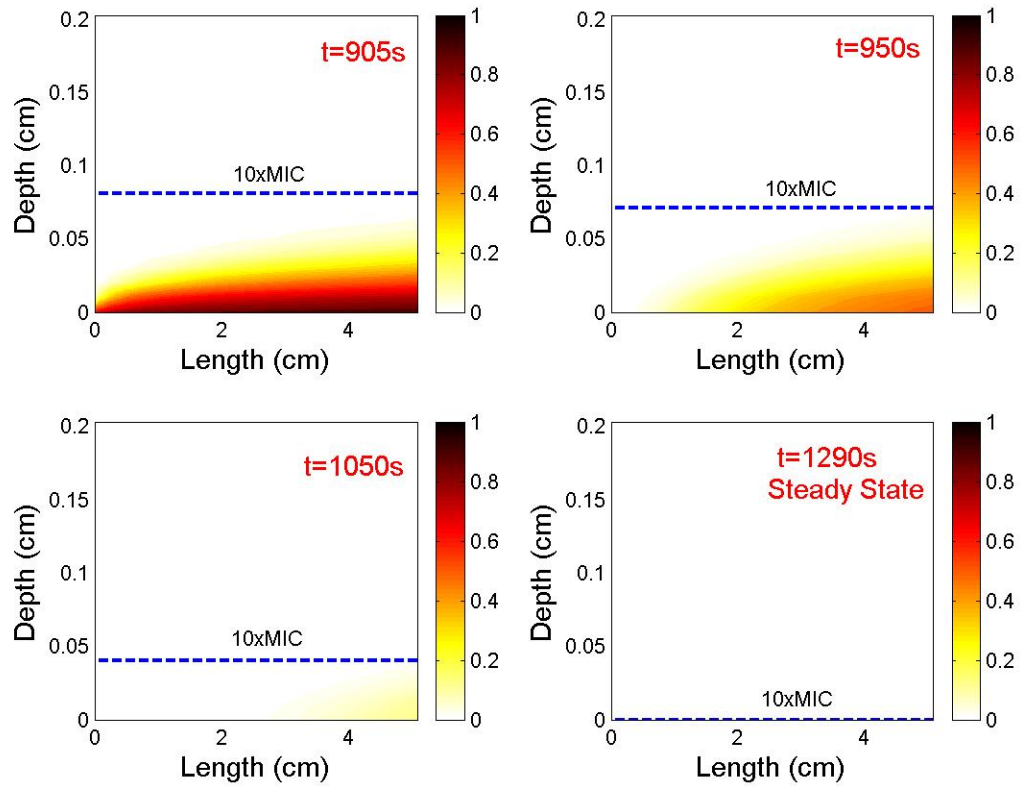


Figure 19: Various time solutions of the domain with ultrasound simulated off. For time values of 1290 seconds to 1800 seconds, the domain did not contain any ciprofloxacin. Dashed lines represent 10 times the MIC value ($MIC=0.125 \mu g/ml$).

CHAPTER 6

DISCUSSION

Bioacoustic Effect

The bioacoustic effect was defined in Chapter 1 as the synergistic effect of ultrasound and antibiotics on the efficacy of biofilm bacteria [Qian et al., 1997]. The bioacoustic effect is a function of ultrasonic energy (frequency, power density and application time), the specific organism and the specific antibiotic. Several authors have reported this phenomena under specific conditions [Qian et al., 1997; Ackart et al., 1975]. For this reason, it was necessary to consider the bioacoustic effect as a possible killing mechanism of biofilm bacteria grown on UWEB pHEMA coated hydrogels with applied ultrasound. The bioacoustic effect experiments that were conducted in this study were intended to separate the effects of ultrasound and controlled release from hydrogel surfaces. By conducting bioacoustic effect experiments on biofilm bacteria (Appendix A) using low intensity ultrasonic energy from an ultrasonic transducer, it was determined that there was no additional killing of biofilm bacteria compared to samples that were treated with low concentrations of ciprofloxacin alone (Figure 25). Additional bioacoustic effect experiments were performed using the Branson 200 ultrasonic cleaning bath on planktonic cells that were in stationary phase, which have

similar metabolic activity to biofilm cells. The rationale for conducting these experiments is that the same ultrasonic bath was used in the controlled release experiments in conjunction with UWEB pHEMA hydrogels. The results of these experiments also indicate that there is no additional bioacoustic effect related killing compared to samples that were treated with ciprofloxacin concentrations ranging from $0.5 \mu\text{g/ml}$ to $50 \mu\text{g/ml}$ (Figure 8). It was, therefore, concluded that the reduction in viable cells and the change in statistical parameters reported in biofilm growing on hydrogels with controlled release was a function only of the presence of high concentrations of ciprofloxacin released into the bulk fluid and not coupled with additional killing due to any bioacoustic effect. These data suggest that the bioacoustic effect may not be universal but vary with bacterial species, antibiotic and experimental setup.

It was suggested by Qian et al. [1999] that bioacoustic effect related killing is a function of the rate limiting step of antibiotic action against a particular organism. At least three steps necessary for an antibiotic to kill the bacteria in a biofilm have been identified. First, the antibiotic must penetrate through the biofilm to the surface of the cell wall. Second, the antibiotic must pass through the cell membrane into the bacteria. Finally, the antibiotic must bind to its target inside the cell. The mode of action of ciprofloxacin against *P. aeruginosa* is different than the mode of action of gentamicin, which is the antibiotic used by Qian et al. [1999] in bioacoustic effect studies. Gentamicin binds to the ribosome of *P. aeruginosa*, interfering with protein synthesis. Ciprofloxacin binds to the DNA gyrase of *P. aeruginosa*, which

interferes with cell reproduction. The difference in the mode of action between the two antibiotics may explain why the expected bioacoustic effect related killing was not observed for *P. aeruginosa* biofilms in conjunction with ciprofloxacin.

UWEB pHEMA Hydrogels

Antibiotic Release

It was seen from the results of the controlled release experiments that the UWEB pHEMA coated hydrogels loaded with 11.1 mg/ml of ciprofloxacin, released the antibiotic passively in the absence of ultrasound, but showed spikes in release when ultrasound was applied. This result corroborated previously reported data [Kwok et al., 2001]. The results from the antibiotic release experiments were for short time periods, seven days to examine background release and four hours to examine controlled release. These experiments were created and conducted in a manner that examined the initial release properties of the smart polymer hydrogels. Further research must be conducted in order to understand the long term release properties of the UWEB pHEMA hydrogels. Longer term studies in conjunction with mathematical models would be able to predict the overall lifetime of the product based on the surface properties of the hydrogel, when ultrasound is applied and the overall application time.

The integrity of the hydrogel surface structure also had an effect on their release properties. In the early stages of this research, UWEB provided hydrogels that were

polymerized onto glass discs and then coated with methylene chains. It became evident that if the pHEMA delaminated from the glass discs, ciprofloxacin would leach uncontrollably from the exposed pHEMA surface. This method was, therefore, discontinued. Cracks formed on the surface of the hydrogels due to dehydration would also promote uncontrolled leaching. The hydrogels used throughout this research were visually inspected to ensure that there were no major defects on the surface.

Biofilm Prevention

In order to determine the effectiveness of UWEB pHEMA hydrogels against biofilm bacteria, it was necessary to develop a set of experiments that would separate the effects of ultrasound and antibiotic release. The four system configurations that were developed (Figure 5), were modelled after the experimental methods that were discussed by Qian et al. [1997] for bioacoustic effect studies. The reported method included an untreated biofilm sample, a sample with ultrasound application only, a sample with antibiotic application only and a sample with both antibiotic and ultrasound application. In applying this method to the UWEB hydrogel experiments, antibiotic treatment was applied using ciprofloxacin loaded hydrogels. Control experiments without antibiotic treatment included hydrogels that were not loaded with ciprofloxacin. This method provided consistency between the UWEB hydrogel experiments and the bioacoustic effect studies discussed in connection with this research.

Studies using UWEB pHEMA coated hydrogels have shown that the ultrasound induced ciprofloxacin release from the hydrogel surface interferes with the natural

growth process of *P. aeruginosa* biofilms. Biofilms grown on three different controls (hydrogel system configurations: without ciprofloxacin without ultrasound, without ciprofloxacin with ultrasound and with ciprofloxacin without ultrasound) were not statistically different after 72 hours of growth. However, after 72 hours of biofilm growth on the surface of a coated UWEB pHEMA hydrogel with ultrasound applied for 20 minutes every 24 hours after inoculation, the biomass of the biofilm was significantly reduced ($p < 0.001$). This trend along with qualitative biofilm image analysis indicates that the accumulation of biofilm is significantly reduced due to the controlled release of ciprofloxacin.

Although the biofilms grown on UWEB hydrogels loaded with ciprofloxacin were substantially reduced over a three day observation period, they were not totally eradicated. Once a biofilm has been established, it becomes increasingly difficult to eliminate. In the biofilm prevention experiments, ultrasonically controlled release of ciprofloxacin was induced every 24 hours after inoculation. This time period allowed the biofilms to establish on the surface of the hydrogel before treatment. Irrespective of the hydrogel configuration, biofilms formed and were statistically indistinguishable after 24 hours of growth. This method was useful in determining the change in biofilms after they were treated over the course of the three day observation period. However, future studies might include immediate ultrasound application after inoculation to determine if biofilm formation can be eliminated altogether with an immediate dose of ciprofloxacin. It would be beneficial to approach the problem in

this way since *P. aeruginosa* cells would be in a planktonic state and more susceptible to ciprofloxacin.

Effluent Collection

The fifth step of biofilm formation discussed in Chapter 1 was detachment and dispersion of planktonic cells back into the surrounding bulk fluid. It was theorized that biofilm detachment and dispersion might be augmented by introducing ultrasonic pressure waves into a flowing system where biofilm was already well established. In clinical applications, this effect could facilitate the spreading of infection and be detrimental to the health of the patient. The results from the effluent collection studies indicate that the viability of *P. aeruginosa* cells in the effluent collected during ultrasound application was significantly reduced for experiments that included ciprofloxacin loaded hydrogels as opposed to experiments that included hydrogels without ciprofloxacin (Table 2). It was observed that over the three day observation period, the effluent from experiments that included hydrogels without ciprofloxacin yielded increasing absorbance readings and increasing CFU. It was also observed that for experiments that included ciprofloxacin loaded hydrogels, the absorbance of the effluent remained constant over the three day observation period. In addition, there were no CFU detected in the effluent of hydrogel systems loaded with ciprofloxacin. The implication of these results is that ciprofloxacin released from the hydrogel during ultrasound application had action not only against biofilm bacteria growing on the surface, but also against detached cells that were washed downstream of the biofilm.

Bacteria that are detached from hydrogel systems that do not contain ciprofloxacin were viable and have the potential to form new biofilms downstream.

Model of Antibiotic Release

The model of antibiotic release outlined in Chapter 5 was intended to provide insight into the dynamic nature of ciprofloxacin release from UWEB hydrogel surfaces. The model was investigated by initiating the release of ciprofloxacin into a flowing fluid for 900 seconds (simulating ultrasound on), and then discontinuing the release of drug into the domain, thereby, allowing the drug to be washed downstream for 900 seconds (simulating ultrasound off). Upon reaching steady state at 162 seconds, there was not a significant ciprofloxacin concentration in the upper 60% of the gap between the plates ($C < 10xMIC$) (Figure 17 and Figure 18). The ciprofloxacin concentration profile was developed only in the lower 40% of the gap between the plates closest to the hydrogel surface. It should be noted, however, that biofilm formation will occur only on surfaces. The thickest biofilms observed and discussed in Chapter 4 were on the order of 100 μm thick. At a distance of 102 μm from the hydrogel surface, which would also be the substratum for biofilm attachment, the average concentration across the length of the domain was 66% of C_0 . Using the solubility of ciprofloxacin in water as C_0 , this concentration was approximately 230 $\mu\text{g/ml}$, 1840 times the minimum inhibitory concentration for planktonic *P. aeruginosa* reported in Chapter 3.

The results from the numerical model have some very practical applications. This model can be used to predict ciprofloxacin concentration levels as a function of time. For example, one might desire a ciprofloxacin concentration of 10xMIC at a distance of 800 μm from the hydrogel surface. Using the model input parameters from Table 6, the model predicted that an average ciprofloxacin concentration of 10xMIC at a distance of 800 μm from the lower boundary was achieved after 87 seconds of ultrasound application. The results of this model were limited to a very specific geometry, however, they can be used to predict an estimated ultrasound application time.

The results of this model were intended only to demonstrate some of the governing transport characteristics of a specific system. There are, however, some concepts that have been intentionally left out of the model for simplification, but may be considered in future efforts to model the drug release. This model assumes that there was no mixing of ciprofloxacin that may be generated by the presence of ultrasonic pressure waves and subsequent acoustic cavitation. The extent of the mixing would be very difficult to determine empirically, but might be determined experimentally. The boundary conditions used for the hydrogel surface may also need to be examined. This model assumes a constant concentration boundary when ultrasound was initiated and a no mass flux boundary when ultrasound was arrested. The exact mechanism of release from the surface of the hydrogel is unknown and the permeability of the membrane type coating needs to be further investigated. The no mass flux condition

when ultrasound was not present, does not provide a mechanism for background release or leaching. Although it was shown that background release was minimal, it still might need to be considered. Finally, this model does not provide a mechanism for ciprofloxacin depletion in the hydrogel. Certainly, if this model was observed over a longer time period, this concept would need to be included.

Industrial Relevance

Infections occurring in response to the implant of an orthopaedic device, or infections related to general reconstructive surgery are a common problem in hospitals today and are being held responsible for the decline of systemic health in some patients. The problem with some of the commercially available drug delivery systems for orthopaedic applications is that they are prone to uncontrollable leaching. The UWEB pHEMA ultrasonically responsive hydrogel system eliminates this problem. When ultrasound is not being applied to the system, it exhibits zero-order release kinetics. This in itself would provide a mechanism to regulate the drug concentration being delivered to a local implant site. When ultrasound is applied, the release of the drug from the surface of the hydrogel spikes significantly. This property would give clinicians and patients control over the amount of drug delivered to the site of infection without invasive surgery or bombarding the patients system with high concentrations of antibiotics, which can themselves be toxic.

Antibiotics are generally considered low-risk pharmaceuticals compared with their medical benefit, but several dangers with respect to the over use of antibiotics have emerged in recent years. The first of these dangers is the emergence of drug resistant organisms. This phenomena was first observed with the discovery of the first antibiotic, penicillin [Roberts, 2002]. The second danger is antibiotic toxicity. Antibiotic toxicity refers to adverse drug reactions that some patients experience in response to high doses of antibiotic treatment [Rouveix, 2003]. The use of controlled drug delivery systems would minimize these dangers. The UWEB pHEMA hydrogel systems would introduce high antibiotic concentrations to a local site of infection. Although the antibiotic concentrations are high locally, they would be relatively low compared to the amount of drug that would be required systemically, thereby, controllably minimizing the total amount of drug exposure.

The UWEB pHEMA hydrogel systems have been developed primarily to be used as a coating for implant surfaces. Methods must be developed to polymerize the pHEMA polymer to implant surfaces, load the coating with antibiotics and then coat the surface with ordered methylene chains. The hydrogels investigated in this research were all flat stand alone systems. As the use of these hydrogels moves from the lab to clinical applications, the processes by which they are manufactured and molded will need to be subsequently developed.

It should also be noted here that the innovative technology of the UWEB pHEMA hydrogel systems may also have a broader scope of applications than those suggested

by this research. The loading of insulin for controlled delivery mediated by ultrasound has also been investigated by UWEB [Kwok et al., 2001]. In theory any molecule that can sufficiently move through the pHEMA matrix, could be loaded into the hydrogel and retained by the ultrasonically responsive barrier membrane.

CHAPTER 7

CONCLUSIONS

1. *There was no bioacoustic effect related killing of Pseudomonas aeruginosa cells in the presence of ciprofloxacin and specific ultrasound.*

The ultrasound source used throughout this research was a Branson 200 43 kHz ultrasonic bath. The bioacoustic effect was investigated using this bath, multiple ciprofloxacin concentrations and *P. aeruginosa* cells in stationary phase. There was no bioacoustic effect related killing observed.

2. *The background release of ciprofloxacin from UWEB pHEMA hydrogels is altered when the hydrogels are coated with ordered methylene chains.* The background release rate of non-coated pHEMA hydrogels loaded with ciprofloxacin decayed exponentially over three days until the ciprofloxacin in the hydrogel was depleted. The background release of ciprofloxacin from coated UWEB pHEMA hydrogels reached a constant zero-order release rate after one day and continued over the six day observation period. These experiments were conducted in a bulk fluid environment, and the fluid was exchanged after every measurement.

3. *Release of ciprofloxacin from UWEB pHEMA hydrogels under flow conditions is significantly increased in the presence of specific ultrasound.* The Branson 200 43 kHz ultrasonic bath was used to demonstrate the

ultrasonically controlled release of ciprofloxacin from UWEB pHEMA hydrogel surfaces. These experiments were conducted under flow conditions. A mathematical model of antibiotic release was created to demonstrate the interaction between fluid flow, advection and diffusion.

4. *The viability and overall health of Pseudomonas aeruginosa biofilms are significantly altered due to the controlled release of ciprofloxacin from the surface of UWEB pHEMA hydrogels.* *P. aeruginosa* biofilms were grown on four different system configurations for three days. The system configurations included: hydrogels without ciprofloxacin without ultrasound, hydrogels without ciprofloxacin with ultrasound, hydrogels with ciprofloxacin without ultrasound and hydrogels with ciprofloxacin with ultrasound (Ultrasound applied for 20 minutes every 24 hours after inoculation). Biofilm colonies were observed and recorded using a Confocal Scanning Laser Microscope (CSLM). Statistical biofilm parameters were found using the software package COMSTAT. The average biomass parameter for three of the hydrogel system configurations was compared to the average biomass of biofilm grown on hydrogels without ciprofloxacin without ultrasound application. After one day of biofilm growth, there was no significant difference between any of the biofilms grown on any of the hydrogel system configurations. After three days of growth, biofilms treated with ultrasonically controlled release of ciprofloxacin showed a significant reduction in biomass compared with the control ($p < 0.001$).

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APPENDICES

APPENDIX A

BIOACOUSTIC EFFECT: TRANSDUCER

The experiments and results that are reported here pertain to bioacoustic effect studies conducted with a Panametrics V318 500 kHz ultrasonic immersion transducer. The experimental setup was constructed in similar fashion to experiments that have been reported in past literature. Over the course of these studies it became clear that the maximum power output of this 500 kHz immersion transducer did not initiate controlled release from the surface of the UWEB pHEMA hydrogels. For this reason, the use of the 500 kHz immersion transducer was abandoned as a means to initiate controlled release from hydrogel surfaces. Therefore, The results reported here do not have immediate applications and interpretations concerning the controlled release of ciprofloxacin from hydrogel surfaces

Methods

Transducer Calibration

A NTR TNU100A ceramic tip needle hydrophone was used to calibrate a Panametrics V318 500 kHz immersion transducer. A calibration tank was constructed using a ten gallon tank with brackets to hold both the transducer and the hydrophone. It was determined that the power density for these experiments would be measured with respect to spatial peak intensity and temporal peak intensity (I_{sptp}) measured in units of W/cm^2 . It was first necessary to find the spatial peak voltage of the transducer. This was achieved by systematically moving the face of the transducer in front of the hydrophone in a water medium. The transducer was held in place

with a section of PVC pipe with the same inside diameter as the outside diameter of the transducer. This assembly was mounted on a piece of steel which was suspended from two horizontal positioning stages. The stages were supplied with positioning knobs that moved lead screws with 20 threads per inch. For two revolutions of the positioning knob, the stage travelled horizontally $1/10$ of an inch. The stages were stacked together allowing for two inches of maximum travel horizontally. The steel plate which held the transducer/PVC assembly was suspended from the stages using five threaded rods, which also had 20 threads per inch. Four of these rods were on the corners of the steel plate, and had two nuts per rod above the plate used to lock the plate in place. The fifth threaded rod was in center of the plate and had a coupling nut on the lower face of the plate, used to position the plate vertically. Two revolutions of the coupling nut would raise the transducer in front of the hydrophone $1/10$ of an inch. The five vertical positioning rods were cut to allow for two inches of travel vertically in front of the hydrophone in the plane of the transducer face. Figure 20 shows a schematic of the calibration tank.

Because the transducer was unfocused, an axial distance of five inches was arbitrarily selected to determine the maximum spatial output voltage of the hydrophone, which was related to the maximum output pressure and power density of the transducer by a calibration constant. A Hewlett Packard HP 33120A function generator was used to generate a 500 kHz burst of fifty cycles. A fifty cycle burst was chosen instead of continuous wave to minimize the effect of reflected ultrasound off the sides

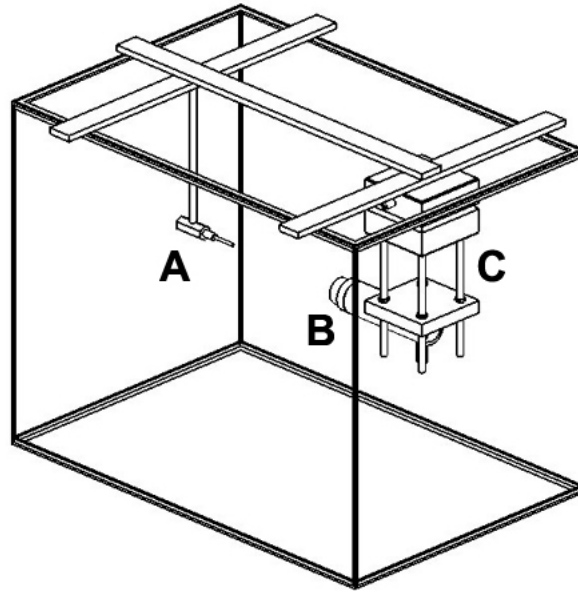


Figure 20: Calibration tank used to calibrate a Panametrics V318 immersion transducer. The tank incorporates a NTR ceramic tip needle hydrophone (A), a Panametrics V318 immersion transducer (B) and a positioning assembly (C).

of the calibration tank. This signal was amplified by an ENI 240L broadband power amplifier to give an output voltage of 20 Vp-p. The transducer was connected to the amplifier and the impedance of the connecting cables was assumed to be insignificant. The output voltage from the transducer and the input voltage from the hydrophone were measured using a Hewlett Packard HP 55120A oscilloscope. The oscilloscope and the trigger on the function generator were connected so that when the function generator was triggered, the oscilloscope would record data at the same time. Figure 21 shows the configuration of the calibration tank, function generator, amplifier, and oscilloscope.

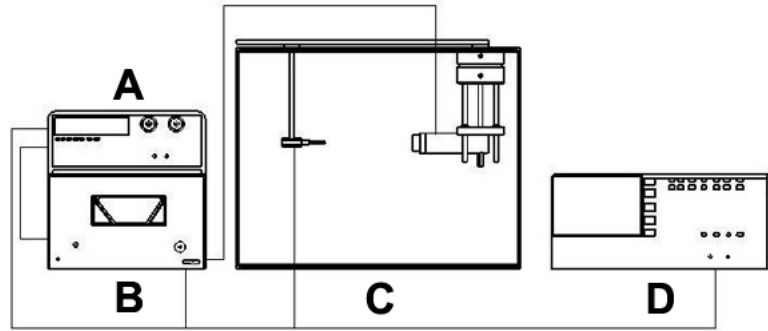


Figure 21: Configuration used to find the spatial peak voltage of the immersion transducer. The system includes a HP 33120A function generator (A), a ENI 240L power amplifier (B), the calibration tank described in Figure 20 (C) and a HP 55120A oscilloscope (D).

The transducer was then systematically moved in front of the hydrophone at intervals of $1/10$ of an inch in both horizontal and vertical directions perpendicular to the transducer axis. Three bursts were given to the transducer and the input voltage of the hydrophone was recorded at each position. These three voltages were then averaged to give one data point per position of the transducer. The data points were mapped to find the maximum output voltage position of the transducer.

The transducer assembly was then locked in this position in order to calibrate the transducer at different spatial peak temporal peak power density levels. Calibration constants were provided with the needle hydrophone. For a 500 kHz waveform, the calibration constant was $5.21 \times 10^{-2} \text{ V}^2\text{cm}^2/\text{W}$. The hydrophone voltage inputs required for power densities ranging from $1 \text{ mW}/\text{cm}^2$ to $370 \text{ mW}/\text{cm}^2$ were determined using the calibration constant. The output voltage of the transducer was adjusted until the hydrophone input voltage agreed with the calculated value and the output

voltage of the transducer was recorded. This output voltage would be the voltage applied to biofilm at the specified power density. This procedure was performed at several axial positions including 5 inches and 2 inches.

Precautions were taken to ensure that the power input to the transducer did not exceed the maximum power level specified by the manufacture which was 0.125 W. The power supplied to the transducer was given by Panametrics by the equation

$$P_{tot} = \frac{(D) (V_{RMS})^2 \cos(\Phi)}{Z} \quad (7.1)$$

where

$$\begin{aligned} D &= \text{Duty Cycle} \\ V_{RMS} &= \text{RMS Input Voltage} \\ \Phi &= \text{Phase Angle} \\ Z &= \text{Electrical Impedance} \end{aligned}$$

The phase angle and electrical impedance were supplied by Panametrics with values of -85° and 450 ohms respectively. If the maximum power output of the transducer exceeded 0.125 W, then the duty cycle, which is the percentage of time in a toneburst that the transducer is excited, was reduced accordingly.

Transducer Experimental Setup

P. aeruginosa biofilms were grown for 48 hours in a CDC reactor (Figure 22). The CDC reactor consists of eight suspended rods each containing three 0.5 inch polycarbonate coupons vertically aligned. The CDC reactor is a continuously stirred tank reactor (CSTR) stirred with a teflon stir blade. Influent media was pumped into

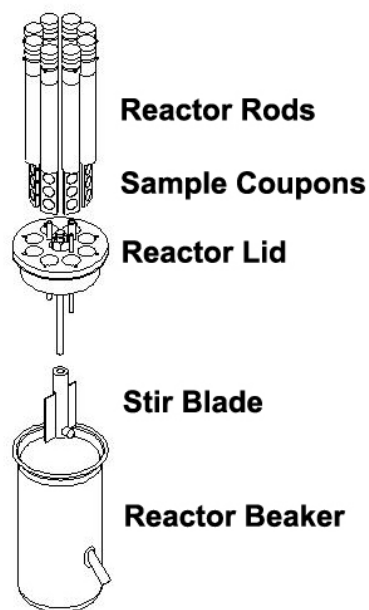


Figure 22: CDC reactor configuration incorporating eight suspended rods and 24 sample coupons in a continuously stirred tank.

the top of the reactor and exited through a port on the side of the reactor. Eight polycarbonate coupons were loaded into the center position of the suspended rods. The outward facing surfaces of the coupons were covered with aluminum foil to ensure biofilm growth only on the interior faces of the reactor coupons. The reactor was then autoclaved to ensure a sterile environment.

Bacterial batch cultures were prepared as above. The reactor was then inoculated with 3 ml of the batch inoculum into 500 ml of full strength LB broth. The reactor was continuously stirred 24 hours using a magnetic stir plate and the stir bar provided with the reactor. After 24 hours of continuous stirring, a continuous flow of 11 ml/min

1/50 strength LB broth was turned on for another 24 hours. At the end of 24 hours of continuous flow, the biofilm in the reactor was then treated and sampled.

Two of the coupons in the reactor were used as controls. The rods containing these coupons were placed in 500 ml of full strength LB broth for one hour. Two of the rods were placed in 500 ml of full strength LB broth with a defined concentration of ciprofloxacin. Two of the rods containing coupons underwent ultrasound treatment for one hour by placing them in another tank that incorporated the ultrasonic transducer, designed to deliver ultrasound energy to the biofilm on the coupons (Figure 23). For these two rods the tank was filled with full strength LB broth. The final two rods also underwent ultrasonic treatment for one hour, but were immersed in the same defined ciprofloxacin concentration as the previous two rods. Altogether there were two coupons that were used as controls, two coupons that received antibiotic treatment only, two coupons that received ultrasound treatment only and two coupons that received both antibiotic and ultrasound treatment.

After each of the coupons underwent the respective treatment, they were removed from the reactor rods and placed in 10 ml of buffer solution in test tubes. These tubes were then sonicated in a higher intensity ultrasonic bath Fisher Scientific FS15 ultrasound bath for five minutes to remove the bacteria from the surface of the coupons. These solutions were then plated out to five 90% reduction dilutions on full strength LB agar plates. The plates were incubated overnight and counted to determine if there was any bioacoustic effect.

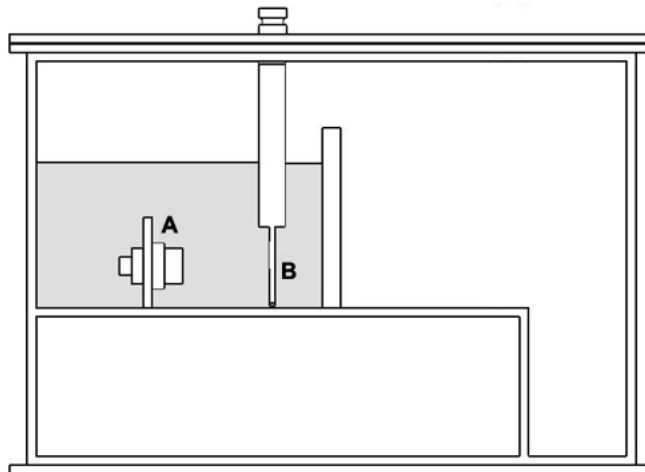


Figure 23: Tank used to sonicate biofilm grown on CDC reactor coupons incorporating the Panametrics V318 immersion transducer (A) and a biofilm sample coupon held by a CDC reactor rod (B).

Results

Transducer Calibration

The Panametrics V318 immersion transducer was calibrated using the methods described above for the spatial peak, temporal peak (I_{sptp}) output voltage. Figure 24 shows a surface plot of the recorded output voltages with the maximum output voltage (input voltage of the hydrophone) occurring near the center axis of the transducer. The calibration tank was then locked in place and calibrated for I_{sptp} power densities ranging from 1 mW/cm^2 to 370 mW/cm^2 . For all power densities considered, a full duty cycle was sufficient to keep the total input power to the transducer less than the 0.125 W maximum, except in the cases where the power density was 100 mW/cm^2

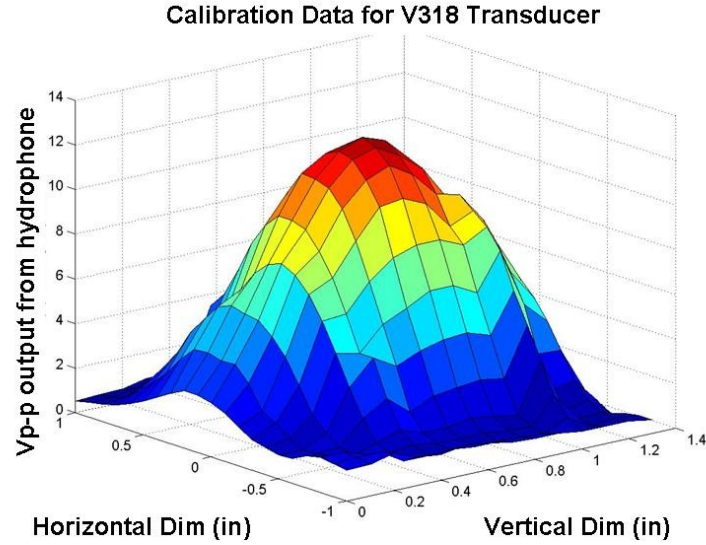


Figure 24: Surface plot of the peak to peak output voltage of the Panametrics V318 immersion transducer at an axial distance of 5 inches.

and 370 mW/cm^2 at a distance of two inches from the face of the transducer. For these Isptp intensities, the required input voltage(RMS) was 46 volts and 80.3 volts which required the duty cycles to be reduced to 0.3 and 0.1 respectively.

Bioacoustic Effect: Transducer

There were no bioacoustic effects observed using the Panametrics V318 immersion transducer and biofilm grown for 48 hours in the CDC reactor. None of the experiments showed a significant $\log_{10}$ reduction when ultrasound was applied for one hour. Figure 25 shows the results from two of these experiments. The experiment displayed in Figure 25 with $2.5 \mu\text{g/ml}$ of ciprofloxacin and 100 mW/cm^2 Isptp, there was approximately a $1.0 \log_{10}$ reduction in CFU when the antibiotic concentration was added compared to the control. There was no further significant reduction when

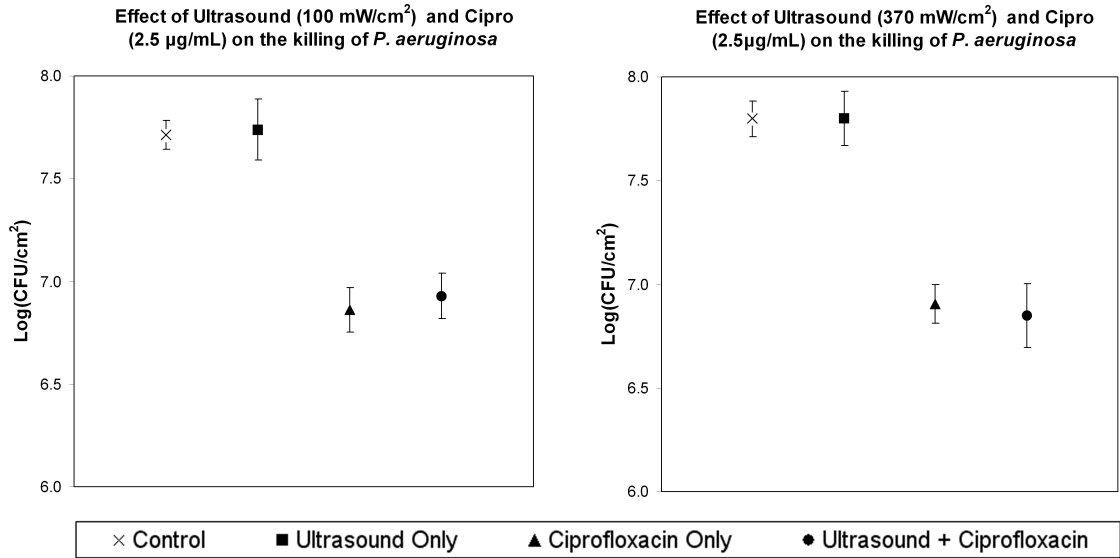


Figure 25: The effects of ultrasound generated by a Panametrics V318 immersion transducer in the presence of 2.5 µg/ml of ciprofloxacin. Plots shown are for individual experiments with two samples representing each data point. There was no bioacoustic effect observed under these conditions.

ultrasound was applied for one hour. Similar results are displayed in Figure 25 for the experiment using 370 mW/cm² Isptp. Error bars in Figure 25 indicate the standard deviation over two sampled coupons.

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a V318 immersion transducer observed on 6/19/2003

Coupon Area	1.267	cm ²
Flowrate	10	ml/min
Dilution Volume	10	ml
Drop Volume	10	μl
Face Distance	5	in
I _{sptp}	10	mW/cm ²
Duty Cycle	1	
US time	30	min
Cip Conc	0.625	μg/ml

Coupon	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀	Std Dev
1-Control	1	3	17	24	25	1.74E+06	6.24	6.28	0.0793
1-Control	2	3	24	31	26	2.13E+06	6.33		
2-Control	1	3	19	19	20	1.53E+06	6.18	6.17	
2-Control	2	3	21	19	15	1.45E+06	6.16		
3-US only	1	N/A	N/A	N/A	N/A	N/A	N/A N/A	0.0000	
3-US only	2	N/A	N/A	N/A	N/A	N/A	N/A		
4-US only	1	3	15	15	12	1.10E+06	6.04	6.73	
4-US only	2	4	19	43	37	2.60E+07	7.42		
5-Cip only	1	2	9	8	7	6.31E+04	4.80	4.80	0.4057
5-Cip only	2	2	5	8	11	6.31E+04	4.80		
6-Cip only	1	2	28	28	31	2.29E+05	5.36	5.37	
6-Cip only	2	2	32	29	32	2.45E+05	5.39		
7-US+Cip	1	2	3	10	5	4.74E+04	4.68	4.66	0.8683
7-US+Cip	2	2	7	3	7	4.47E+04	4.65		
8-US+Cip	1	3	5	12	6	6.05E+05	5.78	5.89	
8-US+Cip	2	3	12	13	13	1.00E+06	6.00		

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a V318 immersion transducer observed on 6/26/2003

Coupon Area	1.267	cm ²
Flowrate	10	ml/min
Dilution Volume	10	ml
Drop Volume	10	μl
Face Distance	5	in
I _{sptp}	100	mW/cm ²
Duty Cycle	0.3	
US time	30	min
Cip Conc	0.625	μg/ml

Coupon	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀	Std Dev
1-Control	1	3	10	18	13	1.08E+06	6.03	6.03	0.0234
1-Control	2	3	10	13	18	1.08E+06	6.03		
2-Control	1	3	11	14	14	1.03E+06	6.01	6.00	
2-Control	2	3	10	11	16	9.73E+05	5.99		
3-US only	1	3	7	9	9	6.58E+05	5.82	5.73	0.0880
3-US only	2	3	5	6	6	4.47E+05	5.65		
4-US only	1	3	14	6	9	7.63E+05	5.88	5.86	
4-US only	2	3	11	6	9	6.84E+05	5.84		
5-Cip only	1	2	17	22	17	1.47E+05	5.17	5.18	0.1565
5-Cip only	2	2	20	18	20	1.53E+05	5.18		
6-Cip only	1	2	27	36	27	2.37E+05	5.37	5.40	
6-Cip only	2	2	35	29	36	2.63E+05	5.42		
7-US+Cip	1	2	23	25	22	1.84E+05	5.27	5.21	0.0000
7-US+Cip	2	2	16	16	22	1.42E+05	5.15		
8-US+Cip	1	N/A	N/A	N/A	N/A	N/A	N/A		
8-US+Cip	2	N/A	N/A	N/A	N/A	N/A	N/A		

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a V318 immersion transducer observed on 8/13/2003

Coupon Area	1.267	cm ²
Flowrate	10	ml/min
Dilution Volume	10	ml
Drop Volume	10	μl
Face Distance	5	in
I _{sptp}	100	mW/cm ²
Duty Cycle	0.3	
US time	30	min
Cip Conc	0.625	μg/ml

Coupon	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀	Std Dev
1-Control	1	4	4	4	12	5.26E+06	6.72	6.67	0.0000
1-Control	2	4	6	5	5	4.21E+06	6.62		
2-Control	1	N/A	N/A	N/A	N/A	N/A	N/A		
2-Control	2	N/A	N/A	N/A	N/A	N/A	N/A		
3-US only	1	3	5	4	7	4.21E+05	5.62	5.69	0.3587
3-US only	2	3	9	7	6	5.79E+05	5.76		
4-US only	1	4	2	6	5	3.42E+06	6.53	6.20	
4-US only	2	3	9	10	9	7.37E+05	5.87		
5-Cip only	1	3	7	9	8	6.31E+05	5.80	5.76	0.2988
5-Cip only	2	3	7	5	8	5.26E+05	5.72		
6-Cip only	1	4	8	8	8	6.31E+06	6.80	6.18	
6-Cip only	2	3	4	4	6	3.68E+05	5.57		
7-US+Cip	1	3	10	7	7	6.31E+05	5.80	5.80	0.0360
7-US+Cip	2	3	7	9	8	6.31E+05	5.80		
8-US+Cip	1	3	9	9	10	7.37E+05	5.87	5.85	
8-US+Cip	2	3	9	8	9	6.84E+05	5.84		

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a V318 immersion transducer observed on 10/13/2003

Coupon Area	1.267	cm ²
Flowrate	11	ml/min
Dilution Volume	10	ml
Drop Volume	10	μl
Face Distance	2	in
I _{sptp}	100	mW/cm ²
Duty Cycle	0.3	
US time	60	min
Cip Conc	2.5	μg/ml

Coupon	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀	Std Dev
1-Control	1	5	10	6	6	5.79E+07	7.76	7.71	0.0068
1-Control	2	5	6	6	5	4.47E+07	7.65		
2-Control	1	5	5	6	6	4.47E+07	7.65	7.72	
2-Control	2	5	8	8	7	6.05E+07	7.78		
3-US only	1	5	10	3	3	4.21E+07	7.62	7.78	0.0623
3-US only	2	5	10	10	13	8.68E+07	7.94		
4-US only	1	5	7	4	5	4.21E+07	7.62	7.69	
4-US only	2	5	10	7	5	5.79E+07	7.76		
5-Cip only	1	4	17	11	10	1.00E+07	7.00	6.93	0.1024
5-Cip only	2	4	9	9	10	7.37E+06	6.87		
6-Cip only	1	4	6	7	8	5.52E+06	6.74	6.79	
6-Cip only	2	4	11	8	7	6.84E+06	6.84		
7-US+Cip	1	4	8	6	8	5.79E+06	6.76	6.86	0.0925
7-US+Cip	2	4	10	15	10	9.21E+06	6.96		
8-US+Cip	1	4	11	9	18	1.00E+07	7.00	6.99	
8-US+Cip	2	4	10	13	14	9.73E+06	6.99		

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a V318 immersion transducer observed on 10/30/2003

Coupon Area	1.267	cm ²
Flowrate	11	ml/min
Dilution Volume	10	ml
Drop Volume	10	μl
Face Distance	2	in
I _{sptp}	370	mW/cm ²
Duty Cycle	0.1	
US time	60	min
Cip Conc	2.5	μg/ml

Coupon	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀	Std Dev
1-Control	1	5	11	12	9	8.42E+07	7.93	7.84	0.0647
1-Control	2	5	9	8	5	5.79E+07	7.76		
2-Control	1	5	7	6	8	5.52E+07	7.74	7.75	
2-Control	2	5	7	7	8	5.79E+07	7.76		
3-US only	1	5	10	10	11	8.16E+07	7.91	7.90	0.1371
3-US only	2	5	7	9	13	7.63E+07	7.88		
4-US only	1	5	5	5	6	4.21E+07	7.62	7.70	
4-US only	2	5	9	8	6	6.05E+07	7.78		
5-Cip only	1	4	11	12	18	1.08E+07	7.03	6.95	0.0654
5-Cip only	2	4	7	10	11	7.37E+06	6.87		
6-Cip only	1	4	9	10	11	7.89E+06	6.90	6.86	
6-Cip only	2	4	8	5	12	6.58E+06	6.82		
7-US+Cip	1	4	8	8	9	6.58E+06	6.82	6.82	0.0447
7-US+Cip	2	4	8	8	9	6.58E+06	6.82		
8-US+Cip	1	4	6	6	7	5.00E+06	6.70	6.88	
8-US+Cip	2	4	17	17	10	1.16E+07	7.06		

APPENDIX B

BIOACOUSTIC EFFECT: ULTRASONIC BATH DATA

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a Branson 200 ultrasonic bath observed on 2/19/2004

Batch Volume	10	ml
Dilution Volume	10	ml
Drop Volume	10	μ l
I_{sptp}	2-3	W/cm ²
US time	20	min
Cip Conc	5	μ g/ml

Batch	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀
1-Control	1	6	4	2	5	3.67E+08	8.56	8.63
1-Control	2	6	8	2	5	5.00E+08	8.70	
2-US only	1	6	5	5	1	3.67E+08	8.56	8.66
2-US only	2	6	3	7	7	5.67E+08	8.75	
3-Cip only	1	5	4	6	4	4.67E+07	7.67	7.65
3-Cip only	2	5	3	6	4	4.33E+07	7.64	
4-US+Cip	1	5	6	4	4	4.67E+07	7.67	7.64
4-US+Cip	2	5	5	5	2	4.00E+07	7.60	

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a Branson 200 ultrasonic bath observed on 2/21/2004

Batch Volume	10	ml
Dilution Volume	10	ml
Drop Volume	10	μ l
I_{sptp}	2-3	W/cm ²
US time	20	min
Cip Conc	5	μ g/ml

Batch	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀
1-Control	1	6	7	4	9	6.67E+08	8.82	8.89
1-Control	2	6	13	6	8	9.00E+08	8.95	
2-US only	1	6	9	8	11	9.33E+08	8.97	8.90
2-US only	2	6	5	9	6	6.67E+08	8.82	
3-Cip only	1	4	20	19	23	2.07E+07	7.32	7.29
3-Cip only	2	4	15	22	19	1.87E+07	7.27	
4-US+Cip	1	4	9	8	13	1.00E+07	7.00	7.06
4-US+Cip	2	4	13	13	13	1.30E+07	7.11	

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a Branson 200 ultrasonic bath observed on 3/12/2004

Batch Volume	10	ml
Dilution Volume	10	ml
Drop Volume	10	μ l
I_{sptp}	2-3	W/cm ²
US time	20	min
Cip Conc	5	μ g/ml

Batch	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀
1-Control	1	6	9	5	9	7.67E+08	8.88	8.93
1-Control	2	6	11	12	6	9.67E+08	8.99	
2-US only	1	6	11	7	8	8.67E+08	8.94	8.87
2-US only	2	6	8	5	6	6.33E+08	8.80	
3-Cip only	1	4	24	17	22	2.10E+07	7.32	7.22
3-Cip only	2	4	7	14	18	1.30E+07	7.11	
4-US+Cip	1	4	8	10	16	1.13E+07	7.05	7.07
4-US+Cip	2	4	15	10	12	1.23E+07	7.09	

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a Branson 200 ultrasonic bath observed on 3/25/2004

Batch Volume	10	ml
Dilution Volume	10	ml
Drop Volume	10	μ l
I_{sptp}	2-3	W/cm ²
US time	20	min
Cip Conc	5	μ g/ml

Batch	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀
1-Control	1	5	31	37	33	3.37E+08	8.53	8.53
1-Control	2	5	35	31	34	3.33E+08	8.52	
2-US only	1	5	29	33	30	3.07E+08	8.49	8.49
2-US only	2	5	27	32	36	3.17E+08	8.50	
3-Cip only	1	5	9	11	12	1.07E+08	8.03	8.03
3-Cip only	2	5	14	9	10	1.10E+08	8.04	
4-US+Cip	1	5	11	8	8	9.00E+07	7.95	7.95
4-US+Cip	2	5	7	9	10	8.67E+07	7.94	

Bioacoustic Effect Data for ciprofloxacin and *P. aeruginosa* using a Branson 200 ultrasonic bath observed on 4/1/2004

Batch Volume	10	ml							
Dilution Volume	10	ml							
Drop Volume	10	μ l							
I_{sptp}	2-3	W/cm ²							
US time	20	min							
Cip Conc	5	μ g/ml							
Batch	Plate	Dilution	Drop 1	Drop 2	Drop 3	CFU/cm ²	Log ₁₀ CFU/cm ²	Avg Log ₁₀	
1-Control	1	6	12	11	14	1.23E+09	9.09	9.12	
1-Control	2	6	13	17	12	1.40E+09	9.15		
2-US only	1	6	11	13	9	1.10E+09	9.04	9.08	
2-US only	2	6	16	12	11	1.30E+09	9.11		
3-Cip only	1	4	23	23	26	2.40E+07	7.38	7.38	
3-Cip only	2	4	24	25	23	2.40E+07	7.38		
4-US+Cip	1	4	18	17	17	1.73E+07	7.24	7.23	
4-US+Cip	2	4	14	18	18	1.67E+07	7.22		

APPENDIX C

BIOFILM STATISTICAL PARAMETERS: COMSTAT DATA

Without Cip, Without US - COMSTAT DATA - 5/26/04 - 5/28/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	5	2.495	3.175	1.569	48.400
	13	0.454	0.655	1.753	24.600
	18	0.608	0.973	1.739	24.600
	20	2.790	3.559	1.576	57.600
	22	0.341	0.484	1.809	21.000
	25	0.387	0.518	1.824	19.800
	32	15.598	19.830	1.390	109.600
	34	0.722	1.016	1.729	20.400
	37	2.308	3.274	1.620	47.600
	41	2.191	3.147	1.667	45.600
	61	1.022	1.252	1.675	27.600

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	7	7.294	8.093	1.589	74.800
	11	4.719	7.207	1.307	52.400
	13	22.321	31.034	1.274	105.600
	16	1.104	1.785	1.774	46.000
	18	43.444	59.332	0.578	119.500
	24	4.330	6.680	1.413	53.200
	29	0.870	1.219	1.794	31.200
	32	3.724	5.836	1.388	53.200
	36	2.881	4.586	1.395	42.800
	39	20.060	25.587	1.147	93.200
	43	2.199	2.819	1.661	39.200

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	7.285	14.725	1.387	91.200
	16	2.319	3.332	1.534	47.200
	19	5.479	5.909	1.249	39.600
	23	12.111	18.525	1.191	105.200
	26	8.798	10.540	1.384	77.200
	28	11.664	14.678	1.275	97.600
	32	12.725	17.599	1.511	123.500
	35	12.663	15.407	1.070	71.600
	37	5.335	5.962	1.477	59.200
	39	1.061	1.359	1.629	25.200

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

Without Cip, Without US - COMSTAT DATA - 8/04/04 - 8/06/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	0.141	0.239	1.941	24.500
	5	0.119	0.341	1.940	24.500
	7	0.055	0.060	1.984	14.800
	9	0.070	0.076	1.975	14.800
	11	0.077	0.086	1.981	15.000
	13	0.329	0.473	1.923	24.500
	15	0.093	0.120	1.971	19.600
	17	0.332	0.494	1.911	24.500
	19	0.198	0.318	1.950	24.500
	21	0.171	0.193	1.953	14.800

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2	0.378	0.544	1.909	30.500
	4	0.406	0.495	1.856	24.500
	6	0.619	0.725	1.916	34.500
	8	0.504	0.712	1.865	27.500
	10	0.288	0.366	1.937	27.500
	12	0.359	0.410	1.921	22.000
	14	0.434	0.503	1.940	31.200
	16	0.385	0.455	1.919	26.000
	18	0.646	0.934	1.860	36.600
	20	0.196	0.217	1.931	18.500

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2	2.608	3.295	1.717	47.500
	5	1.952	3.233	1.697	57.500
	7	2.564	4.789	1.543	46.000
	9	2.695	4.194	1.626	55.500
	11	1.951	2.923	1.655	44.500
	13	0.646	1.013	1.806	28.500
	15	2.062	3.297	1.710	52.000
	17	2.247	3.689	1.759	52.000
	19	0.670	0.972	1.903	41.500
	22	4.236	5.537	1.613	58.500

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

Without Cip, Without US - COMSTAT DATA - 8/11/04 - 8/13/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2	0.852	1.115	1.747	30.500
	4	0.694	0.881	1.804	37.000
	6	1.010	1.228	1.760	37.000
	8	0.592	0.671	1.861	29.500
	10	0.528	0.679	1.807	29.500
	13	0.595	0.742	1.817	29.500
	15	0.683	0.949	1.727	19.000
	17	0.728	0.856	1.814	31.000
	19	0.460	0.573	1.829	25.000
	21	0.512	0.598	1.903	30.500

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	1.605	1.920	1.707	34.800
	5	1.829	2.773	1.681	52.800
	7	0.811	1.114	1.848	44.400
	9	0.966	1.526	1.806	44.400
	11	1.200	1.539	1.715	29.500
	13	0.639	0.857	1.824	29.500
	15	0.960	1.373	1.855	46.800
	17	1.847	2.601	1.651	39.600
	19	1.466	1.814	1.736	33.000
	21	1.728	2.471	1.706	44.400

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2	2.381	4.315	1.405	44.800
	4	2.892	3.977	1.641	41.300
	6	5.123	9.792	1.273	51.800
	8	2.998	4.999	1.689	53.900
	10	4.784	6.392	1.335	53.200
	12	6.720	8.970	1.478	46.200
	14	3.220	5.674	1.571	45.500
	15	1.300	2.132	1.807	35.700
	16	9.035	14.079	1.167	58.100
	18	5.091	9.955	1.524	58.100

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

Without Cip, With US - COMSTAT DATA - 6/9/04 - 6/11/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	2.551	3.075	1.764	47.600
	4	0.118	0.183	1.943	20.000
	6	0.541	0.673	1.910	24.400
	8	0.631	0.961	1.837	30.800
	10	0.839	1.124	1.860	30.800
After US	2	1.458	1.910	1.720	37.600
	4	0.596	0.742	1.849	22.000
	6	0.420	0.660	1.928	31.600
	8	0.245	0.328	1.875	12.400
	11	1.722	2.410	1.823	46.000

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	6	1.113	1.429	1.773	37.200
	8	7.477	8.593	1.639	74.000
	10	6.967	7.998	1.371	42.800
	15	3.981	4.648	1.520	34.000
	17	5.329	6.712	1.639	66.500
After US	2	4.689	6.055	1.685	65.500
	4	1.444	1.646	1.832	35.600
	7	1.205	1.747	1.791	36.400
	9	1.001	1.250	1.854	34.000
	11	2.068	2.554	1.673	30.400

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	0.431	0.902	1.767	16.000
	4	1.605	2.406	1.813	44.800
	6	2.222	3.354	1.741	60.000
	9	8.396	11.794	1.342	75.200
	13	7.078	8.446	0.993	37.600
After US	2	2.499	4.908	1.394	47.200
	4	6.603	10.630	1.442	95.400
	6	0.659	1.039	1.700	19.600
	8	30.666	44.694	0.794	101.400
	10	1.323	1.865	1.714	22.400

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

Without Cip, With US - COMSTAT DATA - 8/4/04 - 8/6/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	0.430	1.016	1.881	45.500
	4	0.135	0.169	1.952	18.000
	6	0.059	0.065	1.979	14.400
	8	0.185	0.264	1.909	22.000
	10	0.060	0.069	1.971	16.400
After US	2	0.073	0.081	1.981	16.500
	4	0.088	0.107	1.967	20.000
	6	0.058	0.065	1.975	16.000
	8	0.059	0.070	1.967	16.000
	10	0.170	0.212	1.947	24.500

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	3	0.567	0.644	1.882	25.000
	5	0.193	0.219	1.925	20.000
	7	1.688	2.180	1.774	48.600
	10	0.873	0.887	1.693	19.500
	12	1.035	1.534	1.849	43.800
After US	2	0.554	0.753	1.884	36.500
	4	0.544	0.683	1.913	36.500
	6	0.560	0.730	1.896	29.500
	9	1.046	1.743	1.749	35.500
	11	0.822	0.947	1.699	19.500

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	7.508	11.784	1.364	61.500
	3	0.801	1.084	1.840	26.000
	5	3.036	5.612	1.651	63.500
	6	1.096	1.810	1.854	49.500
	11	1.891	2.502	1.842	49.500
After US	3	8.381	12.865	1.308	61.500
	5	0.972	1.654	1.867	49.500
	8	1.771	2.196	1.873	49.500
	11	6.021	10.881	1.124	49.500
	13	0.543	0.728	1.920	38.500

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

Without Cip, With US - COMSTAT DATA - 8/11/04 - 8/13/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	1.224	1.496	1.707	26.500
	4	0.546	0.657	1.835	32.000
	6	0.533	0.642	1.802	25.500
	8	0.932	1.073	1.800	36.000
	10	0.392	0.444	1.870	29.000
After US	2	0.546	0.672	1.839	29.000
	4	0.302	0.362	1.891	23.500
	7	0.956	1.132	1.835	38.000
	10	0.390	0.470	1.845	15.500
	12	0.571	0.717	1.811	30.500

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	3	3.791	5.914	1.213	48.000
	5	3.382	4.822	1.485	48.000
	7	3.297	4.499	1.313	48.000
	9	1.828	2.379	1.635	33.600
	11	1.837	2.380	1.673	45.000
After US	4	2.462	3.888	1.686	53.400
	6	1.312	1.775	1.696	35.400
	8	1.633	2.542	1.655	41.400
	10	1.909	2.619	1.707	34.800
	13	1.128	1.580	1.778	31.800

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	2.380	4.309	1.405	44.100
	5	5.116	9.771	1.273	51.100
	7	6.269	10.080	1.388	74.200
	9	2.991	4.982	1.689	53.200
	12	6.720	8.970	1.478	46.200
After US	3	4.780	6.386	1.335	52.500
	5	3.201	5.639	1.571	44.800
	7	9.060	14.120	1.167	58.800
	9	15.071	20.120	1.097	67.900
	11	5.045	9.870	1.524	57.400

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

With Cip, Without US - COMSTAT DATA - 6/9/04 - 6/11/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	4	0.092	0.108	1.964	14.400
	6	0.243	0.326	1.893	14.400
	8	0.105	0.121	1.966	16.800
	10	1.374	2.018	1.809	42.000
	12	0.153	0.173	1.940	15.200
	14	0.410	0.966	1.927	49.200
	16	0.417	0.561	1.931	32.400
	18	0.514	0.655	1.915	36.800
	20	0.200	0.245	1.931	24.400
	22	0.180	0.253	1.944	25.200

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2 6.000	15.678	1.576	138.000	
	5	1.161	1.520	1.837	37.200
	7	4.044	5.096	1.656	47.200
	9	0.890	1.167	1.893	45.200
	11	7.192	10.466	1.585	71.500
	14	0.845	0.974	1.895	30.000
	16	7.285	9.306	1.581	61.600
	18	2.578	3.785	1.825	62.000
	20	0.186	0.241	1.969	29.600
	23	0.186	0.229	1.957	21.200

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2	0.924	1.230	1.872	33.600
	4	2.429	3.261	1.806	59.000
	9	2.883	3.783	1.752	74.400
	11	2.022	2.527	1.820	51.500
	13	27.746	43.486	0.885	109.000
	15	1.059	2.132	1.847	44.400
	17	1.208	1.820	1.854	51.500
	19	4.283	6.217	1.762	67.000
	21	1.414	2.533	1.802	43.000
	23	1.010	1.112	1.888	29.200

Units: Biomass(μm), Avg Thick(μm)

Rough. Coeff(dimensionless), Max Thick(μm)

With Cip, Without US - COMSTAT DATA - 6/16/04 - 6/18/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	0.925	1.033	1.881	32.000
	6	0.564	0.700	1.923	37.000
	10	1.653	2.094	1.838	63.600
	12	0.655	0.836	1.939	45.000
	14	1.269	2.070	1.866	53.400
	16	0.381	0.426	1.932	21.200
	17	0.180	0.275	1.960	29.500
	19	0.836	1.104	1.855	28.500
	21	0.328	0.431	1.917	23.000
	23	0.467	0.579	1.940	32.500

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	6 4.808	7.557	1.605	63.000	
	10	4.531	7.620	1.716	88.800
	14	3.280	6.885	1.719	74.500
	16	2.732	6.399	1.731	82.000
	18	2.207	3.309	1.706	39.500
	20	0.866	1.096	1.834	25.500
	22	1.680	2.802	1.774	51.500
	24	0.682	0.802	1.851	23.200
	27	2.779	3.648	1.752	44.000

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	6.799	13.359	1.591	107.400
	8	5.724	8.112	1.508	77.000
	10	18.078	32.898	0.471	106.200
	12	8.206	16.574	1.427	143.200
	14	1.042	3.909	1.815	87.000
	16	12.016	32.088	0.996	124.000
	19	1.080	2.426	1.779	37.000
	21	0.760	1.222	1.890	52.000
	23	1.313	2.059	1.838	39.500

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

With Cip, Without US - COMSTAT DATA - 7/21/04 - 7/23/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	0.449	0.510	1.834	23.600
	5	1.118	1.446	1.817	41.600
	7	0.537	0.741	1.867	31.200
	9	0.887	1.157	1.812	39.200
	11	0.311	0.377	1.915	29.600
	13	0.379	0.448	1.886	22.400
	15	0.794	1.092	1.853	34.000
	17	0.387	0.471	1.902	25.600
	19	0.194	0.231	1.934	19.200

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	3	3.917	5.491	1.795	74.500
	5	0.615	0.893	1.882	33.600
	7	6.333	13.528	1.592	93.000
	9	2.361	3.150	1.836	56.000
	11	0.307	0.455	1.950	33.600
	13	0.653	1.070	1.888	42.400
	15	0.999	1.573	1.853	49.000
	17	0.842	1.502	1.862	49.000
	19	3.195	5.347	1.706	69.000
	21	0.415	0.494	1.927	27.600

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
	2	3.712	11.842	1.531	82.500
	4	2.340	2.791	1.834	49.500
	6	13.590	21.834	1.327	119.700
	8	1.258	4.018	1.810	88.200
	10	2.642	3.762	1.738	51.500
	12	15.174	23.505	1.125	114.100
	14	14.053	23.184	1.293	98.000
	16	3.913	4.565	1.745	55.800
	18	2.045	2.923	1.844	52.400
	20	2.088	3.136	1.774	52.200

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

With Cip, With US - COMSTAT DATA - 6/2/04 - 6/4/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	3	0.122	0.240	1.954	18.600
	5	0.034	0.054	1.981	14.100
	8	0.032	0.059	1.986	21.900
	10	0.171	0.485	1.941	36.000
	13	0.041	0.064	1.979	15.000
After US	3	0.018	0.038	1.980	8.400
	5	0.006	0.007	1.992	3.900
	7	0.117	0.246	1.952	26.700
	9	0.013	0.025	1.990	11.700
	11	0.014	0.020	1.989	6.600

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	4	0.022	0.035	1.994	13.500
	6	0.070	0.124	1.982	22.800
	8	0.015	0.026	1.994	9.900
	11	0.002	0.002	1.999	8.700
	16	0.014	0.044	1.991	13.500
After US	3	0.020	0.033	1.993	11.100
	5	0.012	0.017	1.995	10.200
	7	0.008	0.011	1.997	7.500
	10	0.015	0.030	1.992	11.100
	12	0.005	0.007	1.997	6.900

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	4	0.012	0.021	1.996	11.700
	6	0.004	0.006	1.998	7.500
	8	0.004	0.006	1.998	6.600
	10	0.006	0.009	1.997	7.200
	12	0.007	0.009	1.997	6.900
After US	3	0.004	0.005	1.998	5.400
	6	0.001	0.002	1.999	3.600
	8	0.002	0.002	1.999	4.500
	10	0.004	0.006	1.998	7.800
	14	0.001	0.002	1.999	2.700

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

With Cip, With US - COMSTAT DATA - 6/23/04 - 6/25/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	3	0.521	0.617	1.912	22.000
	5	3.186	4.251	1.810	66.800
	7	6.783	9.998	1.560	66.800
	9	6.488	7.952	1.623	66.800
	11	2.585	3.775	1.797	51.200
After US	4	6.211	8.659	1.652	74.500
	6	2.356	3.411	1.839	66.800
	8	0.555	0.658	1.912	27.200
	10	1.044	1.214	1.882	36.000
	12	3.374	4.884	1.745	55.000

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	0.357	0.552	1.930	23.600
	4	1.549	1.989	1.854	47.600
	6	0.664	1.092	1.926	47.600
	8	0.936	1.643	1.892	47.600
	10	0.707	0.811	1.894	23.600
After US	2	0.354	0.592	1.925	29.600
	4	0.604	0.826	1.866	29.600
	8	1.836	2.355	1.822	44.800
	10	0.410	0.662	1.936	33.600
	12	0.652	0.775	1.908	33.600

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	3	0.111	0.170	1.971	20.800
	5	0.078	0.102	1.978	15.600
	7	0.229	0.279	1.949	15.600
	9	0.105	0.171	1.973	19.600
	14	0.165	0.306	1.965	26.400
After US	3	0.307	0.429	1.953	26.400
	5	0.091	0.132	1.975	20.000
	7	0.062	0.098	1.979	20.000
	9	0.389	0.610	1.944	30.400
	11	0.044	0.058	1.987	14.800

Units: Biomass(μm), Avg Thick(μm)

Rough. Coeff(dimensionless), Max Thick(μm)

With Cip, With US - COMSTAT DATA - 7/28/04 - 7/31/04

Day 1	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	2.293	3.974	1.748	50.500
	4	3.169	4.071	1.747	70.500
	6	0.472	0.517	1.872	28.500
	8	0.228	0.240	1.892	12.800
	10	0.664	0.733	1.866	29.500
After US	3	0.937	1.251	1.854	41.500
	5	1.041	1.176	1.826	41.500
	7	1.991	2.685	1.802	57.000
	10	0.375	0.474	1.829	23.500
	14	0.207	0.245	1.906	15.500

Day 2	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	3	0.379	0.509	1.857	23.500
	5	0.191	0.252	1.923	23.500
	7	0.183	0.241	1.934	23.500
	10	0.144	0.146	1.948	19.000
	12	0.122	0.130	1.954	15.500
After US	2	0.210	0.286	1.926	25.500
	4	0.069	0.080	1.958	12.500
	6	0.238	0.286	1.916	18.000
	8	0.161	0.189	1.932	18.000
	12	0.248	0.294	1.928	25.500

Day 3	Series	Biomass	Avg Thick	Rough. Coeff.	Max Thick
Before US	2	0.143	0.172	1.946	16.000
	4	0.221	0.276	1.883	16.000
	6	0.269	0.308	1.878	16.500
	8	0.121	0.130	1.935	13.500
	10	0.132	0.150	1.934	13.500
After US	3	0.081	0.130	1.970	16.000
	5	0.109	0.116	1.929	13.000
	7	0.140	0.166	1.945	16.000
	9	0.128	0.149	1.936	10.500
	12	0.074	0.077	1.954	11.000

Units: Biomass(μm), Avg Thick(μm)Rough. Coeff(dimensionless), Max Thick(μm)

APPENDIX D

ADVECTION-DIFFUSION MODEL MATLAB CODE

```
function[C,Cg,x,y]=ADI_pp_advecdiff(on,C0,dPdx,mu,H,L,D,Con,nX,nY,time,t_step);
```

```
%ADI_pp_advecdiff is an alternating direction implicit (ADI) scheme
%that solves the advection diffusion equation in two dimensional
%space for molecular concentrations between two parallel plates.
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
%ADI_pp_advecdiff outputs:
```

```
%C - 3 dimensional concentration array in space and time
```

```
%Cg - corrected C array for graphing purposes
```

```
%x - length vector
```

```
%y - height vector
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
%User defined input parameters:
```

```
%on - if =1 lower plate boundary set to constant concentration Con
```

```
%      if =0 lower plate boundary set to no mass flux
```

```
%C0 - initial concentration matrix
```

```
%dPdx - pressure drop per unit length
```

```
%mu - dynamic viscosity
```

```
%H - domain height
```

```
%L - domain length
```

```
%D - molecular diffusion coefficient
```

```
%Con - concentration at lower boundary when on=1
```

```
%nX - number of nodes in the length direction
```

```
%nY - number of nodes in the height direction
```

```
%time - total simulation time
```

```
%t_step - number of time steps computed
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
%%%%%%%%%Define dimensional parameters dx, dy and dt%%%%%%%%%
```

```
dx=L/(nX-1); dy=H/(nY-1); dt=time/t_step;
```

```
%%%%%%%%%Define initial C array of proper size%%%%%%%%%
```

```
C=zeros(nY,nX,t_step);
```

```
%%%%%%%%%Build the x and y vectors%%%%%%%%%
```

```
x=zeros(nX,1); y=zeros(nY,1); x(1)=0; y(1)=0;
```

```
for i=2:nX
```

```
    x(i)=x(i-1)+dx;
```

```
end for i=2:nY
```

```
    y(i)=y(i-1)+dy;
```

```
end
```

```
%Solve the for the velocity profile in the domain using%
```

```
%%%%%%%%% x-momentum equation %%%%%%%%%%
```

```
v=zeros(nY,1);
```

```
for i=2:nY-1
```

```
    y(i)=y(i-1)+dy;
```

```
    v(i)=(-1/(2*mu))*dPdx*(y(i)^2)*((H/y(i))-1);
```

```
end V=zeros(nY,nX); for j=1:nX
```

```
    V(:,j)=v;
```

```
end
```

```
%%%%%%%%%Create initial C_dt vector from the initial%%%%%%%%%
```

```
%%%%%%%%% concentration matrix %%%%%%%%%%
```

```
C_dt=zeros(nX*nY,1);
```

```
k=1; for i=1:nY
```

```
    for j=1:nX
```

```
        C_dt(k)=C0(i,j);
```

```
        k=k+1;
```

```
    end
```

```
end
```

```
%Build the index vector for horizontal travel through%
```

```
%%%%%%%%% the domain %%%%%%%%%%
```

```

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%Indexes are assigned to the nodes in the domain in the
%following way:
%   on=0           on=1
%   1 2 3         1 2 3
%   8 9 4         8 9 4
%   7 6 5         10 10 10
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
index=1; for i=2:nX-1
    index=[index;2];
end index(nX)=3; for j=2:nY-1
    index=[index;8];
    for k=2:nX-1
        index=[index;9];
    end
    index=[index;4];
end if on==1
    index=[index;10];
elseif on==0
    index=[index;7];
end if on==1
    for m=2:nX-1;
        index=[index;10];
    end
elseif on==0
    for m=2:nX-1
        index=[index;6];
    end
end if on==1
    index=[index;10];
elseif on==0
    index=[index;5];
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%Create the A matrix coeffs for horizontal travel%%%
aa=V(1,:)'; for i=2:length(v)
    aa=[aa;V(i,:)]';
end

aa=-aa/(dx); bb=(2/dt)-aa; cc=D/(dy^2); dd=((2/dt)-(2*D/(dy^2)));
ee=-cc; ff=((2/dt)+(2*D/(dy^2))); gg=-aa; hh=(2/dt)+aa;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%Transform index vector and coefficients%%%
%%%to vertical travel%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
index_v=zeros(nX*nY,1); index_mat=zeros(nY,nX);
aa_v=zeros(nX*nY,1);

k=1; for i=1:nY
    for j=1:nX
        index_mat(i,j)=index(k);
        k=k+1;
    end
end k=1; for i=1:nX
    for j=1:nY
        index_v(k)=index_mat(j,i);
        aa_v(k)=-V(j,i)/(dx);
        k=k+1;
    end
end

bb_v=(2/dt)-aa_v; gg_v=-aa_v; hh_v=(2/dt)+aa_v;

%%%Build the A_half matrix for solving t+1/2 time steps%%
%%%with horizontal domain travel %%%%%%%%%%%%%%%%%%
A_half=zeros(nY*nX,nX*nY); for i=1:nX*nY

```

```

m=i-1;

if index(i)==1
    A_half(i,i)=bb(i);
elseif index(i)==2
    A_half(i,i)=bb(i);
    A_half(i,m)=aa(i);
elseif index(i)==3
    A_half(i,i)=bb(i);
    A_half(i,m)=aa(i);
elseif index(i)==4
    A_half(i,i)=bb(i);
    A_half(i,m)=aa(i);
elseif index(i)==5
    A_half(i,i)=bb(i);
    A_half(i,m)=aa(i);
elseif index(i)==6
    A_half(i,i)=bb(i);
    A_half(i,m)=aa(i);
elseif index(i)==7
    A_half(i,i)=bb(i);
elseif index(i)==8
    A_half(i,i)=bb(i);
elseif index(i)==9
    A_half(i,i)=bb(i);
    A_half(i,m)=aa(i);
elseif index(i)==10
    A_half(i,i)=1;
end
end

%%%%Build the A_step matirx for solving t time steps%%%%
%%%%%% with horizontal domain travel %%%%%%%%%
A_step=zeros(nY*nX,nX*nY); for i=1:nX*nY
    m=i-1;
    n=i+1;

    if index_v(i)==1
        A_step(i,i)=ff;
        A_step(i,n)=ee+ee;
    elseif index_v(i)==2
        A_step(i,i)=ff;
        A_step(i,n)=ee+ee;
    elseif index_v(i)==3
        A_step(i,i)=ff;
        A_step(i,n)=ee+ee;
    elseif index_v(i)==4
        A_step(i,i)=ff;
        A_step(i,m)=ee;
        A_step(i,n)=ee;
    elseif index_v(i)==5
        A_step(i,i)=ff;
        A_step(i,m)=ee+ee;
    elseif index_v(i)==6
        A_step(i,i)=ff;
        A_step(i,m)=ee+ee;
    elseif index_v(i)==7
        A_step(i,i)=ff;
        A_step(i,m)=ee+ee;
    elseif index_v(i)==8
        A_step(i,i)=ff;
        A_step(i,m)=ee;
        A_step(i,n)=ee;
    elseif index_v(i)==9

```



```

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%Build the b_step vector%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
b_step=zeros(nX*nY,1);

for i=1:nX*nY
    m=i-nY;

    if index_v(i)==1
        b_step(i)=hh_v(i)*C_dt_step(i);
    elseif index_v(i)==2
        b_step(i)=(gg_v(i)*C_dt_step(m))+(hh_v(i)*C_dt_step(i));
    elseif index_v(i)==3
        b_step(i)=(gg_v(i)*C_dt_step(m))+(hh_v(i)*C_dt_step(i));
    elseif index_v(i)==4
        b_step(i)=(gg_v(i)*C_dt_step(m))+(hh_v(i)*C_dt_step(i));
    elseif index_v(i)==5
        b_step(i)=(gg_v(i)*C_dt_step(m))+(hh_v(i)*C_dt_step(i));
    elseif index_v(i)==6
        b_step(i)=(gg_v(i)*C_dt_step(m))+(hh_v(i)*C_dt_step(i));
    elseif index_v(i)==7
        b_step(i)=hh_v(i)*C_dt_step(i);
    elseif index_v(i)==8
        b_step(i)=hh_v(i)*C_dt_step(i);
    elseif index_v(i)==9
        b_step(i)=(gg_v(i)*C_dt_step(m))+(hh_v(i)*C_dt_step(i));
    elseif index_v(i)==10
        b_step(i)=Con;
    end
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%Solve for the time t concentration vector%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
C_dt=A_step\b_step;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%Transform C_dt back to horizontal travel%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
C_mat_step=zeros(nY,nX);

k=1; for i=1:nX
    for j=1:nY
        C_mat_step(j,i)=C_dt(k);
        k=k+1;
    end
end

k=1; for i=1:nY
    for j=1:nX
        C_dt(k)=C_mat_step(i,j);
        k=k+1;
    end
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%Assign the C matrix the solution values calculated%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%by the ADI scheme%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
C(:, :, z)=C_mat_step;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%End of time step%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%Correct the C array for graphical purposes%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
Cg=zeros(nY,nX,t_step); for k=1:t_step
    for i=1:nY
        Cg(i, :, k)=C(nY+1-i, :, k);
    end
end
end

```