

Antibacterial Perfluorocarbon Ventilation: A Novel Treatment Method for Bacterial Respiratory Infections

by

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LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APV	antibacterial perfluorocarbon ventilation
ARDS	acute respiratory distress syndrome
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming unit
COPD	chronic obstructive pulmonary disease
DNA	deoxyribonucleic acid
DPI	dry powder inhaler
EELV	end-expiratory lung volume
ETT	endotracheal tube
FDA	food and drug administration
FOV	field of view
I:E	inspiratory-to-expiratory time ratio
IL-1	interleukin-1
IL-6	interleukin-6
IM	intramuscular
IP	intraperitoneal
IV	intravenous
LV	liquid ventilation
MMAD	mass median aerodynamic diameter
mRNA	messenger ribonucleic acid
MW	molecular weight
PA	<i>Pseudomonas aeruginosa</i>

PBS phosphate-buffered saline
PEG polyethylene glycol
PFC perfluorocarbon
PLV partial liquid ventilation
pMDI pressurized metered dose inhaler
Syk spleen tyrosine kinase
TLV total liquid ventilation
TSB tryptic soy broth
TNF tumor necrosis factors

ABSTRACT

Antibacterial Perfluorocarbon Ventilation: A Novel Method of Treatment for Pulmonary Bacterial Infections

by

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Bacterial respiratory infections significantly contribute to the morbidity and mortality associated with lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis. These patients feature abnormal mucus production and rheology that can impair host immune defenses, ultimately leading to chronic lung infection. Inhaled antibiotic delivery is currently used to treat these patients; however, its effectiveness is limited by an intrinsic dependence on airflow within the lung. Poor ventilation due to mucus plugging and lung damage restricts antibiotic delivery to the most burdened regions of the lung. In order to address these shortcomings, this research proposes a novel method of treatment entitled antibacterial perfluorocarbon ventilation (APV). During APV the lungs are filled with a breathable liquid [perfluorocarbon (PFC)] containing emulsified, micron-scale droplets of aqueous antibiotic. Such delivery has removed dependence on airflow and is thus capable of achieving more spatially

uniform delivery. APV should also be able to actively remove infected mucus from the airways as well as promote a return to normal lung function via anti-inflammatory properties of PFC.

This work represents an in-depth analysis and characterization of the emulsions used during APV. Initial efforts evaluated the feasibility of the emulsion's use during liquid ventilation as well as its ability to effectively kill the tenacious bacterial biofilms found in the airways during infection. Following studies utilized both *in vitro* and *in vivo* methods to better understand the effects of emulsion formulation on the pharmacokinetics and availability of delivered drug and any potential cytotoxicity associated with the emulsion. A rat model of bacterial respiratory infection was developed and used at multiple points throughout this work to assess the potential treatment benefits of APV.

Great strides were made in developing and optimizing the emulsion. The emulsions have been shown to be an adequate ventilation medium and a viable means of pulmonary drug delivery during APV. Final efforts resulted in a promising emulsion formulation that exhibited no cytotoxic effects and drastically improved drug availability relative to those previously assessed *in vivo*. Further *in vivo* work is required to determine if this optimized emulsion formulation provides a treatment benefit over inhaled antibiotics.

CHAPTER 1

Introduction

1.1 Motivation and Objectives

Bacterial respiratory infections are a major contributor to the exacerbation or progression of lung diseases such as cystic fibrosis (CF),^{4,5} chronic obstructive pulmonary disease (COPD),^{1,6} and bronchiectasis.⁷ Diseases such as these feature irregular mucus production and impaired mucus clearance that can promote bacterial infections as well as make them exceedingly difficult to eradicate.^{7,8} Chronic airway infection typically leads to significant airway inflammation which can further impair mucus clearance and ultimately accelerate declining lung function. Current treatment for bacterial respiratory infections typically includes the delivery of antibiotics via systemic administration or inhaled aerosolized delivery. Limited diffusion of antibiotics from the systemic circulation into the endobronchial space results in many infections being refractory to the relatively low intrapulmonary antibiotic concentrations achieved with systemic administration.⁹ Inhaled treatment achieves delivery directly to lung and is thus able to produce higher pulmonary antibiotic concentrations while limiting systemic exposure.¹⁰ However, multiple aspects of aerosolized delivery limit its effectiveness and leave substantial room for improvement, especially in the setting of preexisting lung disease. Early impaction of aerosolized drug particles in the

delivery device, mouth, and proximal airway can lead to significant inefficiency and poor penetration of the lower and peripheral airways.¹¹ Additionally, mucus plugging and poor ventilation during lung disease often results in minimal aerosolized delivery to the most burdened regions of the lung.¹¹⁻¹³ Inadequate antibiotic delivery can result not only in the inability to clear the infection but also the development of antibiotic-resistant bacteria.¹⁴

The goal of this research was to develop a novel method of pulmonary antibiotic delivery to address the previously described shortcomings of current therapy. More specifically, our focus was to develop a means of delivery via filling of the lungs with a liquid, thereby removing any dependence on airflow. One means of achieving this is through the use of liquid perfluorocarbon (PFC). Liquid PFCs exhibit the unique ability to uniformly distribute within the lung while also maintaining gas exchange. Our devised treatment, termed antibacterial perfluorocarbon ventilation (APV), is the filling and subsequent ventilation of the lungs with aqueous antibiotics emulsified within PFC. Such an application represents a relatively unexplored means of treatment and a new use of the specific emulsifiers used in this work. Therefore, the primary focus of this work was to characterize the effects of the formulation of the emulsion on varying aspects of its use during APV in an effort to optimize treatment. These aspects included the emulsion's physical properties, bactericidal ability, pharmacokinetics and efficiency as a means of drug delivery, and biocompatibility.

1.2 Lung Disease and Infection

Acute lower respiratory infections alone cause more disease and death than any other infection in the United States.¹⁵ Although many of these cases can be effectively treated with systemic or inhaled antibiotics, improved treatment is still needed in the case of infection during

lung disease. Lung diseases such as COPD, CF, and bronchiectasis feature changes in the respiratory mucus and airway environment that render these patients particularly susceptible to bacterial infections and limit the effectiveness of current antibiotic therapy.

Under normal physiological conditions, the liquid lining the airway surface is formed by a biphasic film consisting of an aqueous sol (or periciliary) layer immediately atop of the airway epithelial cells followed by a more gel-like mucus layer. The sol layer is a purely viscous layer with a depth of approximately 5-10 μm .^{8,16} It surrounds the cilia of the epithelial cells, acting as a lubricant to allow their high-frequency beating. The mucus layer is composed of water, ions, lipids, proteins, and high molecular weight glycoproteins called mucins. Mucins have a rod-like structure, approximately 500-900 nm long and 10 nm wide, and are cross-linked into an entangled three-dimensional network by disulphide bonds.¹⁷ It is this network that gives mucus its non-Newtonian, viscoelastic behavior, exhibiting characteristics of both an elastic solid and viscous fluid depending on the magnitude of shear stress experienced. Such a fluid tends to resist flow, or yielding, until a finite yield stress is exceeded at which point the polymer-like network of mucins is thought to begin to break down as flow is induced. The thickness of the mucus layer is thought to be quite variable but is commonly estimated to be 0.5-10 μm .^{8,18} The mucus layer along with the movement of the underlying cilia represent a critical part of the innate immune system in the airways. When inhaled particulate or pathogens deposit on the surface of the airways, they become entrapped in the adhesive mucus layer. The beating cilia lining the epithelium then propel the overlying mucus toward the oropharynx where it can be expectorated or swallowed.

The mucus environment is significantly altered during lung disease. COPD is a progressive lung disease characterized by reduced air flow in and out of the lungs due to loss of lung tissue elasticity, alveolar tissue breakdown, airway inflammation, or hypersecretion of mucus. COPD is

most commonly caused by smoking, affects approximately 13 million adults in the United States,¹⁹ and is the third leading cause of death amongst Americans.²⁰ In the case of CF, a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein causes impaired ion movement across the epithelium, resulting in the hyperabsorption of water at the airway surface. Mucus dehydration during both CF and COPD causes an increase in the relative fraction of glycoproteins, resulting in a significant increase in mucus viscosity and adhesivity.^{8, 21,}
²² It is believed that elevated levels of DNA in the mucus due to inflammatory cell necrosis also induce changes in rheology during such diseases.^{8, 23} These rheological abnormalities result in significantly impaired mucociliary transport and inefficient cough clearance.⁸ Additionally, during these diseases an inflammation-induced conversion of water-secreting serous cells to mucus-secreting cells causes a hypersecretion of mucus,²⁴ resulting in a mucus thickness much larger than that of healthy individuals. This also causes a depletion of the serous layer, resulting in mucus encroaching upon the cilia of the epithelial cells,^{24, 25} further impairing clearance of the abnormally thick mucus already present in excessive amounts. The inability to properly clear mucus represents a critical breakdown in the innate immunity of the lungs and, as such, leaves these patients especially vulnerable to respiratory infection.

Bacterial airway infection is present in 50-60% of COPD patients with an exacerbation.^{26,}
²⁷ Exacerbations during COPD consist of acute increases in cough and dyspnea that require medical treatment and typically occur one to four times annually.¹ These exacerbations are a significant cause of morbidity and mortality^{26, 28} as well as an enormous source of healthcare costs.^{6, 29} Approximately 70% of the \$24 billion estimated annual costs of COPD are related to exacerbations.³⁰ Although the relationship between infection and COPD exacerbation has long been disputed, recent studies suggest that infection causes exacerbation due to increased host

inflammatory events.^{1, 26, 31} It has been shown that the presence of *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* (*PA*), and *Staphylococcus aureus* in the airways results in increased risk of an exacerbation during COPD.³¹ The inflammatory response during such an episode leads to increased airway secretions, pulmonary edema, inhibited gas exchange, and an overall decrease in lung function.³¹

Bacterial infections are even more prevalent in the case of CF patients, with infections typically beginning during childhood.⁵ Common and early acquisition of infection for CF patients is thought to be due to impaired mucociliary clearance, increased binding ability of bacteria to CF respiratory epithelial cells, and impaired ingestion of bacteria by CF host immune cells.⁵ Bacterial respiratory infections during CF can be due to a variety of organisms, with some of the most common being *PA*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Stenotrophomonas maltophilia*.³² *PA* is widely considered the most common and damaging bacterial pathogen during CF.^{10, 33} *PA* is a Gram-negative bacterium found in moist settings throughout the environment. Most CF patients are chronically infected with *PA* by early adulthood³⁴ and it is nearly impossible to eradicate.³⁵ *PA* infection is one of the primary contributors to the short life expectancy of CF patients, with one study showing more than five times lower mortality rates for uninfected patients as compared to infected patients.³⁶ *PA* is thought to enter the lower airways through the mouth or nasal passage and then bind to components of the mucus layer residing on top of the cilia.³⁴ *In vitro* studies have shown that *PA* has the unique ability (not observed in other pathogens evaluated) to adhere to mucins as well as exhibit a chemotactic response to CF mucin.³⁷ After the initial infection, *PA* typically undergoes a phenotypic adaptation in which the organism converts to a mucoid variant characterized by the copious formation of exopolysaccharide alginate-coated microcolonies^{11, 12}, commonly referred to as the biofilm mode of growth.^{33, 34} This form of *PA* is

nearly uniformly present during chronic lung infection in CF patients.³⁴ Growth within the thick biofilms has been shown to grant a significant degree of resistance against antibiotics to the *PA* cell, with organisms within biofilm withstanding 20 to 1,000 times greater concentrations of antibiotic than that required to kill the nonmucoid form.^{38, 39} Additionally, the alginate matrix provides a degree of protection against the native immune response as well.⁴⁰

Although the inflammatory response is an integral part of innate immunity, it can often play a detrimental role during lung disease or injury. During both COPD and CF, a change in mucus rheology and production impairs host respiratory defense mechanisms, thereby presenting an opportunity for bacterial pathogens to colonize within the airways. These chronic or recurrent bacterial infections result in periods of prolonged inflammation with significantly increased neutrophil presence in the airways and pulmonary circulation.⁶ During binding and phagocytosis of a pathogen, neutrophils release a number of enzymes to aid in the degradation of the foreign matter in a process known as “respiratory burst.” The release of these enzymes results in the production of reactive oxygen byproducts that can be harmful to host bodily tissues. Consequently, much of the epithelial damage present during COPD and CF can be attributed to neutrophil-derived proteases and oxidants.^{41, 42} This damage to the respiratory mucosa, along with increased secretions, further hinders mucociliary clearance, and thus perpetuates infection. Such a cycle is described by the “vicious circle” hypothesis (Figure 1.1).

Ultimately, this series of persistent infection and inflammation in the airways may lead to bronchiectasis, a condition characterized by permanent dilation of the larger airways, progressive smaller airway obstruction, and interstitial pneumonia.⁷ Bronchiectasis affects over 110,000 American adults⁴³ and can be due to a variety of causes including a compromised immune system

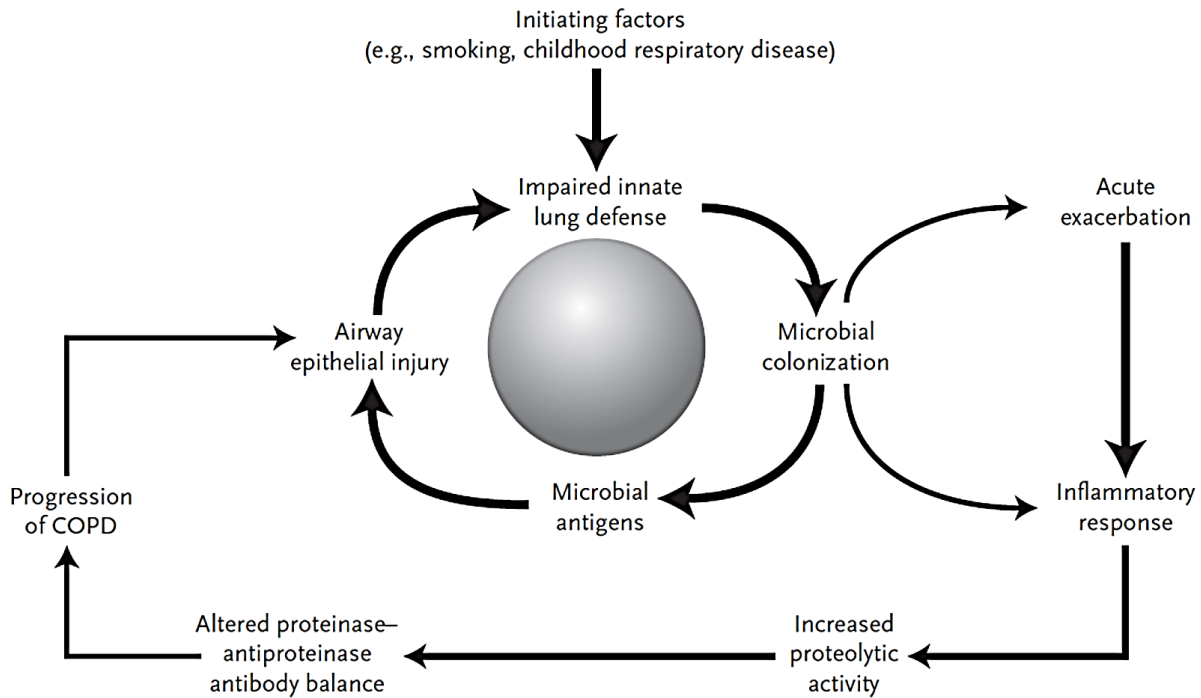


Figure 1.1: Schematic of the "vicious circle" hypothesis.¹

(such as with AIDS) or impaired mucociliary clearance (such as with CF and COPD). The total annual medical cost of treating these patients is \$630 million.⁴³

1.3 Current Antibiotic Treatment

The type of antibiotic to be used during respiratory infection treatment is dependent both on the bacterial strains present as well as the intended delivery method. The most commonly used antibiotics for treatment of infections during CF over the past 20 years are tobramycin, azithromycin, and aztreonam lysine.³² The method of action for tobramycin and azithromycin is similar and involves binding to bacterial ribosomes and thereby inhibiting the translation of mRNA in to essential proteins. Alternatively, aztreonam lysine inhibits synthesis of vital polymers in the bacterial cell wall. In order for these, or any, antimicrobial agent to be effective, the agent must reach the site of infection and remain there for a sufficient length of time.¹⁰ This task is an

especially challenging one in the case of respiratory infections, particularly for infections overlaid with preexisting lung disease.

Systemic [intravenous (IV)] administration and inhaled delivery are currently the primary means of antibiotic delivery during bacterial respiratory infection. Systemic delivery grants the largest degree of freedom with regards to drug selection, as nearly all antibiotics can be delivered in this manner. However, systemic delivery has several disadvantages with regards to effective delivery to the site of infection. When administered IV, antibiotics must first travel throughout the bloodstream before arriving at the pulmonary circulation. Thus, delivery may be limited in any disease or injury in which pulmonary blood flow is compromised. Antibiotics are believed to move from the blood into the bronchial space via diffusion along a concentration gradient.⁴⁴ Once in the lung, the antibiotics must then penetrate respiratory mucus to reach the infection. Due to the effect of sputum binding on antibiotic availability to the bacteria, endobronchial antibiotic concentrations of 10 to 25 times the minimum inhibitory concentration are required in order to be effective.^{45, 46} In addition, many of the antibiotics used (including aminoglycosides such as tobramycin) have exhibited poor diffusion across lipid membranes³⁴ and penetration of the respiratory sputum^{46, 47}. For these reasons, large IV doses must be given in order to be effective at the site of infection, resulting in high serum concentrations that can increase the potential for nephrotoxicity⁴⁸ and ototoxicity⁴⁹.

Inhaled aerosolized delivery of antibiotics offers an attractive alternative to systemic administration in that delivery is specifically targeted at the organ of interest. During such delivery, a suspension of drug-containing solid particles or liquid droplets in air (or other gases) is inhaled by the patient for delivery to the airways. A variety of devices have been created to achieve inhaled delivery. Devices that deliver a specific mass of drug for each actuation of the device are typically

referred to as metered dose inhalers. Such delivery is achieved with liquids via a pressurized metered dose inhaler (pMDI) which uses liquefied gas propellants (typically hydrofluoroalkanes) to emit droplets of liquid drug that can be inhaled by the patient. Metered doses of dry drug formulations are achieved with a dry powder inhaler (DPI). These devices rely on the patient's own inspiratory effort to pull drug from the dosing chamber and as such require fairly high inspiratory flows to be used effectively. In contrast to metered dose delivery devices, continuous delivery devices, often referred to simply as nebulizers, deliver aerosolized drug at a constant flow while the patient continuously breathes over periods of up to 30 minutes. Aerosolization of the liquid drug in this setting is typically achieved through the use of an air jet, ultrasonic sound waves, or a vibrating mesh. Nebulizers tend to offer a wider variety of deliverable drugs than metered dose inhalers but also tend to be larger and less portable. Nebulizers also tend to be less efficient than metered dose inhalers due to drug emitted during exhalation that is not inhaled, although some newer nebulizers have incorporated features to pause delivery during exhalation. The first inhaled antibiotics were delivered via a nebulizer, with aerosolized tobramycin being the first approved by the Food and Drug Administration (FDA) in 1997. Currently approved nebulized antibiotics include aztreonam lysine and colistimethate sodium (colistin) in addition to tobramycin. Parenteral formulations of gentamicin, ceftazidime, and amikacin are also commonly used "off-label" via nebulized delivery.⁵⁰ As previously mentioned, nebulizers tend to require long delivery times (up to 30 minutes) and thus are often considered a significant inconvenience to patients. Additionally, nebulizers require frequent and thorough cleaning in order to maintain proper functionality and avoid microbial growth. Issues such as these are thought to contribute to poor patient adherence and thus affect the practical effectiveness of nebulized treatment. Dry powder drug formulations used with DPIs require shorter treatment times (one to two breaths) and much less cleaning and

thus represent an attractive alternative to nebulized delivery. Dry powder antibiotic formulations of both tobramycin and colistin have recently been developed and are currently approved for use in CF patients. As one may expect with a more targeted delivery approach, inhaled antibiotics have been shown to produce higher intrapulmonary concentrations while limiting absorption into the blood relative to systemic administration. One study showed that inhaled nebulized antibiotics produced as much as 14 times greater antibiotic concentrations in the sputum with seven times lower systemic levels relative to IV delivery.¹⁰ A study comparing nebulized tobramycin with a dry power formulation (delivered via a DPI) showed larger lung depositions, similar central to peripheral distributions, and similar serum profiles for the powder formulation relative to nebulized tobramycin.³

Although inhaled antibiotics have shown benefits over systemic treatment and been proven effective against many cases of infection, significant shortcomings still exist, especially in the setting of underlying lung disease. The use of inhaled delivery in lung disease (primarily CF and COPD) patients is inherently flawed due to its innate dependence on airflow. Poor ventilatory capacity due to both irregular physiology and mucus production significantly hinders effective delivery to the most diseased regions of the lung. In the case of CF, mucus plugs are primarily composed of negatively charged glycoproteins and DNA, causing positively charged aminoglycosides (such as tobramycin) to potentially bind to these compounds and be rendered unavailable.^{51, 52} During chronic infection with *PA*, bacteria is thought to reside in airway generations all the way down to the bronchioles.¹¹ Thus, the high sputum production and mucus plugging typically present within the bronchi of the infected host often prevents distal drug deposition in the regions of highest infection.^{11, 13, 53} By relying on the convection of air to distribute the delivered drug throughout the lung, inhaled delivery preferentially treats the well-

ventilated, typically less burdened, regions of the lung. Along the same lines, it has been shown that a lower forced expiratory volume in CF patients correlates with a smaller proportion of delivered drug reaching the periphery.¹³

The narrow range of aerosolized particle size required to effectively penetrate the lower airways presents further challenges.^{11, 54} Aerosolized particles should be in the size range of 1-5 μm in order to deposit in the lower airways.⁵⁵ Particles with a mass median aerodynamic diameter (MMAD) of greater than 5 μm tend to precipitate within the conducting airways.⁵⁴ Particles with a MMAD of less than 1 μm have a greater chance of being exhaled.⁵⁵ Even if the average particle diameter is 2-4 μm , a larger range of particle sizes is always present, resulting in less than ideal deposition. Studies quantifying aerosol drug deposition in the lung are quite variable but have shown that 25-95% of emitted drug never reaches the lung, with the majority deposited in the oropharynx and delivery device.^{11, 13, 56, 57} Deposition tends to be even worse in very young children with reported values as low as 2% lung deposition.⁵⁸ Patients on a mechanical ventilator also present additional challenges in achieving effective inhaled delivery. Lung deposition of nebulized drug during mechanical ventilation is approximately 20% of the delivered dose, with the majority of drug deposited in the delivery device and ventilator tubing.^{59, 60} Of the drug that does reach the lung during inhaled delivery, the majority is confined to the central lung (defined as the central 2/3 of the total lung), with one study showing only 16% of pulmonary tobramycin being deposited peripherally in CF patients.¹³ The nonuniform intrapulmonary distribution of delivered antibiotics resulting from these challenges can lead to the inability to clear the infection as well as promote the development of antibiotic resistance.^{14, 34} For these reasons, a modified method of antibiotic delivery to the lungs resulting in more spatially uniform distribution with sufficient pulmonary

residence time could significantly improve the treatment of chronic respiratory infections.^{53, 61} One possible means accomplishing this is through the use of PFC as an antibiotic delivery vehicle.

1.4 Liquid Ventilation with Perfluorocarbons

Liquid ventilation (LV) is a respiratory support method that uses liquid PFC as a medium to achieve gas exchange in the lungs. PFCs are clear, odorless liquids composed primarily of covalently bonded carbon and fluorine atoms. Although there are many different types of PFCs suitable for LV, virtually all clinical LV trials have utilized Perflubron (perfluorooctyl bromide).⁶²⁻⁶⁷ Much of the LV work done in animals has also utilized FC-77 (mixture of perfluorocycloether and perfluorooctane).^{2, 68-70} Note that the product name FC-77 was discontinued in 2007 by the manufacturer (3M Inc.) and the same liquid is now sold under the name FC-770. The molecular structures of both Perflubron and FC-77 are shown in Figure 1.2. Due primarily to the extremely strong covalent bonding between carbon and fluorine atoms (C-F bonds are 485 kJ/mol), PFCs

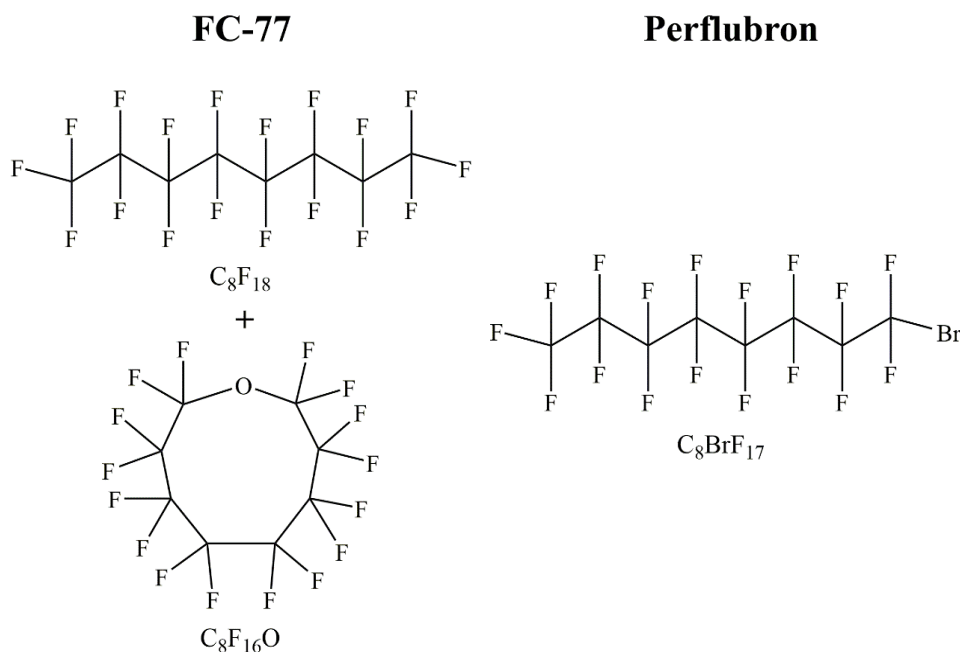


Figure 1.2: Molecular structures of FC-77 and Perflubron.

have high intramolecular forces and tend to be very stable. In a biological environment, this translates to PFCs being bioinert and resisting any type of metabolism or enzymatic changes.⁷¹ PFCs are also nonpolar and tend to have very low intermolecular, or van der Waals, forces. These low intermolecular forces are responsible for many of the properties of PFC critical to LV. First, due to these weak forces, PFCs tend to be quite volatile, with those used during LV typically evaporating at or below physiological temperatures. Second, weak van der Waals forces also give PFCs a very low surface tension (< 20 dyne/cm) which allow them to effectively penetrate the minute spaces of the small airways and alveoli. Lastly, and perhaps most importantly, it is these weak intermolecular forces that make PFC an excellent solvent for gases such as oxygen and carbon dioxide (solubility of ~50 mL O₂ and 140-210 mL CO₂ per dL of PFC).^{71, 72} Like the dissolution of most gases in a liquid, O₂ dissolves in PFC through insertion into an intermolecular site within the solvent (PFC). Therefore, the weak intermolecular forces of PFC facilitate the dissolution of O₂.⁷¹ This is much different in comparison to the binding of O₂ to hemoglobin. The conformational changes undergone by hemoglobin during this process result in a sigmoidal oxyhemoglobin dissociation curve. In contrast, O₂ solubility in PFC is a linear function of the partial pressure of O₂.⁷³ PFCs are also fairly dense (about twice that of water) and not soluble with water or lipids. As such, PFCs are immiscible with virtually all physiological substances other than gases.⁷²

The concept of using liquid PFC to support respiration was first demonstrated in mice nearly 50 years ago.⁷² Early experiences with LV were focused on breathing in hyperbaric conditions and carried out via total body immersion or gravity-assisted ventilation to an intubated animal.⁷² Over time, LV techniques were refined and systems which mechanically assisted the movement of liquid tidal volumes to and from the lungs were developed. In its more recent use,

left to evaporate. The majority of PFC left in the lung evaporates, is exhaled, and cleared from the lung within seven days of instillation.⁷⁴ The rate of evaporative PFC clearance from the lung has been shown to be largest immediately following administration and steadily decline thereafter.⁷⁵ Additionally, clearance tends to occur in the nondependent regions of the lung first.⁷⁵ Although small amounts of PFC may be transported across the lung epithelium into the systemic circulation⁷⁶, this amount has been shown to be less than 1% of the administered dose.⁷⁵ Virtually all delivered PFC is believed to ultimately leave the body via evaporation through the lung or transpiration through the skin.^{72, 76} Even PFC delivered to the systemic circulation in the form of an emulsion for use as an imaging agent or blood substitute has been shown to be cleared via expired air after phagocytosis by reticuloendothelial macrophages.⁷⁷ Although trace amounts of PFC have been shown to preferentially accumulate in fatty tissues and remain for relatively long periods of time, there has been no evidence of any negative consequences.⁷² Along the same lines, the long-term effects and toxicity of PFC have been studied extensively in animals and patients for periods up to 10 years without evidence of adverse effects.⁷⁸⁻⁸¹

Research surrounding LV has evaluated its effectiveness in treating a variety of respiratory conditions over the past 25 years. There is an overwhelming amount of evidence demonstrating improved survival and respiratory function following PLV treatment in the setting of acute respiratory distress syndrome (ARDS), acute lung injury, and respiratory failure in both animals⁸²⁻⁹⁶ and humans^{62-67, 97}. Additionally, treatment utilizing TLV has demonstrated improved outcomes in similar injury or disease settings in animals.^{2, 98-101} The observed improvements in survival rate and lung mechanics following treatment with LV are a result of a variety of mechanisms. The most common observation during LV was a marked improvement in gas exchange.^{2, 62, 63, 67, 82-87, 89-91, 97, 99} The degree of pulmonary shunting in an injured lung has been found to be significantly

decreased following treatment with LV.^{64, 82, 84, 89, 97, 99} This effect is likely due to both the displacement of aqueous exudate in the alveoli impeding gas exchange as well as the recruitment of atelectatic regions. Due to the high density of PFC, as well as its lack of solubility with water, aqueous exudate in the peripheral airways and alveoli is displaced via a buoyant force during LV. The aqueous fluid is typically redistributed throughout the surface area of the lung or transported to the central airways where it can be removed via suctioning.^{64, 94} In addition, the high density and low surface tension of PFCs allow them to penetrate, expand, and recruit previously collapsed or deflated alveoli.^{64, 66, 98} Both of these actions result in increased alveolar surface area available for gas exchange, thereby decreasing shunting. In addition, LV may redistribute pulmonary blood flow from the dependent zones, typically experiencing the greatest degree of consolidation and atelectasis, to the nondependent zones of the lung, thereby improving ventilation-perfusion relationships.⁹⁴ All of these factors likely contribute to the significantly improved gas exchange observed during LV.

Another common observation during treatment utilizing LV is a significant increase in lung compliance.^{63, 64, 66, 67, 82-86, 97-99} Lung compliance can be affected by a number of factors, but surface tension is likely the most relevant regarding the changes observed during LV. The interface between air and the liquid film lining the alveoli results in a surface tension that works to minimize this interfacial area, thereby resisting alveolar expansion (Figure 1.4), largely contributing to the static recoil forces of the lung. In a healthy lung, type II alveolar cells lining the alveoli secrete a phospholipid surfactant that considerably lowers this surface tension, thereby decreasing the work of expanding the lung during inspiration. However, significantly reduced presence and function of endogenous pulmonary surfactants has been shown during lung diseases such as CF and COPD.^{102, 103} During LV, the lungs are filled with PFC and the air-liquid interface is either partially or

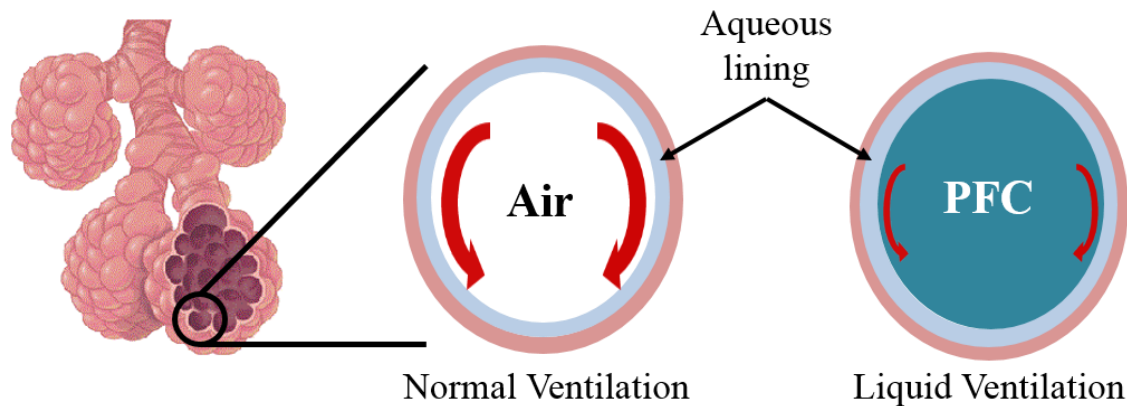


Figure 1.4: Schematic showing reduced resistive effects of interfacial tension on alveolar expansion during liquid ventilation. Red arrows denote forces due to interfacial tension.

completely eliminated, being replaced with an aqueous-PFC interface of a much lower interfacial tension (Figure 1.4). Thus lung volume recruitment is achieved at much lower pressures during LV and lung compliance is increased. In addition, it has been shown that LV may enhance surfactant synthesis and secretion in the lungs relative to conventional gas ventilation¹⁰⁴, possibly also contributing to the improved lung compliance observed during LV. The elimination of the liquid-air interface, along with the low surface tension of PFCs, allows PFC to homogeneously fill and expand dependent regions of the lung that are often minimally ventilated during gas ventilation^{82, 89}, likely also contributing to the enhanced gas exchange associated with LV.

Despite the existence of a large amount of positive data surrounding LV, the use of PFCs during LV remains unapproved by the FDA. The first application of LV in humans was performed as TLV in infants in 1989.¹⁰⁵ In the mid to late 1990s, a number of clinical trials were performed by Alliance Pharmaceutical in an effort to receive FDA approval for the use of Perflubron during PLV in adult ARDS patients.¹⁰⁵ These efforts culminated in a Phase III, multi-center clinical study that evaluated PLV with Perflubron relative to conventional mechanical ventilation in 311 adult ARDS patients.¹⁰⁶ The results of this study showed no improvement in 28-day mortality or number

of ventilator-free days for PLV relative to conventional mechanical ventilation.¹⁰⁶ It should be noted that although PLV showed no benefit over the control group, both groups exhibited better survival than was widely accepted at the time of the trial.¹⁰⁵ Additionally, safety data from the trial showed that Perflubron was well tolerated by patients.¹⁰⁵ Even so, due to the lack of treatment benefit relative to existing methods shown in this study, the FDA ultimately failed to grant approval for Perflubron and Alliance Pharmaceutical subsequently withdrew all effort and funding in this endeavor.¹⁰⁵ Considering the abundance of positive data surrounding LV in animal models and small-scale clinical trials, the negative outcome of Alliance's Phase III trial is somewhat puzzling. The discrepancy between this trial and most other work with LV has been attributed to factors ranging from poor and inconsistent clinical implementation of PLV during the Phase III study to a general lack of congruency between animal disease models and real-world patients.

1.5 Effects of Perfluorocarbons on the Native Immune Response

Numerous studies have shown that PFC exposure can significantly reduce the recruitment, activation, and function of cells involved in the innate immune response in the lungs.^{65, 68, 93, 107-111} This finding is critically important for an application in which patients with a bacterial respiratory infection will undergo LV. A temporary and local reduction of the inflammatory response in the airways of these patients may be conducive to a return to normal mucociliary clearance and respiratory function. However, sustained impairment of the innate immune system could also hinder the patient's ability to control or ultimately clear the infection after treatment. In order to account for these effects, an in-depth understanding of PFC's effects on the innate immune system is vital.

The means by which PFC exposure decreases the innate immune response is still not completely understood. While evaluating the anti-inflammatory effects of PFC *in vivo*, it is sometimes difficult to discern a clear cause for the observed results. It is often unclear whether a suppressed inflammatory response is a direct effect of PFC or rather due to an improved disease state or increased pulmonary function.¹¹¹ For this reason, much of the work investigating the mechanism by which PFC exposure dampens the immune response has been done *in vitro*. Multiple studies have shown that *in vitro* exposure to PFCs for a matter of hours can cause human neutrophils to have a decreased chemotaxis response.^{112, 113} Studies have also shown decreased levels of activation for stimulated human macrophages (measured by levels of pro-inflammatory cytokines such as IL-1, IL-6, and TNF) after exposure to PFC.^{114, 115} Additionally, a two-hour exposure to PFC has been shown to decrease the production of reactive oxygen species by alveolar macrophages.¹¹⁶ A reduction in levels of pro-inflammatory cytokines secreted by stimulated macrophages combined with a decrease in the ability of neutrophils to respond to chemokines likely results in significantly reduced neutrophil recruitment.

However, the underlying mechanism by which PFC exposure causes these effects is still not completely understood. Almost all studies with neutrophil and macrophage exposure to PFC have shown little to no difference in cell viability between PFC-exposed cells and controls. This observation implies that the inhibition of a basic metabolic process causing accelerated cell death and, therefore, decreased levels of activation is an unlikely explanation. One initial speculation proposed that the observed effects could be explained by the presence of a physical coating of PFC surrounding cells, thereby interfering with the interaction between stimulant-containing cells and target cells.¹¹⁷ However, other studies have shown that decreased chemotaxis effects are observed even after PFC-exposed neutrophils were washed with fresh buffer before being exposed to a

stimulus.¹¹² This may suggest that the anti-inflammatory effects are related to a sustained chemical effect rather than the presence of a physical barrier. Another proposed theory is that low levels of PFC diffusing in to the cellular membrane of human leukocytes exert inhibitory effects on transmembrane signaling.¹¹⁸ In support of this theory, one study has shown that the *in vitro* cellular effects of various PFCs increase proportionally with PFC lipid solubility.¹¹⁸ Additionally, by shearing PFC-exposed cells and separating the membrane and cytoplasmic fractions, it was shown that any PFC content present was associated with the membrane fraction.¹¹⁸ This data suggests that PFCs may have a nonspecific effect caused by PFC localizing in the lipid bilayer of the cellular membrane, resulting in a generalized protective or dampening effect on a variety of membrane-associated responses to activation.¹¹⁸ Another study focused on the effects of PFC on the Syk pathway, a signaling system located early in a series of events leading to phagocytosis. Results showed that incubation with PFC reduced tyrosine phosphorylation of Syk, resulting in a corresponding reduction in phagocytosis of opsonized sheep erythrocytes.¹¹² This phenomenon could be explained by PFC-induced alterations at the cell membrane causing disruptions in the transmembrane signaling process leading to Syk phosphorylation.¹¹² Although evidence exists for PFC-induced modifications of the cellular membrane being responsible for the immune effects associated with PFC, skepticism still exists. Multiple studies have observed an attenuated response by PFC-exposed neutrophils to stimulation with phorbol myristate acetate.^{113, 116, 117} Phorbol myristate acetate evokes a cellular response through an intracellular mechanism, more specifically, activation of the protein kinase C pathway. This observation cannot be fully explained by the suggested modified interaction between a stimulant and cellular membrane receptor^{115, 116}, possibly suggesting a combination of both intra- and extracellular effects.

1.6 Perfluorocarbon-Based Drug Delivery

PFC-based pulmonary drug delivery has been explored for a variety of drugs, including vasoactive drugs¹¹⁹, plasmids^{120, 121}, pulmonary surfactant^{85, 92, 122}, and antibiotics¹²³⁻¹²⁸. PFCs represent an attractive medium for drug delivery not only due to their unique application during LV, but also due to their chemical stability. The inert nature of PFCs limits potential interactions with the drug that could hinder the drug's mechanism of action or efficacy. In the case of antibiotic delivery, it has even been shown that the combination of PFC exposure and antibiotics may work synergistically to provide a more effective antibacterial effect than either agent alone.¹²⁹ Note that although multiple studies have concluded that neat PFC has an inherent antibacterial effect of its own,¹²⁹⁻¹³¹ such capabilities remain in question. The bacterial reduction following PFC exposure observed in these studies was likely due to a physical redistribution of the bacterial cells due to surface properties of PFC or decreased bacterial adhesion¹³² rather than bacterial killing. Regardless of its bactericidal ability alone, PFC represents an attractive vehicle for antibiotics to be delivered to the lung. However, the manner in which the antibiotics are added to the PFC phase presents a challenge. Due to the hydrophobic nature of PFC, a simple mixture of PFC and aqueous antibiotics will result in the less dense aqueous phase quickly separating and rising to the liquid surface. The earliest efforts at PFC-based antibiotic delivery used simple mixtures of aqueous drug solutions and PFC, relying solely on bulk flow turbulent mixing to disperse the aqueous phase throughout the PFC. In an attempt to increase dispersion of the drug-containing, aqueous phase, researchers often introduced the aqueous phase before filling the lungs with PFC, termed "top loading"^{126, 127}, or in small boluses during the inspiratory phase of TLV^{124, 128}. Although these creative methods of drug introduction result in a greater initial dispersion, phase separation and coalescence likely still occurs rather quickly. A method of PFC-based delivery able to achieve

sustained dispersion of drug throughout the PFC phase would likely result in increased therapeutic potential. Accordingly, multiple efforts have been made to utilize a drug-in-PFC suspension during LV. The first attempts at such a delivery scheme utilized solid, crystalline antibiotic microparticles suspended within PFC.^{123, 125-127} These suspensions were typically created by spray-drying a mixture of antibiotic, saline, PFC, surfactants, and bulking agents to create porous microparticles with a fluorophilic shell. A schematic of this process is shown in Figure 1.5. The microparticles were then added to PFC to form a stable suspension within a bulk PFC phase. Studies evaluating the pharmacokinetics of LV with these suspensions have shown the treatment's ability to significantly increase pulmonary antibiotic concentration relative to IV or intramuscular (IM) delivery, while still maintaining non-toxic serum concentrations.^{123, 127} Treatment efficacy of these suspensions in the setting of a bacterial respiratory infection has also been evaluated. In one study,

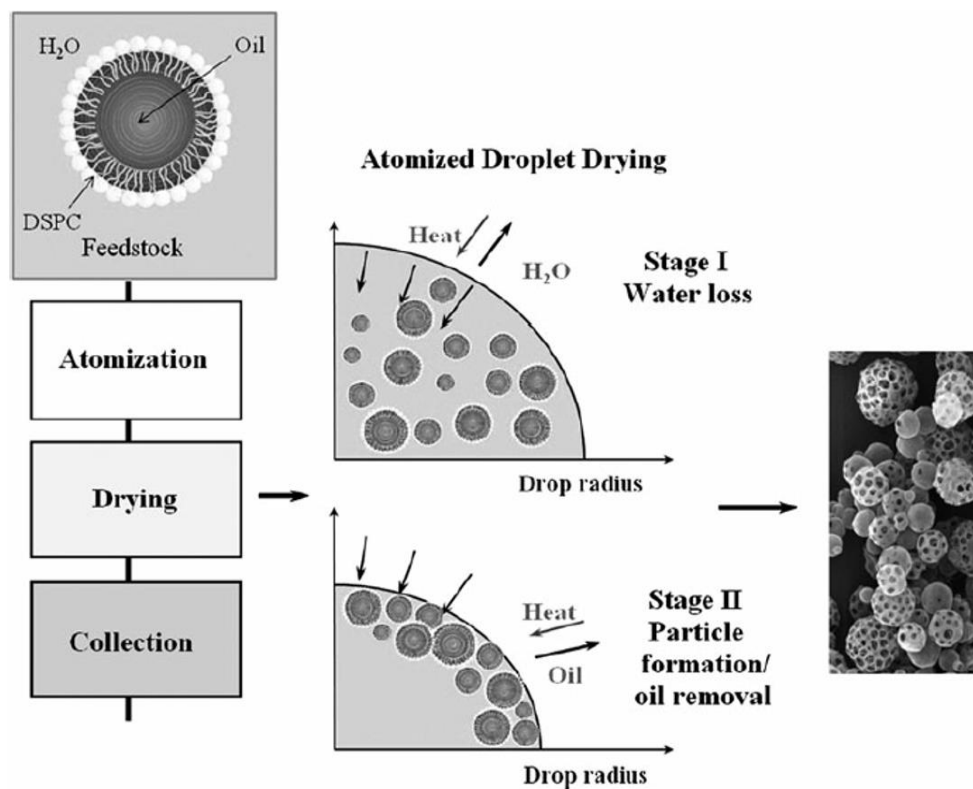


Figure 1.5: Schematic showing the fabrication process for solid, crystalline antibiotic microparticles, termed PulmoSpheres.³

various treatments employing different antibiotic delivery mechanisms were initiated one day after rats were intratracheally inoculated with *Streptococcus pneumoniae*.¹²⁶ Treatment with the microparticle-PFC suspensions showed significantly increased survival over a 10-day period relative to IM delivered antibiotics alone, but no advantage over other forms of antibiotic administration coupled with LV (IM or addition of unemulsified antibiotics to PFC).¹²⁶ This suggests that the survival benefit may have been due to the anti-inflammatory properties of PFC or perhaps better oxygenation in consolidated lung regions. Similarly, prophylactic treatment utilizing LV with an antibiotic-PFC suspension was shown to more effectively prevent bacterial respiratory infection in rats relative to delivery via the IM route.¹²⁵

An alternative method of drug addition involves suspending the drug in aqueous form (*i.e.* emulsified aqueous droplets in a continuous PFC phase) rather than as solid particles. The use of aqueous antibiotics rather than solid particles presents both potential advantages as well as new challenges. In theory, if aqueous drug solutions were able to be added during emulsion preparation immediately prior to delivery, a much broader selection of drugs (or even combination of drugs) could be utilized as compared to dry particle suspensions. As previously mentioned, the preparation process required for dry particle suspensions is labor-intensive and thus a desired drug must undergo this individualized process well in advance and likely in a specialized facility. The reduced preparation associated with a liquid-in-liquid emulsion would likely translate to decreased overall costs as well. Additionally, having the drug phase present within the PFC mixture in an aqueous solution may also allow for more controllable kinetics. In the case of either dry particle suspensions or liquid emulsions, once the drug comes in to contact with an aqueous surface in the lung (*i.e.* bacterial biofilms, respiratory mucus, or the epithelium) it will likely be transported into or along that surface via passive diffusion. By having the drug present in a solution within the PFC

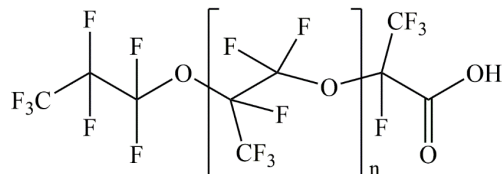
mixture, one is able to control the concentration of that solution and thus better control drug kinetics within the lung. Ultimately this would theoretically allow for more freedom and customization in the treatment process. Conversely, by including an aqueous phase in the PFC mixture, new mechanisms of instability are introduced that were previously absent in the case of dry particle suspensions. The primary stability concern within a suspension of dispersed solids is coagulation, or clumping, of the solid particles. For fluid-fluid dispersions, additional methods of de-mixing may be present. The types of phase separation present can depend on many factors, but the most important are likely the size of the dispersed droplets, the density difference between the two phases, and the solubility of the dispersed phase within the continuous phase. In the case of micron-sized, aqueous droplets within a continuous PFC phase, creaming of the less dense aqueous droplets will result in stratification of the two phases and is likely the most dominant form of phase separation. As creaming stratifies the mixture, bringing the droplets in closer vicinity to each other, the average thickness of PFC film separating each droplet decreases until it reaches a critically thin thickness and droplet coalescence occurs. Ostwald ripening, another de-mixing phenomenon in which smaller droplets dissolve in the continuous phase and redeposit on to larger droplets, could also be present but is more common in mixtures where the dispersed phase has a larger degree of solubility in the continuous phase. Only one study was found to have previously attempted the use of an emulsion of aqueous antibiotics in PFC rather than a suspension of solid particles.¹³³ The emulsions were formed using natural bovine surfactant as the emulsifier. It was shown that PLV with such emulsions in healthy rabbits resulted in significantly higher intrapulmonary antibiotic concentrations and lower peak serum antibiotic concentrations relative to IV administration.¹³³ The bactericidal capacity and stability of such emulsions were not evaluated.

1.7 Antibacterial Perfluorocarbon Ventilation

In order to address the shortcomings of current antibiotic therapy for respiratory bacterial infections, we propose a treatment termed APV. During APV, the lungs are filled with an emulsion containing a disperse phase ($\leq 5\%$ by volume) of aqueous antibiotics within PFC liquids. The initial dispersion of aqueous antibiotics during preparation of the emulsion is achieved mechanically by sonicating a mixture of the two phases. In order to stabilize the aqueous droplets, an emulsifier is added to the mixture prior to sonication. Due to the hydrophobic and lipophobic nature of PFC, the selection of suitable surfactants able to stabilize a water-in-PFC emulsion is extremely limited.¹³⁴ The fluorosurfactants used in this work utilize a fluorinated synthetic oil based on hexfluoropropylene oxide, more specifically Krytox 157FS oil (DuPont, Wilmington, DE). In addition to the unmodified molecule, a copolymer form utilizing a central polyethylene glycol (PEG) block was also used. The PEGylated forms of the molecule (referred to as Krytox-PEG) were formed by converting Krytox 157FS, a perfluoroether with carboxylic acid functionality, to an acid chloride which is then reacted with polyoxyethylene diamine.¹³⁴ The molecular structures of both the unmodified Krytox 157FS and Krytox-PEG are shown in Figure 1.6.

Two different forms of the Krytox 157FS molecule were used in this work: a high molecular weight form ($n = 41$, MW = 7,250 Da) and a low molecular weight form ($n = 13$, MW = 2,500 Da). The high and low molecular weight forms of Krytox 157FS will be referred to simply as FSH and FSL, respectively, in the remainder of this work. The PEGylated forms of these molecules contained different size PEG blocks. The Krytox-PEG polymer utilizing FSH contained a smaller PEG chain ($m = 22$, MW = 1,000 Da) while the Krytox-PEG polymer utilizing FSL contained a larger PEG chain ($m = 75$, MW = 3,350 Da). The Krytox-PEG polymers utilizing FSH

Krytox 157FS



Krytox-PEG

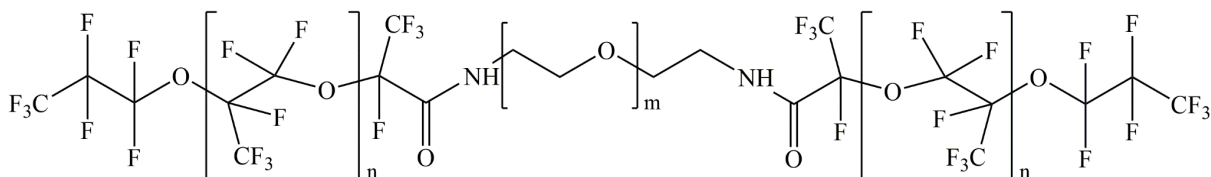


Figure 1.6: Molecular structures of the fluorosurfactants used as an emulsifier in the water-in-PFC emulsions utilized during APV.

and FSL will be referred to simply as FSH-PEG and FSL-PEG, respectively, in the remainder of this work. The total molecular weights for the FSH-PEG and FSL-PEG copolymers are 15,500 and 8,350 Da, respectively. FSL-PEG was used in combination with equal masses of FSL in order to increase surface activity and produce a more stable emulsion, while FSH-PEG was able to be used alone. Similarly structured Krytox-PEG copolymers have exhibited favorable biocompatibility when used to encapsulate mammalian cells or small multicellular organisms within aqueous microcompartments surrounded by PFC.^{135, 136}

Similar to traditional LV, APV could be performed either as total APV or partial APV. During total APV, the patient would be sedated, intubated, and the lungs completely filled with emulsion. The lungs would then be tidally ventilated with emulsion for a relatively short period of time (< 2 hours) to ensure appropriate gas exchange while delivering antibiotic and breaking up and removing infected mucus. During this time, the lungs would be completely filled with emulsion and thus the airway surfaces throughout the entire lung would be in constant contact with the emulsion. During partial APV, the lung would only be partially filled with emulsion

(approximately end-expiratory volume, ~10-20 mL/kg). The lung would then be gas ventilated using a conventional ventilator. This would provide gas exchange but with a lesser degree of tidal emulsion flow within the airways relative to total APV. In this setting, the entire lung would experience contact with the emulsion at the end of each expiration while some of the larger conducting airways would be gas filled during the rest of the respiratory cycle. In both cases, at the conclusion of treatment some portion of emulsion would be suctioned from the lungs with the remainder left in the lung and allowed to evaporate under normal gas ventilation. This would leave behind an antibiotic dose that is applied directly to the lungs with full access to dependent and previously occluded airways. Due to the need for the patient to be sedated and possibly given a paralytic, APV would likely be a one-time treatment used in conjunction with systemic or inhaled antibiotics. There is no question that inhaled antibiotic delivery presents a much less invasive and more convenient method of antibiotic treatment relative to APV. As such, inhaled delivery should still be attempted prior to APV in virtually all cases. APV, however, represents a more drastic line of treatment that could significantly improve morbidity and mortality in severe patients in which inhaled delivery is simply insufficient. Accordingly, APV would be best suited for patients already on a ventilator due to the need for the patient to be intubated during therapy. Respiratory bacterial infections in CF, COPD, and bronchiectasis are common and often exacerbate the disease state to the point that mechanical ventilation is required. Such patients groups could greatly benefit from APV with very little added procedures or discomfort.

APV, both partial and total, could enhance current antibiotic therapy in several ways. First, antibiotic is delivered directly to the source of infection, allowing for higher concentrations in the lung and lower systemic concentrations and risk of toxicity. While inhaled antibiotics have been shown to produce much higher concentrations in the lung relative to systemic administration¹⁰,

this technique can only deliver antibiotics to areas of the lung receiving effective gas ventilation. Due to PFC's unique ability to homogeneously fill the lung, APV should result in more uniform distribution of antibiotic throughout the lung, including dependent, atelectatic, and edematous regions typically unreachable via inhaled antibiotics. Second, the previously discussed anti-inflammatory properties of PFC may promote lung healing and a return towards normal mucociliary clearance. Lastly, the tidal flow of liquid emulsion during APV should actively remove infected mucus from airway walls. Considering the significant role that mucus plays in the progression of infection during lung disease, the debridement of abnormally thick mucus and biofilms from the airways should be a primary aim of treatment.

The general mechanisms by which mucus is cleared during the cough reflex have been studied and are well understood. Numerous studies have examined the interaction between airflow (both laminar and turbulent) and a mucus-like layer.^{18, 137-143} During such a scenario, when the speed of airflow over the stationary mucus phase reaches a critical value, the result is an instability that is manifested via standing or propagating waves on the mucus surface.^{18, 141, 144} As the height of such waves continues to grow into the airway lumen, the effective cross-sectional area of the local lumen is decreased and the air velocity increased. Increased air velocity results in increasing values of local shear stress at the mucus interface that will eventually exceed the mucus yield stress thereby inducing flow. Mucus flow results in crests of mucus moving downstream and growing in height until nearly blocking the lumen cross-section, resulting in an extreme rise in pressure and a catastrophic clearance event in which the yield stress is exceeded for a large volume of mucus.¹⁴¹ *In vitro* work with mucus or mucus simulants along with mathematical modeling have led to the understanding of how such clearance relates to the mucus physical properties and flow environment. Mucus detachment increases with shear magnitude and duration, and mucus

thickness.¹⁴⁰ Mucus detachment is also greater in unhealthy, more elastic mucus than in healthy, less elastic mucus.^{22, 140} Thus, thick layers of tenacious mucus will bunch and detach under shear that only causes thin, healthy mucus to flow along the airway. This is in part why coughing does little to remove normal mucus but is effective against thick mucus during disease. Additionally, mucus clearance is impaired by high surface tension at the mucus-air interface, which stabilizes mucus and prevents it from detaching.¹⁸

During tidal ventilation with the emulsion, mucus detachment is expected to occur in a manner similar to that during a cough. Thus, the removal of mucus during APV is dependent on shear stress, surface tension, and gravitational forces acting on the mucus, as well as the viscosity, elasticity, and thickness of the mucus layer.^{18, 22, 142, 143} A schematic showing these effects can be seen in Figure 1.7. Several of these factors differ from the cough scenario during APV, with many of these changes likely resulting in enhanced mucus removal. First, the emulsion would apply a level of shear stress to the airways comparable to that experienced during a cough, but the duration of the sustained stress would be significantly increased. Typical coughs have an initial flow rate

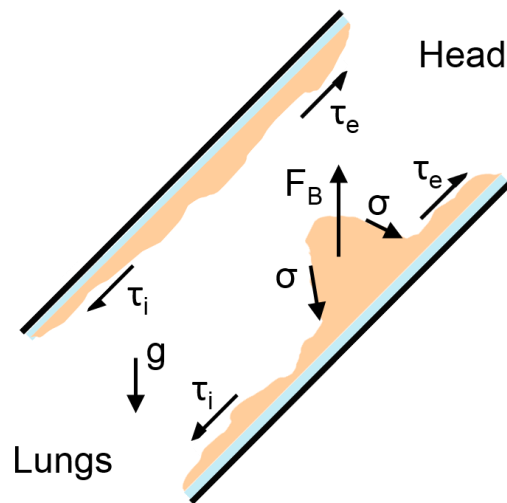


Figure 1.7: Schematic of forces acting on mucus in airway during APV where F_B is the buoyant force, τ_i and τ_e are the flow-induced shear stress during inspiration and expiration, respectively, σ are forces due to aqueous-emulsion interfacial tension, and g denotes the direction of gravity.

of 7-8 L/s for 30-50 ms followed by 3 L/s for 200-500 ms, resulting in a short duration of turbulent flow with a typical tracheal wall shear stress of 1.75 Pa.^{145, 146} During total APV with typical TLV parameters [respiratory rate = 7 breaths/min, tidal volume = 15 mL/kg, inspiratory-to-expiratory time ratio (I:E) = 1:2 or 1:4], calculated Reynolds numbers (using Equation 1 below and assuming a PFC viscosity of 1.3 cP and density of 1.8 g/mL, a 70-kg patient, and human airway measurements¹⁴⁷) range from near zero in the small airways to approximately 13,000-50,000 in the trachea, representing both turbulent and laminar flows. For turbulent flow and assuming a smooth surface at the mucus interface, the flow-induced shear stress at the tracheal wall can be calculated according to Equation 2,¹⁴⁸

$$Re = \frac{\rho u d}{\mu} \quad (1) \quad \tau_w = \frac{0.023 \rho u^2}{Re^{0.2}} \quad (2)$$

where Re is Reynolds number, ρ is PFC density, u is the average linear velocity of PFC flow, d is the tracheal diameter, μ is the dynamic viscosity of PFC, and τ_w is shear stress at the tracheal wall. Assuming the previously mentioned PFC and ventilation parameters, the calculated tracheal wall shear stress at inspiration and expiration, respectively, would be approximately 17.5 and 1.4 Pa for I:E = 1:4 and 7.0 and 2.0 Pa for I:E = 1:2. The shear stresses during APV are up to an order of magnitude larger than those during a cough and applied for a much longer duration, with even the shorter inspiratory phase lasting more than an order of magnitude longer than the high flow phase of a cough.

In addition, a lesser shear stress will be necessary to detach mucus during APV, as the mucus-PFC interfacial tension is reduced relative to the mucus-air interfacial tension. Although the mucus-air interfacial tension in a healthy individual can be as low as 20 dyne/cm¹⁴⁹, this value is typically much greater during disease, with values of 72 and 81 dyne/cm measured for mucus from patients with CF and chronic bronchitis.¹⁵⁰ This is similar to the surface tension of an air-

water interface (72 dyne/cm) due to the aqueous nature of the mucus. The interfacial tension of a water-PFC interface (analogous to mucus-PFC interface) is approximately 45 dyne/cm.¹⁵¹ The reduced interfacial tension between mucus and the emulsion should allow for instabilities at the mucus surface to occur more readily, thereby allowing for easier release of mucus into the bulk fluid.¹⁸

The effect of this interfacial tension can also be shown in terms of work of adhesion. Pillai et al investigated the work of adhesion of mucus, or the reversible work per unit area required to separate mucus from a substrate.¹⁵² The work of adhesion can be determined from the interfacial tensions between the multiple phases present and can be described for a gas-filled and PFC-filled lung according to Equations 3 and 4,¹⁵²

$$W = \gamma_{mucus-air} + \gamma_{epithelium-air} - \gamma_{mucus-epithelium} \quad (3)$$

$$W = \gamma_{mucus-PFC} + \gamma_{epithelium-PFC} - \gamma_{mucus-epithelium} \quad (4)$$

where W is the work of adhesion to separate mucus from the epithelium and γ is the interfacial tension between two phases. Assuming that both the mucus and epithelium can be described as aqueous surfaces, the interfacial tension between PFC and these surfaces ($\gamma_{mucus-PFC}$ and $\gamma_{epithelium-PFC}$) should be reduced relative to their counterparts in the gas-filled lung ($\gamma_{mucus-air}$ and $\gamma_{epithelium-air}$).

Thus, from Equations 3 and 4, it can be seen that the work required to detach mucus from the epithelium should be less in a PFC-filled lung relative to a gas-filled lung. Once in the bulk fluid, mucus can be transported out of the lungs. This movement will be aided by the buoyant force acting upon the mucus. The density of PFC (1.7-1.9 g/mL) is nearly twice that of mucus. Thus mucus movement will improve if a patient is positioned with the head above the lungs, allowing the buoyant force to push mucus from the dependent airways towards the larger, conducting

airways. As a result of these factors, total APV should be able to wash thick, viscoelastic mucus from the lungs. During previous experiences with TLV in sheep with severe ARDS, 100-300 mL of thick, proteinacious fluid was removed within ventilator traps after a few hours of ventilation.² Mucus removal during partial APV should still be possible, although perhaps less effective. Due to the compressibility of gas, the tidal flow of emulsion will be decreased. However, buoyant forces and low interfacial tension will still promote the movement of mucus into the larger conducting airways, where it can be removed via suction. This was observed during PLV as respiratory support during ARDS, albeit with a less tenacious mucus.^{63, 64} Ultimately, active mucus removal should allow antibiotics to penetrate previously plugged airways during APV and subsequent inhaled treatments, resulting in more uniform antibiotic distribution.

At the same time, this technique should not remove significant amounts of endogenous surfactant. First, pulmonary surfactant is not soluble in PFC.¹⁵³ Second, it should flow along the airway rather than detach: the surfactant layer is only about 0.1 μm thick, inelastic, and has a far lower viscosity than mucus.¹⁵⁴ Moreover, alveolar surfactant will experience very low shear due to the extremely low flow rates experienced at this generation of the airways. Accordingly, studies clearly demonstrate that surfactant is not removed after up to three hours of TLV, and lung compliance returns to normal after PFC evaporation.^{153, 155} Multiple studies have transitioned animals to conscious, spontaneous breathing with normal lung function after up to three hours of TLV.^{153, 155} Thus, there should not be enough surfactant depletion to cause respiratory distress during less than two hours of APV.

Although PFC has previously been examined as a means of pulmonary antibiotic delivery, our approach differs in multiple ways. None of the previous approaches have delivered antibiotic within a true emulsion using an appropriate emulsifier. Previous attempts to deliver antibiotics via

PFC have used a simple mixture of PFC and aqueous antibiotics, a spray-drying process resulting in a suspension of solid microparticles in PFC, or aqueous antibiotics emulsified in PFC using natural bovine surfactant. Delivery of the two phases without an added stabilizing process or agent relies upon turbulent mixing to disperse the drug and likely fails to sustain therapeutic antibiotic concentrations in a large portion of the lung. The solid microparticle suspensions used in previous studies entail a costly and timely spray-drying process that has failed to show improved treatment efficacy over unstabilized mixtures of the two phases.¹²⁶ The use of a liquid emulsion can likely decrease the cost of such a treatment as well as allow for delivery of a wider array of drugs with more controllable kinetics. Previous attempts to formulate an emulsion of aqueous antibiotics and PFC used natural bovine surfactant (composed of phospholipids) as the emulsifier.¹³³ Due to the lipophobic nature of PFC, natural pulmonary surfactant is not an ideal emulsifier, likely resulting in a somewhat unstable emulsion. Lastly, our focus on removing lung exudates should also maximize this treatment. PLV and TLV mobilize lung exudates for removal, either by suction (PLV) or within ventilator mucus traps (TLV).^{2, 63, 64, 153} However, no one has utilized this aspect of LV to treat bacterial respiratory infections. For these reasons, we believe our approach differs from previous attempts and presents the potential for significantly improved treatment.

1.8 Summary of Study

The goal of this work was to investigate the use of tobramycin-loaded, water-in-PFC emulsions during LV (termed APV) as a means of antibiotic delivery and therapy for bacterial respiratory infection patients. Several different aspects of APV were evaluated using both *in vitro* and *in vivo* methods. Initial work evaluated the fluid mechanical aspects of the emulsion. The physical properties of liquid PFCs are integral to their use during LV. Thus, evaluating the effects

of the emulsification process on these physical properties is a critical first step in the development of APV. In the next phase of this work, we evaluated the bactericidal capacity of such emulsions against clinically relevant biofilms and how these effects vary with emulsion formulation. *In vivo* methods were then used to evaluate the pharmacokinetics of antibiotic delivery via APV with varying emulsion formulations. Finally, following a lack of observed treatment benefit in a bacterial respiratory infection model in rats, simplified *in vitro* methods were used in an attempt to gain a better understanding of the physical transfer of drug from the emulsion to aqueous surfaces with which it comes in to contact. The results from such *in vitro* studies have resulted in significantly optimized, more effective emulsion formulations to be used during APV.

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CHAPTER 2

Initial Characterization of a Water-in-Perfluorocarbon Emulsion for the Pulmonary Delivery of Tobramycin

2.1 Introduction

Antibacterial perfluorocarbon ventilation (APV) is a proposed treatment that aims to address the shortcomings of inhaled antibiotic delivery. This current treatment is perhaps most lacking in the setting of bacterial respiratory infection during preexisting lung disease, such as is commonly the case during cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD). During APV, the lung is filled with liquid perfluorocarbon (PFC) containing emulsified aqueous antibiotic (*i.e.* a water-in-PFC emulsion) and ventilated for a short period (< 2 hours). Such treatment would allow for improved penetration and distribution of delivered antibiotic as well as the ability to actively detach and remove infected mucus from the airways. In order to maintain uniform spatial distribution of aqueous drug within the immiscible PFC, FSL-PEG and FSL (molecular structures shown in Figure 1.6) are used as emulsifiers and added to the mixture prior to mechanical dispersal of the aqueous phase. Previous work with this fluorosurfactant focused on a water-in-PFC-in-water emulsion.¹ Thus, the physical properties of the water-in-PFC emulsions to be used during APV are largely unknown.

Additionally, the application of the emulsions in this work also differs. Previous emulsions utilizing the fluorosurfactants were primarily intended for IV administration¹ rather than during liquid ventilation (LV). As discussed in sections 1.4 and 1.7, the abilities of PFC to provide adequate respiratory support as well as effectively remove mucus during LV are largely dependent on its physical and rheological properties. Therefore, it is imperative to understand any potential changes that are induced in these properties as a result of the emulsification process. Such an evaluation will indicate the feasibility and mucus removal efficacy of using the emulsions during APV. In addition to providing a means of respiratory support and mucus removal, the emulsion must also be capable of effective antibiotic delivery. As discussed in section 1.2, bacterial biofilms represent a key pathological feature of chronic airway infections^{2,3} and a significant impediment to effective antibiotic therapy.^{4,5} Much of the therapeutic potential of APV rests on the ability of the tobramycin-loaded emulsions to achieve effective delivery to the lungs and eradication of bacteria in a biofilm setting.

The general aim of the work presented in this chapter was to perform a comprehensive initial evaluation of a tobramycin-loaded, water-in-PFC emulsion in order to explore the feasibility and potential efficacy of APV. In the current study, we characterized the stability, rheology, and anti-biofilm characteristics of an emulsion consisting of aqueous tobramycin in a PFC vehicle. In addition, initial pharmacokinetic work was performed in order to compare antibiotic delivery via the emulsion to that of aerosolized delivery. To be effective in its intended application, the emulsion must (i) be effective against typical airway biofilm pathogens such as *Pseudomonas aeruginosa* (*PA*), (ii) be stable over the intended two-hour duration of treatment, and (iii) maintain surface tensions, aqueous interfacial tensions, and viscosity similar to those of neat PFC. Low surface tension and aqueous interfacial tension are necessary for the liquid to penetrate otherwise

plugged airways and aid in mucus detachment.⁶ The viscosity of PFCs has significant effects on the ability to adequately ventilate the lungs during LV⁷ and the shear stress-induced disruption of infected mucus lining the airways.

2.2 Methods

2.2.1 Emulsion Preparation

The FSL-PEG fluorosurfactant was synthesized as previously described elsewhere.¹ The emulsion was prepared by first dissolving 6 mg FSL (Krytox 157FSL, CAS # 51798-33-5; Dupont, Wilmington, DE, USA) and 6 mg FSL-PEG copolymer in approximately 0.6 mL of PFC liquid. The PFC used in all experiments was perfluorocycloether/perfluorooctane (FC-770; 3M Inc., St. Paul, MN, USA). FC-770 has been used in previously published studies on partial liquid ventilation (PLV).⁸⁻¹⁰ For eventual clinical translation, a PFC with a higher level of purity and larger amount of documented *in vivo* safety data would be used. Next, 125 μ L of 0.4, 4, or 40 mg/mL tobramycin (Abraxis Pharmaceutical Products, Schaumburg, IL, USA) in sterile saline was added. The mixture was emulsified via sonication (Model 450, 20 kHz, 3.2 mm diameter microtip; Branson Ultrasonics, Danbury, CT, USA) at 200 W/cm² for 60 seconds in continuous mode. PFC was then added to the emulsion to a final volume of 5 mL and was sonicated for an additional 10 seconds to disperse the aqueous droplets in the PFC phase. Additional PFC was then added to the emulsion to create the varying aqueous volume percentages examined. The primary emulsion formulation parameters that will be referred to throughout this work are aqueous volume percentage (V_{aq}), aqueous tobramycin concentration (C_{aq}), and fluorosurfactant concentration (C_{fs}). The V_{aq} and C_{aq} define the percentage of aqueous volume within the emulsion and the concentration of tobramycin within that aqueous phase, respectively. Thus, the total mass of

tobramycin within any particular volume of emulsion is defined by both V_{aq} and C_{aq} . C_{fs} describes the total mass of fluorosurfactant (sum of FSL-PEG and FSL) used to emulsify the aqueous phase within the emulsion. Thus, values for C_{fs} are given as the mass of fluorosurfactant per aqueous volume used in the emulsion (*i.e.* units of mg/mL H₂O) throughout this work.

2.2.2 Particle Size and Number

The number and size distribution of aqueous droplets in an emulsion with $V_{aq} = 2.5\%$ and $C_{fs} = 96$ mg/mL H₂O were acquired via photomicrography using a hemocytometer (Brightline, Hausser Scientific, Horsham, PA, USA). Images were digitally acquired using Spot Advanced Software (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and processed in MATLAB (The MathWorks Inc., Natick, MA, USA) to determine the number of droplets per volume and their size distribution. To determine emulsion stability, analysis was repeated one, two, and 24 hours after preparation. Five different optical fields of view (FOV), each containing a minimum of 500 droplets/FOV, were analyzed to determine the droplet number density and size distribution at each time point. This manual droplet counting/sizing method has been used with similarly sized emulsions and shown to produce results not statistically different from those obtained using a Coulter counter.^{1, 11}

2.2.3 Emulsion Viscosity

The dynamic viscosities of neat PFC and emulsions with $C_{fs} = 96$ mg/mL H₂O and V_{aq} values ranging from 0.3 to 2.5% were measured using a cone and plate rheometer (AR 1000, TA Instruments, New Castle, DE, USA). Samples were interrogated at 37° C over a range of shear rates from 20 to 500 s⁻¹, capturing a range of values estimated to be achieved at the airway

epithelium during total liquid ventilation. Three repeated measurements were taken for each set of conditions examined.

2.2.4 Air and Aqueous Interfacial Tensions

The surface tension and aqueous interfacial tension of neat PFC and an emulsion with $C_{fs} = 96$ mg/mL H₂O and $V_{aq} = 2.5\%$ were measured using a DuNouy ring tensiometer with a platinum-iridium ring (6 cm circumference, Interfacial Tensiometer; CSC Scientific Company Inc., Fairfax, VA, USA). Immediately after emulsion preparation, 10-15 mL of emulsion or neat PFC was placed in a petri dish and allowed to reach room temperature (22-25° C) before being used for measurements. For aqueous-emulsion or aqueous-PFC interfacial tension measurements, 10 mL of de-ionized, filtered water was introduced on top of the emulsion or neat PFC. Three repeated measurements were taken for each set of conditions examined.

2.2.5 Anti-Biofilm Activity

PA strain ATCC 27853, was generously provided by David McLeod (Gilead Laboratories, Foster City, CA, USA). This strain was selected for its biofilm forming capacity. Biofilms were grown on solid phase transfer pin lids as described elsewhere¹² and as summarized in Figure 2.1. Bacteria were first grown in tryptic soy broth (TSB) with 1% glucose at 37° C on a gyratory shaker. A solution of approximately 10⁸ colony-forming units (CFU) per mL of mid-log growth *PA* in growth medium was then placed into wells of a 96-well plate (150 uL/well). A solid phase transfer pin lid (Nunc-TSP 445497; Nunc, Rochester, NY, USA) was then placed such that pins were immersed in the bacterial broth. Trays were incubated for 18 hours at 37° C without shaking. Pin-

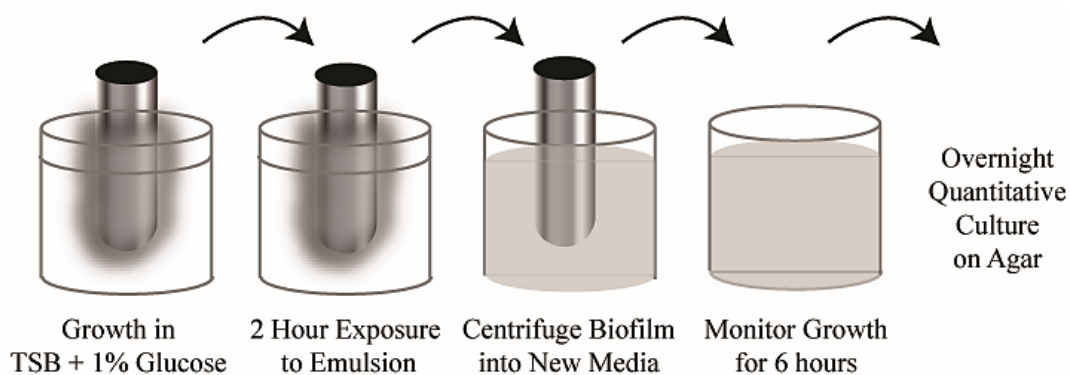


Figure 2.1: Sequence of steps in biofilm exposure experiment.

adherent biofilms were then exposed to the emulsions, neat PFC, aqueous antibiotics, or growth medium by transferring the lid to a new 96-well plate containing the exposure medium (200 μL /well). All emulsions in Table 2.1 were examined. After two hours of exposure, biofilms were centrifuged for 20 minutes at $840 \times g$ into fresh growth medium (TSB with 1% glucose). Because centrifugally released biofilm material could not be reliably separated into individual cells to perform CFU counting, bacterial viability from recovered biofilms was quantified as the change in broth turbidity over six hours. LB agar (Lennox L agar; Life Technologies, Carlsbad, CA, USA) cultures were used to confirm complete biofilm killing in cases where no change in broth turbidity

C_{fs} [mg/mL H ₂ O]	V_{aq} [%]	C_{aq} [mg/mL]	Total Tobramycin Content [mg/mL]
96	0.25	0.4	1
96	1.00	0.4	4
96	2.50	0.4	10
96	0.25	4.0	10
96	1.00	4.0	40
96	2.50	4.0	100
96	0.25	40.0	100
96	1.00	40.0	400
96	2.50	40.0	1,000

Table 2.1: Formulations of all emulsions examined for anti-biofilm activity.

occurred over six hours of observation. Sixteen wells were used for each type of exposure, resulting in 16 repeated measurements for each set of conditions examined.

2.2.6 Pharmacokinetic Characterization

Specific pathogen-free, male Sprague Dawley rats [$n = 20$, weight 401 ± 14 g (mean \pm standard deviation); Charles River Laboratories, Wilmington, MA, USA] were divided into the following groups: partial APV (referred to simply as APV in the remainder of Chapter 2) treatment ($n = 10$) and aerosolized treatment ($n = 10$). All rats were initially anesthetized with a mixture of ketamine hydrochloride [50 mg/kg, intraperitoneal (IP); Hospira Inc., Lake Forest, IL, USA] and xylazine hydrochloride (5 mg/kg, IP; Lloyd Laboratories, Shenandoah, IA, USA). Rats receiving aerosolized treatment were then placed in a prone position and orally intubated with a 16 gauge angiocatheter. Aerosolized delivery was accomplished via a Microsprayer Aerosolizer (Model IA-1B; Penn-Century, Wyndmoor, PA, USA). Prior to intubation, the length of the angiocatheter was trimmed to ensure that the Microsprayer nozzle was sufficiently exposed when the Microsprayer was fully inserted into the angiocatheter. Following intubation with the trimmed angiocatheter, the Microsprayer was fully inserted into the angiocatheter and tobramycin (15 mg/kg in 250 μ L of sterile saline) was delivered intratracheally. When performed in this manner, the nozzle tip was measured to be approximately 10 mm proximal to the carina (Figure 2.2). Although efforts were made to synchronize actuation of the Microsprayer to inspiration, the rats often became temporarily apneic following intubation and thus the aerosol was sometimes delivered in the absence of inspiratory or expiratory flow. Following delivery, rats were extubated and allowed to recover under supplemental oxygen provided via a nose cone. For rats receiving APV, intravenous (IV) access was acquired via the lateral tail vein following initial anesthesia. Sedation was

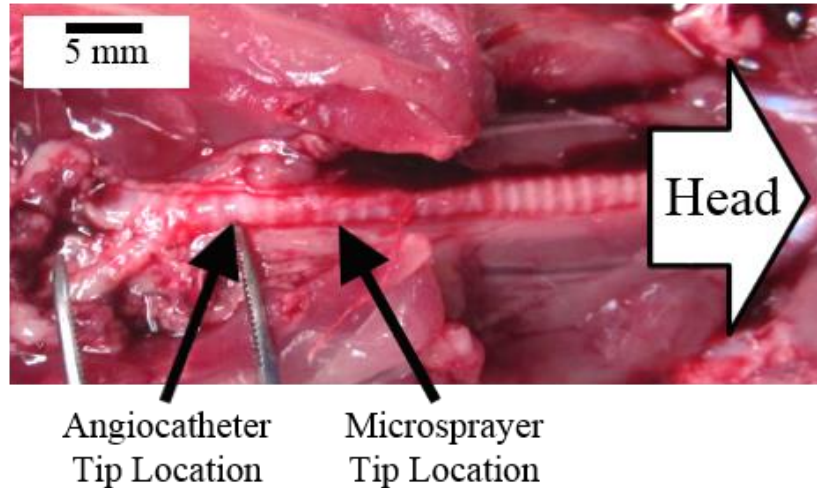


Figure 2.2: Image of intubated rat trachea showing angiocatheter (16 gauge, 1.77” long) and Microsprayer (Model IA-1B) tip locations relative to carina during treatment.

maintained with a constant IV infusion of ketamine hydrochloride (0.6 mg/kg/min). Heart rate and arterial oxygen saturation were monitored via a pulse oximeter (VetOx Plus 4800; HESKA, Loveland, CO, USA). Rat body temperature was monitored and maintained with a homeothermic blanket system (Model 507220F; Harvard Apparatus, Holliston, MA, USA). Rats were orally intubated with a 16 gauge angiocatheter and connected to a ventilator (Model 683; Harvard Apparatus, Holliston, MA, USA). Ventilation was carried out with a tidal volume of 9 mL/kg, a respiratory rate of 50-80 breaths/min, a positive end-expiratory pressure of 3 cmH₂O, and a fraction of inspired oxygen of 1. Respiratory rate was adjusted to maintain peak inspiratory pressures less than 30 cmH₂O. Preoxygenated emulsion ($V_{aq} = 2.5\%$, $C_{aq} = 40$ mg/mL, and $C_{fs} = 96$ mg/mL H₂O) was instilled (15 mL/kg) via a port connected to the angiocatheter, resulting in a delivered tobramycin dose of 15 mg/kg. When performed in this manner, the angiocatheter tip was measured to be approximately 5 mm proximal to the carina (Figure 2.2). The emulsion was prepared no more than 20 minutes before use and was instilled slowly over a period of less than three minutes without interrupting gas ventilation. Gas ventilation was continued for 60 minutes

following delivery of the emulsion, after which the rats were either euthanized or allowed to recover under supplemental oxygen.

Rats were euthanized with pentobarbital sodium (175 mg/kg, IP, Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI, USA) and the lungs removed aseptically at either one hour (n = 5 for each treatment group) or four hours (n = 5 for each treatment group) following the delivery of tobramycin. In the four-hour groups, blood samples were drawn via the lateral tail vein at 30, 60, 120, and 240 minutes following the delivery of tobramycin. Serum tobramycin concentration measurements were performed via an immunoassay by the Department of Pathology within the University of Michigan Hospital (Ann Arbor, MI, USA). Lung tissue was homogenized with 10 mL of sterile saline and the presence of active tobramycin in the homogenate measured via a microbiological assay.

The microbiological assay used in this work utilizes the agar well diffusion method that has been previously described by many researchers.¹³⁻¹⁵ Molten LB agar was dispensed (30 mL/dish) into square, polystyrene petri dishes with a grid (Model FB0875711A; Thermo Fisher Scientific, Pittsburgh, PA, USA) on a flat surface. Note that accurately dispensed volumes are important because the resulting agar depth affects assay consistency. The dishes were allowed to cool at room temperature and stored at 4° C if not used immediately after preparation. Next, the agar surface of each plate was inoculated with approximately 500 µL of mid-log growth (optical density = 0.12-0.14) *PA* in TSB, ensuring that the entire agar surface was coated with bacterial solution. After allowing the plates to dry at room temperature, wells with a radius of approximately 3.4 mm were made within the agar for each plate. Samples of the lung tissue homogenate were then loaded into wells (75 µL/well) within the inoculated agar. Standard solutions of known tobramycin concentration were also prepared and loaded into wells in an identical fashion. A set

of standards was included on each individual plate along with the experimental sample to be measured in order to minimize measurement variability due to differences in agar thickness between plates. Following loading of the samples, agar plates were incubated at 37° C for 24 hours before circular inhibition zones were imaged and measured. Examples of inhibition zones from such a plate are shown in Figure 2.3A. Measurement of inhibition zone areas was performed via ImageJ (US National Institutes of Health, Bethesda, MD, USA). The concentration of the unknown samples was then determined by interpolation from the inhibition zones of the standard solutions. Similar to previously described methods¹³, linear regression analysis of the standards was obtained by plotting the log transformation of the known tobramycin concentrations as a function of inhibition zone radii (Figure 2.3B). Standards curves typically had an $R^2 \geq 0.98$. The lower limit of detection using these methods was 5 $\mu\text{g/mL}$. Any result below this limit was assigned a value of 2.5 $\mu\text{g/mL}$. Each sample was measured in triplicate and the resulting values averaged to produce a single concentration for each trial. Five separate trials with independently prepared emulsions used in different rats were performed for each formulation evaluated. In addition to samples from rats receiving treatment, lung tissue from untreated rats and rats receiving an emulsion containing only sterile saline in the aqueous phase were examined as negative controls.

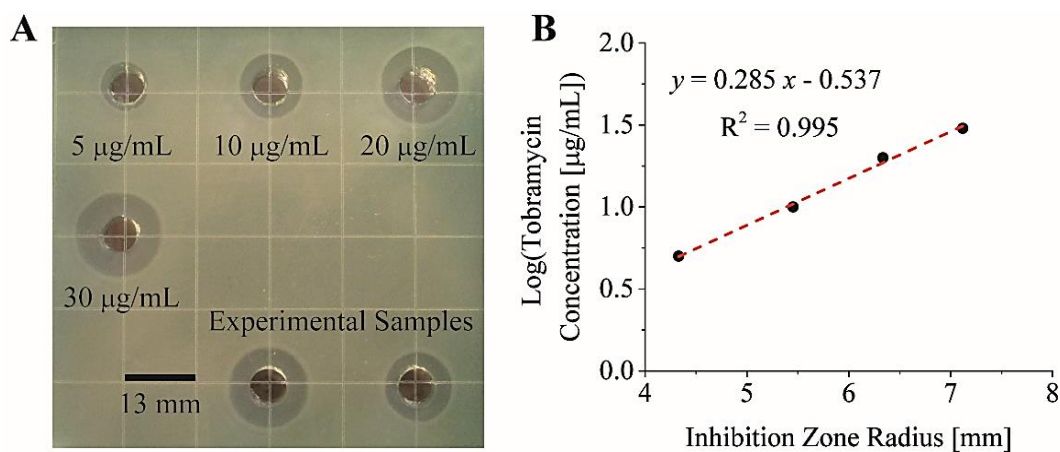


Figure 2.3: (A) Example of inhibition zones resulting from the agar well diffusion method. (B) Example of standard curve used for interpolation during agar well diffusion method.

2.2.7 Statistical Analysis

SPSS 22 (IBM Corporation, Armonk, NY, USA) was used to perform one- or two-way ANOVA and *post hoc t*-tests on comparisons of interest. The Tukey-Kramer method was applied to differentiate significant differences between groups. Repeated-measures ANOVA was used to determine the effect of treatment mode on serum tobramycin concentration with time after delivery used as the independent, repeated variable. Differences were deemed significant for $p < 0.01$ unless otherwise specified. All subsequent reported values are given as mean \pm standard deviation unless otherwise specified.

2.3 Results

2.3.1 Emulsion Physical Properties

The aqueous droplet diameter, aqueous droplet number density, emulsion viscosity, emulsion surface tension, and aqueous-emulsion interfacial tension data are shown in Figure 2.4. Emulsion with $V_{aq} = 2.5\%$ and $C_{fs} = 96$ mg/mL H₂O had an initial mean aqueous droplet diameter of 1.9 ± 0.2 μm and droplet number density of $3.5 \pm 1.7 \times 10^9$ droplets/mL. Aqueous droplet diameter was shown to be stable, with no significant change between zero and 24 hours. Aqueous droplet number density was shown to significantly change over time, exhibiting a nearly half-log decrease over 24 hours. The emulsions and neat PFC exhibited Newtonian behavior with constant viscosities over the range of shear rates examined ($20 - 500$ s^{-1}). No significant differences in viscosity were seen between neat PFC and emulsions. Over all of the shear rates tested, the ranges of average viscosities were $1.22 - 1.32 \times 10^{-3}$, $1.21 - 1.34 \times 10^{-3}$, and $1.26 - 1.37 \times 10^{-3}$ Pa·s for emulsions with 2.5, 0.3, and 0% (neat PFC) aqueous volume, respectively. Lastly, the mean surface tensions of neat PFC and emulsion ($C_{fs} = 96$ mg/mL H₂O and $V_{aq} = 2.5\%$) were $15.0 \pm 0.1 \times 10^{-3}$

and $14.6 \pm 0.6 \times 10^{-3}$ N/m, respectively. The mean aqueous interfacial tensions were $46.7 \pm 0.3 \times 10^{-3}$ and $26.9 \pm 11.0 \times 10^{-3}$ N/m ($p < 0.01$) for neat PFC and emulsion ($C_{fs} = 96$ mg/mL H₂O and $V_{aq} = 2.5\%$), respectively.

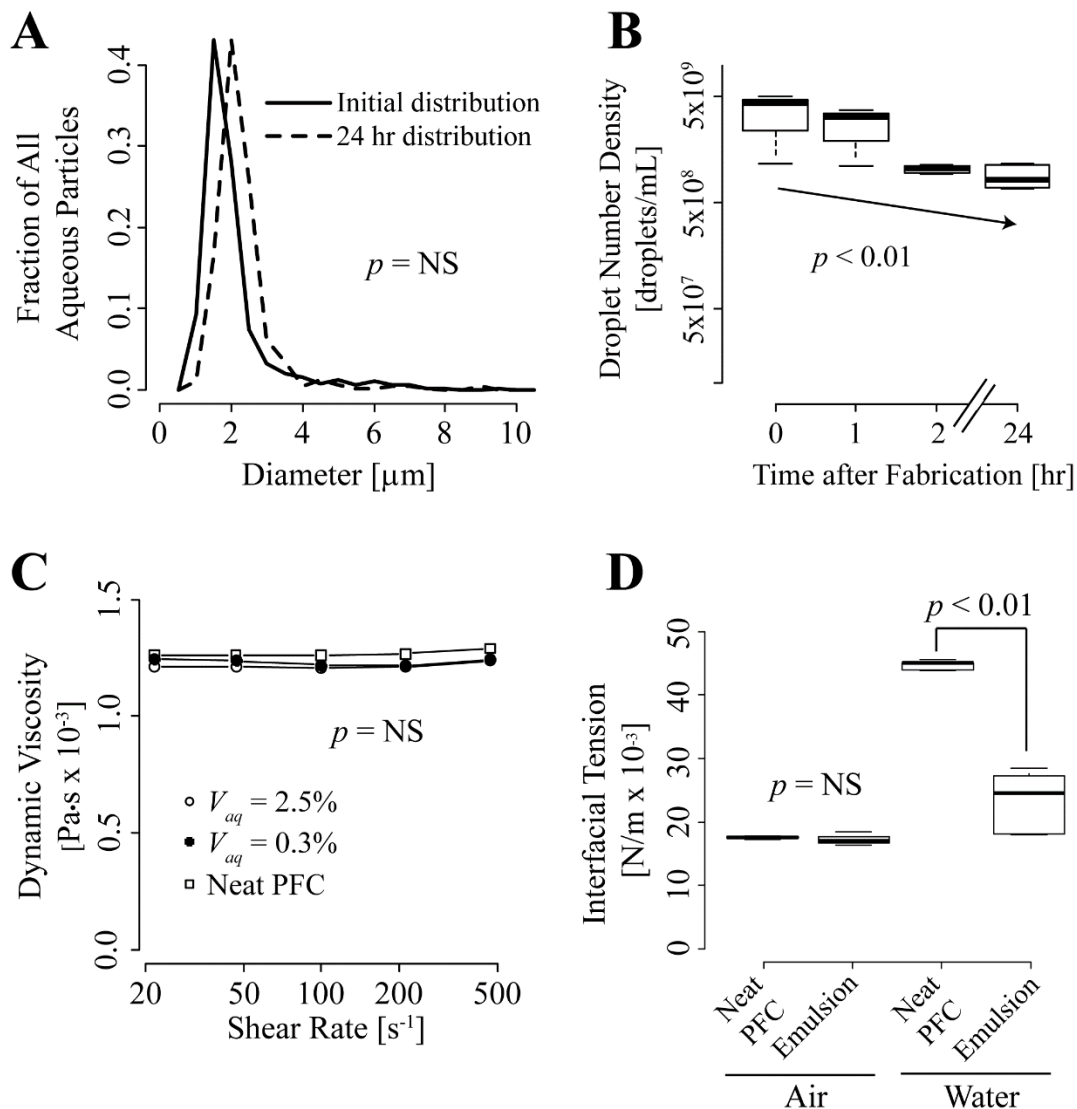


Figure 2.4: (A) Size distribution of aqueous droplets in emulsion both initially and 24 hours post-preparation. (B) Droplet number density over time. (C) Viscosity over differing shear rates for neat PFC and emulsions of varying V_{aq} . (D) Air- and aqueous-interfacial tensions for neat PFC and emulsion.

2.3.2 Anti-Biofilm Capacity

A typical biofilm recovered from an immersed pin is shown in Figure 2.5A. As shown in Figure 2.5B, tobramycin delivered via emulsion was capable of significantly limiting or altogether preventing growth in biofilm-derived *PA* after a two-hour exposure. C_{aq} values of at least 4 mg/mL were necessary to reduce the proliferation of biofilm-derived bacteria following exposure, and bactericidal effects were achieved only with $C_{aq} = 40$ mg/mL at the highest V_{aq} (2.5%). Under these conditions, the total emulsion concentration of tobramycin that the biofilm was exposed to was 1,000 $\mu\text{g/mL}$ and 8 of 16 replicates had no growth on follow-up culture at 24 hours. It is

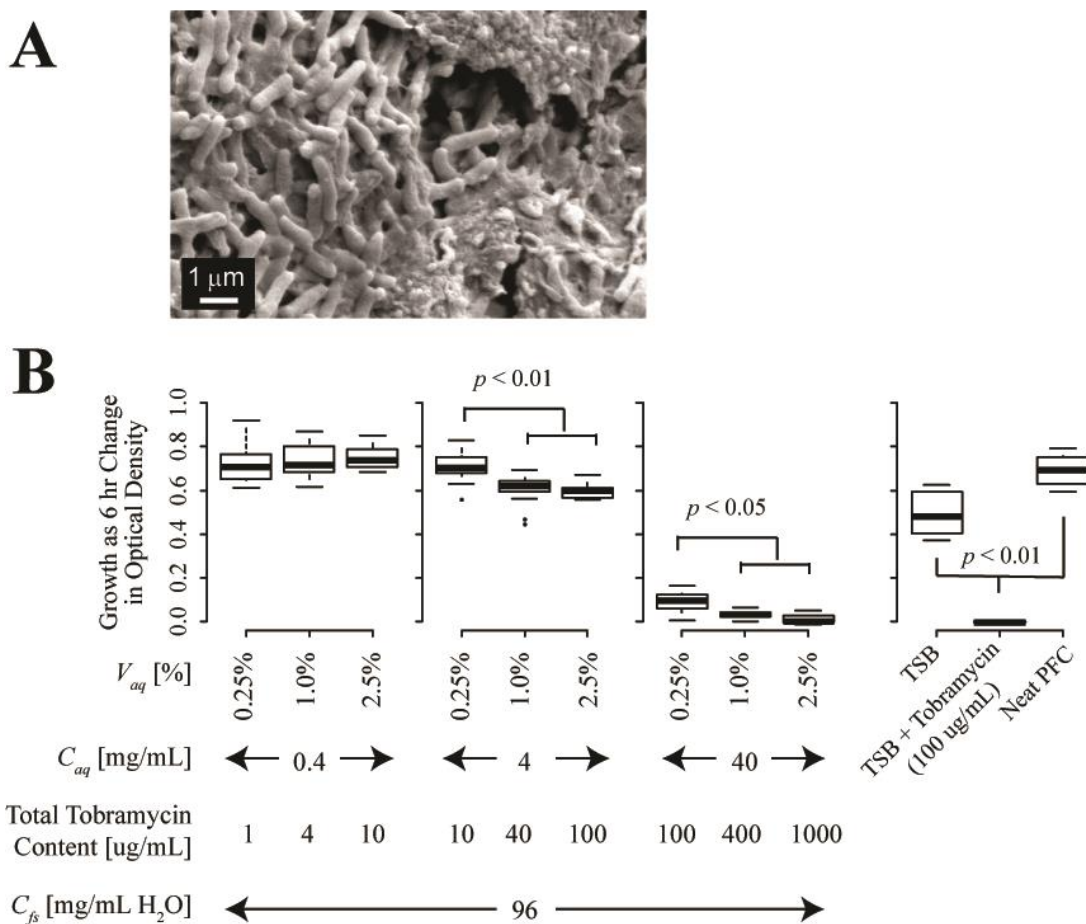


Figure 2.5: (A) Scanning electron micrograph (10,000 x) of typical biofilm from exposure experiment. (B) Six-hour biofilm growth after two-hour exposure to emulsions with varying C_{aq} and V_{aq} . $n = 16$ for each condition.

important to note that a mean inhibitory concentration cannot be determined in a traditional way with this emulsion, as bacterial growth is likely a function of both V_{aq} and C_{aq} rather than solely of the total overall tobramycin concentration.

2.3.3 Pharmacokinetics

Lung tissue from an untreated rat and a rat receiving ventilation with an emulsion containing only saline in the aqueous phase produced no distinguishable zones of inhibition. As shown in Figure 2.6A, tobramycin delivered via APV resulted in significantly higher concentrations in the lung tissue at both one and four hours post-delivery relative to aerosolized treatment. Mean lung tissue homogenate tobramycin concentrations at one hour post-delivery were 197 ± 36 and 78 ± 18 $\mu\text{g/mL}$ for APV and aerosolized treatment, respectively. Mean lung tissue homogenate tobramycin concentrations at four hours post-delivery were 82 ± 14 and 7 ± 5 $\mu\text{g/mL}$ for APV and aerosolized treatment, respectively. Furthermore, three of five measurements in the aerosolized group at four hours post-delivery resulted in concentrations below the lower limit of

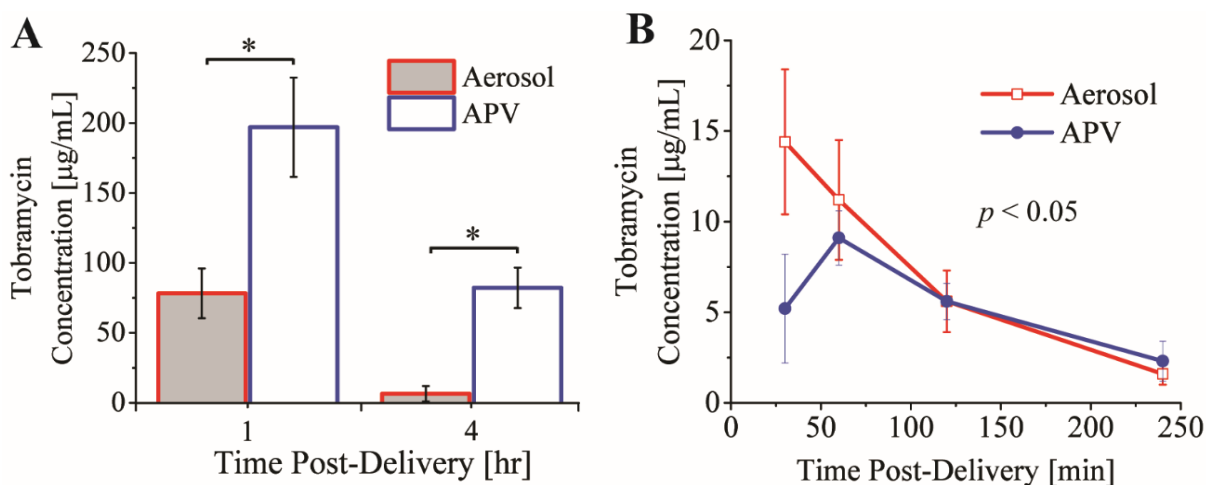


Figure 2.6: Lung tissue homogenate (panel A) and serum (panel B) tobramycin concentrations at varying time points following tobramycin delivery via APV ($C_{fs} = 96$ mg/mL H₂O FSL-PEG+FSL, $V_{aq} = 2.5\%$, and $C_{aq} = 40$ mg/mL) and aerosolized delivery. Error bars represent standard deviations, $n = 5$ for each condition, and significant ($p < 0.01$) differences are denoted by an asterisk.

detection (5 $\mu\text{g}/\text{mL}$). A two-way ANOVA showed a statistically significant effect of treatment mode (APV or aerosolized delivery) ($p < 0.01$) and time post-delivery (one or four hours) ($p < 0.01$) on lung tissue homogenate tobramycin concentrations. Serum tobramycin concentrations from rats receiving tobramycin delivered via APV or aerosolized treatment are shown in Figure 2.6B. A repeated-measures ANOVA showed that tobramycin delivery via APV produced significantly ($p < 0.05$) lower serum concentrations relative to aerosolized delivery. Measured peak serum tobramycin concentrations for APV and aerosolized treatments were 9.1 ± 1.5 and 14.4 ± 4.0 $\mu\text{g}/\text{mL}$ and occurred at 60 and 30 minutes, respectively.

2.4 Discussion

In the current work, we demonstrated the feasibility of formulating a water-in-PFC emulsion containing tobramycin and its effectiveness as an antibiotic delivery vehicle. In order for this emulsion to be effective, it must be relatively stable over the likely application duration (< 2 hours), preserve the viscosity and low interfacial tensions of PFC that allow for effective ventilation, and be bactericidal. Our emulsion has met these conditions. The sonication process for generating the emulsion is straightforward and could readily be performed just prior to administration, similar to the activation of ultrasound contrast agents [*e.g.* Definity (Lantheus Medical Imaging) or Optison (GE Healthcare)] or preparation of drug-laden emulsions used in chemoembolization.¹⁶

Aqueous droplet size was sufficiently stable over a period of 24 hours while a significant decrease in aqueous droplet number density was observed over the same period. Multiple destabilization phenomena, including Ostwald ripening and coalescence, could be responsible for these observations. Coalescence of aqueous droplets within the emulsion would directly increase

mean droplet size as well as decrease droplet number density. During Ostwald ripening, larger droplets tend to grow in size at the expense of smaller droplets which decrease in size. Since the optical methods used to size the droplets was unable to measure nanometer-sized droplets, it is possible that Ostwald ripening caused some small, but initially countable, droplets to become undetectable at later time points. This would cause even a small increase in mean droplet size to correlate to a large decrease in the measurable droplet number density.

The viscosity of the emulsion compared to that of neat PFC was not substantially different. This is not surprising given the low volumetric fraction of aqueous phase in the emulsion (0.3-2.5%). The V_{aq} range examined was determined by considering the targeted mass of drug to be delivered relative to the lung fill volumes used in these trials (end-expiratory lung volume). The surface tension of the emulsion did not significantly differ from that of neat PFC, while the aqueous-emulsion interfacial tension was shown to be considerably less than the aqueous-PFC interfacial tension. This behavior is likely due to the addition of fluorosurfactant when preparing the emulsion. Due to their hydrophilic and fluorophilic moieties, the fluorosurfactants likely accumulate at the emulsion-aqueous interface causing a decrease in the interfacial tension. These results show that the emulsion exhibits the low surface tension of PFCs that allow them to penetrate small, branching airways and alveoli during LV and an even lower aqueous interfacial tension that should enhance mucus removal.^{17, 18} First, a low aqueous-PFC interfacial tension allows the PFC to more effectively penetrate airways that are partially or fully plugged with mucus. Second, it reduces the adhesivity of the mucus during LV, resulting in mucus detachment at lower flow rates and shear stresses.^{6, 17}

As expected, the tobramycin concentration needed within the aqueous phase to achieve bacterial killing against biofilms was substantially higher than the measured planktonic mean

bactericidal concentration against this strain (25 $\mu\text{g}/\text{mL}$, data not shown). The reasons are likely twofold. First, antibiotic activity against biofilms is known to be reduced. Second, the physical means of antibiotic delivery from the droplets to the biofilm is not yet understood and may be reducing antibiotic effectiveness.

It is worth noting that while the aqueous phase concentrations of tobramycin are quite high, the total dose administered to a patient using this technology is similar to currently used doses. If the highest C_{aq} (40 mg/mL) and V_{aq} (2.5%) in our study were to be used clinically, a 70-kg patient undergoing PLV with 15 mL/kg of the emulsion (the dose used in previous clinical trials) would receive approximately one gram of tobramycin instilled into the lung. However, at the completion of therapy, a significant amount of the emulsion would be drained from the lung. Conservatively, estimating that 10 mL/kg is left behind, the delivered dose would be 700 mg, only slightly larger than the currently recommended 600 mg daily inhaled dose.¹⁹ However, the fraction of the administered dose actually delivered to the lungs may be higher during APV than aerosolized delivery due to aerosolized drug loss via exhaled drug as well as deposition in the oropharyngeal region and delivery device. It should be noted that the *in vivo* efficacy of different emulsion formulations has not yet been determined. The ideal V_{aq} , C_{aq} , and fill volumes are all likely to change based on their *in vivo* efficacy.

Based on these results, delivery of the same tobramycin dose via emulsion may lead to lower toxicity than aerosolized delivery. Pulmonary retention of the delivered tobramycin was significantly greater for APV than aerosolized delivery (2.5 times larger at one hour post-delivery and 10 times larger at four hours post-delivery). As a result, peak serum concentrations resulting from APV were smaller and occurred at a later time point relative to aerosolized treatment, suggesting delayed drug absorption into the pulmonary circulation. Clearance of tobramycin from

the systemic circulation after approximately 60 minutes post-delivery appears to be similar between the two modes of treatment. The difference in pharmacokinetics observed between the two routes of administration could also be due in part to a difference in the mass of drug reaching the lungs. Aerosolized delivery in this study was performed with the Microsprayer nozzle positioned within the rat trachea. Although this technique likely results in much greater lung deposition than shown with clinical devices in humans, it may still result in some drug deposition in the trachea, thereby potentially reducing the fraction of drug reaching the lungs.

The physical means by which antibiotic is transferred from aqueous droplets within the emulsion to the aqueous surfaces of the lung (epithelial lining fluid or biofilm mass) is not yet understood and warrants further investigation. The bactericidal effects resulting from biofilm exposure to emulsions as well as the presence of tobramycin in the systemic circulation following APV treatment indicate that the antibiotic is available and delivery is achieved to some degree. A better understanding of this process would allow for easier optimization of the emulsion and therapy in order to achieve maximum treatment benefit.

In conclusion, the emulsion assessed in this study exhibits a stable droplet size over a period of 24 hours, maintains the same viscosity as neat PFC, retains a low surface tension, creates an even lower aqueous interfacial tension than that of neat PFC, and is capable of significantly lowering bacterial growth in an *in vitro* setting. In addition, *in vivo* delivery of tobramycin via APV results in enhanced pulmonary retention as well as delayed absorption into the systemic circulation relative to aerosolized delivery. The next phase of this work will provide a more in-depth evaluation of the *in vivo* effects of varying the emulsion formulation in an effort to further optimize the emulsion for effective treatment. It is important to note that the current study did not evaluate the effects of C_{fs} , and instead used excess fluorosurfactant to ensure emulsion stability.

Considering the unknown long-term biocompatibility of the fluorosurfactants, a better understanding of the effects of C_{fs} and the degree to which it can be reduced while still retaining the favorable properties of the emulsion is a primary aim of the following chapter.

2.5 References

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CHAPTER 3

Effects of Emulsion Formulation on the Pharmacokinetics of Pulmonary Tobramycin Delivery via Antibacterial Perfluorocarbon Ventilation

3.1 Introduction

As discussed in section 1.3, the current standard of pulmonary antibiotic treatment is inhaled delivery. The pharmacokinetics of such delivery have been studied in many settings and are well understood.¹⁻⁴ Clinical treatment strategy utilizing inhaled antibiotic delivery is in large part based off of such data. Initial *in vivo* work with antibacterial perfluorocarbon ventilation (APV) was positive, but further development of APV requires a greater understanding of the temporal patterns of drug delivery and removal from the lung. However, the pharmacokinetics of these emulsions are complicated and not yet fully understood. It was hypothesized that the pharmacokinetics during APV are a function of the aqueous volume percentage (V_{aq}), aqueous antibiotic concentration (C_{aq}), and fluorosurfactant concentration (C_{fs}). The V_{aq} and C_{aq} are defined as the percentage of aqueous phase in the emulsion and the antibiotic concentration within that aqueous phase, respectively. Thus, together they define the total amount of drug delivered to the lungs upon initiating APV. The fluorosurfactant is responsible for maintaining emulsion stability and avoiding droplet coalescence and phase separation. C_{fs} , therefore, is likely to affect the

transport of antibiotic to the lung parenchyma and pulmonary capillaries. Previous studies (discussed in Chapter 2) indicate that *in vitro* bacterial killing of *Pseudomonas aeruginosa* (PA) biofilms is optimized by maximizing C_{aq} , with a far smaller increase in killing with increasing V_{aq} . However, the *in vivo* setting is a vastly different scenario with a more complex geometry and multiple transport processes continually affecting antibiotic presence and availability. Therefore, this study sought to determine the effect of V_{aq} , C_{aq} , and C_{fs} on antibiotic delivery to the lung via APV.

As previously mentioned, APV can be performed as either partial or total APV. Partial APV was chosen for the following study for multiple reasons. During total APV emulsion is constantly cycled out of the lung, through a large extracorporeal circuit containing large-surface area devices (*e.g.* oxygenator and heat exchanger), and back into the lung. During such a process, it is likely that some degree of drug deposition occurs within the extracorporeal circuit. Additionally at the conclusion of total APV, some portion of emulsion must be removed from the lung in order to transition to partial APV. Processes such these make the determination of a total dose of drug delivered to the lung difficult and variable. Partial APV on the other hand involves delivery of a finite volume of emulsion (and thus a finite mass of drug) directly to the lungs. Although during partial APV in a human some portion of emulsion would be suctioned from the lung, emulsion delivered in this work was not recovered. Suctioning liquid from the airways of a rat has proven to be technically difficult and highly inconsistent. Thus, partial APV treatment (referred to in the remainder of the chapter simply as APV) without recovery of emulsion was used in this work in order to reduce treatment variability and allow for more accurate determination of delivered drug doses.

Because the fluorosurfactant enables the emulsification of the antibiotics within the perfluorocarbon (PFC) phase, there is a minimum C_{fs} value below which the dispersion of drug is not maintained following sonication. Phase separation within the emulsion diminishes the ability of APV to achieve uniform pulmonary drug distribution and thus limits its therapeutic potential. Although results from Chapter 2 showed sufficient stability over a 24-hour period, these trials evaluated emulsions utilizing a single, relatively large, C_{fs} value (96 mg/mL H₂O). In order to better understand the effects of C_{fs} on emulsion stability as well as establish an appropriate range of C_{fs} values to be examined *in vivo*, a preliminary *in vitro* study was performed to determine the minimum value of C_{fs} that exhibited sufficiently stable drug dispersion.

3.2 Methods

3.2.1 Emulsion Preparation

Tobramycin-loaded, water-in-PFC emulsions were prepared similar to those methods described in section 2.2.1. Briefly, equal masses of FSL-PEG and FSL were first dissolved in 250-500 μ L of PFC. Similar to previous *in vitro* work with the emulsions, the PFC used in all experiments was perfluorocycloether/perfluorooctane (FC-770; 3M Inc., St. Paul, MN, USA). Next, 100-500 μ L of 20-100 mg/mL tobramycin (X-Gen Pharmaceuticals Inc., Horseheads, NY, USA) in sterile saline was added to the PFC solution. The mixture was then sonicated (Model S-450D, 20 kHz, 3.2 mm diameter microtip; Branson Ultrasonics, Danbury, CT, USA) at 200 W/cm² for 20 seconds. The mixture was then added to a larger amount of PFC in order to bring the total volume to 10 mL and re-sonicated at 200 W/cm² for 60 seconds. Emulsion was consistently prepared in 10-mL batches in an attempt to minimize preparation variability from batch to batch.

As in Chapter 2, C_{fs} refers to the total amount of fluorosurfactant used and is discussed in terms of fluorosurfactant mass per aqueous volume (*i.e.* units of mg/mL H₂O).

3.2.2 *In Vitro* Assessment of Emulsion Drug Dispersion

The spatial distribution of tobramycin-containing, aqueous droplets within a column of emulsion was evaluated over a period of four hours following emulsion preparation in order to evaluate the effect of C_{fs} on emulsion stability. Emulsions with fixed V_{aq} (2.5%) and C_{aq} (40 mg/mL) were examined with varying C_{fs} (10, 30, or 96 mg/mL H₂O). A graduated cylinder (polystyrene, 12.7 mm inner diameter) was filled with 30 mL of freshly sonicated emulsion to a height of approximately 24 cm. The graduated cylinder was modified to feature access ports with a needle (18 gauge) protruding through the cylinder wall into the center of the column lumen at heights of 4, 10, and 16 cm from the base. A schematic of the graduated cylinder can be seen in Figure 3.1. At 0, 30, 120, and 240 minutes following emulsion preparation, a 200- μ L sample of emulsion was drawn from each height. In order to quantify the presence of active tobramycin in each sample of emulsion, the samples were diluted with 4 mL of sterile water and re-sonicated at 200 W/cm² for two minutes, resulting in an inverted emulsion (continuous aqueous phase with dispersed PFC phase). The phase inversion was visually confirmed by the observation of PFC droplets accumulating at the bottom of the continuous aqueous phase. The inverted emulsion was then centrifuged at 3,000 x *g* for 20 minutes in order to separate the PFC and aqueous phase. The presence of active tobramycin in the separated aqueous phase was then quantified via the microbiological assay described in section 2.2.6. Each sample was measured in triplicate and the resulting values averaged to produce a single concentration for each trial. Three separate trials with independently prepared emulsions were performed for each formulation evaluated.

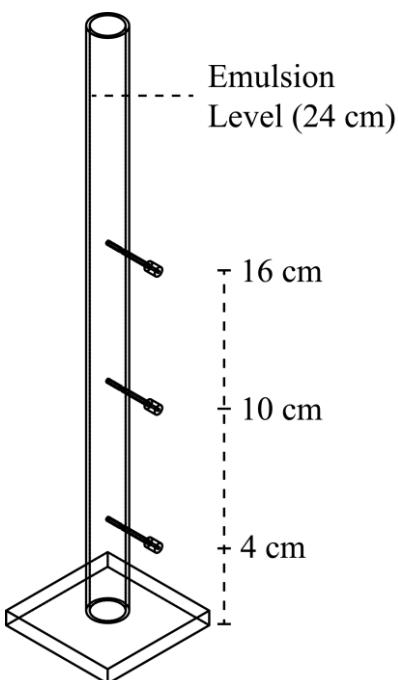


Figure 3.1: Schematic of the graduated cylinder used during *in vitro* assessment of emulsion drug dispersion showing height of access ports.

All emulsion formulations evaluated had a total emulsion tobramycin concentration of 1 mg/mL. In theory, if the tobramycin were homogeneously distributed throughout the emulsion and all tobramycin were recovered during the emulsion inversion process, the assayed aqueous phase would have a tobramycin concentration of 50 $\mu\text{g}/\text{mL}$. Experimental values from this study were normalized by this concentration and are thus reported as a percentage of the theoretical tobramycin content.

3.2.3 Pharmacokinetic Evaluation

Pulmonary delivery of tobramycin (15 mg/kg) was achieved in specific pathogen-free, male Sprague Dawley rats [n = 40, weight 416 ± 16 g (mean \pm standard deviation); Taconic, Hudson, NY, USA] via either intratracheal aerosolized delivery or APV with various emulsion formulations. APV and aerosolized delivery were performed with methods similar those outlined

in section 2.2.6 with minor modifications incorporated in an attempt to improve treatment efficacy or experimental accuracy. Differences between the methods used for each study will be mentioned in the following description. During the pharmacokinetic trials discussed in Chapter 2, the process of repeated intraperitoneal (IP) sedation following recovery (necessary for blood sampling) appeared to cause some distress to the rats. Thus, in the current study intravenous (IV) access was acquired on all rats and a constant IV infusion of ketamine hydrochloride (1 mg/kg/min) was used to maintain sedation for the entire experiment. Vital signs were monitored and body temperature was maintained for all rats as previously described in section 2.2.6. Rats receiving aerosolized treatment were orally intubated with a 16 gauge angiocatheter and placed in a supine position (prone position used during Chapter 2 trials). Intratracheal aerosolized delivery of tobramycin (40 mg/mL aqueous tobramycin concentration, 15 mg/kg) was performed as previously described. Following delivery, rats were extubated and provided supplemental oxygen via a nose cone until euthanasia.

In order to ensure complete and accurate delivery of emulsion during APV, the current trials used a tracheal tie to create a seal around the angiocatheter within the trachea. Following sedation and IV access, the neck was shaved and cleaned with alcohol and a 1 cm midline incision made. The trachea was isolated and a tie positioned loosely around it. The rat was then orally intubated with a 16 gauge angiocatheter and connected to a ventilator (Model 683; Harvard Apparatus, Holliston, MA, USA). Immediately after beginning ventilation the tie was tightened around the trachea, creating a seal around the angiocatheter within it. Ventilation was carried out with the same parameters previously described in section 2.2.6. Preoxygenated emulsion was instilled (15 mL/kg) via a port connected to the angiocatheter, resulting in a delivered dose of 15 mg/kg of tobramycin. Unlike the treatment methods described in section 2.2.6, instillation of

emulsion was performed with the rat a supine position. Filling in a supine position is more similar to what would be clinically implemented and has been shown to result in the most homogenous distribution within the lungs.⁵ The emulsion was prepared no more than five minutes before use and was instilled during gas ventilation in successive aliquots (2-3 mL/aliquot) with each aliquot instilled over a period of approximately 60 seconds. Gas ventilation was continued following delivery of the emulsion until euthanasia.

The delivery methods and emulsion formulations used are summarized in Table 3.1. In order to evaluate the isolated effect of V_{aq} and C_{aq} , emulsions with constant C_{fs} (30 mg/mL H₂O) and varying V_{aq} (1, 2.5, and 5%) were assessed. C_{aq} was varied inversely with V_{aq} in order to maintain a constant delivered dose of tobramycin. Similarly, in order to evaluate the effect of C_{fs} , emulsions with constant V_{aq} (2.5%) and C_{aq} (40 mg/mL) and varying C_{fs} (30 or 96 mg/mL H₂O) were assessed. Additionally, tobramycin delivery via a combination of PFC and aqueous tobramycin without fluorosurfactant was evaluated using a modified APV procedure. Due to the immiscibility of the two phases in the absence of fluorosurfactant, the emulsion delivery procedure described above was slightly modified in order to achieve accurate and consistent doses. For this group, neat PFC was instilled in an identical fashion to that described for emulsion delivery during APV. Next, the angiocatheter was temporarily disconnected from the ventilator and the aqueous

Delivery Method [-]	C_{fs} [mg/mL H ₂ O]	V_{aq} [%]	C_{aq} [mg/mL]	Total Tobramycin Content [mg/mL]
APV	30	1.0	100.0	1
APV	30	2.5	40.0	1
APV	30	5.0	20.0	1
APV	96	2.5	40.0	1
Aerosol + PFC	0	2.5	40.0	N/A
Aerosol	N/A	N/A	40.0	N/A

Table 3.1: Summary of delivery methods and corresponding emulsion formulations used during pharmacokinetic evaluation.

tobramycin phase intratracheally delivered via the Microsprayer. As with the aerosolized delivery group, the angiocatheters used in this treatment were appropriately trimmed in order to ensure uninhibited aerosolized delivery. The rats were then reconnected to the ventilator and the remainder of the experiment carried out as described above.

In all experiments, blood samples were drawn via the lateral tail vein (opposite side of the tail relative to IV infusion) at 30, 60, 120, and 240 minutes following delivery of tobramycin. In treatment groups showing peak serum concentrations at 30 minutes post-delivery, treatment was repeated with blood samples drawn at 10, 20, and 30 minutes post-delivery in order to better determine the time and magnitude of peak concentrations. A hematocrit measurement was taken at the time of each blood sampling and was ultimately used to normalize serum tobramycin measurements to account for hemodilution due to IV fluid delivery. Five rats were analyzed for each set of conditions evaluated.

Rats were euthanized with pentobarbital sodium (175 mg/kg, IP, Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI, USA), the trachea immediately tied off with suture, and the lungs and trachea removed intact. The lungs were then thoroughly homogenized with sterile saline (25 mL/kg bodyweight). Following brief centrifugation, supernatant from homogenized lung tissue samples was collected, serially diluted, and the presence of active tobramycin measured via the microbiological assay previously described in section 2.2.6. Any result below the lower limit of detection (5 µg/mL) was assigned a value of 2.5 µg/mL. Serum tobramycin concentration measurements were performed via an immunoassay by the Laboratory Medicine Department within Allegheny General Hospital (Pittsburgh, PA, USA).

3.2.4 Statistical Analysis

SPSS 22 (IBM Corporation, Armonk, NY, USA) was used to perform all statistical analysis. A one-way ANOVA and *post hoc* analysis was performed on the peak serum tobramycin concentrations and lung tissue tobramycin concentrations. Due to unequal variances between groups, the Games-Howell method was used to determine significant differences. A mixed model analysis was performed to examine differences in the serum tobramycin concentrations with time post-delivery used as the repeated-measure variable. A similar analysis was also used in the *in vitro* stability studies to examine differences in tobramycin concentrations, using time post-preparation and height as the repeated-measure variables. All error bars in presented figures represent standard deviations.

3.3 Results

3.3.1 Emulsion Drug Dispersion

The formation of a distinct aqueous layer of varying sizes was observed at the top of the emulsion column between 0 and 30 minutes post-preparation for all trials performed. Figure 3.2 shows tobramycin content for emulsions ($V_{aq} = 2.5\%$ and $C_{aq} = 40$ mg/mL) with C_{fs} values of 10, 30, and 96 mg/mL H₂O. In Figure 3.2A, tobramycin content is shown as a function of height for each C_{fs} value and time point examined. Figure 3.2B shows the same data as a function of time for each C_{fs} value and height examined. Results showed a significant ($p < 0.01$) effect of C_{fs} on emulsion tobramycin content. Tobramycin content resulting from a C_{fs} of 10 mg/mL H₂O was significantly ($p < 0.01$) lower than that for C_{fs} values of both 30 and 96 mg/mL H₂O. Emulsion utilizing a C_{fs} value of 10 mg/mL H₂O showed insufficient dispersion of antibiotic even immediately following preparation and was thus not evaluated during the *in vivo* pharmacokinetic

experiments. Tobramycin content for C_{fs} values of 30 and 96 mg/mL H₂O did not significantly differ from each other. No significant effect was shown for time post-preparation or height on tobramycin content.

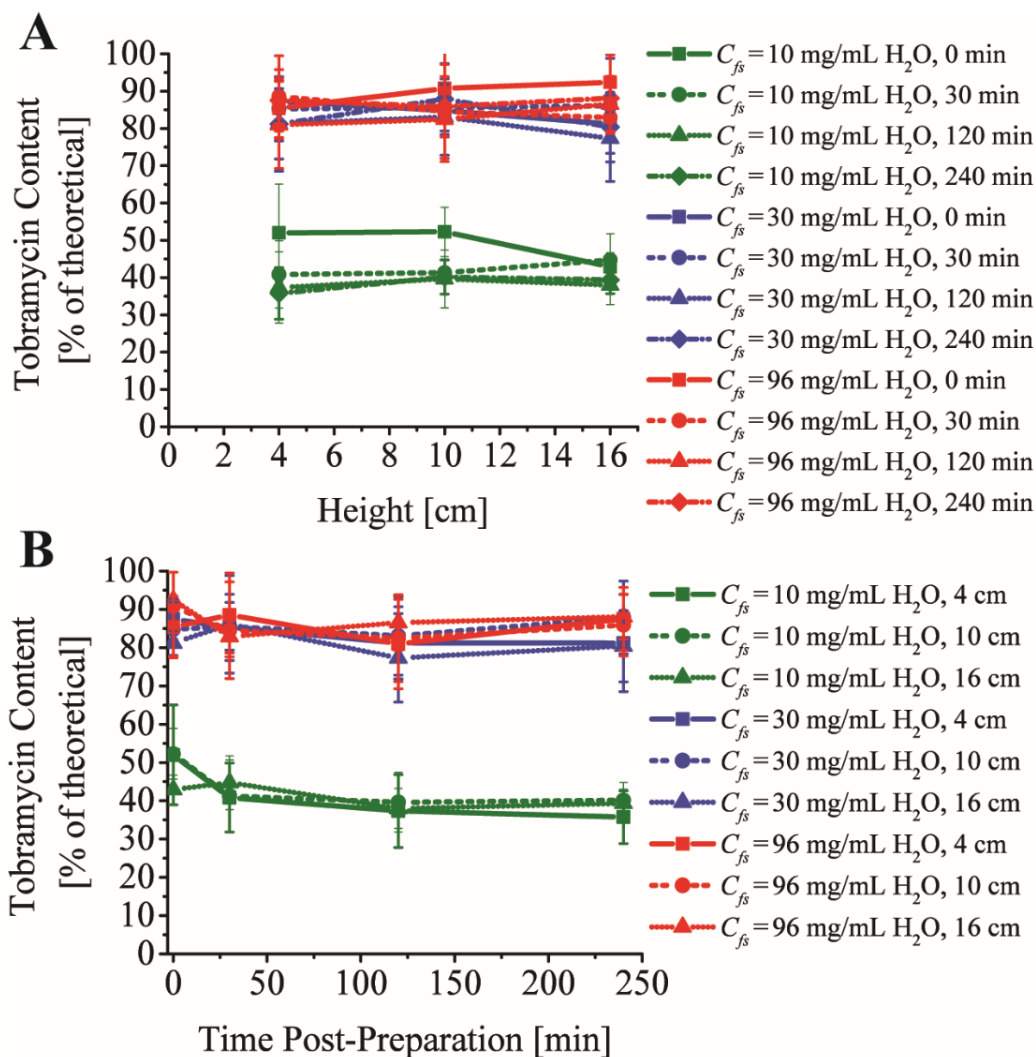


Figure 3.2: Tobramycin content within the emulsion (normalized by theoretical value) as a function of height from the base of the column for varying C_{fs} values and times post-preparation (panel **A**) and as a function of time post-preparation for varying C_{fs} values and heights from the base of the column (panel **B**). $n = 3$ for each condition evaluated.

3.3.2 Pharmacokinetic Effects of Emulsion Formulation

Lung tissue homogenate tobramycin concentrations at four hours post-delivery for all treatments evaluated are shown in Figure 3.3. Two of the five aerosolized delivery trials and all five of the aerosolized delivery in combination with PFC trials resulted in concentrations below the lower limit of detection (5 $\mu\text{g/mL}$). All APV treatments resulted in significantly (either $p < 0.01$ or $p < 0.05$, see Figure 3.3 for distinction) greater pulmonary tobramycin concentrations relative to aerosolized delivery at four hours post-delivery. As shown in Figure 3.3A, pulmonary

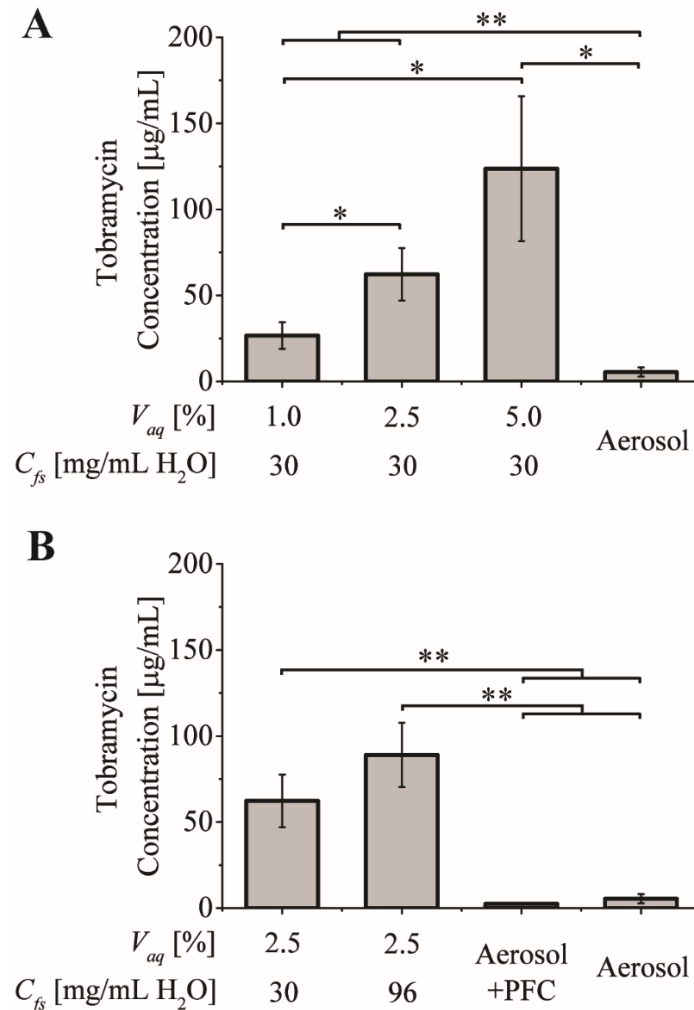


Figure 3.3: Effect of V_{aq} (panel **A**) and C_{fs} (panel **B**) on tobramycin concentration of lung tissue homogenate at four hours post-delivery. $n = 5$ for each condition and statistically significant differences are denoted by an asterisk (* for $p < 0.05$, ** for $p < 0.01$).

tobramycin concentrations increased with increasing V_{aq} (decreasing C_{aq}), with the largest V_{aq} (5%) resulting in 22 times greater concentration than aerosolized delivery. As shown in Figure 3.3B, increased C_{fs} also resulted in larger pulmonary tobramycin concentrations, although the difference between the two C_{fs} values examined was not significant ($p = 0.14$).

Serum tobramycin concentrations for all treatments are shown in Figure 3.4. A V_{aq} of 5% resulted in significantly ($p < 0.01$) lower serum tobramycin concentrations relative to all other groups shown in Figure 3.4A. Similarly, the largest value of C_{fs} (96 mg/mL H₂O) had significantly ($p < 0.01$) lower serum tobramycin concentrations than all other groups shown in Figure 3.4B. The time and magnitude of peak serum concentrations for all treatments evaluated are shown in Table 3.2. Peak serum concentrations for all APV groups occurred at a later time point than for both aerosolized delivery alone and in combination with PFC (60 minutes post-delivery for APV groups vs. 10 minutes post-delivery for aerosol groups). Peak serum concentrations resulting from aerosolized delivery or aerosolized delivery in combination with PFC were greater than all those

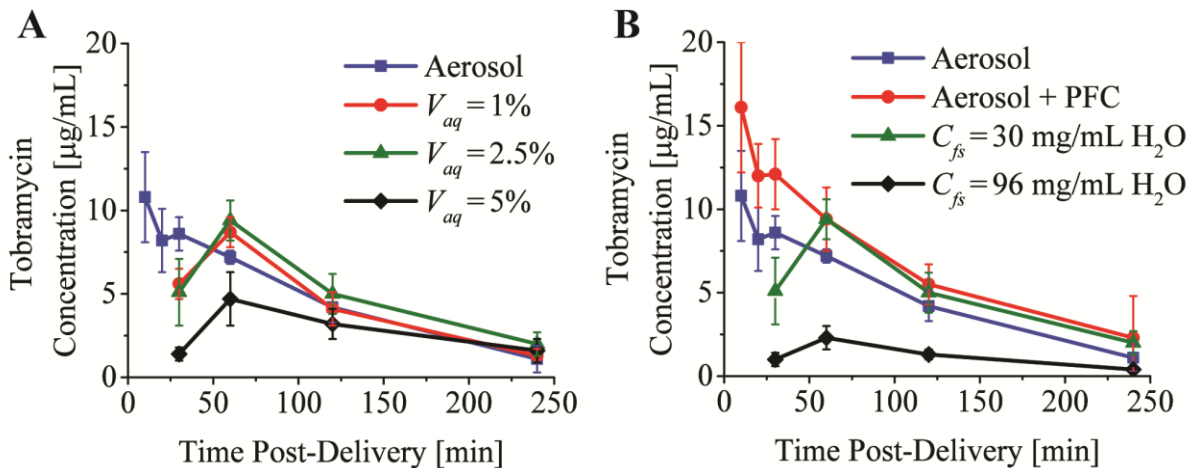


Figure 3.4: (A) Serum tobramycin concentration out to 240 minutes following tobramycin delivery via aerosolized delivery and APV with $C_{fs} = 30$ mg/mL H₂O and varying V_{aq} . (B) Serum tobramycin concentration out to 240 minutes following tobramycin delivery via aerosolized delivery, aerosolized delivery with PFC, and APV with $V_{aq} = 2.5\%$ and varying C_{fs} values. $n = 5$ for each condition evaluated.

Delivery Method [-]	C_{fs} [mg/mL H ₂ O]	V_{aq} [%]	Time to Peak Serum Conc [min]	Peak Serum Conc [μg/mL]
APV	30	1.0	60	8.7 ± 0.9
APV	30	2.5	60	9.4 ± 1.2
APV	30	5.0	60	4.7 ± 1.6
APV	96	2.5	60	2.3 ± 0.7
Aerosol + PFC	0	2.5	10	16.1 ± 3.9
Aerosol	N/A	N/A	10	10.8 ± 2.7

Table 3.2: Time and magnitude of peak serum concentrations following tobramycin delivery. Concentration values are reported as mean ± standard deviation.

produced by APV, although not significantly different in all cases. At the same C_{fs} value (30 mg/mL H₂O), a V_{aq} of 5% produced significantly ($p < 0.01$) lower peak serum concentrations than the 1% and 2.5% V_{aq} groups and aerosolized delivery. Similarly, a C_{fs} of 96 mg/mL H₂O produced significantly ($p < 0.01$) lower peak serum concentrations relative to a C_{fs} of 30 mg/mL at the same V_{aq} (2.5%), aerosolized delivery, and aerosolized delivery in combination with PFC.

3.4 Discussion

Previous work has proven APV to be a viable means of pulmonary antibiotic delivery as well as shown the bactericidal ability of the used emulsions against *PA* biofilms. However, the effects of emulsion formulation on the resulting antibiotic delivery have yet to be fully investigated. Understanding these effects is a vital step in optimizing the emulsion formulation for safe and effective treatment. In this study we sought to evaluate the effects of emulsion formulation (V_{aq} , C_{aq} , and C_{fs}) on the pharmacokinetics of antibiotic delivery via APV. Additionally, an *in vitro* study was also performed to assess the effects of C_{fs} on emulsion stability.

As the long-term biocompatibility of the fluorosurfactants used (or rather any available fluorosurfactants) has not been evaluated, there is warranted caution regarding their use in the

lung. This only highlights the importance of fully understanding the effects of the fluorosurfactant in order to determine the degree to which it can be minimized without impairing effective drug delivery. Results from this work suggest that a C_{fs} value as low as 30 mg/mL H₂O (a nearly 70% reduction from previous APV *in vivo* trials discussed in Chapter 2) still exhibits uniform drug dispersion up to four hours following preparation. As noted in previous work with APV, the emulsion preparation is straightforward and could be performed immediately prior to administration, similar to processes for drug-laden emulsions used in chemoembolization.⁶ Thus, even the reduced value of C_{fs} (30 mg/mL H₂O) evaluated should be able to maintain uniform drug dispersion for the entirety of the intended treatment duration (< 2 hours). This reduction of C_{fs} caused a slight decrease in pulmonary antibiotic retention, but the effect of this retention on the anti-biofilm activity of the emulsion is not yet known.

Similar to work discussed in Chapter 2, the presence of pulmonary antibiotics following treatment was evaluated by measuring the tobramycin concentration of homogenized lung tissue via a microbiological assay. A significant advantage of this detection method is the assurance that the measured tobramycin content is still active and able to exert its intended bactericidal effect. The most clinically relevant measure of antibiotic presence in the lung is likely the aqueous concentration at the airway surfaces, however, such a measurement is difficult to perform in rats. Due to the complex environment present within the airways during APV, it is difficult to speculate how the tobramycin concentrations of lung tissue homogenate relate to the concentrations encountered at the aqueous surfaces of the lung. A greater understanding of the physical transfer of drug from the emulsion to the aqueous surfaces which it encounters would likely best be accomplished in a more controlled, *in vitro* setting and is a primary aim of work discussed in coming chapters. When interpreting the pharmacokinetic results of this work it is also important

to note that emulsion was not suctioned from the lungs prior to euthanasia and subsequent tissue tobramycin measurements. This differs from the clinical scenario in which some portion of the emulsion, along with dislodged mucus and biofilm, would be suctioned from the lungs within two hours of delivery. The volume of emulsion remaining in the lungs at the time of euthanasia was not measured in this work. However, a previous study evaluating partial liquid ventilation (PLV) with neat PFC in similarly sized rats noted that the addition of 0.8 mL of PFC every 30 minutes was needed to maintain a constant PFC volume in the lungs.⁷ Although the evaporative loss rate for PFC during PLV is known to decrease with time following delivery, clinical studies have shown that the rate remains fairly constant up to eight hours post-delivery.⁸ The average amount of emulsion instilled during this study was 6.2 mL. Thus, assuming a near-constant evaporative rate over the duration of the experiment (four hours), one would expect the PFC phase of the emulsion to have largely evaporated at the time of euthanasia and tissue tobramycin measurement. In addition, although efforts were not made to recover or measure any PFC that may have been present in the homogenized tissue, PFC was not typically observed during homogenization or the subsequent analysis.

Similar to the pharmacokinetic work discussed in Chapter 2, increased pulmonary antibiotic concentrations were measured following APV (all emulsion formulations) relative to aerosolized delivery. Aerosolized delivery in combination with PFC showed low retention similar to aerosolized delivery alone. During such delivery, the aerosol droplets likely initially deposited on the epithelium or PFC surface. Any further dispersion of the aqueous drug would have relied solely on the mixing induced by tidal gas flow once ventilation was resumed. These results suggest that the increased retention of delivered drug observed during APV is a result of the initial dispersion of the aqueous drug throughout the PFC, the presence of the fluorosurfactant, or the

combination of the two. Although ventilation with PFC has been shown to alter the distribution of pulmonary perfusion,⁹ the similar lack of retention between aerosolized delivery alone and in the presence of PFC would suggest that PFC-induced differences in pulmonary blood flow are likely not the primary cause of the retention associated with APV. Regarding effects of emulsion formulation, increasing either V_{aq} or C_{fs} was shown to increase pulmonary retention, although the effect of V_{aq} was much stronger. A five-fold increase in V_{aq} (and corresponding five-fold decrease in C_{aq}) resulted in a nearly identical increase in pulmonary tobramycin content four hours post-delivery. In the case of C_{fs} , an approximately three-fold increase resulted in a less than 50% increase in pulmonary retention. These results indicate that increased V_{aq} in combination with decreased C_{aq} slows diffusion of tobramycin from the airways into the pulmonary circulation. Antibiotics are believed to move between the bronchial space and blood at a rate dependent on the concentration gradient between the two compartments.¹⁰ During APV, as aqueous droplets come in to contact and combine with the aqueous lining of the airways, the local antibiotic concentration near the epithelium increases thus increasing diffusion into the blood. The rate at which this occurs is determined by both the frequency of droplet deposition as well as the antibiotic concentration within each droplet (*i.e.* C_{aq}). An increase in V_{aq} increases the number of droplets and as such could increase the frequency of droplet deposition. However, these results suggest that increased V_{aq} does not increase droplet deposition enough to compensate for the decreased antibiotic mass within each droplet, leading to an overall slowed diffusion for increased V_{aq} and decreased C_{aq} .

Although not as profound as with V_{aq} , increases in C_{fs} also showed increased pulmonary antibiotic retention at four hours post-delivery. Additionally, serum tobramycin concentrations resulting from the largest C_{fs} value (96 mg/mL H₂O) were significantly lower than those at 30 mg/mL H₂O, further suggesting delayed antibiotic absorption into the pulmonary circulation for

increased C_{fs} . The cause of this diminished absorption at increased values of C_{fs} is not yet fully understood. *In vitro* assessment of emulsion stability showed no difference between C_{fs} values of 30 and 96 mg/mL H₂O (both emulsions exhibited uniform antibiotic distribution) and thus suggests that the observed pharmacokinetic effect of C_{fs} is likely not due to gravity-driven phase separation. However, it is possible that coalescence and creaming is altered for emulsion in the airways at physiological temperatures and under constant ventilation as compared to our *in vitro* experiment. Increased phase separation within emulsion in the airways for the 30 mg/mL H₂O group could accelerate movement of emulsified droplets out of the bulk of the emulsion and in to contact with the epithelial surfaces through which tobramycin will ultimately leave the lungs. Alternatively, the diminished absorption effects could be due to the accumulation of fluorosurfactant at the interface between the emulsion and airway aqueous lining. The fluorosurfactant molecules possess both hydrophilic and fluorophilic moieties and thus aggregate at aqueous-PFC interfaces. It is possible that some amount of fluorosurfactant accumulates at the boundary of the lung aqueous lining and impairs either the deposition of droplets or the diffusion of tobramycin following deposition. Further in-depth studies are needed to fully understand the effects of fluorosurfactant on drug transport from the emulsion in a physiological setting.

Considering the results of this study, the optimal emulsion formulation for APV may utilize a significantly reduced C_{fs} value as compared to previous *in vivo* work. Additionally, pulmonary retention of the delivered antibiotic can be most effectively increased by increasing V_{aq} and decreasing C_{aq} to achieve the desired delivery dose. However, it is unknown whether the rate of tobramycin delivery from the airway to the tissue has a significant effect on anti-biofilm activity and thus treatment efficacy. Studies comparing rapid, nearly complete delivery of the antibiotic must be compared to slower, controlled delivery. Whatever the outcome, the ability to reliably

control such kinetics represents an attractive quality of pulmonary drug delivery via water-in-PFC emulsions. There are multiple factors to consider when choosing an optimal V_{aq} and C_{aq} for treatment. As C_{aq} is decreased, a scenario could be approached in which the aqueous concentration of antibiotics in the lung during APV is less than that required for bacterial killing. Previous *in vitro* work (discussed in Chapter 2) has shown that emulsions utilizing C_{aq} values as low as 4 mg/mL (five-fold smaller than the lowest C_{aq} value used in the present study) were still able to reduce proliferation of biofilm-derived bacteria following exposure. Additionally, as V_{aq} is increased, larger aqueous volumes will be delivered to the lung during APV. Therefore caution should be used when determining V_{aq} as to not increase this value to the point of inhibiting normal respiratory function. If the V_{aq} values evaluated in this study (1-5%) were to be used clinically during partial APV with a 15 mL/kg dose of emulsion (dose used in previous clinical trials of PLV), a 70-kg patient would receive approximately 10-50 mL of aqueous antibiotic delivered to the lung. However, at the conclusion of treatment, some portion of the emulsion would be suctioned from the lung. Conservatively estimating that only 1/3 of the emulsion volume would be removed from the lung, 7-35 mL of aqueous antibiotic would ultimately remain in the lung. During currently used nebulized tobramycin treatment for cystic fibrosis patients, approximately 10 mL of aqueous antibiotics are delivered to the lung on a daily basis.¹¹ Considering that such treatment is generally well tolerated without supplemental oxygen or ventilatory support, the range of V_{aq} values evaluated in this work are likely within reason for a patient on mechanical ventilation.

The current study has confirmed the use of APV as a viable means of pulmonary antibiotic delivery and taken critical steps in further developing this emerging technology. APV has demonstrated the ability to significantly increase the pulmonary retention of delivered antibiotics relative to currently used methods in a predictable and controllable manner. However, the

therapeutic impact of this increased retention must be further investigated to ultimately optimize the emulsion formulation for effective APV treatment.

3.5 References

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CHAPTER 4

***In Vitro* Evaluation of the Effects of Emulsion Formulation on Cytotoxicity and Drug Availability**

4.1 Introduction

The emulsions used during antibacterial perfluorocarbon ventilation (APV) consist of aqueous, drug-containing droplets dispersed in a continuous perfluorocarbon (PFC) phase ($\geq 95\%$ by volume) via the use of fluorosurfactants. The fluorosurfactant molecules serve as an emulsifier to stabilize the aqueous droplets and therefore must possess both hydrophilic and fluorophilic moieties in order to assemble at the aqueous-PFC interface. In the absence of such an emulsifier, the aqueous droplets almost instantaneously coalesce and rise out of solution (due to buoyancy) following sonication of the two phases, thereby hindering the ability to achieve spatially uniform drug delivery during APV. In support of this, previous *in vitro* work evaluating the stability of such emulsions (discussed in Chapter 3) has shown that as fluorosurfactant concentration (C_{fs}) is decreased below some critical value, the ability to maintain a spatially uniform distribution of drug is lost.

Although previous work has characterized the pharmacokinetics of APV (discussed in Chapter 3), the physical process by which drug is transferred from aqueous droplets within the emulsion to the aqueous surfaces within the lung (*i.e.* epithelial lining fluid, biofilm, or mucus) is

still not completely understood. Because tobramycin is insoluble in PFC, the drug will not be available to diffuse into an aqueous surface in contact with the emulsion until a droplet has moved sufficiently close enough to the liquid interface to coalesce with it. It is likely that some portion of droplets undergo this process during filling of the lung at the initiation of APV. Following the filling process, droplet motion within the emulsion-filled lung is likely dominated by convective forces induced by the tidal fluid movement of continuous ventilation. As PFC evaporates during ventilation, the droplet number density (*i.e.* the number of droplets within a finite volume of emulsion) increases and contact between the droplets and airway surfaces would be expected to increase.

If the emulsion is left to completely evaporate within the lung, one would expect virtually all tobramycin within the emulsion to be brought in to contact with an aqueous surface and ultimately be available for diffusion into a biofilm mass or the systemic circulation. However, previous studies evaluating the pharmacokinetics of APV in healthy rats (discussed in Chapter 3) have produced seemingly contradictory findings. Results from these studies showed that APV resulted in significant amounts of tobramycin remaining in the lungs at four hours post-delivery (up to 22 times that of aerosolized delivery), yet serum concentrations at the same time point were approaching zero, similar to those for aerosolized delivery. Although the volume of emulsion in the lung at the time of euthanasia (four hours post-delivery) was not explicitly measured, as discussed in section 3.4, the majority of emulsion is expected to have evaporated by this time. Similarly, treatment efficacy studies evaluating APV (to be discussed in Chapter 5) have also produced unexpected results. Although relatively large amounts of tobramycin remain in the lung long after delivery, APV has failed to show a reduction in pulmonary bacterial load in a rat model

of respiratory infection. Such findings raise questions regarding the availability of drug delivered via the emulsion during APV.

One possible explanation for these observations is the impairment of droplet deposition or drug diffusion following deposition by the accumulation of fluorosurfactant at the interface between the emulsion and the aqueous surface for intended delivery. As previously discussed, the fluorosurfactant molecules are specifically designed to effectively assemble at aqueous-PFC interfaces (*i.e.* the droplet boundary) and prevent or delay the coalescence of neighboring droplets. However, it is likely that this same effect is induced to some degree at the boundary between the emulsion and aqueous surfaces in the lung. Previous *in vitro* and *in vivo* work with the emulsion has provided compelling evidence in support of such phenomena. During rheological characterization of the emulsion (discussed in Chapter 2), the interfacial tension between the emulsion and an aqueous phase resting on top of it was shown to be significantly reduced as compared to that of neat PFC. This effect was attributed to the accumulation of fluorosurfactant at the interface between the emulsion and bulk aqueous phase. Additionally, previous pharmacokinetic results showed increased pulmonary retention and decreased systemic absorption for tobramycin delivered via emulsions utilizing larger C_{fs} . Thus, it appears that the fluorosurfactant used in the emulsion may have significant effects on the availability of delivered drug within the lung. Considering this, the ideal C_{fs} for any particular fluorosurfactant to be used during APV should be determined with not only biocompatibility and emulsion stability in mind, but also drug availability. With regards to emulsion stability and drug availability, the optimal C_{fs} may lie between some low value at which significant phase separation begins to occur and a higher value at which the fluorosurfactant limits tobramycin delivery.

The primary goal of this study was to evaluate the effects of C_{fs} on drug availability and cytotoxicity for two different fluorosurfactant types in a controlled, *in vitro* setting. More specifically, we aimed to determine the ideal fluorosurfactant type and corresponding non-toxic C_{fs} that optimizes the diffusion of tobramycin from the emulsion into an aqueous surface in contact with the emulsion. A previously unused fluorosurfactant with a varied molecular structure was explored in this study. The fluorosurfactants used in all previously described APV work are based off of the low molecular weight form of Krytox 157FS, referred to as FSL (MW = 2,500 Da). As mentioned in section 1.7, FSL-PEG exhibited insufficient surface activity to stabilize a water-in-PFC emulsion when used alone, thus it was used in combination with equal masses of the unmodified molecule (FSL) in this work. The structure of these molecules can be seen in Figure 1.6. In addition to this previously used fluorosurfactant combination, a modified form of the fluorosurfactant, termed FSH-PEG, was also evaluated. FSH-PEG is synthesized in an identical manner as that for FSL-PEG (described in section 1.7) but utilizes the larger molecular weight form of Krytox 157FS, referred to as FSH (MW = 7,250 Da), as well as a lower molecular weight PEG block (MW = 1,000 Da). Ultimately, these modifications resulted in an increase in the fluorophilic portion of the molecule relative to the hydrophilic portion. This is most easily recognized by comparing the ratios of the molecular weight of the fluorophilic blocks to the hydrophilic block within each polymer. Such ratios are 1.5:1 and 14.5:1 for FSL-PEG and FSH-PEG, respectively. As a result, FSH-PEG proved to be surface active enough to be used alone rather than in combination with an unmodified form of the molecule as was done with FSL-PEG.

As shown in experiments discussed in Chapter 3, as C_{fs} is decreased for a particular fluorosurfactant type, a critical concentration will be reached below which aqueous drug will begin to separate out of solution. In the case of phase separation, the amount of drug dispersed throughout

the bulk of the emulsion may be significantly diminished. Thus, when interpreting the diffusion of drug from an emulsion sample taken from a bulk volume, one must consider the mass of drug present within the sample in addition to the relative availability of the drug. In order to differentiate between decreased drug diffusion due to impairment caused by fluorosurfactant versus diminished diffusion caused by the absence of drug, a secondary experiment quantifying the presence of tobramycin within each emulsion sample was also included.

As previously mentioned, the fluorosurfactant type and corresponding C_{fs} used during APV are also expected to have significant effects on biocompatibility. Although fluorosurfactants similar to those used in this work have exhibited favorable biocompatibility in cell-based applications (discussed in section 1.7), APV's use of the molecules differs in significant ways. Previous applications were not related to the lung and thus did not evaluate relevant cell types. In addition, during APV, epithelial cells will likely be in regular contact with PFC containing dissolved fluorosurfactant. During previous uses of the fluorosurfactant, cells were primarily contained within aqueous vesicles emulsified in PFC.^{1, 2} Due to the varying solubility of the fluorosurfactants in the two phases as well as its tendency to assemble at an aqueous-PFC interface, such differences could significantly affect cytotoxicity. Therefore, when determining an optimal fluorosurfactant type and concentration, effects on emulsion stability and drug availability must be considered in combination with potential cytotoxic effects. Thus, in addition to evaluating effects on drug availability, a separate experiment investigating the cytotoxicity of the fluorosurfactants was also performed.

Lastly, the effects of aqueous volume percent (V_{aq}) and aqueous tobramycin concentration (C_{aq}) on tobramycin availability were also assessed. Previous pharmacokinetic results (discussed in Chapter 3) have shown significant effects of these parameters on the pulmonary retention of

delivered tobramycin. Thus, in order to ultimately determine an optimal emulsion formulation, the effects of V_{aq} and C_{aq} on drug emulsification and availability must be assessed in the same manner as those of fluorosurfactant type and concentration.

The availability of emulsified tobramycin for varying emulsion formulations was assessed by measuring its ability to readily diffuse into an agar surface in contact with the emulsion. In conjunction with this experiment, identical emulsion samples underwent a similar process as that described in section 3.2.2 in order to quantify the presence of tobramycin. The measured drug content of the emulsion samples serves to clarify the cause of the observed drug diffusion for any particular emulsion formulation. That is, in order to completely interpret the observed drug diffusion for a particular emulsion sample taken from a larger volume, one must know whether drug content has been diminished due to emulsion instability and phase separation prior to sampling. The cytotoxicity of the fluorosurfactants was evaluated with human alveolar epithelial cells using common *in vitro* techniques. The evaluation of these varying aspects should afford a comprehensive understanding of the effects of emulsion formulation and provide the insight needed to ultimately determine an optimal fluorosurfactant type, C_{fs} , V_{aq} , and C_{aq} to assess in further *in vivo* treatment efficacy studies.

4.2 Methods

4.2.1 Emulsion Preparation

Tobramycin-loaded, water-in-PFC emulsions were prepared with the same equipment and sonication settings as those described in section 3.2.1 using two different fluorosurfactant types. Similar to previous studies, emulsions utilizing a combination of equal masses of FSL-PEG and FSL were assessed. Although synthesized in an identical manner, it should be noted that a new

batch of FSL-PEG was used for the current study relative to all previous work with the emulsions. Emulsions using FSH-PEG, a previously unevaluated fluorosurfactant, were also assessed. As mentioned in section 1.7, the FSH-PEG polymer was synthesized in an identical manner to that for FSL-PEG but utilizes different sized perfluoroether and PEG polymer blocks. The fluorosurfactant concentrations and molarities discussed in this work take in to account the total amount fluorosurfactant used for any particular emulsion formulation. Thus, in the case of emulsion formulations using FSL-PEG and FSL, the specified concentrations or molarities are based on the sum of the masses or moles of each molecule. Similar to previous work with APV, the PFC used in all experiments was perfluorocycloether/perfluorooctane (FC-770; 3M Inc., St. Paul, MN, USA).

4.2.2 Evaluation of Drug Emulsification and Availability

The relative availability of emulsified tobramycin to an aqueous surface in contact with the emulsion was quantified via the commonly used agar well diffusion method. During such a measurement, a liquid sample of unknown antibiotic concentration is dispensed into a well within inoculated agar and allowed to completely evaporate during subsequent incubation. The antibiotic within the sample diffuses into the agar and creates a circular zone of inhibited bacterial growth with a radius proportional to the unknown antibiotic concentration. When performed in conjunction with samples of known antibiotic concentrations, a concentration for the unknown sample can then be determined via interpolation.

Initially, in order to isolate the effects of fluorosurfactant type and concentration, emulsions with fixed V_{aq} (2.5%) and C_{aq} (40 mg/mL) were examined with C_{fs} values of 0.5-96 mg/mL H₂O for each of the two fluorosurfactant types. Following this evaluation, the effects of V_{aq} and C_{aq}

were assessed by examining emulsions with $C_{fs} = 2$ mg/mL H₂O of FSH-PEG, $V_{aq} = 0.5$ -2.5%, and $C_{aq} = 40$ -200 mg/mL. The specific emulsion formulations used are shown in Table 4.1. Similar to previous studies, V_{aq} and C_{aq} were varied inversely to maintain a constant total tobramycin content of 1 mg/mL of emulsion.

Fluorosurfactant Type [-]	C_{fs} [mg/mL H ₂ O]	V_{aq} [%]	C_{aq} [mg/mL]	Total Tobramycin Content [mg/mL]
FSL-PEG+FSL	0.5	2.5	40	1
FSL-PEG+FSL	2.0	2.5	40	1
FSL-PEG+FSL	4.0	2.5	40	1
FSL-PEG+FSL	10.0	2.5	40	1
FSL-PEG+FSL	30.0	2.5	40	1
FSL-PEG+FSL	96.0	2.5	40	1
FSH-PEG	0.5	2.5	40	1
FSH-PEG	2.0	2.5	40	1
FSH-PEG	4.0	2.5	40	1
FSH-PEG	10.0	2.5	40	1
FSH-PEG	30.0	2.5	40	1
FSH-PEG	96.0	2.5	40	1
FSH-PEG	2.0	0.5	200	1
FSH-PEG	2.0	1.0	100	1

Table 4.1: Emulsion formulations evaluated during assessment of drug emulsification and availability.

For each formulation evaluated, approximately 10 mL of emulsion was prepared in a 15-mL conical tube and allowed to sit at room temperature without agitation for five minutes before sampling. Three samples (75 μ L/sample) of emulsion were taken from the middle height of the emulsion (midway between the conical bottom and emulsion surface). Samples were taken from the middle height of the emulsion in order to assess the effects of creaming and phase separation. Previous emulsion stability evaluation (discussed in Chapter 3) has shown that drug content at the middle height of the emulsion should be indicative of content throughout the bulk of the emulsion. Emulsion samples were placed directly into wells within previously inoculated agar. LB agar

plates inoculated with *Pseudomonas aeruginosa* (PA) were prepared using those methods described in section 2.2.6. Aqueous samples of known tobramycin concentration were also loaded into identical wells for later comparison. Plates were then incubated at 37° C for 24 hours, thereby allowing the sample volume (either emulsion or aqueous tobramycin) within each well to completely evaporate. Following incubation, circular inhibition zones produced by tobramycin that had diffused into the agar were imaged and measured as described in section 2.2.6. Bacterial inhibition zones produced by some emulsion formulations had a secondary zone of affected growth outside of the inner zone of completely inhibited growth. An example of this effect can be seen in Figure 4.1A. This same effect was observed for some aqueous tobramycin solutions as well. For both emulsion and aqueous tobramycin solutions, this phenomenon was observed primarily for those trials resulting in larger inhibition zones. A similar effect has also been observed in previous studies by other investigators using the agar well diffusion method.³ Measurement of the inner inhibition zone was used for all further analysis. Interpolation from the inhibition zones of the standard solutions was then used to determine equivalent aqueous tobramycin concentrations (*i.e.*

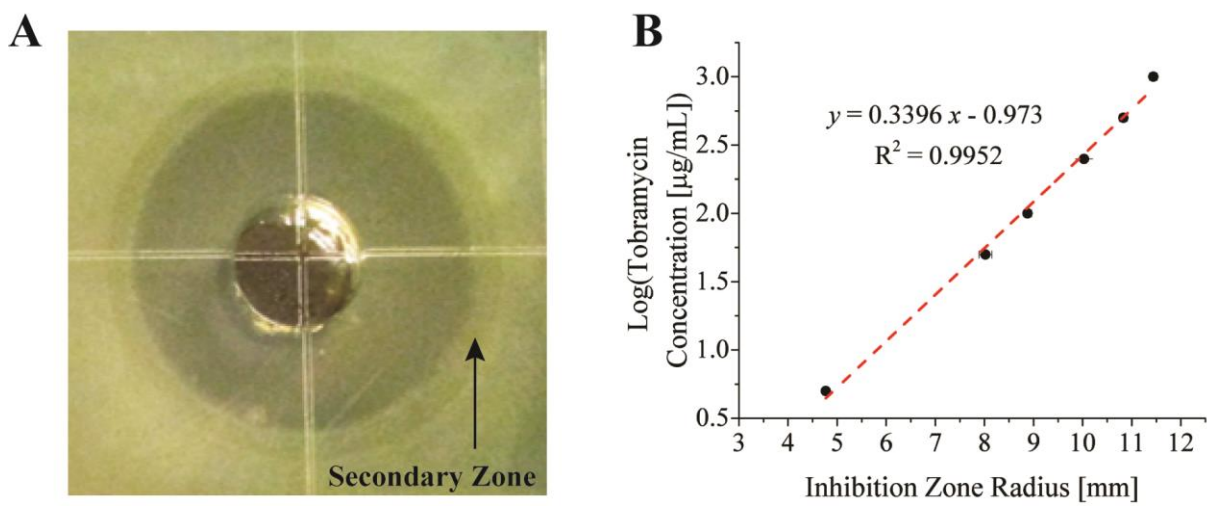


Figure 4.1: (A) Example of dual bacterial inhibition zones observed during evaluation of drug emulsification and availability. (B) Standard curve used during availability assessment ($n = 3$). Note x -error bars are included but may be too small to distinguish.

the aqueous tobramycin concentration resulting in an identical inhibition area) for each emulsion evaluated. Similar to methods described in Chapter 2, linear regression analysis of the aqueous standards was obtained by plotting the log transformation of the known tobramycin concentrations versus inhibition zone radii. However, different from methods described in Chapter 2, a separate set of standards was not included on each agar plate for each measured sample. The larger size of inhibition zones in the current experiment required the aqueous standards to be performed on a separate plate from the emulsion samples. Although such a modification may be expected to increase measurement variability, consistency among standards was high as can be observed by the small error bars (x -direction) in the plot of standard solutions in Figure 4.1B. The set of aqueous standards were measured in triplicate on separate agar plates. An aqueous tobramycin solution with a concentration of 5 $\mu\text{g/mL}$ produced the smallest measurable inhibition zone. Emulsion samples resulting in immeasurably small inhibition zones were assigned an equivalent aqueous tobramycin concentration of 2.5 $\mu\text{g/mL}$. Each prepared emulsion was tested in triplicate and averaged to produce a single measurement for each trial. Three separate trials were performed for each emulsion formulation evaluated.

In order to further confirm the role of fluorosurfactant on drug availability, a modified exposure procedure from that described above was also performed. Samples from an emulsion with a formulation exhibiting the optimal combination of emulsion stability and drug availability were taken in an identical manner as that described above. Prior to the addition of samples to the agar wells, the wells were filled (75 $\mu\text{L/well}$) with a solution of FSH-PEG dissolved in PFC (2.4 mg/mL , contained no drug or aqueous phase) and allowed to rest at room temperature for 10 minutes. The concentration of FSH-PEG in PFC used for the pre-exposure is equivalent to an emulsion ($V_{aq} = 2.5\%$) with the maximum C_{fs} value examined (96 $\text{mg/mL H}_2\text{O}$) with no aqueous

phase present. Following the exposure, the FSH-PEG in PFC solution was thoroughly aspirated from each well and the emulsion samples added. Agar plates were then incubated and processed in an identical manner as that described above.

A decreased inhibition zone in the experiments described above represents a decrease in the mass of tobramycin diffusing into the agar. The decreased diffusion could be due to either direct effects of the fluorosurfactant or decreased drug content due to phase separation occurring within the bulk emulsion volume prior to sampling. In order to distinguish between decreased diffusion due to impaired drug availability versus decreased drug presence caused by phase separation, the presence of tobramycin within the emulsion was quantified in conjunction with all diffusion trials described above. This evaluation used the identical agar well diffusion method described above following an initial phase inversion and separation process (similar to that used during drug dispersion evaluation described in section 3.2.2). More specifically, two samples (240 $\mu\text{L}/\text{sample}$) of emulsion were taken in an identical manner as that described above (taken five minutes post-sonication from middle height). The samples were then diluted with 8 mL of sterile water and re-sonicated at 200 W/cm^2 for one minute, resulting in an inverted emulsion (continuous aqueous phase with dispersed PFC phase). The inverted emulsion was then centrifuged at 3,000 $\times g$ for 20 minutes in order to separate the PFC and aqueous phases. Samples from the aqueous phase were then loaded into wells (75 $\mu\text{L}/\text{well}$) within the inoculated agar along with standard solutions of known tobramycin concentration and assayed as described above. Separate sets of aqueous standards were included on each individual plate with each sample measured. Each sample was measured in triplicate and the resulting values averaged to produce a single concentration. The concentration for each of the two samples for a given emulsion were averaged to produce a single value for each trial and three separate trials performed for each emulsion formulation. All emulsion

formulations evaluated had a total emulsion tobramycin concentration of 1 mg/mL. In theory, if the tobramycin were homogeneously distributed throughout the emulsion and all tobramycin was recovered during the emulsion inversion process, the assayed aqueous phase would have a tobramycin concentration of 30 µg/mL. Experimental values from this study were normalized by this concentration and are thus reported as a percentage of the theoretical tobramycin content. Samples from emulsions using either fluorosurfactant type at the largest C_{fs} value (96 mg/mL H₂O) with sterile saline in the aqueous phase were also examined via the methods described for both drug emulsification and availability as negative controls.

4.2.3 Cytotoxicity Evaluation

The relative cytotoxicity of the fluorosurfactants was evaluated using human alveolar basal epithelial cells (A549 cells; Sigma Aldrich, St. Louis, MO, USA). Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37° C in an incubator with 5% CO₂. A 96-well plate (Nunc MicroWell, Model 266120; Thermo Scientific, Waltham, MA, USA) was seeded at 10,000 cells/well (200 µL/well complete growth medium) and allowed to grow overnight. Following overnight growth, the growth medium was aspirated from each well and replaced with either sterile phosphate-buffered saline (PBS), neat PFC, fluorosurfactants dissolved in PFC, or emulsion containing sterile PBS in the aqueous phase (200 µL/well). The PFC used in all cytotoxicity experiments was filtered with a 0.2 µm-filter prior to use in order to reduce the probability of contamination.

Initial evaluation utilized solutions of fluorosurfactant dissolved in PFC rather than emulsions containing an aqueous phase to ensure that emulsion stability did not affect the toxicity

measurements. In an emulsion, lower C_{fs} would lead to greater phase separation, transporting surfactant up and away from the cells and thus exaggerating reductions in toxicity at lower C_{fs} . Both FSL-PEG+FSL and FSH-PEG were evaluated at molar concentrations of 10^{-8} to 10^{-3} mol/L. For FSL-PEG+FSL, these concentrations are the sum of the molar concentrations of each FSL-PEG and FSL in mixtures containing equal masses of each molecule. Ultimately, cytotoxicity evaluation was repeated for an emulsion with the formulation exhibiting the optimal combination of emulsion stability and drug availability.

Following addition of the exposure medium, the plates were returned to the incubator for a period of six hours. After incubation, the exposure medium was removed and replaced with complete growth medium (200 μ L/well) and the cells allowed to grow overnight. Approximately 18 hours following the conclusion of the exposure, the viability of the cells was assessed using an alamar blue-based cell viability assay (alamarBlue; Life Technologies, Carlsbad, CA, USA). Following the addition of alamarBlue, the cells were returned to the incubator for a period of four hours prior to performing absorbance measurements at wavelengths 570 and 600 nm. Preliminary studies (data not shown) showed that a four-hour incubation time following the addition of alamarBlue combined with a seeding density of 10,000 cells/well resulted in the greatest assay sensitivity. The percent reduction of added alamarBlue was calculated from measured absorbance values according to the manufacturer's instructions and is an indicator of cellular proliferation and metabolic activity (increased reduction indicates increased viability). The viability of cells exposed to fluorosurfactants dissolved in PFC or emulsion was normalized by the viability of those cells exposed to neat PFC within the same 96-well plate. Eight wells were used for each type of exposure and ultimately averaged to produce a single measurement for each trial. Four independent trials

were performed for each condition evaluated resulting in four independent measurements. Control exposures (PBS and neat PFC) were repeated with each individual experiment.

4.2.4 Statistical Analysis

SPSS 22 (IBM Corporation, Armonk, NY, USA) was used to perform all statistical analysis. A two-way ANOVA was used to determine significant effects for the tobramycin content and availability data. The same analysis was used in the cytotoxicity evaluation to determine the significance of the effects of fluorosurfactant type and C_{fs} on cell viability. The Bonferroni method was applied to determine significance between comparisons of interest. An independent t -test was used to determine the effect of pre-exposing the agar well to FSH-PEG on tobramycin availability. An identical analysis was also performed to compare cell viability of cells exposed to emulsion to that of cells exposed to neat PFC. All subsequent reported values are given as mean \pm standard deviation and all error bars in presented figures represent standard deviations.

4.3 Results

4.3.1 Emulsion Formulation Effects on Drug Emulsification and Availability

Samples from negative control emulsions with 96 mg/mL H₂O of either fluorosurfactant type containing no tobramycin produced no bacterial inhibition zones. Emulsion tobramycin content and relative availability are shown in Figure 4.2A and 4.2B, respectively, for emulsions ($V_{aq} = 2.5\%$ and $C_{aq} = 40$ mg/mL) using either FSH-PEG or FSL-PEG+FSL. In both fluorosurfactant groups, tobramycin content at the lowest C_{fs} (0.5 mg/mL H₂O) resulted in values less than the lower limit of detection in two of three trials. During evaluation of drug availability,

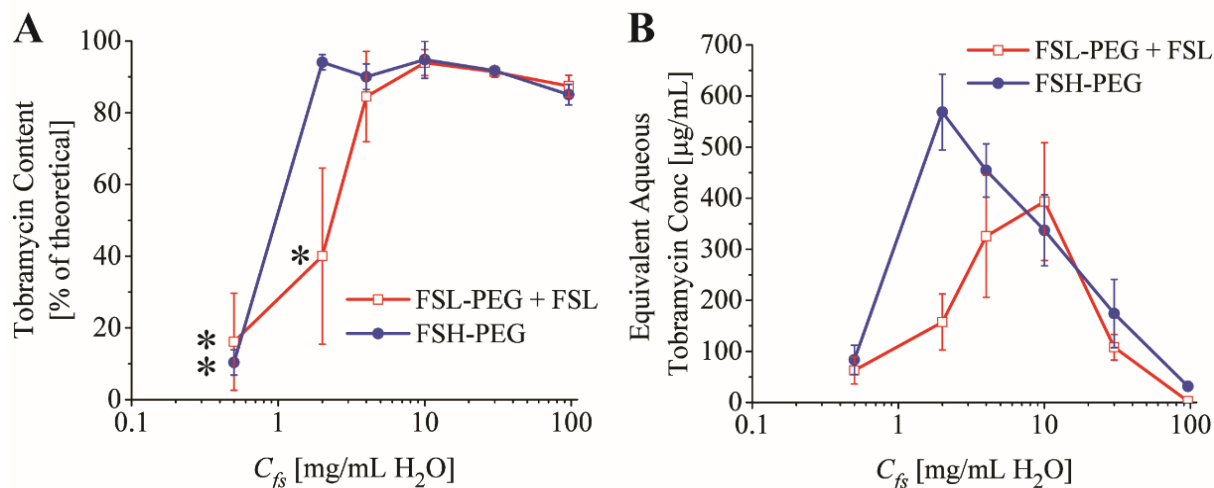


Figure 4.2: Tobramycin content (normalized by theoretical value; shown in panel **A**) and availability (as indicated by equivalent aqueous tobramycin concentration; shown in panel **B**) as a function of C_{fs} for emulsions utilizing $V_{aq} = 2.5\%$, $C_{aq} = 40$ mg/mL, and FSL-PEG+FSL or FSH-PEG. $n = 3$ for each condition and an asterisk denotes statistically significant ($p < 0.01$) differences.

samples from emulsion using FSL-PEG+FSL at the largest C_{fs} (96 mg/mL H₂O) produced immeasurably small inhibition zones in all three trials.

Tobramycin content (Figure 4.2A) was greater than 80% of the theoretical value at the larger C_{fs} values examined for both fluorosurfactant types. However, content diminished as C_{fs} decreased. For emulsions utilizing FSL-PEG+FSL, tobramycin content was shown to significantly decrease ($p < 0.01$) at $C_{fs} \leq 2$ mg/mL H₂O. For FSH-PEG, tobramycin content was significantly decreased ($p < 0.01$) only for the lowest C_{fs} value examined (0.5 mg/mL H₂O). Because of the greater stability at lower C_{fs} , tobramycin content was found to be statistically greater ($p < 0.01$) for the FSH-PEG emulsions.

Tobramycin availability as indicated by drug diffusion (Figure 4.2B) was lowest at both high and low C_{fs} , reaching a peak at intermediate values. The largest measured tobramycin availability had an equivalent aqueous tobramycin concentration of 393 ± 115 µg/mL at $C_{fs} = 10$

mg/mL H₂O for FSL-PEG+FSL and 569 ± 74 $\mu\text{g/mL}$ at $C_{fs} = 2$ mg/mL H₂O for FSH-PEG. Tobramycin availability was found to be statistically greater ($p < 0.01$) for emulsions utilizing FSH-PEG as compared to those utilizing FSL-PEG+FSL when considering all values of C_{fs} . Measured tobramycin availability was lowest at the maximum C_{fs} value examined (96 mg/mL H₂O) for both fluorosurfactant types.

Pre-exposure of the agar well lumen to FSH-PEG in PFC significantly decreased ($p < 0.01$) tobramycin availability in the emulsion with optimal availability ($C_{fs} = 2$ mg/mL H₂O of FSH-PEG, $V_{aq} = 2.5\%$, and $C_{aq} = 40$ mg/mL; Figure 4.3). Equivalent aqueous tobramycin concentrations for the trials with and without pre-exposure to FSH-PEG were 264 ± 25 and 569 ± 74 $\mu\text{g/mL}$, respectively. This result confirms that the fluorosurfactant diminishes drug availability.

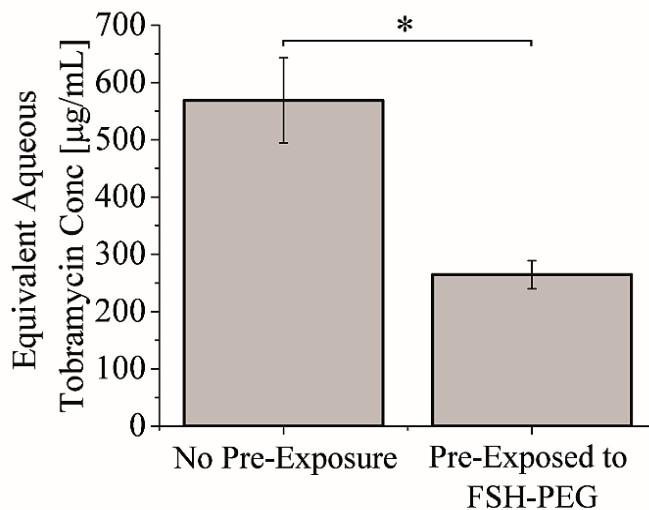


Figure 4.3: Tobramycin availability (as indicated by equivalent aqueous tobramycin concentration) for emulsion ($C_{fs} = 2$ mg/mL H₂O of FSH-PEG, $V_{aq} = 2.5\%$, and $C_{aq} = 40$ mg/mL) with and without pre-exposure of the agar well to FSH-PEG in PFC (2.4 mg/mL). $n = 3$ for each condition and an asterisk denotes a statistically significant ($p < 0.01$) difference.

Lastly, to determine an overall optimal formulation, we examined the effect of varying V_{aq} and C_{aq} on tobramycin content and availability (Figure 4.4A and 4.4B, respectively) in emulsion using the fluorosurfactant type and C_{fs} exhibiting optimal emulsification and availability ($C_{fs} = 2$

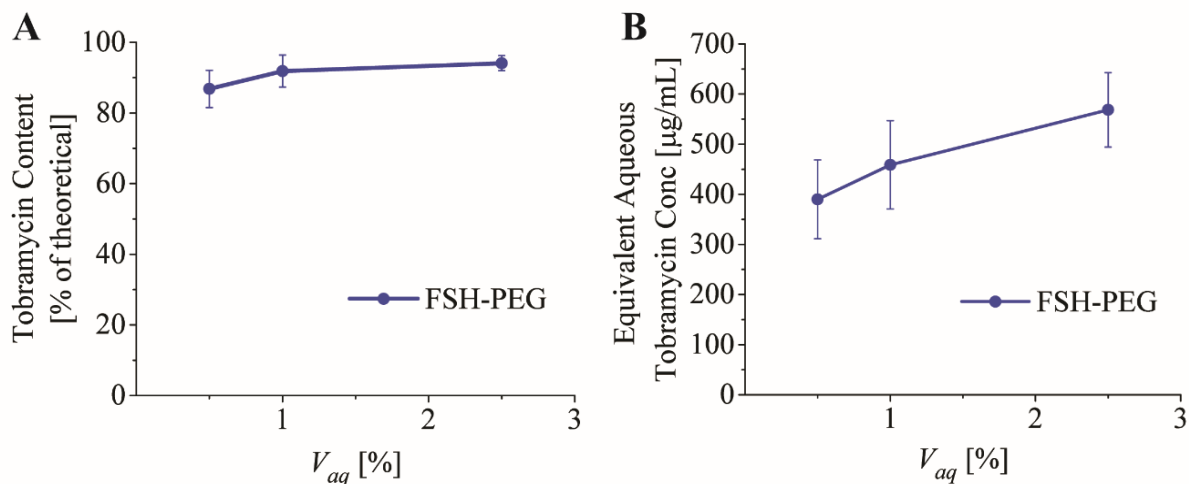


Figure 4.4: Tobramycin content (normalized by theoretical value; shown in panel **A**) and availability (as indicated by equivalent aqueous tobramycin concentration; shown in panel **B**) as a function of V_{aq} for emulsions utilizing FSH-PEG at $C_{fs} = 2$ mg/mL H₂O. C_{aq} was varied inversely to V_{aq} to maintain a total tobramycin content of 1 mg/mL. $n = 3$ for each condition.

mg/mL H₂O of FSH-PEG). Tobramycin content was greater than 85% for all V_{aq} and C_{aq} values examined and not significantly different at varying V_{aq} ($p = 0.18$). Tobramycin availability appears to increase with increasing V_{aq} , but this effect was not deemed significant ($p = 0.09$).

4.3.2 Cytotoxic Effects of Fluorosurfactants

Cytotoxicity studies demonstrated increased growth for alveolar epithelial cells exposed to neat PFC versus PBS. Cells exposed to neat PFC and PBS resulted in $83 \pm 9\%$ and $35 \pm 5\%$ alamarBlue reduction, respectively. Thus, it should be noted that subsequently reported cell viability results greater than 42% of that for cells exposed to neat PFC represent increased growth relative to PBS-exposed cells.

Cell viability following exposure to varying concentrations of either FSH-PEG in PFC or equal masses of FSL and FSL-PEG in PFC are shown in Figure 4.5. Figure 4.5A shows the fluorosurfactant concentrations in terms of molar concentration while Figure 4.5B shows the same data in terms of mass concentration. Cell viability is significantly greater ($p < 0.01$) for FSH-PEG

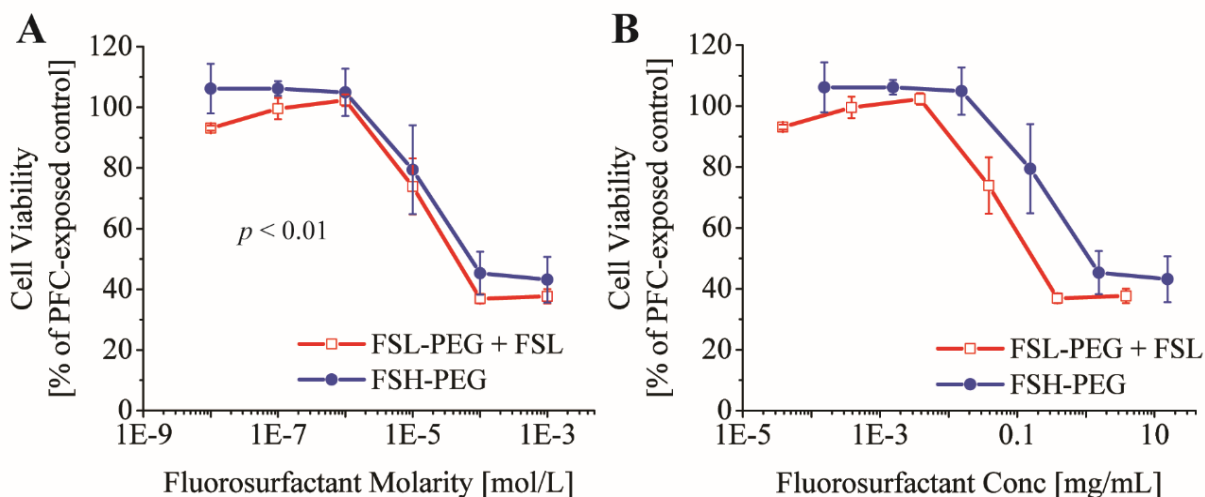


Figure 4.5: Cell viability (normalized by that of cells exposed to neat PFC) as a function of (panel **A**) fluorosurfactant molarity and (panel **B**) fluorosurfactant concentration for FSL-PEG+FSL and FSH-PEG. $n = 4$ for each condition.

as compared to FSL-PEG+FSL based on molar concentration. Cell viability was greater than 90% of that for cells exposed to neat PFC for both fluorosurfactant types at molarities of 10^{-6} M and lower. Cell viability began to decrease at higher concentrations of fluorosurfactants before reaching a viability of approximately 35-45% at molarities of 10^{-4} M or greater for both types. The transition in to the cytotoxic region occurs at slightly larger mass concentrations for FSH-PEG due to its larger molecular weight relative to FSL-PEG and FSL (15,500 Da for FSH-PEG versus 8,350 Da for FSL-PEG and 2,500 Da for FSL). As can be seen in Figure 4.5B, viability begins to decrease at fluorosurfactant concentrations of 0.02 and 0.004 mg/mL for FSH-PEG and FSL-PEG+FSL, respectively. Thus, when determining an optimal C_{fs} for either fluorosurfactant type based on drug emulsification and availability, FSH-PEG affords a larger non-toxic range of mass concentrations to be used.

Lastly, viability for cells exposed to emulsion with the formulation exhibiting optimal emulsification and availability ($C_{fs} = 2$ mg/mL H₂O of FSH-PEG) containing 2.5% PBS was $97 \pm 10\%$ of that for cells exposed to neat PFC. An independent t -test comparing the percent reduction

of alamarBlue of cells exposed to the emulsion to that of cells exposed to neat PFC showed no significant difference ($p = 0.5$), indicating that this emulsion formulation is not cytotoxic in this setting.

4.4 Discussion

In vivo studies (discussed in Chapters 3 and 5) have shown that although APV significantly increases the pulmonary retention of delivered tobramycin, it does not affect bacterial growth in the lung during infection. Such findings warrant investigation in to the availability of drug within the water-in-PFC emulsions used. Thus, the primary goal of this work was to explore the effects of fluorosurfactant type and concentration on the availability of emulsified tobramycin to an aqueous surface analogous to that encountered in the lungs. In order to achieve this, a simplified, *in vitro* scenario was used in which emulsion was loaded into a cylindrical agar well, thereby creating a large surface area of contact between the emulsion and a primarily aqueous surface. The ability of antibiotics to freely diffuse into such a surface in a reliable manner has been commonly utilized and documented for over 40 years in microbiological assays.³⁻⁵ Similar to standard agar well diffusion methods, the degree of drug diffusion from emulsion into the agar was measured via inhibited growth of bacteria (*PA*) that had been seeded on to the agar prior to emulsion loading. Such a measurement technique ensures that the delivered drug being measured is still active and capable of exerting its intended bactericidal effect. Following inhibition zone measurements, the degree of drug diffusion for each emulsion formulation was equated to the aqueous tobramycin concentration producing an equivalent inhibition zone. As mentioned in previous chapters, the most clinically relevant measure of drug in the lungs is likely the drug concentration at the aqueous, liquid surface of the airways or biofilm. Such a measurement is difficult, if possible at all, to carry

out in an emulsion-filled lung. Thus, the measurement technique described determines the analogous aqueous tobramycin concentration resulting in the same degree of drug diffusion in an identical setting. In order to confirm that any observed differences in drug diffusion were due to impaired availability rather than a lack of drug presence due to phase separation, tobramycin content of identical samples was measured concurrently. It should be noted that although tobramycin content was only evaluated at a single height within the emulsion at five minutes post-preparation, previous work evaluating drug dispersion (discussed in Chapter 3) has shown that such a sample should be indicative of drug content throughout the bulk of the emulsion up to four hours post-preparation.

Results from this work have shown that the presence of fluorosurfactant significantly impairs the availability and delivery of emulsified tobramycin to an aqueous surface such as agar. The availability of emulsified tobramycin was shown to be lowest at the largest C_{fs} values examined and increased with decreasing C_{fs} until reaching a maximum at C_{fs} values of 10 and 2 mg/mL H₂O for FSL-PEG+FSL and FSH-PEG, respectively. Within these ranges, tobramycin content of the emulsion did not vary significantly and was greater than 85% of the theoretical value. As C_{fs} was decreased further, measured tobramycin availability decreased as did the tobramycin content within the samples. Thus, the measured decrease in tobramycin diffusion at C_{fs} values less than 10 and 2 mg/mL H₂O for FSL-PEG+FSL and FSH-PEG, respectively, is due to a lack of drug rather than impaired availability. These effects are illustrated in Figure 4.6. Note that phase separation (indicated by a decrease in tobramycin content within samples) was observed for FSL-PEG+FSL only at $C_{fs} < 10$ mg/mL H₂O, whereas a previous evaluation (discussed in Chapter 3) showed significant phase separation at $C_{fs} = 10$ mg/mL H₂O for FSL-PEG+FSL. This minor discrepancy can likely be attributed to batch to batch variability of the fluorosurfactant. In

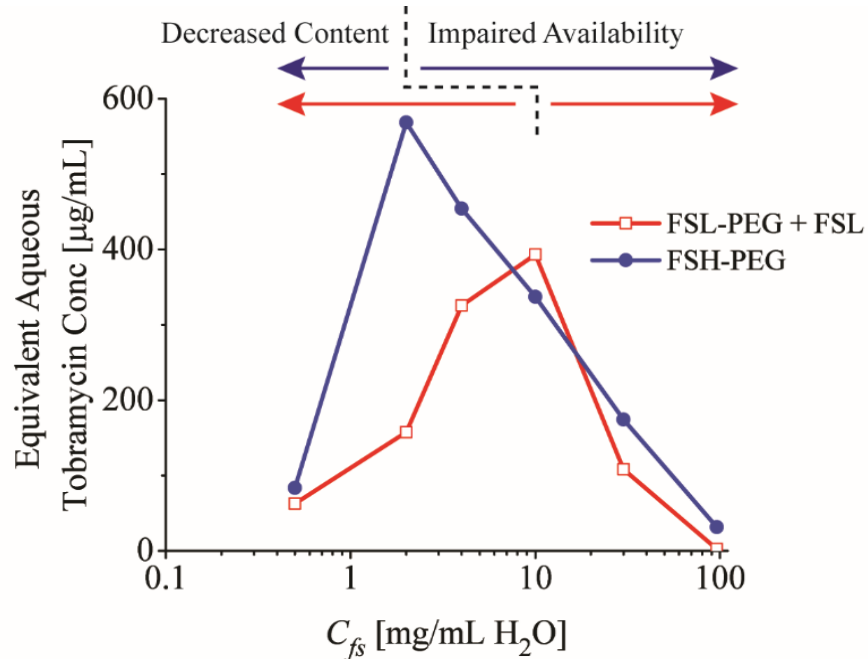


Figure 4.6: Tobramycin availability as a function of C_{fs} showing proposed causes of decreased drug diffusion observed over varying ranges of C_{fs} for each fluorosurfactant type. Note error bars have been removed for clarity.

a separate experiment, the exposure of dissolved fluorosurfactant (FSH-PEG) to the agar surface prior to emulsion loading for a period of only ten minutes reduced the relative availability and diffusion of emulsified tobramycin by greater than 50%. Such an observation further confirms the accumulation of fluorosurfactant at an aqueous surface in contact with the emulsion and its significant role in impairing subsequent drug delivery to that surface. In contrast to the profound effects of C_{fs} , changes in V_{aq} and C_{aq} within the ranges evaluated ($V_{aq} = 0.5-2.5\%$ and $C_{aq} = 40-200$ mg/mL) were shown to have relatively small effects on drug availability.

The intended effect of the fluorosurfactants is to assemble at the aqueous-PFC boundary of the drug-loaded droplets and delay their coalescence and creaming, thereby maintaining homogenous drug dispersion. Thus, it is reasonable to expect that increased levels of fluorosurfactant may also prevent droplet deposition on and coalescence with an aqueous surface in contact with the emulsion. However, even when left to completely evaporate within the agar

well, maximum drug diffusion from the emulsion was shown to be equivalent to an aqueous tobramycin concentration of approximately 570 $\mu\text{g/mL}$. Thus, either a portion of the measured drug content (approximately 940 $\mu\text{g/mL}$ for formulation with maximum diffusion) was ultimately unavailable to freely diffuse into the agar or the general diffusional capability of tobramycin is hindered. In either case, the effects of fluorosurfactant on drug availability are likely beyond those of inhibited droplet deposition. Due to the active nature of emulsified tobramycin following recovery via the described phase inversion and centrifugation process, the formation of a chemical bond between tobramycin and fluorosurfactant molecules is an unlikely explanation for the observed lack of availability. Rather a more likely explanation is the impairment of mass transfer into an aqueous surface due to the fairly quick (effects shown after only 10 minutes of pre-exposure) aggregation of fluorosurfactant. Similar effects were observed *in vivo* during APV pharmacokinetic trials evaluating tobramycin delivery via emulsion utilizing FSL-PEG+FSL at $C_{fs} \geq 30 \text{ mg/mL H}_2\text{O}$. Results from these studies showed relatively large amounts of drug remaining in the lung, seemingly unavailable for systemic absorption, at a time point at which the PFC phase of the emulsion is expected to have largely evaporated. Thus, the effects of impaired drug diffusion into an agar surface measured in this work are likely indicative of a similar phenomenon occurring in the lung during APV.

The cytotoxicity evaluation carried out in the current work showed a concentration-dependent cytotoxic effect for fluorosurfactants dissolved in PFC. Cells exposed to FSL-PEG+FSL and FSH-PEG at concentrations of 10^{-6} M or less exhibited growth similar to that of cells exposed to neat PFC, while exposure to larger concentrations resulted in decreased cell viability. Exposure to FSH-PEG was shown to produce significantly higher cell viability relative to FSL-PEG+FSL when considering all molarities evaluated. Because drug content and

availability data comparing the two fluorosurfactant types is in terms of fluorosurfactant mass rather than molarity, the cytotoxicity data should be considered in the same manner. The relatively larger molecular mass of FSH-PEG as compared to FSL-PEG and FSL translates to an even greater advantage for FSH-PEG when comparing cell viability at similar mass concentrations of fluorosurfactant. Although this initial evaluation was performed with a mixture of fluorosurfactants and PFC without an aqueous phase, emulsion with $V_{aq} = 2.5\%$, $C_{fs} = 2$ mg/mL H₂O of FSH-PEG, and sterile PBS in the aqueous phase was also evaluated. Emulsion exposure exhibited no cytotoxic effects, showing cell viability statistically unchanged from that of cells exposed to neat PFC. It is also worth noting that the viability of cells exposed to the emulsion was greater than that predicted (using Figure 4.5B) for an equivalent amount of fluorosurfactant dissolved in PFC. This effect is likely due to the accumulation of fluorosurfactant at the droplet interfaces within the bulk of the emulsion thereby resulting in reduced fluorosurfactant exposure to the cells located at the boundary of the well.

The optimal emulsion formulation that will ultimately maximize the treatment efficacy of APV should maintain a spatially uniform distribution of drug while simultaneously maximizing drug availability to an aqueous surface such as a biofilm. The results of this work indicate that the effect of C_{fs} on these two parameters may be working against each other to some degree. Thus, the optimal emulsion formulation must balance these opposing effects by utilizing the minimum concentration of fluorosurfactants necessary to maintain suitable drug dispersion in order to also maximize drug availability. This is accomplished for FSL-PEG+FSL at a C_{fs} value in the range of 4-10 mg/mL H₂O and at a lower value of 2 mg/mL H₂O for FSH-PEG. Ultimately, FSH-PEG resulted in emulsions with significantly greater drug availability as well as lower cytotoxicity relative to FSL-PEG+FSL, the fluorosurfactant used during all prior APV work. Furthermore,

emulsion utilizing FSH-PEG at the optimal C_{fs} value (2 mg/mL H₂O) exhibited no measurable cytotoxic effects. Thus, future work towards the advancement of APV should utilize FSH-PEG as the sole fluorosurfactant component until an alternative molecule is shown to be favorable. Further efforts towards optimizing APV treatment should evaluate the treatment efficacy of APV utilizing the optimized emulsion formulations discussed in this work. Additionally, the current cytotoxicity evaluation represents positive initial work towards ensuring the safety of such emulsions, but should be followed by more in-depth, *in vivo* biocompatibility studies.

4.5 References

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CHAPTER 5

***In Vivo* Treatment Efficacy of Antibacterial Perfluorocarbon Ventilation in a Rat Model of Bacterial Respiratory Infection**

5.1 Introduction

5.1.1 Study Motivation and Design

In vitro evaluation of the emulsions used during antibacterial perfluorocarbon ventilation (APV) discussed in Chapter 2 has shown effective killing of *Pseudomonas aeruginosa* (PA) biofilms following a two-hour emulsion exposure. However, the more relevant metric of APV's bactericidal capacity lies in the treatment's ability to effectively eradicate bacteria in an *in vivo* setting of bacterial respiratory infection. Accordingly, the primary goal of the work presented in this chapter was to evaluate the ability of APV to reduce pulmonary bacterial load in a rat model of chronic PA lung infection relative to that of conventional aerosolized tobramycin.

The ideal animal model for this work would mimic the disease characteristics clinically observed in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) patients during infection. This is primarily the presence of mucoid PA growth (*i.e.* biofilms) in the airways, increased production of thick respiratory mucus by the host, as well as a severe airway inflammatory response. Multiple approaches have been taken to develop such a disease model in

rats. The simplest method used to inoculate the airways is via intratracheal delivery of mobile bacteria in a buffered solution.¹⁻³ However, experience with this model has shown that it often results in either rapid clearance of the pathogen or acute sepsis and death.⁴ Thus, it is more representative of an acute bacterial challenge than the chronic infection seen clinically during CF or COPD. In order to better achieve the characteristics of a chronic infection, models have been developed in which the bacteria is embedded into some form of protective matrix prior to delivery. Impregnation of bacteria into agar beads was first introduced nearly 40 years ago⁵ and has since been used extensively.⁶⁻⁸ More recently, a similar method has utilized bacteria entrapped in alginate microspheres.⁹⁻¹² Use of bacteria within agar or alginate provides an additional barrier between the bacteria and the pulmonary environment, thereby typically resulting in a less immediate immune response and more prolonged infection. A study comparing the agar and alginate methods has shown that both result in comparable infections, but also notes that the alginate method may offer a theoretical advantage in that the coating substance is more chemically similar to the alginate produced by *PA* in a biofilm.¹³ Thus, the following work utilized the alginate microsphere method of inducing lung infection in rats.

The most clinically relevant method of evaluation would be to intervene with treatment only after the bacteria has been introduced to the airways and given sufficient time to acclimate as well as evoke a host response (*i.e.* inflammation and mucus production). However, many previous studies evaluating the effectiveness of perfluorocarbon (PFC) mediated treatment in this setting have implemented treatment either prior to or immediately following (≤ 4 hours) the delivery of bacteria to the airways.^{1, 14, 15} Such studies likely evaluate the treatments' ability to disrupt the delivered bacteria's initial attachment and colonization rather than the more relevant treatment of

the underlying pathology of bacterial infection during lung disease. Considering this, all treatment intervention in this work was initiated no earlier than 48 hours following bacterial delivery.

Although APV could ultimately be performed as either partial APV (lungs partially filled with emulsion and ventilated with gas) or total APV (lungs completely filled and tidally ventilated with emulsion), only partial APV was assessed in the current work. There are multiple reasons for the decision to focus solely on partial APV. First, a period of partial APV is always present during total APV while transitioning a patient back to gas ventilation. Thus, any efficacy shown for partial APV should be indicative of that for total APV to some degree. Second, partial APV is unarguably a simpler approach. Gas mechanical ventilation in rats has been used extensively and the effects of ventilation parameters are well established and understood. Although work has been done to characterize the effects of liquid ventilation parameters as well, the methods and equipment to carry out such a procedure are less standardized. An initial evaluation and characterization of APV is more easily accomplished in the simplest setting (*i.e.* partial APV as opposed to total APV). Lastly, previous work characterizing the pharmacokinetics of APV (discussed in Chapter 3) utilized partial APV. Thus, the results of the current work would be most easily and accurately interpreted if performed in a similar manner. However, there are also drawbacks to the use of partial APV rather than total APV. During total APV virtually all surfaces within the lung are in constant contact with the emulsion. Conversely, during partial APV the upper airways are gas filled during the majority of the respiratory cycle. As PFC evaporates during treatment, an even greater portion of the lung will lose contact with the emulsion and thus have limited access to the delivered antibiotics. Although this might be addressed by intermittently altering the positioning and inclination of the lungs during ventilation, the experimental setup used in this work did not allow for easy manipulation of the rat's position relative to the ventilator.

The work discussed in this chapter is composed of two different studies evaluating the efficacy of partial APV in different modes of treatment at varying time points throughout the development of APV. Ultimately, the partial APV treatments implemented during either study were both shown to be unsuccessful in eradicating pulmonary bacteria during infection. In addition to describing the studies and resulting data, the remainder of this chapter will discuss the motivation behind the study designs as well as the proposed reasoning for their lack of success. Chapter 5 also serves to review and validate the alginate microsphere method used for induction of a *PA* respiratory infection in rats. Methods outlining a refined method of both inoculum preparation and delivery are described in detail. Review and validation of such a model may prove useful for future work towards further developing APV.

5.1.2 Earlier Efficacy Trials (APV + Aerosol)

The earlier efficacy trials to be discussed were performed following initial physical and bactericidal characterization of the emulsions (discussed in Chapter 2), prior to the pharmacokinetic studies (discussed in Chapter 3) and assessment of drug availability (discussed in Chapter 4). *In vitro* bactericidal evaluation showed emulsions utilizing increased C_{aq} to be most effective in killing *PA* biofilms. Thus, the emulsion formulations used during early efficacy trials utilized maximized C_{aq} (with minimized V_{aq}) and were expected to be similarly effective against *in vivo PA*. In an effort to recreate the clinical setting to the greatest degree possible, earlier studies evaluated partial APV in conjunction with subsequent aerosolized tobramycin over a multi-day course of treatment. As discussed in section 1.7, APV is envisioned as an adjunct treatment to be used in combination with subsequent aerosolized delivery. Initial treatment with APV should provide a therapeutic lavage of the lungs in which the airways are mechanically ridded of a large

portion of mucus and biofilm while simultaneously receiving an evenly distributed loading dose of antibiotics. Theoretically, clearance of the airways should enhance subsequent inhaled delivery which is often hampered by mucus plugging and the resulting decreased airflow.¹⁶ Lastly, the earlier trials also evaluated treatment utilizing two different lung fill volumes. As mentioned above, the lack of complete and constant contact between the emulsion and surfaces of the lung is believed to be a significant disadvantage of partial APV. In order to confirm this hypothesis, a relatively large and small lung fill volume were each evaluated. In theory, partial APV can be carried out with the lungs filled with emulsion to any volume up to end-expiratory lung volume (EELV). Using EELV should result in the entire lung in contact with the emulsion at the end of each exhalation while any smaller volume will leave portions of the lung unfilled throughout the entire respiratory cycle. The lung fill volume will also likely affect the amount of oxygen transfer achieved during ventilation, the degree of liquid tidal flow in the airways, as well as the time course of emulsion evaporation. These earlier trials will be referred to as “APV + aerosol” trials in the remainder of this chapter.

5.1.3 Later Efficacy Trials (APV Alone)

Later efficacy trials were performed following evaluation of the pharmacokinetic effects of emulsion formulation (discussed in Chapter 3) but still prior to assessment of emulsified drug availability or varying fluorosurfactant types (discussed in Chapter 4). The pharmacokinetic study showed reduced pulmonary residence times for tobramycin delivered via emulsions utilizing decreased V_{aq} with increased C_{aq} (such as those described above for APV + aerosol trials). Thus, the later efficacy trials utilized increased V_{aq} with decreased C_{aq} relative to the APV + aerosol trials in an effort to increase pulmonary drug retention and bacterial killing. Both efficacy trials were

performed prior to assessment of FSH-PEG and thus FSL-PEG+FSL was used as the sole fluorosurfactant for all work discussed in this chapter. The C_{fs} used during both trials (30 mg/mL H₂O) was chosen primarily with biocompatibility and emulsion stability in mind given what we understood at the time. Due to the observed lack of benefit of aerosolized delivery following partial APV in the APV + aerosol trials, a simplified, single-dose treatment procedure was evaluated. Lastly, in an effort to ensure complete delivery of the intended drug dose to the airways, a tracheal tie was implemented during partial APV in the later trials in order to create a seal around the angiocatheter (used as an endotracheal tube) within the airway. These later efficacy trials will be referred to as “APV alone” trials in the remainder of this chapter.

5.2 Methods

5.2.1 Preparation of Bacterial Inoculum

The chronic *PA* respiratory infection rat model used in both efficacy studies was induced using a slightly modified version of a previously established alginate microsphere technique.^{10, 13} The same *PA* strain used in all previous APV work (ATCC 27853) was used for all inoculation procedures. Bacteria was grown for 6-8 hours in tryptic soy broth (TSB) supplemented with 1% glucose (approximately 40 mL) at 37° C on a gyratory shaker to mid-log growth. The optical density of the resulting solution was measured and a volume of bacterial solution containing approximately 5.5×10^8 colony forming units (CFU) (approximately 1-10 mL depending on the solution’s phase of growth) was then centrifuged at $3,725 \times g$ at 4° C for 20 minutes. Following centrifugation, supernatant was discarded and the bacteria were re-suspended in approximately 10 mL of sterile phosphate-buffered saline (PBS). The previously described centrifugation and re-suspension step was repeated once more in order to ensure removal of growth medium from the

bacterial suspension. Next, a small sample (100 μ L) of the bacteria in PBS solution was taken for serial dilution and quantitative culture in order to ultimately confirm the amount of bacteria delivered to each rat. Quantitative cultures were performed on this solution rather than the final solution because serial dilution of the microsphere solution was shown to be inaccurate and unreliable. Following the final re-suspension of bacteria in PBS, 2.5 mL of bacterial suspension was added to 7 mL of previously prepared sodium alginate solution. The sodium alginate solution was prepared by dissolving sodium alginate (Alginic acid sodium salt, CAS # 9005-38-3; Sigma Aldrich, St. Louis, MO, USA) in sterile saline to a concentration of 11 mg/mL and autoclaving the resulting mixture.

The solution of sodium alginate and bacteria was loaded into a sterile 10-mL syringe and thoroughly mixed via repeated inversion. Next, the solution was forced through a small-gauge, polyethylene tubing with an inside diameter of 0.58 mm (BD Intramedic Polyethylene Tubing, Model 427411; Becton Dickinson, Franklin Lakes, NJ, USA) while a jet of air was aligned coaxial to the tubing to blow off the droplets. Droplets were blown into a sterile flask containing 40 mL of a cross-linking solution of 0.1 M CaCl_2 in TRIS-HCl buffer (0.1 M, pH 7.0). The alginate microspheres were allowed to cure for one hour in the calcium solution under continuous stirring at room temperature. Following curing, the alginate solution was centrifuged at $3,725 \times g$ at 4°C for five minutes in order to separate the microspheres out of solution. Following centrifugation, the supernatant was discarded and the microspheres re-suspended in 25 mL of sterile PBS. The solution was again centrifuged at the same settings, supernatant discarded, and the microspheres re-suspended in sterile PBS to a final volume of 8 mL. The final solution contained 9.625 mg/mL of sodium alginate and approximately 10^5 - 10^6 CFU/mL. The resulting solution was then stored at 4°C until being delivered to rats within two hours of preparation.

5.2.2 Inoculation Procedure

Specific pathogen-free, male Sprague Dawley rats (weighing approximately 400 grams) were initially anesthetized with a mixture of ketamine hydrochloride [50 mg/kg, intraperitoneal (IP); Hospira Inc., Lake Forest, IL, USA] and xylazine hydrochloride (5 mg/kg, IP; Lloyd Laboratories, Shenandoah, IA, USA). Rats were placed in a prone position and orally intubated with a 16 gauge angiocatheter. Immediately following intubation, a length of small-gauge, polyethylene tubing with an inside diameter of 0.58 mm was inserted through the angiocatheter such that the tip of the tubing extended approximately 2 mm past the end of the angiocatheter within the trachea. From Figure 2.2, one can see that such placement results in the end of the tubing resting approximately 2-3 mm proximal to the carina. The appropriate length of tubing was measured and marked prior to the procedure in order to ensure correct positioning within the angiocatheter. Also prior to the procedure, 200 μ L of freshly prepared inoculum was loaded into a length of approximately 24 inches of tubing and a 5-mL syringe full of air attached to one end of the tubing via a 24 gauge angiocatheter. Following placement of the tubing in the trachea, the syringe was used to evacuate the contents of the tubing, thereby intratracheally delivering the inoculum. Inoculum doses of 10^4 - 10^6 CFU/rat were used throughout the trials discussed in this chapter. Experiences during this work would suggest that the ideal bacterial dose is approximately $6 \times 10^4 - 1 \times 10^5$ CFU/rat. Such a dose tends to result in low mortality while also producing a lasting infection. Earlier trials provided supplemental oxygen following inoculum delivery and relied on the rat's own respiratory efforts to maintain normal breathing. This method ultimately resulted in frequent apneic episodes following inoculum delivery and increased overall mortality at this point in the model. Therefore, later trials incorporated a short period (approximately 30 seconds) of low tidal volume mechanical ventilation following inoculum delivery in order to

resuscitate breathing. Following delivery of the inoculum and resumed spontaneous breathing, rats were provided supplemental oxygen until observed to be ambulatory at which point they were returned to housing with free access to food and water. All rats remained in housing for a period of 48 hours before undergoing treatment.

5.2.3 Emulsion Preparation

Tobramycin-loaded, water-in-PFC emulsions were prepared with the same equipment and sonication settings as those described in section 3.2.1. All emulsions evaluated in the current work used equal masses of FSL-PEG and FSL. Similar to previous chapters, C_{fs} refers to the total amount of fluorosurfactant used. Larger C_{aq} values were used in this work than in previous chapters. While preparing the aqueous tobramycin phase of emulsions utilizing $C_{aq} = 1,000$ mg/mL, the solution had to be warmed to 37° C using a heated water bath in order to achieve complete dissolution of the tobramycin. Similar to previous work with APV, the PFC used in all experiments was perfluorocycloether/perfluorooctane (FC-770; 3M Inc., St. Paul, MN, USA).

5.2.4 Antibacterial Perfluorocarbon Ventilation and Aerosolized Treatment

Two different partial APV (referred to in the remainder of Chapter 5 simply as APV) treatment efficacy studies are discussed in the remainder of Chapter 5. The primary difference between the two studies is the timing and number of treatments received by each rat following inoculation. APV + aerosol trials evaluated APV in conjunction with subsequent aerosolized tobramycin at two different lung fill volumes (3 and 15 mL/kg). Previous studies have found the larger volume used (15 mL/kg) to be approximately equivalent the EELV of a rat.^{2, 17} The emulsion used for both lung fill volumes utilized $C_{aq} = 1,000$ mg/mL and $C_{fs} = 30$ mg/mL H₂O of FSL-

PEG+FSL. The large and small lung fill volume treatments used emulsion with V_{aq} values of 0.1 and 0.5%, respectively, resulting in a total delivered tobramycin dose of 15 mg/kg for both cases. Following APV, five smaller maintenance doses of tobramycin (2.5 mg/kg) were delivered via aerosolized delivery every 8-12 hours, resulting in three full days of treatment.

In order to compare APV to conventional treatment, a second treatment group received only aerosolized tobramycin. The aerosolized treatment group received identical delivered doses at the same time points as the APV treatment groups. Thus, the only difference between the groups was the delivery method for the larger, initial tobramycin dose. All rats were euthanized with pentobarbital sodium (175 mg/kg, IP, Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI, USA) approximately 24 hours following final treatment. A timeline describing the experiment is shown in Figure 5.1.

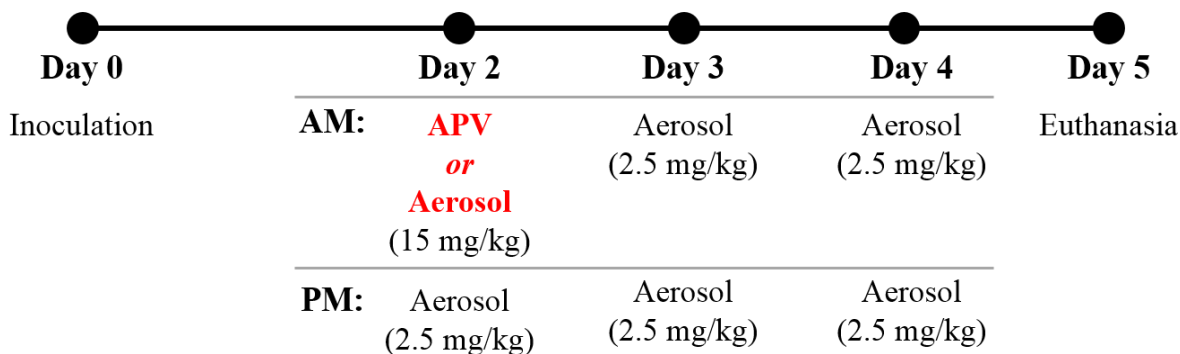


Figure 5.1: Timeline showing treatment schedule used during APV + aerosol trials.

Additionally, a negative control group of inoculated rats receiving no tobramycin was also included. The negative control group was anesthetized in an identical manner to and at the same time points as the APV and aerosolized treatment groups. Although data sets may include rats from multiple rounds of inoculation (*i.e.* inoculated on different dates with independently prepared inoculum), an approximately equal number of rats within each round was randomly assigned to receive APV, aerosolized delivery, or no treatment. APV treatment with large and small lung fill

volumes were evaluated separately from each other (each performed in rounds with only aerosolized treatment and negative controls). The total number of rats assigned to each treatment group for each set of trials is given with the results in section 5.3.2.

APV alone trials (the later efficacy study performed) evaluated pulmonary bacterial load at eight hours following a single APV treatment (also initiated at 48 hours post-inoculation). Although the goal of these trials was to evaluate pulmonary bacterial presence immediately post-treatment, necropsy was delayed eight hours post-delivery in an attempt to allow adequate time for tobramycin to leave the lungs via systemic absorption. Tobramycin still present in the lung tissue at the time of homogenization may affect the accuracy of subsequent quantitative cultures. APV during these trials utilized a single emulsion formulation with $V_{aq} = 2.5\%$, $C_{aq} = 40$ mg/mL, and $C_{fs} = 30$ mg/mL H₂O of FSL-PEG+FSL. A lung fill volume of 15 mL/kg was used, resulting in a total delivered tobramycin dose of 15 mg/kg (same as for APV + aerosol trials). A second treatment group received an identical delivered tobramycin dose via aerosol at the same time point. A negative control group of inoculated rats receiving no tobramycin was again included. During these trials, a throat swab (Peel Pouch Dryswab Fine Tip, Model MW113; Medical Wire and Equipment Co., Wiltshire, England) was performed immediately prior to tobramycin delivery and cultured to confirm the presence of *PA* in the airways. Following swabbing of the back of the throat, the swab tip was cut off into a microcentrifuge tube containing 1 mL sterile water. The tube was allowed to sit at room temperature for 30 minutes and then vortexed for 30 seconds before a sample (100 μ L/sample) was plated on *PA*-selective agar (Cetrimide Agar Base; Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 37° C overnight. In contrast to the trials discussed above, the negative control treatment group received no sham anesthesia. Throat swabs on the negative control group were performed immediately prior to euthanasia. All rats in the APV

alone trials were euthanized with pentobarbital sodium (175 mg/kg, IP) at eight hours post-treatment.

APV treatment for both studies was performed according to those methods described in section 2.2.6 with some minor differences between APV + aerosol and APV alone trials. Due to the need to recover animals following treatment in the APV + aerosol trials, no neck incision was made during intubation or tie placed around the trachea. Preliminary studies (data not shown) showed that such a process significantly increased mortality during a recovery procedure. For the APV alone trials, APV-treated rats were kept under sedation for the entire eight hours between treatment and euthanasia and thus a tracheal tie was used. For APV + aerosol trials, emulsion was instilled with the rat in a prone position, while a supine position was used for the APV alone trials. For the APV + aerosol trials, gas ventilation was continued for a period of approximately one hour following emulsion instillation at which point intravenous sedation was ceased and the tail wound closed with skin adhesive. Rats were then weaned off gas ventilation and extubated once spontaneous breathing was observed. Supplemental oxygen was given until rats were ambulatory at which point they were returned to housing with free access to food and water. For APV alone trials, gas ventilation was continued until euthanasia at eight hours following emulsion instillation.

Aerosolized delivery of tobramycin (either 15 mg/kg or 2.5 mg/kg for maintenance doses in APV + aerosol trials) was performed according to those methods described in section 2.2.6 with minor differences between APV + aerosol and APV alone trials. Aerosolized delivery was achieved with the rat in a prone position during APV + aerosol trials, while a supine position was used for APV alone trials. Following delivery, rats were immediately extubated and provided supplemental oxygen. Once rats were observed to be ambulatory, they were returned to housing

with free access to food and water until further treatment (during APV + aerosol trials) or euthanasia.

5.2.5 Evaluation of Pulmonary Bacterial Load Post-Treatment

A sterile necropsy was performed following euthanasia in all trials in order to remove the lungs for measurement of remaining pulmonary bacterial load. The excised lungs were immediately added to sterile saline (approximately 25 mL/kg bodyweight) and thoroughly homogenized (LabGEN 7b Series Portable Homogenizer; Cole Parmer, Vernon Hills, IL, USA) for further processing. The homogenizer was immersed in ethanol followed by three separate rinses in sterile water in between samples in order to avoid cross contamination. A sample (100 μ L) of the tissue homogenate was then serially diluted and cultured on *PA*-selective agar in order to quantify the amount of viable bacteria present. Agar plates were incubated overnight at 37° C before counting was performed. The lower limit of detection using these methods was 20 CFU/mL. Any samples resulting in no measurable bacterial growth were assigned a value of 10 CFU/mL.

5.2.6 Statistical Analysis

A log transform was performed on all pulmonary bacterial load measurements prior to further analysis. A one-way ANOVA was performed on the log transformation of the data in order to determine the effect of treatment group on pulmonary bacterial load remaining at the time of euthanasia. All figures describing pulmonary bacterial load in the remainder of this chapter present the mean and standard deviations (represented by error bars) of the log transformation of the original bacterial measurements.

5.3 Results

5.3.1 Respiratory Infection Model

The alginate microsphere methods described in this work were shown to produce a consistent bacterial respiratory infection with reasonably low mortality. Inoculum doses of 6×10^4 - 1×10^5 CFU/rat were found to result in a survival rate of greater than 70% while larger doses resulted in significant mortality, typically within the 48 hours immediately post-inoculation. Rats were sacrificed at either two (APV alone trials) or five (APV + aerosol trials) days post-inoculation in the collection of trials discussed. In the time following inoculation, rats were typically observed to be lethargic with decreased appetite and moderately labored breathing. Porphyrin staining around the eyes and nose, a common indicator of stress or sickness in rats, was often observed. At the time of necropsy, viscous, mucus-like exudate was often observed in the airway or angiocatheter (used as endotracheal tube). Examples of mucus-filled angiocatheters at the time of necropsy are shown in Figure 5.2A and 5.2B. As seen in Figure 5.2C, lungs from infected rats were also commonly observed to have signs of pulmonary edema.

The pulmonary bacterial load of negative control rats receiving no treatment at two and five days post-inoculation is shown in Table 5.1. It is important to note that because pulmonary bacterial load values can vary over orders of magnitude, large outliers can have much greater effects on the mean values of the raw data than the mean values of the log transformed data. Although not measured in all trials, the total homogenized lung volume for most trials was found to be approximately 13 mL. Using this value, the total lung bacterial load for rats at two and five days post-inoculation can be estimated to be approximately 6.8×10^6 and 2.0×10^7 CFU, respectively. Note that negative control rats sacrificed at five days post-inoculation (APV + aerosol trials) were sham anesthetized twice per day for the three days prior to sacrifice while those

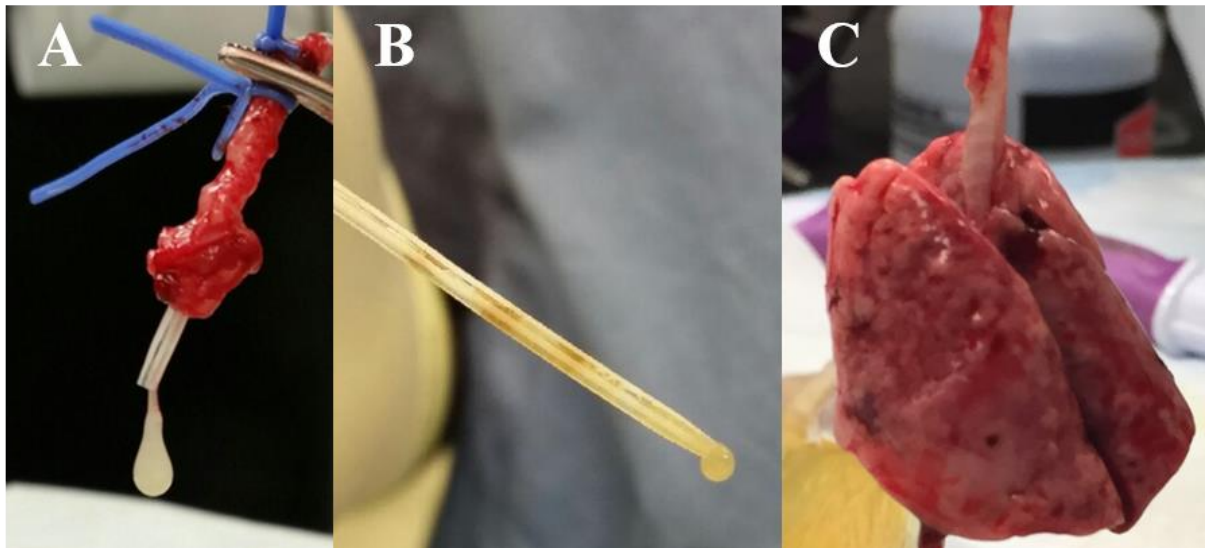


Figure 5.2: (A and B) Mucus-like secretions from infected rats observed in angiocatheter (used an endotracheal tube) at time of euthanasia. (C) Typical lungs removed from infected rats showing signs of pulmonary edema (darker red regions).

Time Post-Inoculation [days]	<i>n</i> [-]	Mean Bacterial Load [CFU/mL]	Median Bacterial Load [CFU/mL]	Log Transform [log(CFU/mL)]
2	15	5.2×10^5	1.2×10^5	5.1 ± 0.9
5	14	1.5×10^6	8.0×10^3	3.9 ± 1.5

Table 5.1: Pulmonary bacterial load of negative control (untreated) rats at two and five days post-inoculation. Log transform values are given as mean \pm standard deviation.

ethanized at two days post-inoculation (APV alone trials) were essentially untouched following infection. APV + aerosol trials had one negative control rat with no measurable pulmonary bacterial load while APV alone trials showed viable pulmonary bacterial measurements for all negative controls. Furthermore, the throat swab method used in APV alone trials proved to be a reasonably reliable means of confirming infection prior to treatment, producing a positive culture for rats with viable pulmonary bacteria in 14 of 15 trials (93% of trials) for negative controls, or 31 of 32 trials (97% of trials) overall.

5.3.2 Treatment Effect on Pulmonary Bacteria

The pulmonary bacterial load of rats at five days post-inoculation (APV + aerosol trials) following varying three-day tobramycin treatments is shown in Figure 5.3. APV for each lung fill volume is shown compared to only those negative controls and aerosolized-treated rats that were performed within the same rounds of inoculation. Figure 5.3A shows pulmonary bacterial load for evaluation of APV treatment utilizing the smaller lung fill volume (3 mL/kg). One rat from each the aerosolized and APV treatment groups produced no measurable pulmonary bacteria. Figure 5.3B shows pulmonary bacterial load for evaluation of APV treatment utilizing the larger lung fill volume (15 mL/kg). One rat from each the aerosolized treatment and negative control groups produced no measurable pulmonary bacteria. A one-way ANOVA performed separately on each data set (shown in Figure 5.3A and 5.3B) showed no significant effect of treatment method on pulmonary bacterial load for the small ($p = 0.9$) or large ($p = 0.8$) lung fill volume trials.

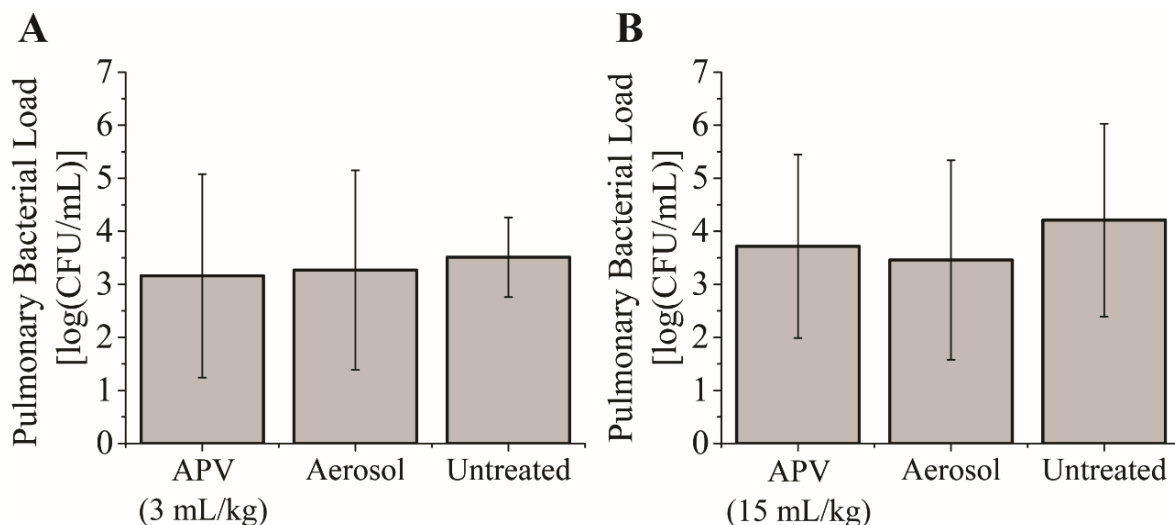


Figure 5.3: (A) Pulmonary bacterial load for groups receiving no treatment ($n = 6$), aerosolized tobramycin ($n = 5$), or APV with 3 mL/kg lung fill volume ($n = 10$). (B) Pulmonary bacterial load for groups receiving no treatment ($n = 8$), aerosolized tobramycin ($n = 5$), or APV with 15 mL/kg lung fill volume ($n = 8$).

Figure 5.4 shows the pulmonary bacterial load of rats at two days post-inoculation (APV alone trials) eight hours following varying methods of single-dose treatment. Again, APV is shown in contrast with only those negative controls and aerosolized-treated rats that were performed within the same round of inoculation. All rats produced measurable pulmonary bacteria and positive pre-treatment throat cultures. Although pulmonary bacterial load was observed to be lower for rats receiving aerosolized treatment relative to other groups, the effect of treatment method was not deemed significant ($p = 0.06$) for this small-sample data set.

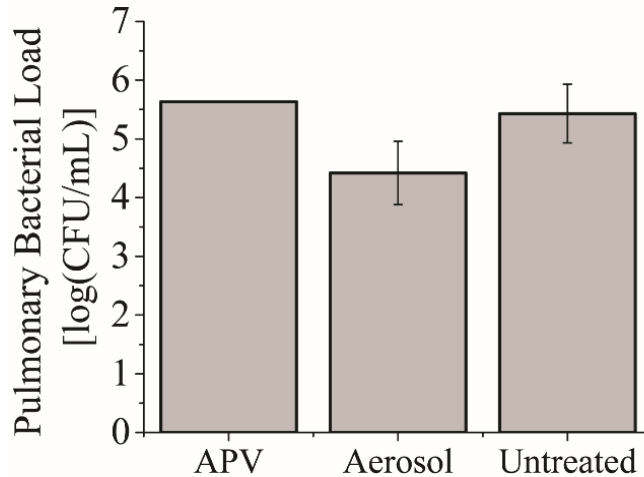


Figure 5.4: Pulmonary bacterial load for groups receiving no treatment ($n = 3$), aerosolized tobramycin ($n = 3$), or APV ($n = 2$).

5.4 Discussion

The current findings have failed to provide evidence of a potential advantage for APV with the specified emulsion formulations in eradicating a chronic *PA* respiratory infection. Considering the increased understanding of the emulsions that has been gained since these trials, the observed lack of success is likely predominantly due to the use of suboptimal emulsion formulations. The selection of emulsion formulations to be used during the efficacy trials was largely driven by results from the *in vitro* biofilm exposure experiments (discussed in Chapter 2) and

pharmacokinetic studies (discussed in Chapter 3). The failed translation of the positive results observed in each of these studies to the *in vivo* treatment scenario can likely be attributed to multiple factors. Initial studies evaluating biofilm killing capacity likely overestimated the bactericidal ability of the emulsion due to a less than ideal experimental setup. During such experiments biofilms were immersed from the top surface of the emulsion (schematic of experimental setup shown in Figure 2.1). In this orientation, any creaming occurring during the emulsion exposure increases contact between the aqueous drug and biofilm surface. Thus, the positive bactericidal results from this experiment could have been partly a result of a small amount of separated aqueous drug rather than completely indicative of the killing ability of the bulk of the emulsion. Additionally, these experiments neglected the effect of systemic absorption of the drug. Pharmacokinetic studies (discussed in Chapter 3) later showed that increased C_{aq} values, as used in the APV + aerosol efficacy trials, results in quicker and larger drug absorption into the systemic circulation, thereby removing it from the site of infection. Accordingly, lower C_{aq} values (in combination with larger V_{aq} values) were implemented during the APV alone efficacy trials in order to prolong exposure between the drug and pulmonary bacteria. The primary oversight of this reasoning was that it neglected to take in to account the availability of the measured pulmonary drug in the emulsified state in which it exists within the lungs. The method of pulmonary tobramycin measurement used during the pharmacokinetic trials likely increased the drug's availability during measurement relative to its prior emulsified state. More specifically, the dilution and homogenization process used during tissue tobramycin measurement likely increased tobramycin availability in a manner similar to that achieved during the phase inversion and separation process used to quantify tobramycin content discussed in Chapters 3 and 4. Thus, some portion of the pulmonary tobramycin measured during the pharmacokinetic experiments may have

been unavailable to contribute to APV's *in vivo* antibacterial effect. Ultimately, these effects were not fully brought to light until the studies assessing drug availability discussed in Chapter 4. During these studies it was shown that the presence of fluorosurfactant can significantly impair the availability of emulsified drug to a surface in contact with the emulsion. Both efficacy trials implemented emulsions utilizing FSL-PEG+FSL at $C_{fs} = 30$ mg/mL H₂O. As can be seen in Figure 5.5, emulsion utilizing these parameters (containing a loaded tobramycin mass of 1,000 µg/mL) resulted in an equivalent aqueous tobramycin concentration of 108 µg/mL. Thus, the fluorosurfactant type and concentration used during both efficacy trials likely resulted in a significantly decreased available tobramycin dose, possibly as small as an order of magnitude lower than the intended dose. Considering this, the lack of bactericidal activity observed during the efficacy trials is not necessarily surprising.

Other valuable inferences stand to be gained from the results of these trials. The APV + aerosol efficacy trials showed that APV implemented in combination with subsequent aerosolized

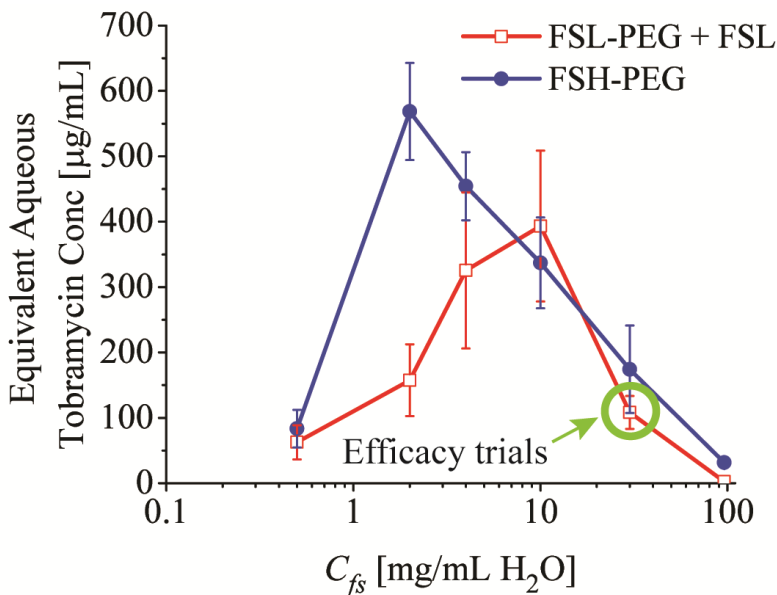


Figure 5.5: Tobramycin availability (reproduced from Chapter 4) showing fluorosurfactant type and C_{fs} used during efficacy trials.

delivery was no more effective than aerosolized delivery alone. Thus, the expected benefits of airway clearance during APV on subsequent aerosolized delivery were likely absent in this study. Multiple factors could have contributed to such an effect. At the conclusion of APV performed on a patient, some portion of the emulsion would be suctioned from the lungs along with any dislodged mucus or biofilm. However, previous experience with rats has shown that suctioning of PFC from the lung is difficult and highly inconsistent. Thus, during APV in the current studies, emulsion was instilled in the lung and left to completely evaporate during subsequent ventilation. Such a technique may dislodge mucus or biofilm but fails to remove it from the airways. In addition, the effect of mucus plugging on the distribution of aerosolized drug seen clinically may differ from that in a rat. As mentioned in Chapter 2, intratracheal placement of the Microsprayer in the rat can sometimes induce apnea, resulting in aerosol delivery in the absence of airflow. The effects of poor ventilation distal to mucus plugging on aerosolized delivery are surely diminished in such a scenario.

The current work also evaluated APV utilizing lung fill volumes of 3 and 15 mL/kg. These values represent either end of the spectrum, resulting in the lung being approximately 20 or 100% filled at the end of expiration. The lack of difference in effectiveness between the two treatments suggests that although contact between the emulsion and lung is an important treatment parameter to be considered during APV, it was likely not the critical issue at the core of APV's observed shortcomings. Alternatively, the lack of difference between the two lung fill volumes is consistent with the diminished treatment effects of impaired drug delivery proposed above. If the available drug dose was as low as previously estimated (approximately 10% of intended dose), reduction of bacterial killing would likely be insignificant regardless of the degree of contact between the emulsion and bacterial surface.

Lastly, although ineffective in resolving the bacterial infection, APV treatment appeared to be well tolerated by the inoculated rats. Mortality during treatment was rare and arterial oxygen saturation (data not shown) during APV was typically observed to be similar to or better than that of unventilated rats on supplemental oxygen. Additionally, rats recovering from APV (during APV + aerosol trials) exhibited similar traits and behavior as those of the other treatment groups.

The disease model developed and refined in preparation for these studies also represents a positive outcome of this work. The methods used were based off of previously established rat models^{10, 13} of *PA* lung infection in which viable bacterial colonies are immersed in a synthetic alginate and delivered intratracheally. The inoculation methods described were shown to produce a pulmonary bacterial infection with reasonably low mortality (< 30%) that was typically unable to be cleared by an untreated rat. Furthermore, the resulting infection was also shown to be unresolved following aerosolized treatment, thereby providing a useful testbed to show potential improvements over currently used therapy. Additionally, the use of the described throat swab technique proved to be a reliable, relatively non-invasive method to confirm the presence of infection prior to treatment. It is recommended that future APV work utilizing a rat disease model use the inoculation methods described in this chapter in conjunction with a throat swab prior to treatment.

The current work represents the most clinically relevant measure of APV's therapeutic potential at varying time points in the midst of its development. Although APV was shown to provide no treatment benefit in either evaluation, the negative outcomes ultimately guided the direction of future efforts towards emulsion optimization. Such efforts have ultimately resulted in a drastically increased understanding of APV and a significantly more optimized emulsion formulation that is yet to be evaluated in an *in vivo* infection model. The tobramycin availability

of the optimal emulsion formulation from work discussed in Chapter 4 ($C_{fs} = 2$ mg/mL H₂O of FSH-PEG, $V_{aq} = 2.5\%$, and $C_{aq} = 40$ mg/mL) is more than five times greater than that of the formulations evaluated in the efficacy evaluation trials described in this chapter. Thus, the optimal emulsion formulation will likely exhibit a greater bactericidal effect in the lung.

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CHAPTER 6

Conclusions, Limitations, and Future Work

6.1 Conclusions

The presented work explored the use of tobramycin-loaded emulsions during antibacterial perfluorocarbon ventilation (APV) as a novel treatment for bacterial respiratory infections. Initial studies evaluated the bacterial killing capacity of such emulsions as well as the feasibility of their use as a liquid ventilation (LV) medium. *In vivo* studies were then used to confirm the ability of APV to successfully deliver tobramycin to the lung and characterize the effects of emulsion formulation on the resulting pharmacokinetics. A chronic bacterial respiratory infection model in rats was used at multiple points throughout this work to evaluate the efficacy of APV treatment relative to currently used treatments. Due in part to poor *in vivo* results, in-depth *in vitro* studies were performed to analyze the effects of emulsion formulation on drug availability and biocompatibility. The aim of this *in vitro* work was further optimization of the emulsion for maximal treatment efficacy and safety. From these studies, it can be concluded that:

1. The fluorosurfactants used in this work (PEGylated perfluoroether copolymers) are capable of sufficiently emulsifying aqueous tobramycin within a naturally immiscible perfluorocarbon (PFC) phase. The presence of the aqueous drug in the form of micron-

scale droplets was confirmed via microscopy. The emulsion preparation process is straightforward and could be performed at the bedside immediately prior to administration.

2. The addition of aqueous tobramycin and fluorosurfactants, along with the remainder of the emulsification process, does not negatively alter the rheological properties of PFC critical to its use as a LV medium. The viscosity and air interfacial tension of the emulsion are not significantly different than that of neat PFC. The aqueous interfacial tension of the emulsion is reduced relative to that of neat PFC, potentially resulting in increased mucus removal capabilities during treatment.
3. Exposure to the tobramycin-loaded emulsions for the intended treatment duration (two hours) results in effective killing of *Pseudomonas aeruginosa* biofilms in a simplified *in vitro* setting. Bactericidal effects in this setting increased for emulsion formulations utilizing increased aqueous tobramycin concentration (C_{aq}), and to a lesser extent, aqueous volume percent (V_{aq}).
4. Partial APV utilizing the emulsions is capable of successful pulmonary tobramycin delivery and the ensuing pharmacokinetics are largely determined by the emulsion formulation. Changes in V_{aq} and C_{aq} were found to have the largest effect on the kinetics of delivered drug. At a constant fluorosurfactant concentration (C_{fs}), increased V_{aq} in combination with decreased C_{aq} results in significantly increased pulmonary drug retention, with formulations examined exhibiting up to 22 times greater retention than aerosolized delivery at four hours post-delivery.
5. The distribution of tobramycin (in the form of small aqueous droplets) throughout the bulk of the emulsion is spatially uniform over the intended treatment duration for emulsion formulations utilizing a sufficiently large C_{fs} . The critical C_{fs} value below which emulsion

instability is present and phase separation occurs is dependent on the fluorosurfactant type, but was shown to range from 2 to 30 mg/mL H₂O for those used in this work.

6. In addition to assembling at the boundary of the aqueous droplets within the emulsion, the fluorosurfactants also appear to accumulate at an aqueous surface in contact with the emulsion (surface for intended delivery) and impede tobramycin transport into such a surface. The availability of drug to diffuse into an aqueous surface thus decreases with increasing C_{fs} , and the ideal C_{fs} for a particular fluorosurfactant type is the minimum value necessary for sufficient emulsification of the loaded aqueous drug. Such a value should result in maximal drug availability while also maintaining homogenous drug dispersion throughout the emulsion.
7. The drug emulsification and availability properties of an emulsion were found to significantly vary with fluorosurfactant type. FSH-PEG exhibited superior properties when compared to the relatively less fluorophilic fluorosurfactant used in initial studies (FSL-PEG+FSL). Emulsions utilizing FSH-PEG resulted in emulsion stability at lower values of C_{fs} while also showing significantly greater drug availability. Stable emulsions using FSH-PEG showed a maximum drug availability at $C_{fs} = 2$ mg/mL H₂O that was greater than five times the availability of emulsions used during *in vivo* treatment evaluation.
8. Partial APV with emulsions utilizing FSL-PEG+FSL at a relatively large C_{fs} (30 mg/mL H₂O) showed no advantage in reducing pulmonary bacterial load in a rat model of chronic lung infection relative to aerosolized tobramycin delivery. Furthermore, neither treatment (APV or aerosolized) produced a significant decrease in pulmonary bacterial growth relative to no treatment. The lack of *in vivo* bactericidal ability observed for APV was likely a result of suboptimal emulsion formulations. More specifically, the type and concentration

of fluorosurfactant used during the efficacy trials likely resulted in poor drug availability to the bacterial surfaces within the lung.

9. The fluorosurfactants used in this work exhibited concentration-dependent cytotoxic effects when exposed to human alveolar epithelial cells in an *in vitro* setting for a period of six hours. FSH-PEG resulted in reduced cytotoxicity as compared to FSL-PEG+FSL. Emulsion utilizing the optimal C_{fs} value for drug emulsification and availability ($C_{fs} = 2$ mg/mL H₂O of FSH-PEG) exhibited no measurable cytotoxic effects as compared to cell exposure to neat PFC.

6.2 Limitations and Future Work

The major limitations of this work primarily resulted from the use of a rat model as opposed to a larger animal species. The use of rats severely limited our ability to evaluate the mechanically-induced mucus and biofilm removal effects of APV. The significant role of abnormal mucus and biofilm presence in the progression of bacterial respiratory infection during lung disease is well established. Mucus removal has been previously noted during LV with neat PFC.¹⁻³ Moreover, there is ample theoretical evidence to expect that ventilation with emulsion during APV should result in equivalent or even greater mucus removal effects (discussed in Section 1.7). Mucus and biofilm removal during initial treatment with APV would not only make an immediate positive impact on the disease state, it should significantly increase the effectiveness of subsequent inhaled treatment. Yet all rat treatment trials performed in this work utilized emulsion delivery without subsequent removal, a setting that does not adequately allow for such effects to be manifested. The small airways of rats make suctioning of emulsion, as well as dislodged mucus and biofilm, technically difficult to perform in a consistent and reliable manner. Future APV work in a larger

animal model (likely rabbits, ferrets, pigs, or sheep) would allow for the use of equipment (*e.g.* cuffed endotracheal tubes) and treatment procedures (*e.g.* bronchoscopy) more akin to those used clinically. Additionally, the use of a larger animal model may afford the opportunity to work with a more relevant disease model as well. Although the rat model discussed in the current work recreates many of the symptoms exhibited during cystic fibrosis (CF) in humans, recent work has made strides in developing genetically-altered CF pig and ferret models that resemble the clinical disease state much more closely.^{4, 5} Pigs generated with mutated cystic fibrosis transmembrane conductance regulator (CFTR) genes even demonstrate impaired pulmonary host defenses as indicated by the spontaneous development of mucus accumulation, bacterial infection, and airway inflammation.⁶

Another limitation within these studies was the lack of an ideal experimental testbed to evaluate the bactericidal capacity of the emulsions used during APV. The ability of the emulsions to effectively eradicate bacteria in the setting of lung infection is integral to the treatment efficacy of APV. Initial studies evaluating the bacterial killing effects of the emulsions (discussed in Chapter 2) used a previously developed biofilm susceptibility assay.⁷ The primary drawback of this experimental setup is that the biofilm-coated pins are immersed in the emulsion from the top surface. Thus, any aqueous drug that was brought to the surface via creaming of the emulsion (which is present to some degree for all emulsion formulations) was brought in to direct contact with the biofilm surface. Additionally, the ratio of bacterial surface area to emulsion volume, and thus drug mass, is likely much smaller in this scenario relative to the physiological one. These effects likely resulted in an overestimation of the bactericidal ability of the varying emulsion formulations examined. In order to address this, later studies (discussed in Chapter 4) used an adaption of the agar well diffusion assay to measure the ability of emulsified drug to be delivered

to an aqueous surface and subsequently inhibit bacterial growth. Although the orientation of contact between the emulsion and delivery surface is improved, the bacteria in this setting greatly differ from that in the airways during infection. As discussed in section 1.2, the biofilm environment found in the airways during respiratory infections affords the bacteria a significant degree of protection against antibiotic therapy. Bacteria seeded on to an agar surface are likely more susceptible to antibiotic exposure. Although a significantly more realistic bacterial environment can be found in the airways of an infected animal, the *in vivo* setting is complicated with numerous factors often left unaccounted for. A more controlled, *in vitro* setting is often ideal during the initial development and optimization phases of a technology such as APV. A much improved *in vitro* experimental setup would expose a large surface area of bacterial biofilm to emulsion in a manner such that buoyancy effects are directing drug away from the surface. Although the physiological scenario would include bacterial surfaces in nearly every orientation relative to gravity, the described setup would represent the most challenging scenario, and thus a useful experimental testbed. The easiest and most relevant method to achieve this may involve emulsion exposure to a portion of an excised infected airway or lung.

The next step for further development of APV is re-evaluation of APV treatment with the optimal fluorosurfactant (FSH-PEG) and emulsion formulation ($C_{fs} = 2$ mg/mL H₂O, $V_{aq} = 2.5\%$, and $C_{aq} = 40$ mg/mL) in the chronic lung infection rat model previously described in Chapter 5. As shown in Chapter 4, the optimized emulsion formulation exhibits greater than five times the drug availability of those formulations previously evaluated in the rat model. Due to the likely diminished benefits of APV to subsequent aerosolized delivery in a rat (discussed in Chapter 5), evaluation should use the simpler approach of a single-dose treatment comparing partial APV and aerosolized delivery. A more clinically relevant evaluation of a multi-day course of treatment

including APV with subsequent aerosolized delivery should be postponed until a larger animal model is available. Similar to previous efficacy trials, pulmonary bacterial load remaining following treatment should be the primary outcome measure. In addition to continuing to optimize the efficacy of APV, further biocompatibility testing should also be pursued in parallel. Initial cytotoxicity results are promising, but more in-depth *in vivo* trials should be performed in rats or a larger animal model. *In vivo* trials should assess lung function as well as liver and kidney function at multiple time points following APV treatment.

Although the current work made great strides in optimizing the emulsion formulation, further optimization is likely possible and should be explored. The current studies have demonstrated the immense importance of nearly every emulsion formulation parameter and established a strong understanding of their corresponding effects on APV treatment. It is worth noting that only a small collection of fluorosurfactants have been evaluated in this application. Although the selection of fluorosurfactants available is somewhat limited, even small changes in the molecular structure and size may have significant effects on treatment potential. Additionally, this work assessed emulsions utilizing only a single type of PFC (FC-770). The current studies represent initial optimization and proof-of-concept work, and thus the use of a single PFC which is readily available and affordable is justified. However, multiple PFCs with varying molecular structures have been used during LV and have even been shown to produce varying cellular responses.⁸ The type of PFC used will ultimately affect its interaction with the fluorosurfactants within the emulsion and thus is a determinant of the resulting emulsion properties. Exploration of the use of other PFC types has the potential to further improve APV treatment and should be pursued.

In a much broader sense, future work should also explore the same basic delivery principles used during APV to deliver other therapeutic agents in other disease states. In theory, drug delivery via ventilation with a water-in-PFC emulsion using the methods developed in this work could be used to achieve spatially uniform delivery of any aqueous soluble drug. Any disease states in which inflammation is a primary concern would also benefit from the inherent anti-inflammatory effects of PFC. Any treatment in which a primary aim is to lavage the lungs and rid the airways of an aqueous media would benefit from the ability of PFCs to displace aqueous fluid. Commonly performed whole-lung lavages of pulmonary alveolar proteinosis patients in order to rid the lung of lipoproteinaceous material may represent an opportunity for application of this technology. Such a lavage could be performed with emulsion in order to simultaneously deliver therapeutic agents (such as granulocyte macrophage colony-stimulating factor) that are typically given via aerosol following lavage. The delivery of similar growth factors via an emulsion could also be utilized during acute respiratory distress syndrome while simultaneously ridding the lungs of aqueous exudate and benefiting from the anti-inflammatory properties of PFC. The general strategy used during APV possesses unique treatment capabilities and likely has the potential to improve the efficacy of respiratory treatments beyond antibiotic therapy.

6.3 References

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