LUNG-DIRECTED DELIVERY OF THERAPEUTICS FOR THE TREATMENT OF PULMONARY FRANCISELLA INFECTION

by

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A Dissertation
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS AND SYMBOLS

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine……DPPC
1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol……..DPPG
All-Glass Impinger……AGI
Ampere……...…A
Brain Heart Infusion……...BHI
Colony Forming Units……...CFU
Electrospray-neutralization……..……..ESN
Francisella tularensis subsp. *LVS*……..…..…..*F. LVS*
Francisella tularensis subsp. *novicida*……..…..*F. novicida*
Immunoglobulin G……..IgG
Institute of Theoretical and Experimental Biophysics……..……ITEB
Intraperitoneal……...……i.p.
Kilo-……k
Lethal Dose……..LD
Lipopolysaccharide……LPS
Live Vaccine Strain……LVS
Micro-……..µ
Milli-……...m
Minimum Inhibitory Concentration……...MIC
Multiple Path Particle Dosimetry……MPPD
Nano-……n
Phosphate Buffered Solution……..PBS
Polyvinylpyrrolidone……..PVP
Siemens……S
Subspecies…….……subsp
Trypic Soy Broth with Cysteine……..TSBC

ABSTRACT

LUNG-DIRECTED DELIVERY OF THERAPEUTICS FOR THE TREATMENT OF PULMONARY FRANCISELLA INFECTION

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George Mason University, 2015

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According to the World Lung Foundation, acute respiratory infections result in 4.25 million deaths each year, are the third largest cause of mortality worldwide, and are the number one cause of death in low- and middle- income countries. These infections require few infectious particles and tend to be serious, difficult to treat, and spread quickly. Despite existing treatments, many researchers work on additional therapy options including new drug development, more effective drug combinations, and developing ways to overcome antibacterial resistance. However, delivery of existing treatments in a novel manner may lead to better therapeutic regimens without the high cost and length of time required for discovery and development of new drugs.

The Institute of Theoretical and Experimental Biophysics in Moscow recently developed a new nanoaerosol generator. This study evaluated this novel technology, which has the potential to enhance therapeutic delivery. First, the analysis of quantum

dots distribution in cryosections of murine lungs demonstrated that nanoaerosols penetrate the alveoli, the site of lower respiratory infections and entry point to the circulatory system. Second, using a pulmonary *Francisella tularensis* subsp. *novicida* infection of BALB/c mice as a model, the generator was used to aerosolize the antibiotic, levofloxacin, and was able to rescue mice more efficiently than traditional delivery methods, including large particle aerosol. In addition, it was found that treatment with nanoaerosols consumes less total volume of therapeutic solutions and is gentler on sprayed material than the aerosolization by a conventional three-jet collision nebulizer. Nanoaerosols can be produced using numerous types of drugs, not just antibiotics, and should be explored further as treatments for additional forms of respiratory disease.

CHAPTER ONE: INTRODUCTION

According to the World Lung Foundation, acute respiratory infections result in 4.25 million deaths each year and are the third largest cause of mortality worldwide [1]. Low- and middle-income countries are hit particularly hard by these diseases where they are often the number one cause of death. Focus tends to settle on pneumonia, influenza, respiratory syncytial virus, and tuberculosis but lethal acute respiratory infections can occur from any number of viruses, bacteria, and fungi. Commonly, these infections require few infectious particles and tend to be serious, difficult to treat, and spread rapidly.

The medical community has tools available to treat many cases of these respiratory infections but new therapies are needed. Many researchers spend their careers in this field to work on new drug development, more efficient drug combinations, and working on ways to overcome resistance to current therapies. However, this research requires significant time and resources so a potentially shorter-term solution should be explored. Improvement to the lung delivery system and expanding the types of treatments delivered to the lungs can vastly improve upon future treatment of pulmonary infections. Delivering existing treatments in a novel manner may lead to better therapeutic regimens that may not only give time for the discovery of new treatments, but lead to new avenues of research on its own.

Aerosolized therapeutics improve upon traditional delivery methods in cases of pulmonary infection due to their ease of administration, access to the large lung surface area, systemic distribution prior to first-pass metabolism, and appropriate localization in the alveoli. [2] The merit of inhalational therapies has been well established in the treatment of cystic fibrosis patients, where the high local concentration but low systemic effects are ideal. [3]

New technology in the production of nanoaerosols allow for further improvement on these treatments due to their deeper penetration into the respiratory tract, enhanced deposition in the alveoli, and requirement for a lesser dose. The ability for the drug to bypass the digestive system and be delivered directly to the targeted location reduces the required dose of many drugs by three to six orders of magnitude when compared to oral routes. [4] In some cases, as with the anti-inflammatory drug, indomethacin, the effective dose is reduced by as much as a million times. [5]

Francisella tularensis can result in an acute respiratory disease and will therefore be used as the infection model for these proposed lung-directed therapies. The United States' Department of Health and Human Services has listed *F. tularensis* as a select agent due to its severe threat to both human and animal health, high degree of contagiousness, ability to be aerosolized, and the lack of a viable vaccine. In addition, *F. tularensis* is a Tier 1 Select Agent due to its history of being developed as a biological weapon by the United States and Soviet Union. Due to increasing cases of naturally acquired antibiotic resistance among pathogens and the possibility of genetically

modified strains created for more nefarious purposes, it is critical that the scientific community investigate new or improved treatments against potential threat agents.

The *Francisella* species are gram-negative, facultatively intracellular, pathogenic bacteria readily found in nature. Four subspecies of *F. tularensis*, the most human relevant of the *Francisella* genus, are currently known: *tularensis*, *holarctica*, *novicida*, and *mediasiatica*. [6] Human outbreaks of *Francisella* infection, known as tularemia, are commonly associated to subspecies *tularensis* in the United States and subspecies *holarctica* in Europe. As with most zoonotic diseases, human outbreaks of tularemia correspond with a similar increase in documented animal cases. The most common animal host in the United States are rabbits; however, *Francisella* infections have been verified in hundreds of mammalian species, as well as numerous fish, birds, and reptile species. [7]

Francisella can be transmitted to humans directly from infected animal hosts or indirectly via insect vectors. In the case of direct transmission, generally agricultural workers are at the highest risk due to an increased likelihood of animal bites and aerosolization of bacteria by manipulating animal products. [8,9] Infected individuals outside of the agricultural field are most likely victims of indirect transmission. Ticks, biting flies, and mosquitos have all been shown to transmit *Francisella* bacteria by feeding on humans after a blood meal on an infected animal host. [7,10] In addition, ticks have also been shown to acquire the bacteria through transovarial transmission, which suggests they may also be reservoirs. [11]

Mice are regularly used as models in infectious disease research due to their common genetic and physiological traits as well as their ability to be easily manipulated and obtained. Approximately 99% of the mouse genome is similar to humans, which is closer than other lower species models. [12] The presence of homologous organ systems makes them invaluable in examining results and extrapolating that information to humans. In addition, their relatively low cost and quick breeding allow researchers to easily obtain them. Perhaps the most convincing argument for the use of mice in research is the ease at which they can be genetically manipulated to select for desired traits needed for study. [12] Different strains are maintained to exhibit these characteristics. This study utilizes Harlan Laboratories' BALB/c strain, which is categorized for use in many fields of biomedical research. Conlan *et al*, among others, have repeatedly shown that BALB/c mice are susceptible to *Francisella* infections although more so to the subspecies *novicida* than the subspecies *LVS*. [13-15]

A very low dose of *Francisella* is required to cause disease by the pulmonary route. As seen in the chart below, in BALB/c mice ten or less individual aerosolized bacteria are usually sufficient to cause disease.

Table 1 Published LD_{50}s of Francisella subspecies via Different Delivery Methods

***Francisella* Strain**	**Delivery Method**	**LD_{50}**	**$100LD_{50}$**	**Source**
novicida	IP	3	$3x10^2$	Lauriano *et al* [16]
	IN	10	10^3	Lauriano *et al* [16]
	Aerosol	10	10^3	Zogaj *et al* [17]
Schu S4	IP	1	100	Fulop *et al* [18]
	IN	33	$3.3x10^3$	Klimpel *et al* [19]
	Aerosol	10	10^3	Conlan *et al* [13]
LVS	IP	1	10^2	Conlan *et al* [13]
	IN	10^3	10^5	Lu *et al* [20]

	Aerosol	10^3	10^5	Conlan *et al* [13]

After infection with a disease-causing dose, there is a three to five day incubation period before an individual exhibits the symptoms of tularemia. Symptoms include fever and malaise, which tends to prevent accurate diagnoses unless that patient describes experiences with wild animals or ticks to their physician. *Francisella* can be transmitted to humans in a number of ways, which can lead to six different forms of tularemia: ulceroglandular, oculgandular, oropharyngeal, gastrointestinal, typhoidal, and respiratory. [6]

The most serious form of tularemia (respiratory) is caused by the introduction of aerosolized *Francisella* directly into the lungs. As few as ten bacteria from the subspecies *tularensis* introduced into the lungs can be fatal. [13] Respiratory tularemia frequently results from lawn mowing or otherwise manipulating contaminated materials in ways that can lead to aerosolization. However, the intentional exposure of humans to respiratory tularemia is a matter of great concern in the field of bioterrorism. [6] As result of this, laboratory workers are also at an increased risk due to potential laboratory mishaps as seen in the outbreak of laboratory-acquired infections during the 50s and 60s.

The major line of defense against bacterial infection in the lungs is the alveolar macrophages. It has been shown that 90% of bronchoalveolar lavage fluid is composed of macrophages and these macrophages are capable of removing up to 10^5 CFU of bacteria in two hours. [21] However, after inhalation of *F. tularensis Schu S4*, alveolar macrophages are quickly infected and ultimately used by the bacteria for systemic

distribution. [21] At 24 hours post infection the bacteria is still contained within the lungs and forms slight hemorrhagic lesions. [22] By 48 hours post infection, the bacteria have left the lungs and spread into the spleen. At this point enlarged spleens are seen as well as occasional enlarged livers and lungs. [22] Bacteria are detectable in the lungs, spleen, liver, kidneys, and blood at 72 hours post infection. Body temperature begins to rise and pyogenic lesions are found in the lung, spleen, liver, and lymph tissue. [22] Clinical signs of infection begin at 96 hours post infection, including piloerection, continual increase in body temperature, decreased appetite and activity, and weight loss. [23] The bacterial burden increases in all organs and necrosis in lymph nodes, hepatitis, splenitis, and interstitial pneumonia are seen. The average time to death in the primate model is 4.5 to 7 days post infection. [22,24]

To address the biological threat of intentionally released aerosolized *Francisella* and increased need for novel or enhanced treatment options within both the public and private sectors, the aforementioned nanoaerosol technology was applied to this study. Nanoaerosolized antibiotics were compared to traditional methods to evaluate potential superiority against pulmonary tularemia in the areas of antibiotic delivery and passive immunization. Additionally, passive immunization is a fairly old but uncommon treatment technique used during the recent Ebola outbreak. Using the same principles of pulmonary delivery it is possible to make this treatment more feasible for a broader range of respiratory diseases, including *Francisella*.

CHAPTER TWO: NANOAEROSOLIZED ANTIBIOTICS

Introduction

Aerosolized therapeutics may improve upon traditional delivery methods in cases of pulmonary infection due to their ease of administration, access to the large lung surface area, and limited systemic distribution. [2] It has been found that the delivery of therapeutics via the pulmonary route bypasses digestive destruction in the stomach and first-pass metabolism in the liver, thus potentially greatly reducing required doses and resulting side effects. The merits of inhalational therapies have been well established in the treatment of cystic fibrosis patients where the high local concentration but low systemic effects are ideal. [3]

Nanoaerosols are defined here as being aerosols comprised of particles that are less than 200 nm in diameter. [25] In comparison, aerosol particles created by a standard 3-jet collision nebulizer are usually between 1 and 5 μm in diameter. [26] New technology in the production of nanoaerosols may allow for further improvement on the treatment of lung-based infections due to the enhanced deposition of therapeutic aerosol in the lower respiratory tract and appropriate localization in the alveoli, which may result in a lower necessary dose.

Despite obvious advantages, progress in biomedical applications of nanoaerosolized drugs has been slow due to numerous technological problems involving nanoaerosol generation and dosimetry. Most methods used in the generation of

nanoaerosol from inorganic materials (such as the sublimation–condensation technique) are not applicable to many therapeutic molecules such as antibiotics or proteins due to their fragility during the evaporation and sublimation process. [27] It has been shown that even relatively mild ultrasound nebulizers are capable of damaging fragile biomolecules and drugs. [28]

Of the different methods summarized in the recent reviews, electrospray atomization provides the most efficient way to turn biological or therapeutic substances into nanoaerosol. [29,30] Noakes *et al* patented the first inhaler that employed electrospray atomization in 1989. [31] Since then, several other designs for nanoaerosol generators have been described but operated on the principle based on Noakes' electrospray atomization followed by charge neutralization by counter-ions generated in ionized air via radioactive isotopes or corona discharge. [32-35] In these neutralization techniques, aerosol particles are exposed to highly reactive radicals, hot molecules, ozone, and oxygen atoms, all of which are destructive to biological molecules.

The Institute of Theoretical and Experimental Biophysics (ITEB) in Moscow developed a new technique for the mild generation of nanoaerosol in which electrosprayed, charged drug nano-clusters are neutralized in gas-phase by counter-ions generated via electrospraying of a volatile solvent, such as ethanol. [36-38] This technology, called electrospray-neutralization (ESN) is based on the electrohydrodynamic atomization of a solution followed by gas-phase neutralization of the electrospray–generated ions and nanoclusters with oppositely charged ions generated via the same technique. It provides a new avenue in the fabrication of a variety of

nanoproducts based on the fact that oppositely charged species are forced to form complexes upon neutralization, while collisions between similarly charged products are inhibited. Unlike the current methods of neutralization with corona, which generates highly reactive radicals, the ESN technology employs less reactive ions and induces less damage to the sprayed material. [33] It has been demonstrated that ESN can produce protein nanoaerosols with almost complete retention of the functional properties of protein molecules, which speaks to its gentle aerosolization process and suitability for use with biologics and therapeutics. [25,39] ESN as a method of nanoaerosol generation has the following advantages: (i) it is universal by being applicable to most soluble drugs, (ii) the particle size and concentration are well controlled by changing electrical parameters, (iii) it is capable of extreme (up to molecular level) atomization, (iv) it is capable of producing charged nanoaerosol particles with enhanced deposition in lungs, (v) it is adaptable to very small volumes (microliters) of solution.

Nanoaerosols generated by other methods have been shown to be an effective way to deliver anti-inflammatory and anti-hypertensive drugs to mice. [4,5,40] Due to these results, it seems logical and potentially beneficial to extend these studies to other therapeutics. In this respect, the new electrospray technology has an advantage over the sublimation-condensation technique used in the anti-inflammatory and anti-hypertensive drug studies because it enables the generation of nanoaerosols from virtually any water or alcohol soluble substance, as long as solution conductivity remains low. To demonstrate the ability to nanoaerosolize useful antibiotic therapeutics and compare treatment

efficiency to traditional delivery methods, a mouse model of pulmonary *Francisella tularensis* subsp. *novicida* (*F. novicida*) infection was used.

The *Francisella* species are gram-negative, facultatively intracellular, pathogenic bacteria readily found in nature that can lead to a lethal infection in humans when as few as ten bacteria are inhaled. [13] Respiratory tularemia has been reported to result from traditional farming methods, lawn mowing, or otherwise aerosolizing contaminated materials. [6] However, the intentional exposure of humans to respiratory tularemia is a matter of great concern in the field of bioterrorism. [6] The United States' Department of Health and Human Services has listed *F. tularensis* as a select agent due to its severe threat to both human and animal health, high degree of contagiousness, ability to be aerosolized, and the lack of a viable vaccine. In addition, *F. tularensis* is a Tier 1 Select Agent due to its history of being developed as a biological weapon by the United States and Soviet Union.

Levofloxacin is a third generation fluoroquinolone antibiotic shown to be highly effective in treating *Francisella* infections (MIC_{90} 0.012 mg/L), despite not being considered the standard treatment. [19,41-43] Cell division is halted by the presence of levofloxacin as it inhibits type II topoisomerase, DNA gyrase, and topoisomerase IV thereby preventing the separation of replicated DNA. Levofloxacin is well tolerated by most individuals, able to reach high blood levels and required MICs, capable of intracellular penetration, and has a lower relapse rate than standard treatments. [42] The MIC_{90} of levofloxacin against *F. tularensis* is 0.012 mg/L. [43] According to the National Institutes of Health, fluoroquinolones, including levofloxacin, can have severe side

effects to the patient including, but not limited to, tendonitis, liver damage, and peripheral neuropathy. [44]

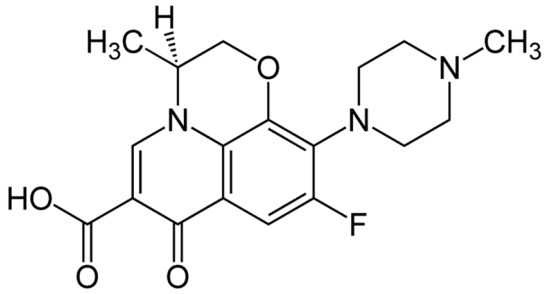

Figure 1 Molecular Structure of Levofloxacin

Ciprofloxacin is second-generation fluoroquinolone active against a wide variety of bacterial pathogens, both Gram-positive and -negative. Cell division is halted by the presence of ciprofloxacin as it inhibits type II topoisomerase, DNA gyrase, and topoisomerase IV thereby preventing the separation of replicated DNA. [45] The MIC_{90} of ciprofloxacin against *F. tularensis* is 0.016 mg/L. [43] Ciprofloxacin includes the same severe side effects warnings as fellow fluoroquinolone, levofloxacin, including tendonitis, liver damage, and peripheral neuropathy. [44]

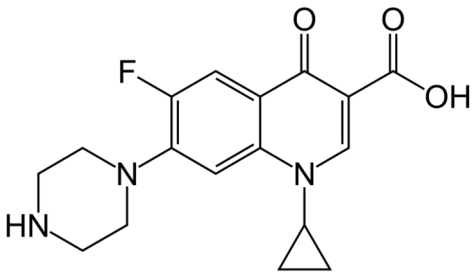

Figure 2 Molecular Structure of Ciprofloxacin

Fosmidomycin is a phosphonate antibiotic produced by organisms of the *Streptomyces* genus. Isoprenoid production is hindered by the presence of fosmidomycin because it inhibits the methylerythritol phosphate (MEP) pathways via the binding of 1-deoxy-D-xylulose 5-phosphate reductoiomerase (MEP synthase). [46,47] The MIC of fosmidomycin against *F. tularensis* is 136 μM. [47] Fosmidomycin has been shown to be an effective antimicrobial against *Francisella* in previous *in vitro* studies and *in vivo* studies involving wax moth caterpillars. [46,47] It has previously been used as an antimalarial drug and was shown to have minimal side effects, affecting mostly the gasterointestinal tract. [48]

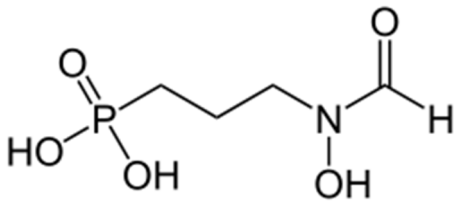

Figure 3 Molecular Structure of Fosmidomycin

In conjunction with the delivery of the aforementioned antibiotics via aerosol, *Francisella* bacteria were delivered via intranasal exposure. Based on preliminary results, nose-only aerosol delivery results in imprecise numbers of bacteria delivered into the lungs between experiments. While antibiotic effectiveness is measured to the nearest

10-factor dose, delivering 100LD_{50} of bacteria via aerosol accurately in every experiment is unlikely and thus the actual number of bacteria delivered is determined retrospectively in each experiment. Intranasal exposure has the potential of losing bacteria but increasing the instillation volume and utilizing an inhaled anesthetic reduces that possibility and increases the accuracy of delivered bacterial dose. [49] It has been shown that intranasal exposure to *Fransicella* results in bacterial build up in the lungs as well as a systemic infection post exposure and most aspects of the disease course are very similar between intranasal instillation and aerosol exposure. [50] Since aerosol exposure to *Francisella* would result in a similar disease course, though through smaller numbers of bacteria potentially delivered directly to the alveoli, this method results in a comparable lung infection.

This study investigated the utility of a nanoaerosol based therapeutic approach using levofloxacin against a murine pulmonary *Francisella* infection as a model. Furthermore, this therapeutic approach was compared in the same animal model to traditional delivery methods: intraperitoneal injection, oral administration, and 3-jet collision jet nebulizer generated aerosols.

Materials and Methods

Deposition modeling

Respiratory deposition probabilities for aerosolized particles in BALB/c mice were calculated using the Multiple Path Particle Dosimetry (MPPD) model, version 3.0 (Chemical Industry Institute of Toxicology, Research Triangle Park, NC), using the mouse model selection, MMAD, particle density of 1.48 g/cm^3, size range from 0.01 to 1

μm as selected parameters. [51,52] Estimated respiratory values of BALB/c mice determined by Flandre *et al* were entered into the program for modeling purposes (Equation 1). [53]

Nanoaerosol generation

The nanoaerosol generator was used in this study as previously described. [25,36] Briefly, a sample suspended or dissolved in water with a conductivity of less than 200 μS/cm was sprayed at a positive potential while ethanol was sprayed at a negative potential. Conductivity was measured with a conductivity meter (Oakton Con 11 Series, Thermo Scientific, Waltham, MA) possessing a modified probe to allow for the measurement of low volume samples. To accelerate atomization, the positively charged capillary had a pressure of between 7 and 9 cm H_2O and a current of 95 nA applied. The pressure and current in the negatively charged capillary were 3.6 cm H_2O and 40 nA, respectively. The volumetric yield of nanoaerosol was 2 liters per minute. Mice were exposed to nanoaerosols for four hours in a conductive whole body exposure unit (described below) attached to the generator output by conductive tubing.

Whole body exposure unit

This specialized chamber was developed at GMU to enable the delivery of nanoaerosols to five mice at a time. The chamber was produced by taking a 1 L acrylic, latched induction chamber (Vetequip, Inc, 941443), with the dimensions 3.75" x 4.5" x 3.75", and drilling out the plastic inlet and outlet ports to be replaced with brass fittings as shown in Figure 4. Then, taping off a "window" on each side to allow for observation of the mice, the inside of the box was coated with Total Ground Carbon Conductive

Coating (MG Chemicals, 838-340g), including the floor and lid, to reduce the deposition of nanoaerosol particles. The outside of the box was coated with Super Shield Silver Coated Copper Conductive Coating (MG Chemicals, 843-140g). Using a piece of copper tape, the lid was connected to the base of the box to ensure connection (not shown). This box can comfortably hold five mice for whole body exposure to nanoaerosols, which are delivered to the box via conductive tubing to the inlet port. Post chamber sampling can be done from the outlet port.

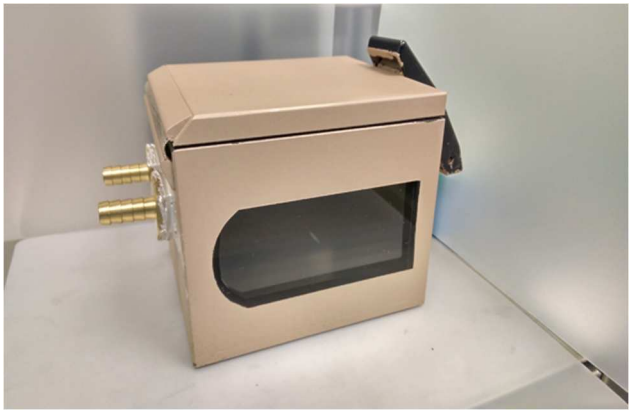

Figure 4 Conductive Whole Body Exposure Chamber

Aerosol sizing

Nanoaerosol particles were sized using a Scanning Mobility Particle Sizer (SMPS, TSI Incorporated, Shoreview, MN) to measure air particle size distribution in the range of 20 to 1000 nm. The SMPS is composed of an Ultrafine Water-based Condensation Particle Counter (model 3786), an Electrostatic Classifier (model 3080), a

Long Differential Mobility Analyzer (model 3081), and the Aerosol Instrument Manager® software (TSI Incorporated, Shoreview, MN).

Quantum dots deposition

6-8 week old female BALB/c mice (Harlan, Frederick, MD) were given a 46 nM quantum dot (20nm Qdot 705, Life Technologies, Grand Island, NY) solution by either a four-hour nanoaerosol spray or a single 40 μL intranasal dose. Controls received 40 μL of PBS intranasally. Mice rested for two hours before being administered a ketamine-xylazine cocktail. While under anesthesia, mice were euthanized and lungs were perfused *in situ* with 10 mL of PBS followed by 20 mL of 4% depolymerized paraformaldehyde. Lungs were harvested and underwent cryosectioning (10 μm thick sections with Thermo Scientific HM550 cryostat, Waltham, MA) and imaged using a confocal microscope (Nikon Eclipse TE2000-U, Melville, NY).

Bacterial strains

F. novicida (ATCC 15482) was obtained from American Type Culture Collection (Manassas, VA). All bacteria were streaked onto tryptic soy agar with 0.1% cysteine (TSAC) or Chocolate Agar (GC Agar II with Isovitalex, BD Biosciences, Franklin Lakes, NJ) plates and single colonies were inoculated into tryptic soy broth with 0.1% cysteine (TSBC) or Brain Heart Infusion, pH 6.8 (BHI) broth (TekNova, Hollister, CA). Cultures were incubated at 37°C overnight with liquid cultures at 250 rpm.

Confocal Immunofluorescence

6-8 week old female BALB/c mice (Harlan, Frederick, MD) were infected with *F. novicida* intranasally. Following euthanasia, lung sections were stained with DAPI to observe the nuclei, FITC phalloidin (green) to observe the cellular actin, and goat anti-

Francisella tularensis affinity purified polyclonal antiserum (DD-33, AB-AG-FTUL, Department of Defense Critical Reagents Program) was used as the primary antibody. The primary antibody was detected using donkey anti-goat IgG (H+L) secondary antibody, Alexa Fluor 594 (red) conjugate (Life Technologies, Fredrick, MD). The blue, green and red images were merged together to produce a composite image.

Murine infection

6-8 week old female BALB/c mice (Harlan, Frederick, MD) were infected intranasally with 50 μL of PBS containing 100LD_{50} of *F. novicida* (approximately 1000 CFU). Mice were examined twice a day for signs of illness or death. Bacterial inoculum concentrations were verified retrospectively via plating on chocolate agar.

Antibiotic treatments

Three hours post infection treatment was initiated and continued once a day for five days by (i) a 100 μL intraperitoneal injection, (ii) a100 μL orally administered dose, (iii) a 0.5 to 2 hour aerosol treatment, or (iv) a four hour nanoaerosol treatment. Aerosol treatment was administered via a three-jet collision nebulizer provided as part of the Biaera whole body exposure system (Biaera Technologies, Hagerstown, MD). Nanoaerosol treatment was administered as described above. Dosages were based on the average weight of all mice in the experimental run. Estimated respiratory values of mice determined by Flandre *et al* were used in conjuction with MPPD to calculate approximate deposition in lungs (Equation 1). [53] Effective doses of intraperitoneal and oral

levofloxacin, ciprofloxacin, and fosmidomycin against *Francisella* were located in the literature and used as a starting point in dose curves (Table 2).

Table 2 Published effective doses of difference antibiotics delivered via traditional delivery methods

Antibiotic	Delivery Route	Delivered Dose	Source
Levofloxacin	Intraperitoneal	6.25 mg/kg/day (13 days)	Klimpel *et al* [19]
	Oral	33 mg/kg/day (10 days)	Nelson *et al* [54]
Ciprofloxacin	Intraperitoneal	40 mg/kg/day (5 days)	Russell *et al* [55]
	Oral	100 mg/kg/day (14 days)	Piercy *et al* [56]
Fosmidomycin	Intraperitoneal	5 mg/kg/day (8 days)	Jomaa *et al* [57]
	Oral	50 mg/kg/day (8 days)	Jomaa *et al* [57]

Aerosol sampling

Aerosol samples from the Biaera aerosol generator were collected in distilled water contained within an all-glass impinger (AGI). Nanoaerosol samples from the nanoaerosol generator were collected on polyvinylpyrrolidone (PVP) filters that were dissolved in 100 μL of distilled water for measurements. PVP filters provide high capturing efficiency and are ideal for sample analysis because they are chemically inert. [58,59] Collected levofloxacin samples from both methods were diluted with distilled water and the antibiotic concentration was measured at a wavelength of 288 nm on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) against a standard curve. Liposome-encapsulated levofloxacin samples were diluted in ethanol as opposed to

distilled water to disrupt the liposome membranes prior to measurements and were measured at 300 nm.

Liposome preparation

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) was acquired from Echelon Biosciences Incorporated (Salt Lake City, UT). Cholesterol was acquired from Sigma-Aldrich (St. Louis, MO). Liposomes containing levofloxacin were prepared from DPPC, DPPG, and cholesterol precursors (2:1:2 molar ratio) using the well-established thin film dehydration/rehydration technique followed by sonication and extrusion to produce small unilamellar vesicles. [60,61] Liposomes were sized using a qNano particle analyzer (iZon, Oxford, United Kingdom) and shown to have a mean and mode diameter of approximately 161 nm and 156 nm, respectively (Figure 5). Levofloxacin concentration within liposomes was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) as described above.

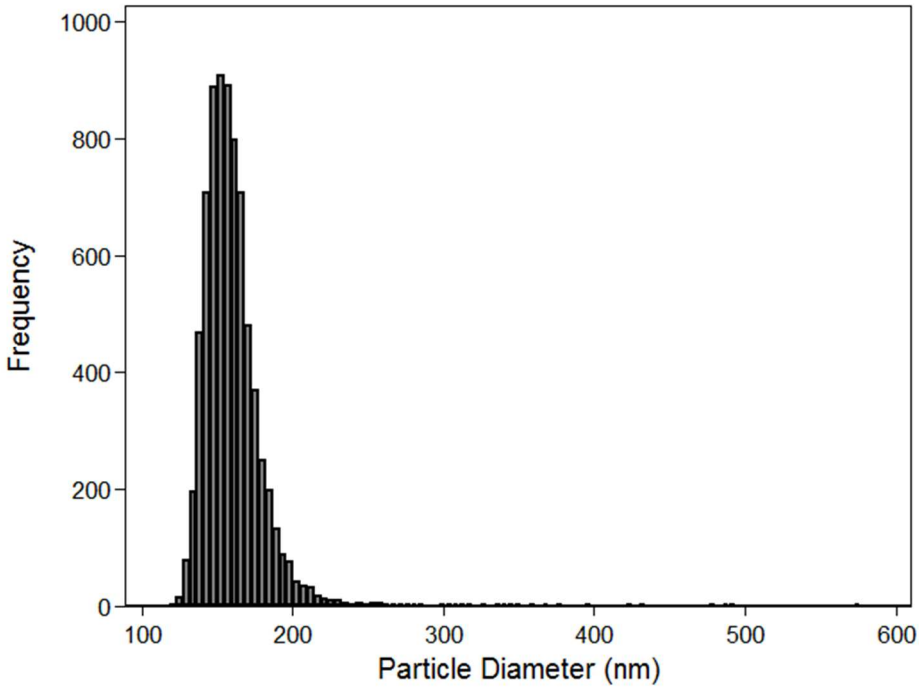

Figure 5 qNano Liposome Analysis

Histopathologic examination

Lungs, livers, and spleens were harvested from four different mice: (1) an uninfected, untreated naïve mouse; (2) an uninfected, naïve mouse treated with liposome-encapsulated water; (3) an infected mouse treated with liposome-encapsulated levofloxacin; and (4) an infected, untreated mouse. Formalin fixed organs were submitted to Experimental Pathology Laboratories, Inc. (Sterling, VA) for processing, hematoxylin and eosin staining, and histopathologic evaluation. Samples were randomly assigned numbers to ensure blind scoring by the pathologist.

Ethics statement

All animal experiments included in this manuscript were approved by and conducted in compliance with regulations of the Institutional Animal Care and Use Committee (protocol #0253) of George Mason University. All experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (2011) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002).

Results and Discussion

MPPD 3.0 software models the deposition of various sized aerosolized particles within a mouse respiratory tract based on extensive data from previously published studies. [51] As seen in Figure 6, large particle aerosols have the highest total deposition in the lungs but a very small percentage of that is deposited in the lower respiratory tract. Small particle aerosols have a lower total deposition but a large portion of these particles is retained in the alveoli. In an effort to maximally target the alveoli for the purpose of increasing treatment efficiency, nano-sized particles should be used.

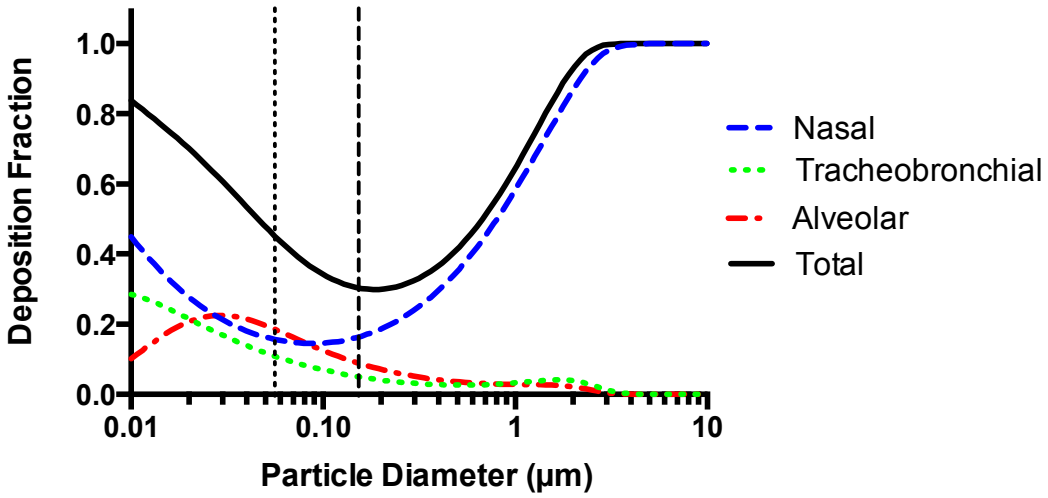

Figure 6 Predicted Respiratory Deposition of Various Sized Particles

Based on this data, it can be seen that particles generated by the 3-jet collision nebulizer, which range from 1 to 5 µm, have a high total deposition but that those particles mostly deposit in the nasal cavity, as there is an extremely low percentage of alveolar and tracheobronchial deposition for particles of that size. [26] Particles generated by the ESN generator fit the nano-size range that is predicted to have less total deposition but a significantly higher deposition percentage in the alveolar and tracheobronchial regions. It is hypothesized that the increased deposition of therapeutics in the lower respiratory tract through the use of the ESN generator will contribute to an improved outcome against pulmonary infections despite the low overall deposition.

Previous studies show that *Francisella* targets alveolar type II epithelial cells and macrophages during pulmonary infections so the ability to deliver therapeutics directly to the alveoli would be beneficial. [62,63] Intranasal delivery of *F. novicida* to the lungs was

verified to result in localization of bacteria in the alveoli (Figure 7A). According to MPPD, the small size of nanoaerosols allow for deeper penetration into the lung, specifically to the alveoli. To evaluate this claim, quantum dots were used to trace deposition in the lungs following nanoaerosol exposure and intranasal instillation. Quantum dots are nanocrystals made of semiconducting material approximately 20 nm in diameter that fluoresce at specified wavelengths and are frequently used as labels. [64] As seen in Figure 7B, the nanoaerosol generator produced nanoaerosolized particles with a geometric mean diameter of 39 nm, which confirms that the generator is capable of producing particles in the nano-sized range. As predicted, sections of the lungs treated with nanoaerosolized quantum dots show that, when compared to intranasal delivery, particles homogenously penetrate deeper into the lower respiratory tract, including the alveoli and lung parenchyma, instead of mostly being deposited in the bronchioles and mucus (Figure 7C, D).

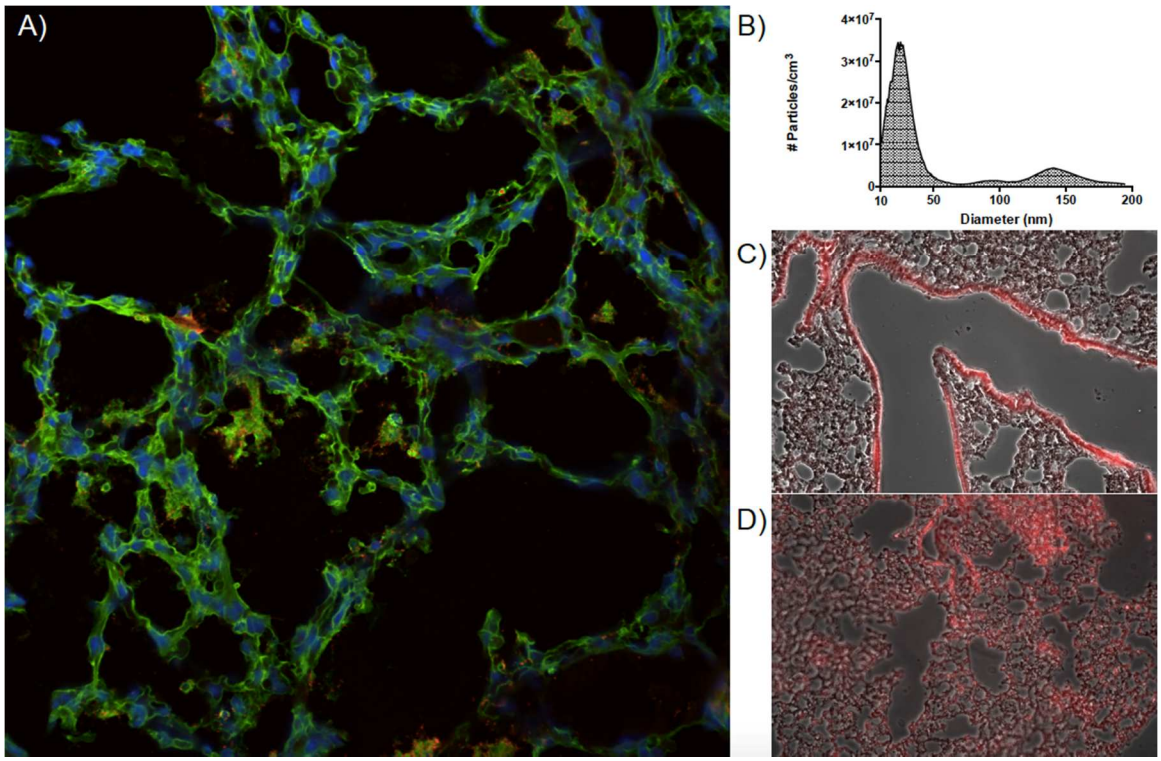

Figure 7 Bacterial and Quantum Dot Deposition

While quantum dots are a good demonstrative tool, this data is only qualitative in nature and the use of nanoaerosols as therapeutics still requires detailed evaluation. *F. tularensis* is a biothreat agent that is known to be susceptible to treatment with levofloxacin, among other antibiotics. [19,41-43] Figure 8A shows that the generator is capable of nanoaerosolizing a 4 mg/mL levofloxacin solution, producing particles with a geometric mean diameter of 56 nm. The MPPD model predicts a total deposition of 43.5% for particles of this size: 17.8% alveolar, 10.3% tracheobronchial, and 15.4% nasal (Figure 6). Two additional small peaks with the mode diameters of 150 and 280 nm are

present in the size distribution histogram and are most likely the result of particle coagulation.

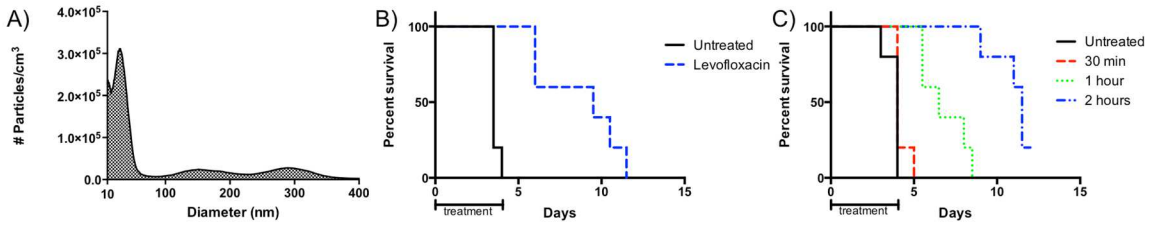

Figure 8 Levofloxacin Nanoaerosol and Standard Aerosol

To determine if the generator is an effective therapeutic delivery tool, mice were infected with 100LD_{50} *F. novicida* and subsequently treated with nanoaerosolized aqueous levofloxacin (4 mg/mL) in a conductive whole-body exposure chamber (described above) for four hours a day for five days. Despite all mice succumbing to infection, the treated mice showed a significant increase in mean time to death ($p < 0.005$) compared to the controls: from 3.6 to 8.7 days. The daily total deposited dose was estimated to be approximately 0.42 mg/kg through the use of sample collection PVP filters and the MPPD deposition predictions. For an example calculation, see Equation 1 below. This calculation shows the equation for estimating the nanoaerosolized levofloxacin dose deposited in each mouse per day and the general principle behind all of the dosing calculations in this study. The spray variable was determined through the use of PVP filters for nanoaerosol treatments and AGI for standard aerosol treatments. The ITV (inspiratory tidal volume) and RR (respiratory rate) variables use mouse

physiological data measured by Flandre *et al*. [53] The mass variable is the average weight of all mice in that particular run of the experiment. The deposition variable is the total deposition percentage as determined by MPPD.

Knowns

$Spray - 146\ {}^{\mu g}/_{hr}$

$Flow\ Rate - 108\ {}^{L}/_{hr}$

$ITV - 0.219\ {}^{mL}/_{breath}$

$RR - 273.1\ {}^{breath}/_{min}$

$Mass - 20\ g$

$$0.219\ {}^{mL}/_{breath} * 273.1\ {}^{breath}/_{mL} = 59.81\ {}^{mL}/_{min}\ or\ 3.588\ {}^{L}/_{hr}$$

$$\frac{146\ {}^{\mu g}/_{hr}}{108\ {}^{L}/_{hr}} = 1.35\ {}^{\mu g}/_{L} * 3.588\ {}^{L}/_{hr} = 4.8438\ {}^{\mu g}/_{hr}$$

$$0.435 * 4.8438\ {}^{\mu g}/_{hr} = 2.107\ {}^{\mu g}/_{hr} * 4\ hr = 8.428\ {}^{\mu g}/_{day}\ \left(\sim 0.42\ {}^{mg}/_{kg}\right)$$

Equation 1 Example Dosing Calculation for Nanoaerosolized Levofloxacin

The deposition fractions modeled by the MPPD program were used to estimate deposition here; however, additional characterization of nanoaerosols is necessary to fully understand and accurately calculate the deposition of the particles. Further analysis must account for numerous unknowns, such as the physical properties of the sprayed material within the aerosol and the effect of charge on aerosol deposition, but such experiments are not within the scope of this study.

The Baiera-driven 3-jet collision nebulizer creates standard aerosols composed of particles between 1 and 5 μm in diameter. [26] In this experiment, the time of exposure was altered to change the delivered dose in order to model the nanoaerosol experiment. The standard aerosol of aqueous levofloxacin (4 mg/mL) deposited approximately 0.98 mg/kg/day to the two-hour treatment group, estimated through the use of an AGI sampler and the MPPD deposition predictions. Of the mice that died in the two hours per day treatment group, the mean time to death of 10.8 days was significantly longer ($p < 0.005$) than the 6.8 days mean time to death of the one hour per day treatment group. The 30 minutes per day treatment group had a mean time to death of 4.2 days and the control group had a mean time to death of 3.8 days (Figure 8C**)**.

For comparison purposes, the lowest effective dose of levofloxacin delivered via intraperitoneal injection and oral administration against $100LD_{50}$ of *F. novicida* was determined to be 3 mg/kg and 33 mg/kg, respectively (Figure 9A, B). A dose of approximately 0.42 mg/kg of nanoaerosolized levofloxacin leads to a mean time to death of 8.7 days compared to the 6.5 days mean time to death of the approximately 0.63 mg/kg dose given via intraperitoneal injection, despite delivering a lower dose. Thus, nanoaerosolized levofloxacin delivered to the lung has more therapeutic value than a higher dose of levofloxacin delivered via intrapertioneal injection.

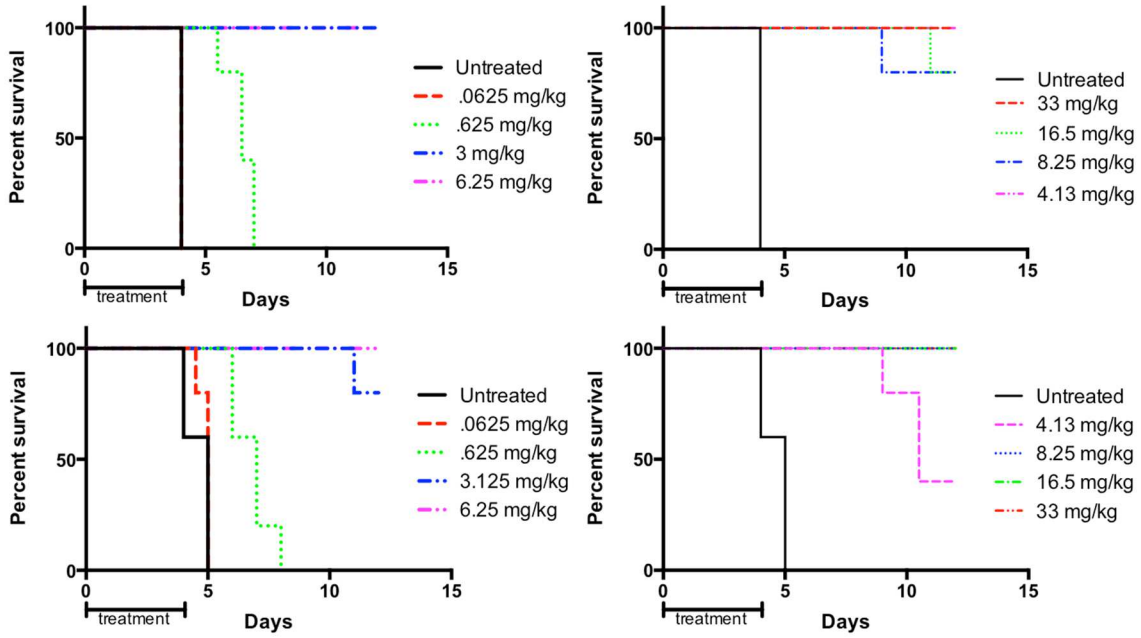

Figure 9 Levofloxacin and Liposomal Levofloxacin Survival Curves from Traditional Delivery Methods

Initial experiments involving traditional delivery methods of intraperitoneal injection and oral administration were repeated with ciprofloxacin and fosmidomycin to determine if these antibiotics could rescue against *F. novicida* infections (Figure 10). Despite showing the capability of rescuing mice against *F. novicida* infections, these antibiotics were ultimately not used in aerosol *in vivo* experiments because they were not as effective as levofloxacin.

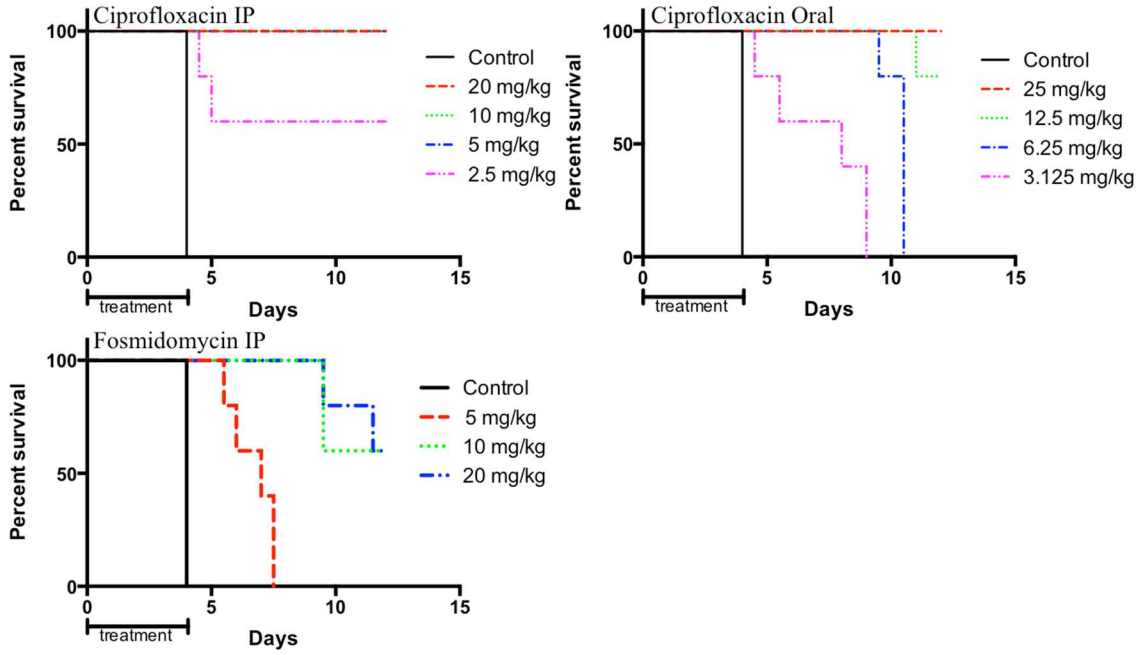

Figure 10 Ciprofloxacin and Fosmidomycin Survival Curves from Traditional Delivery Methods

The two-hour standard aerosol treatment group that received approximately 0.98 mg/kg was not statistically different than the nanoaerosolized treatment group's, which delivered half the dose at ~0.42 mg/kg ($p > 0.005$). These results suggest that the use of nanoaerosols significantly decreases the effective dose of levofloxacin required to rescue mice from a pulmonary *F. novicida* infection by approximately two-fold compared to a standard aerosol treatment. Additionally, the amount of solution required to deliver these doses differs significantly. The generation of nanoaerosol in these experiments requires 40 times less volume of sprayed material than what is needed for the generation of standard aerosol. This conservation of material would be useful for otherwise cost prohibitive therapeutics, such as peptides.

Despite these promising results in regard to dose reduction, the therapy is ultimately not viable unless mice can be rescued from a lethal pulmonary *Francisella* infection. Wong *et al* reported an increase in survival against $10LD_{50}$ *F. LVS* by encapsulating ciprofloxacin in liposomes and delivering them via standard aerosol. The act of encapsulating ciprofloxacin in liposomes brought the survival from 0% with a mean time to death of 8.2 days to 100%. [65] Similarly, Hamblin *et al* reported that aerosolized liposome-encapsulated ciprofloxacin was capable of rescuing mice from a lethal *F. tularensis Schu S4* infection with as little as a single aerosol treatment. [66]

In order to apply this improvement to the study, liposomes containing levofloxacin were prepared from DPPC, DPPG, and cholesterol precursors (2:1:2 molar ratio) using the well-established thin film dehydration/rehydration technique, including sonication and extrusion, to produce small unilamellar vesicles. [60] As seen in Figure 11A, the nanoaerosol generator produced nanoaerosolized particles with a geometric mean diameter of 153 nm, which is larger than the nanoerosolized levofloxacin but still small enough to deliver material to the alveoli. The MPPD model predicts a total deposition of 30.2% for particles of this size: 8.69% alveolar, 4.91% tracheobronchial, and 16.6% nasal (Figure 6). The use of nanoaerosolized liposome-encapsulated levofloxacin increased the percentage of surviving mice to 80% (4/5 mice rescued) and decreased the estimated delivered dose of levofloxacin to approximately 0.35 mg/kg per day using the same exposure time of four hours per day for five days (Figure 11B). The lower delivered dose and decreased percentage delivered to the alveoli compared to the

nanoaerosolized levofloxacin suggests that the liposomes themselves may assist with delivery and uptake.

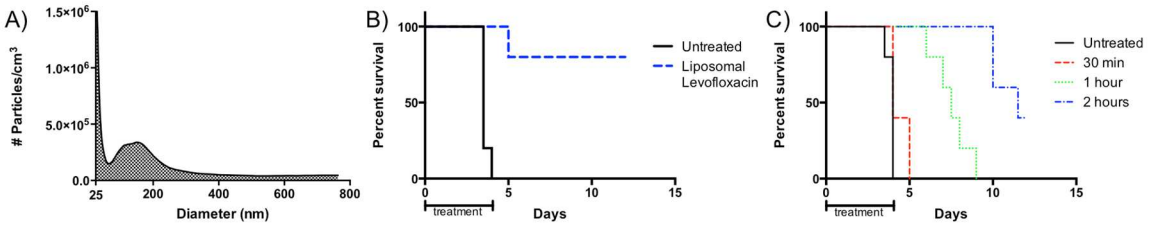

Figure 11 Liposome-Encapsulated Levofloxacin Nanoaerosol and Standard Aerosol

The dose-survival curves were repeated using liposome-encapsulated levofloxacin for the intraperitoneal, oral, and aerosol delivery methods (Figure 9C, D, Figure 11C). These curves were not significantly different from those obtained via treatment with naked levofloxacin despite the fact that the two-hour aerosol treatment increased the daily delivered dose from ~0.98 mg/kg per day of levofloxacin to ~1.3 mg/kg per day of liposome-encapsulated levofloxacin. The lowest effective doses for intraperitoneal injection and oral administration of liposome-encapsulated levofloxacin remained about the same as with naked levofloxacin: 3 mg/kg and 33 mg/kg per day, respectively. These findings are inconsistent with the literature, which asserts that liposomes enhance intraperitoneal and oral treatments; however, perhaps delivering these minimal, barely effective doses of liposome-encapsulated levofloxacin does not make a significant difference when administering this particular treatment by intraperitoneal or oral routes against pulmonary *Francisella* infections.

The lack of a significant change in the standard aerosol dose survival curve between unencapsulated and liposome-encapsulated levofloxacin seemed to contradict the results reported by Wong *et al*. [65,67] To begin to address this, further investigation showed that over 50% of the liposomes had burst and a significant portion of the levofloxacin had reverted back to the unencapsulated form as a result of the 3-jet collision nebulizer aerosolization process. This is consistent with previous reports as to the membrane disruption caused by this type of nebulizer. [67,68] Since the collision nebulizer aerosolization process resulted in such a large reduction in the concentration of intact liposomes, the nanoaerosolized liposomal levofloxacin dose cannot be accurately compared to the dose of liposomes aerosolized by this particular aerosolization process. However, this finding supports the notion that the ESN nanoaerosol generator is gentler on sprayed material than a collision jet nebulizer and would be useful in delivering fragile biological substances.

Finally, it can be concluded from the survival curves that ~0.35 mg/kg of nanoaerosolized liposomal levofloxacin is statistically equivalent in terms of survival to 3 mg/kg liposomal levofloxacin delivered via intraperitoneal injection and 33 mg/kg liposomal levofloxacin by oral delivery ($p > 0.005$). This data is equivalent to a 8-fold reduction in the minimum effective treatment dose compared to the intraperitoneal delivery method and 94-fold reduction compared to the oral delivery method.

The ability for nanoaerosols to reduce the effective dose of levofloxacin needed to rescue mice from a lethal pulmonary *F. novicida* infection makes it a potentially valuable tool against respiratory infections. However, when delivering foreign material into the

lungs, potentially damaging factors must be investigated. In order to examine possible organ damage, the lungs, livers, and spleens of four different mice were sent to a pathologist for evaluation: a *Francisella* infected mouse that remained untreated (A); a *Francisella* infected mouse treated with liposome-encapsulated levofloxacin (B); an uninfected, naïve mouse treated with liposome-encapsulated water (C); and an uninfected, naïve mouse (D). The magnitude of inflammatory or degenerative lesions was graded on a scale of 1 to 5, with Grade 1 being minimal and Grade 5 being severe.

The pathology findings (Table 3) indicate that the infected mouse that was not treated and the infected mouse that was treated with nanoaerosolized liposome-encapsulated levofloxacin both presented with randomly dispersed foci of necrosis in the liver accompanied by subacute inflammation consisting of neutrophils and macrophages. Similar areas of necrosis and inflammation were noted in the lungs and spleen in the infected mouse while the infected, treated mouse exhibited only inflammation, consistent with the mouse's recovery from the infection. The infected mouse that was not treated also showed strong evidence of intracellular bacteria in the lungs and liver.

Table 3 Histopathologic Scoring of Murine Lungs, Livers, and Spleens.

Mouse ID	Findings	Grade
A	**LIVER**	
	Necrosis, random, with subacute inflammation	3
	Intracellular bacteria	P
	SPLEEN	
	Necrosis, with subacute inflammation	4
	LUNG	
	Necrosis, with subacute inflammation	3
	Intracellular bacteria	P
	Hemorrhage and edema	3
	Foreign material, pigmented	3

B	**LIVER** Necrosis, random, with subacute inflammation **SPLEEN** Inflammation, subacute **LUNG** Inflammation, subacute, perivascular Hemorrhage and edema	 3 4 4 3
C	**LIVER** Not remarkable **SPLEEN** Not remarkable **LUNG** Not remarkable	 - - -
D	**LIVER** Infiltrate, mononuclear cell **SPLEEN** Not remarkable **LUNG** Not remarkable	 1 - -

The uninfected mouse and uninfected mouse treated with nanoaerosolized liposome-encapsulated water did not have any remarkable findings in their livers, lungs, or spleens. Table 3 shows the relevant pathology findings in detail. This allows for the conclusion that the nanoaerosol treatment alone does not damage tissue in the lungs or other organs. Interestingly, this data also suggests that nanoaerosolized liposome-encapsulated levofloxacin, despite being able to rescue mice from infection, is not able to prevent tissue damage caused by pulmonary *Francisella* infection.

Nanoaerosolized therapeutics are not limited to levofloxacin. Numerous other antibiotics can by nanoerosolized by the ESN generator. Additionally, antivirals, antifungals, anti tuberculosis drugs, steroids, and anti cancer drugs are being investigated for potential use as nanoaerosolized therapeutics. Despite evidence that nanoaerosolized

liposome-encapsulated levofloxacin and the nanoaerosol process itself are not harmful to the lungs, all other proposed nanoaerosolized treatments must undergo similar pathological scrutiny.

Small particles have been shown to migrate through the mucosal membrane in the nasal passages to the brain. [69-72] Care must be taken to ensure that no toxicity issues occur through this route. Alternatively, this knowledge can be used to specifically target the brain. As seen in Figure 6, increased deposition in the nasal passages is achieved through larger particle sizes. The ESN nanoaerosol generator can be adjusted to allow for the creation of particles outside of the nanoaerosol range to achieve this if desired.

Despite these promising results and numerous potential uses, the ESN nanoaerosol generator is not without limitations. Material sprayed must be soluble in water or alcohol and the solution must have a low conductivity. Due to the small size of the particles, they are unstable and must be used immediately or they will coagulate into large particles. This means individuals must have access to the generator at the time of treatment. Additionally, the creation of a potentially handheld version of the ESN nanoaerosol generator would result in high costs that may not be affordable depending on the circumstance.

Conclusion

It has previously been established that aerosolized therapeutics can improve upon the traditional delivery methods of oral and intraperitoneal injection. However, this study demonstrates that nanoaerosols (less than 200 nm) are potentially the next phase of improvement in drug delivery.

One significant benefit of nanoaerosolized therapeutic agents is the very small total volume required for each treatment. Through the method of ESN nanoaerosol generation, nanoaerosols require 40 times less initial volume to spray than traditional nebulizers. This could be a significant boon for approaches where the required therapeutics are extremely expensive, such as peptides or antibodies.

Due to the difficulty in delivering high doses of therapeutics via nanoaerosols because of the small amount of drug mass contained in the nanometer-sized particles, liposomes are a useful tool in enhancing therapeutic stability and the uptake of the delivered therapeutic in the lungs. Nanoaerosol generation is gentler on sprayed therapeutic compounds than the collision jet nebulizer and therefore is compatible with the use of liposome-encapsulated therapeutics or other fragile materials.

Nanoaerosols have proven to reduce the required effective dose of levofloxacin to rescue mice from a pulmonary *F. novicida* infection. Nanoaerosolized liposome-encapsulated levofloxacin results in an 8-fold reduction compared to the intraperitoneal delivery method and 94-fold reduction compared to the oral delivery method. Nanoaerosolized levofloxacin is also as effective as twice the dose of levofloxacin aerosolized via a 3-jet collision nebulizer. This result is most likely due to the direct delivery of a fraction of the antibiotic to the lower respiratory tract and the alveolar space, which is the site of *Francisella* infection in this model. [62,63] These results illustrate the significant benefit of direct delivery to the site of infection in the alveoli.

In addition, the delivery of nanoaerosols to the lung showed no evidence of causing tissue damage in mice. These results provide encouragement to pursue the

further development of nanoaerosol-based therapeutic delivery, especially for its ability to achieve a therapeutic resolution of infection with a significantly reduced dose and the small net amount of therapeutic used. This technology could assist patients suffering from not only acute respiratory infections but also cystic fibrosis and potentially other systemic diseases, such as diabetes, by enabling pulmonary delivery of medication at a significantly reduced dose, which could lead to a reduction in cost and the number or severity of side effects.

Future studies could explore the range of therapeutics that can be delivered via the nanoaerosol generator and other applications to which the technology can be applied. More detailed studies of the biophysical characteristics of the particles and their deposition could also be performed. In addition, the pharmacokinetics and pharmacodynamics of delivered nanoaerosolized therapeutics should be further investigated in order to fully develop the benefits demonstrated in this study.

CHAPTER THREE: PULMONARY PASSIVE IMMUNIZATION

Introduction

In nature, antibodies of the mother are transferred to the child in order to confer protection against pathogens until the child is capable of manufacturing its own antibodies. The principle of this natural occurrence has been exploited to confer passive immunity between non-related individuals to protect against or treat disease. Since the discovery that specific antibodies can protect against bacteria and toxins in the early 1890s, immune serum has been used as a way to treat infectious diseases. [73] Despite a significant drop in use after the discovery of antimicrobials in the 1930s, it is still used in some instances. [73] In a recently relevant example, eight patients from the Congo that were infected with the Ebola virus received blood transfusions from individuals who had previously recovered from their infection. Only one of the eight patients succumbed to the disease, which, at 12.5%, is significantly lower than the 80% mortality rate normally seen with that strain. [74]

The administration of vaccine by the intramuscular or intradermal route results in a systemic immune response but does not induce a mucosal response. However, the administration of vaccine mucosally activates the immune response in these tissues while still inducing systemic immunity through the lymphoid organs. [21] Systemic immunity against pulmonary *Francisella* results in the removal of bacteria after exiting from the lungs but does not control bacterial replication inside of them. This can lead to

reinfection and is only protective against low doses. [15] Mucosal immunity does not necessarily prevent infection in the lungs and spleen but prevents the bacterial burden from reaching a lethal level. [75]

A major hurdle in the development of a *Francisella* vaccine is the activation of only an antibody response. Immunity against *Francisella* requires both a B and T cell response despite the general belief that humoral immunity is important against extracellular pathogens and cell-mediated immunity is more important against intracellular pathogens. [21,76,77] Many documented cases of immune serum transfer have been shown to rescue naïve individuals against *Francisella* challenge. [18,78-83] If *Francisella* infected, T cell deficient mice are treated with immune serum, the therapeutic effect is lost, which supports the important role of T cells in recovery from *Francisella* infections. [78] However, immune serum with immunoglobin removed was not able to rescue *Francisella* infected, wild type mice, which highlights the necessity of antibody in fighting off infection. [78]

It has been demonstrated that passive immunization (delivered intraperitoneally) with purified antibodies, as opposed to immune serum, directed to *Francisella tularensis* subsp. *LVS* (*F. LVS*) LPS are protective against *F. LVS* intradermal challenge and partially protective against *F. SchuS4* intradermal challenge. [78,84-86] The switch to purified antibodies from serum eliminates the possibility of "serum sickness" previously seen in humans due to antibodies being harvested from other animals. [87] Savitt *et al* showed that 150 μg of the purified anti *Francisella* LPS IgG2a, FB11, when delivered via intraperitoneal injection conferred complete protection against *F. LVS* when given

prophylactically and 50% survival when given therapeutically. In addition, the use of anti *Francisella* LPS IgG2a prophylactically against *F. tularensis Schu S4* resulted in a five-day increase in mean time to death compared to the control. [85] A brief experiment performed by Lu *et al* showed that an IgG2a LPS-binding antibody, that is not as effective as FB11, could rescue mice against 20LD_{50} *F. LVS* when 50 μg was delivered but not against higher infectious doses. [20] No further investigation regarding antibody delivery to the lungs was pursued.

To extend but still conform to previous successful experiments in the literature (such as Savitt *et al*), experiments were run using FB11, a mouse monoclonal IgG2a antibody against *Francisella tularensis* LPS. Experiments were performed to determine the effectiveness of intranasal delivery of this antibody to the lungs against high infectious pulmonary doses of *F. LVS*, including the exploration of lowest effective dose, organ burden, post exposure prophylaxis, and single dose delivery. Additionally a pilot study was performed to determine the effectiveness of intranasal FB11 against *F. tularensis SchuS4*.

Materials and Methods

Bacterial Culture

F. LVS (NR-646, BEI Resources, Manassas, VA) was cultured by streaking a thawed vial on Chocolate Agar (GC Agar II with Isovitalex, BD Biosciences, Franklin Lakes, NJ) and incubating for 48 hours at 37°C and 5% CO_2. Brain Heart Infusion, pH 8.6 (BHI) broth (TekNova, Hollister, CA) was inoculated from growth on aforementioned plate and incubated overnight at 37°C and 200 rpm.

Antibody Verification

A Western blot of FB11 was performed against *F. LVS* (NR-646, BEI Resources, Manassas, VA), *F. novicida* (ATCC 15482, American Type Cell Culture, Manassas, VA), *Escherichia coli* (ATCC 4157, American Type Cell Culture, Manassas, VA), and *Pseudomonas aeruginosa* (ATCC 15692, American Type Cell Culture, Manassas, VA) whole cell lysate.

Antibacterial Assay

F. LVS (NR-646, BEI Resources, Manassas, VA) was added to a 96-well plate at a concentration of $1x10^5$ bacteria (50 μL) per well along with 150 μL of BHI, pH 6.8 broth. 100 μL of 1:2 dilutions from 1 μg/μL to .015625 μg/μL of FB11 diluted in PBS were added to wells. The plate was incubated for 24 hours at 37°C and 5% CO_2. Optical density at 600 nm was measured using PowerWave X Spectrophotometer (BioTek, Winooski, VT).

Murine infection

6-8 week old female BALB/c mice (Harlan, Frederick, MD) were infected intranasally with 50 μL of PBS containing the specified concentration of *Francisella* three hours prior to treatment on day 0. Mice were examined twice a day for signs of illness or death. Bacterial concentration of inoculums were verified via plating. The institutional animal care and use committee approved all animal procedures.

Intranasal Protection Assay

6-8 week old female BALB/c mice were treated intranasally with 50 μL of PBS containing specified concentrations of dialyzed FB11 (Fisher Scientific, Pittburgh, PA) once daily on days 0, 1, 2, 3, and 4, unless otherwise specified.

Organ Burden Assay

Lungs, livers, and spleens were harvested from mice on days 4, 5, and 6 post infection, homogenized, diluted in PBS, and plated on chocolate agar. Plates were incubated for 72 hours at 37°C and 5% CO_2.

Nanoaerosol Protection Assay

6-8 week old female BALB/c mice were treated with nanoaerosolized FB11 through a four-hour exposure in a conductive nanoaerosol chamber (Figure 4) once daily on days 0, 1, 2, 3, and 4. Nanoaerosols generated using a custom generator described by Morozov *et al*. [36]

Results and Discussion

Prior to beginning experiments in mice, it was verified that FB11 only reacts to *F. LVS* LPS and that it does not have antimicrobial properties. A Western blot confirmed that FB11 reacted to *F. LVS* LPS (A) and not *E. coli* (B), *P. aeruginosa* (C), or *F. novicida* (D) LPS with the exception of a light band of unknown source at ~40 kDa in *E. coli* (Figure 12). Additionally, FB11 was found to have no direct antimicrobial effect on *F. LVS* in MIC conditions so treatment *in vivo* would rely on opsonization or immunomodulation (Figure 13).

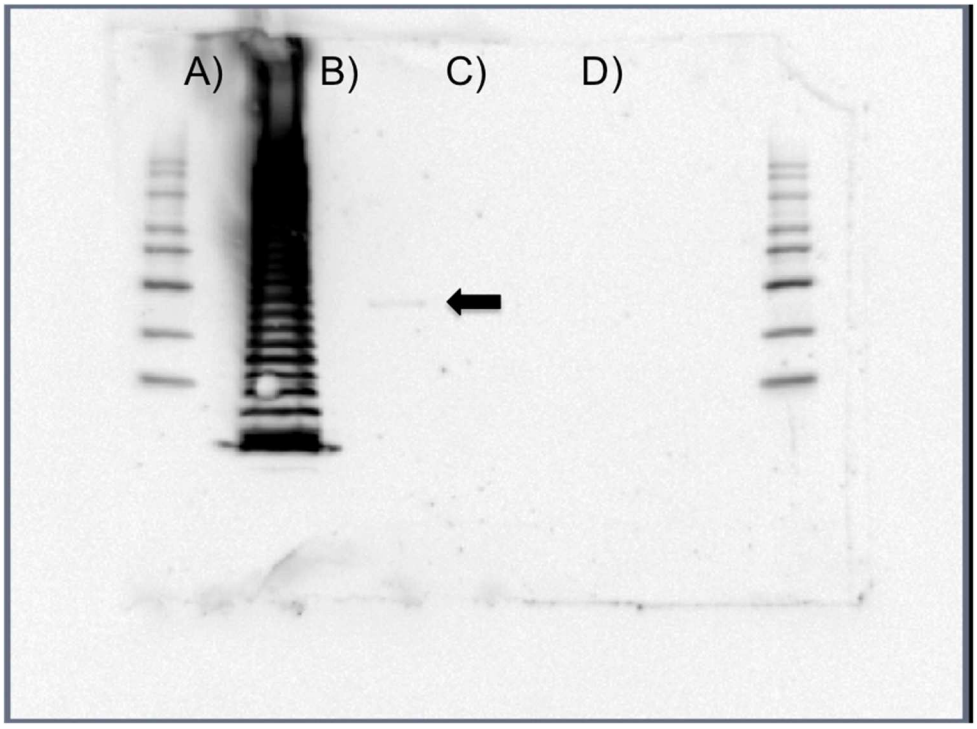

Figure 12 Western Blot of Bacterial Species with FB11

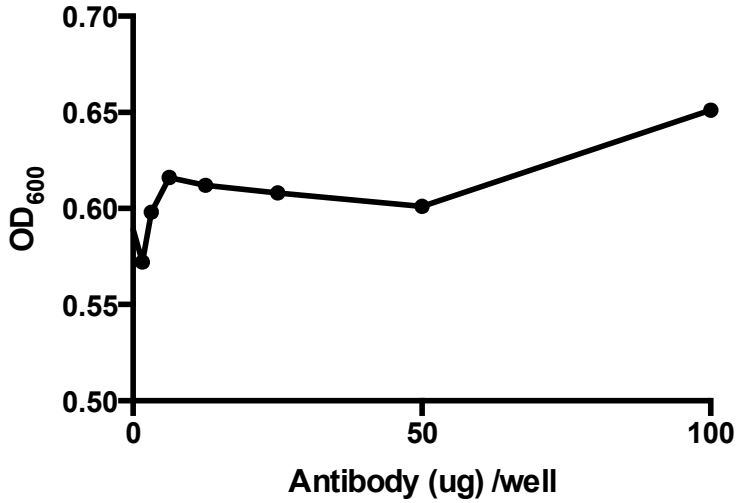

Figure 13 Antibacterial Assay of FB11 against *F. LVS*

In order to determine if IgG delivery to the lungs affects the disease course of a pulmonary *Franicsella* infection, 50 μg of FB11 was intranasally administered for five days against 75LD_{50} of intranasally delivered *F. LVS* (Figure 14). This treatment not only rescued all of the mice, the treated mice barely become ill enough to begin Column E monitoring according their average health score. Column E monitoring is additional monitoring for animals determined to be ill, in pain, or in distress that would otherwise be treated by the veterinary staff if not part of an approved study. The goal of this additional monitoring is to avoid death or severe pain and results in humane euthanasia, if necessary, to avoid those outcomes. A health score of five is the institutional guideline for beginning Column E monitoring. Asterisks signify days of death in control group.

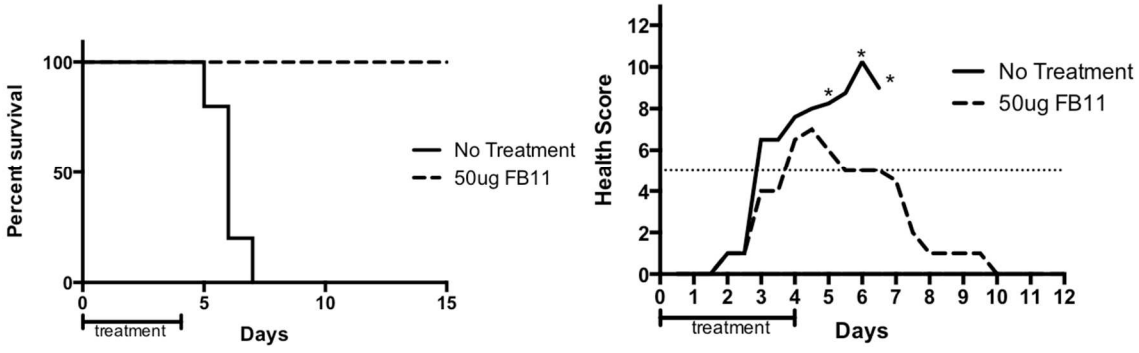

Figure 14 Intranasal FB11 Protection Assay against *F. LVS* and Health Scores

Due to these results, a dose curve experiment was devised to determine the lowest effective intranasal FB11 dose. Mice infected intranasally with 150LD_{50} *F. LVS* were

treated with 25, 12.5, or 6.25 µg of FB11 for five days (Figure 15A). Once again, not only did all treatment groups survive but also they exhibited very limited signs of illness. Additional experiments were run at progressively larger bacterial concentrations and reduced FB11 dose until there was no statistical difference between the control and treatment survival curves. There was 80% survival when mice were given 1 µg of FB11 for five days against $500LD_{50}$ *F. LVS* ($p < 0.005$) but only 20% survival against $1500LD_{50}$ *F. LVS* ($p = 0.052$) (Figure 15B). Assuming that the antibody is approximately 150 kDa and there are 6.02×10^{14} kDa/µg, approximately 9.03×10^{16} molecules of FB11 are delivered in a 1 µg treatment. This means that there were approximately 1.81×10^{11} molecules of FB11 per bacterium in the $500LD_{50}$ challenge and 6.02×10^{10} molecules of FB11 per bacterium in the $1500LD_{50}$ challenge. More than likely this is due to a large loss of FB11 in the nasal and upper respiratory pathways and isn't representative of what reaches the bacteria in the alveoli.

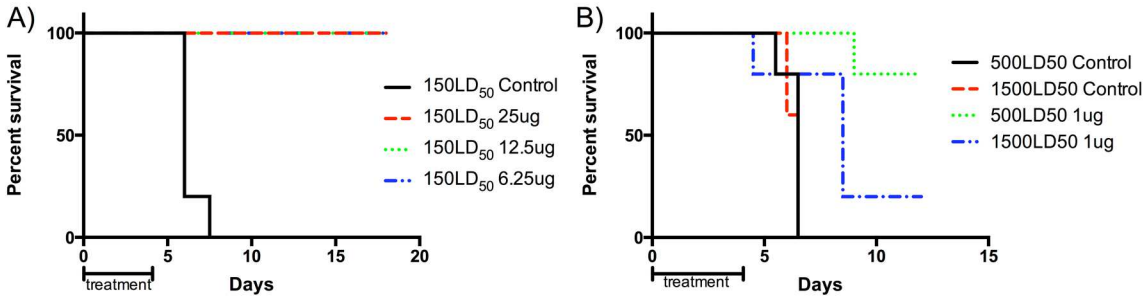

Figure 15 Intranasal FB11 Decreasing Dose Curve against *F. LVS*

Since the treatment began three hours post infection, the antibody opsonizing a large number of initial bacteria could potentially lead to these results. In order to explore this possibility an organ burden assay was performed to verify the infection is taking hold in the body, a single dose experiment was performed to determine if the treatment given three hours post infection, and not the ones given subsequently, is the most important to survival, and a post exposure experiment was performed to determine if the treatment is as effective when the infection is given more time to establish prior to FB11 administration.

For the organ burden assay, mice were intranasally infected with 150LD_{50} *F. LVS* and treated with 10 μg of FB11 intranasally on days 0 through 4. On days 4, 5, and 6, mice were euthanized and their lungs, livers, and spleens were harvested to determine their bacterial burden. These days correspond with the peak in symptoms of illness, with day 4 being the final day of treatment and day 6 being the approximate time to death of the control mice. On day 4, the treated mice had approximately one log less bacteria than the control mice in each organ measured, with the highest being ~10^7 CFU in the lungs (Figure 16A). After the treatment ended on day 4, the control organ burdens continued to rise while the treatment group liver and spleen burdens slowly declined (Figure 16B, C). The lung burden of the treated group remained fairly static but it is not unexpected since they are the last organs to clear (Figure 16D). Mice treated with FB11 three hours post infection are developing a significant infection that is found systemically but they have a log decrease in bacterial burden and appear to follow a shortened disease course.

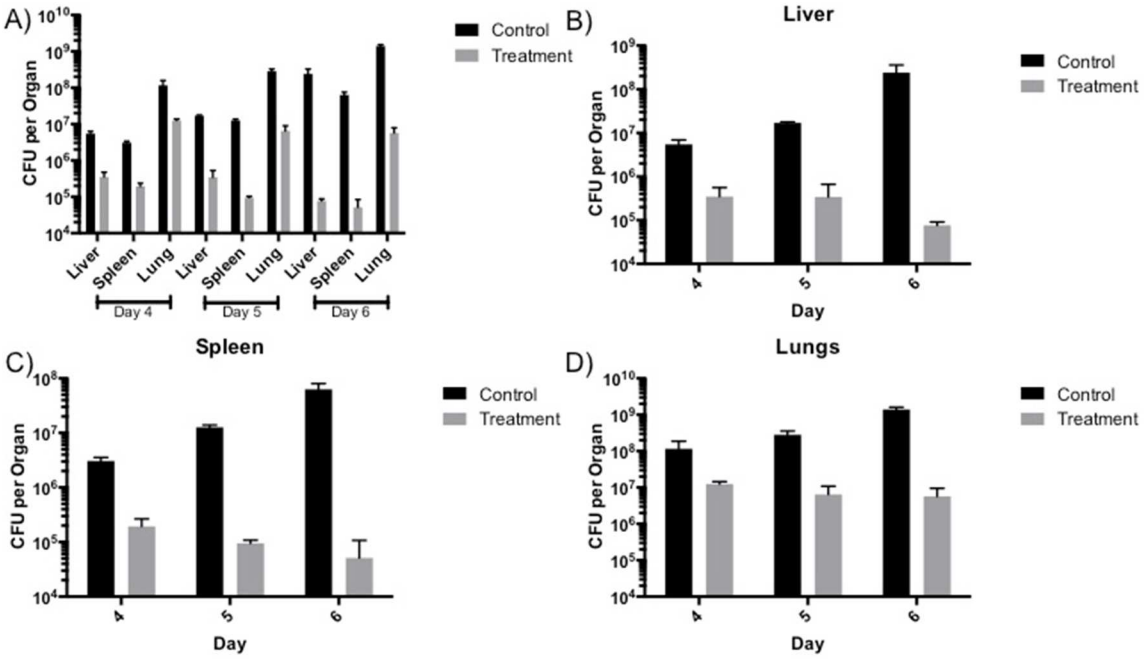

Figure 16 Organ Burden Assay of Intranasal FB11 against *F. LVS*

A single dose study was used to determine if the first treatment given three hours post infection is the most important for survival. Mice were intranasally infected with 500LD_{50} or 1500LD_{50} *F. LVS* and treated with 10 μg of FB11 intranasally three hours post infection on day 0 (Figure 17). Mice generally appeared healthier than simultaneously run treatment groups (1 μg of FB11 for five days); however, both trials resulted in early deaths that cannot be explained. The treatment is known not to be toxic because much higher concentrations were previously given with no adverse effects. If not a random occurrence, it is predicted that, in a small percentage of cases, the single treatment causes bacteria to initiate some form of SOS type response or alter in a way that is quickly fatal to the mouse but is avoided with continual treatment. Additional

studies should be done to investigate this phenomenon. Mice surviving the early stages of infection recovered quickly and overall the single treatment group resulted in a high percentage of survival.

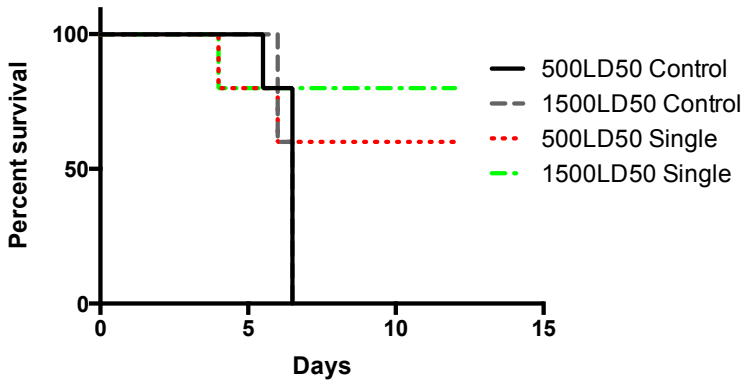

Figure 17 Single Dose of Intranasal FB11 against *F. LVS*

The post-exposure study had mice receive five days of treatment with 10 µg of FB11 intranasally starting at 24, 48, and 72 hours post infection. Mice were infected with $10LD_{50}$, $500LD_{50}$, or $1500LD_{50}$ of *F. LVS*. Mice infected with $10LD_{50}$ of *F. LVS* were rescued despite delaying treatment by 24 hours. The 24 hour delayed treatment against $500LD_{50}$ and $1500LD_{50}$, however, only experienced a significant increase in mean time to death from 6.3 days to 8.3 and 8.7 days, respectively ($p < 0.005$). The 48 and 72 hours delayed treatment groups in the $500LD_{50}$ and $1500LD_{50}$ experiments did not statistically differ from the non-treated mice and are not shown.

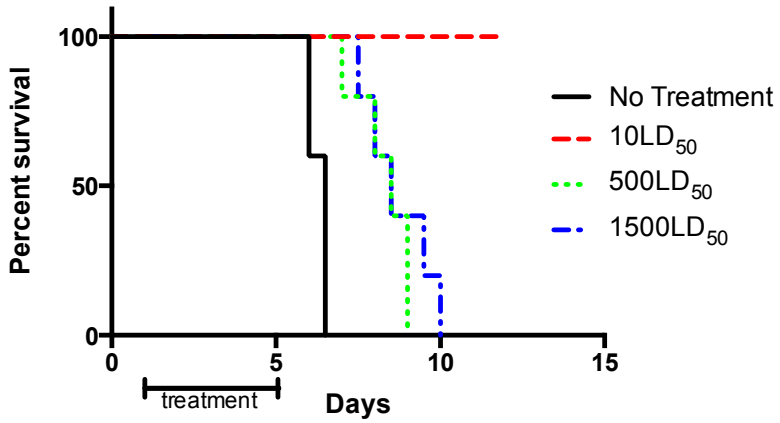

Figure 18 Intranasal FB11 as Post Exposure Treatment

Previous experiments by Savitt *et al* show that FB11 is ineffective against *F. tularensis SchuS4* when injected. These results were reevaluated using intranasal delivery of 50 µg of FB11 once a day for five days against $20LD_{50}$ of pulmonary delivered *F. tularensis SchuS4*. No therapeutic effect by FB11 was seen at this dosing and schedule against *F. tularensis SchuS4* (Figure 19). Increasing the dose of antibody or delivering a pretreatment may result in an increase in mean time to death. However, identifying and evaluating a more effective antibody to use against *F. tularensis SchuS4* would be the best course of action as antibodies are costly and pretreatment in patients is unlikely.

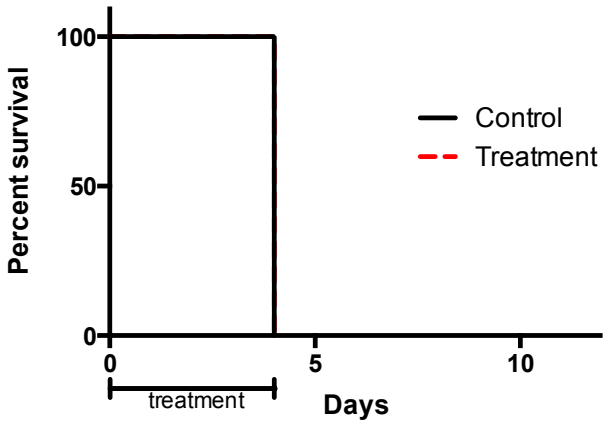

Figure 19 Pilot Study of Intranasal FB11 against *F. tularensis SchuS4*

The utilization of nanoaerosol technology for the delivery of therapeutics has given promising results and proteins have been shown to maintain structure and functionality when delivered in nanoaerosol form via the ESN nanoaerosol generator with relatively high efficiency (~70%). [36] Since FB11 has been shown to be effective against pulmonary *F. LVS* infections at low doses it is a good candidate to evaluate with the nanoaerosol generator in the future.

Conclusion

Passive immunization has been used for decades to treat infectious diseases but has fallen out of common use with the discovery of antimicrobials. The delivery of antibody through the lungs is a relatively new concept that needed to be explored as a potential treatment options against pulmonary diseases.

The anti *Francisella* LPS IgG2A, FB11, was previously shown to rescue mice from an *F. LVS* infection when delivered via intraperitoneal injection. FB11 is now

known to be capable of rescuing mice from a lethal pulmonary *F. LVS* infection when delivered intranasally at a significantly lesser dose than intraperitoneal injection. At $500LD_{50}$ *F. LVS*, 1 μg of FB11 delivered intranasally once daily for five days is sufficient to rescue 80% of mice from a respiratory infection.

Organ burden studies show that the antibody does not prevent infection but lessens the bacterial burden by approximately one log in the lungs, liver, and spleen. Additionally, it was shown that FB11 shortens the disease course as the bacterial burden begins to decrease in the liver and spleen after the treatment ends while the burden rises in the control group.

A single treatment with 10 μg against $1500LD_{50}$ results in an 80% rescue of the mice, which suggests that the first dose, given three hours post infection, is the most important to survival. This may allow for a single initial dose of antibody immediately after exposure followed by antibiotic therapy to enhance the outcome while maintaining lower costs.

Delaying treatment by 24 hours still results in 100% protection against $10LD_{50}$ but only an increased time to death against 500 and $1500LD_{50}$. Delaying treatment by 48 or 72 hours against the higher infectious doses results in no effect. These results, along with the single treatment experiments, suggest that delivering antibody soon after the initial exposure gives the best possible effect.

FB11 was shown to be ineffective against *F. tularensis SchuS4* when injected but it was reevaluated using intranasal delivery. It was found that despite the change in delivery method, FB11 is not capable of rescuing mice against *F. tularensis SchuS4*.

Other antibodies should be identified against this particular *Francisella* strain and used to evaluate pulmonary antibody delivery.

These results support that pulmonary delivery of antibody is valid against pulmonary *Francisella* infections when delivered soon after exposure and should perhaps be combined with other current treatment methods, such as antibiotics. This therapy may be more valuable against other causative agents of acute respiratory diseases that are not intracellular and this topic should be further investigated. Future experiments should use the ESN nanoaerosol generator to deliver antibody to the alveoli to see if that affects the dose requirement and delayed treatment outcomes.

CHAPTER FOUR: CONCLUSIONS

Improvements to the delivery of therapeutics to the lungs, as well as the pulmonary delivery of therapeutics to treat diseases, were investigated.

The ESN nanoaerosol generator created by ITEB was found to be a potentially valuable enhancement upon standard pulmonary delivery techniques. The nanoaerosols produced are capable of depositing into the alveoli, which is the site of infection and entry point into the circulatory system. The generator requires 40 times less initial volume to spray than traditional nebulizers and is gentler on the sprayed therapeutic compound. When using levofloxacin and a pulmonary *F. novicida* infection as a model, it was found that nanoaerosols are approximately twice as effective as standard aerosols, eight times as effective as intraperitoneal injection, and 94 times as effective as oral administration. This highlights the benefits of direct lung delivery to the alveoli during a lower respiratory infection. Additionally, the delivery of nanoaerosols did not result in lung tissue damage, which would make the generator's use unfeasible. Other therapeutics and models should be explored to realize the full potential of this novel technology. More detailed studies of the biophysical characteristics of the particles and their deposition should also be performed. In addition, the pharmacokinetics and pharmacodynamics of delivered nanoaerosolized therapeutics should be further investigated in order to fully appreciate the benefits demonstrated in this study.

Passive immunization has been used for decades to treat infectious diseases. However, the delivery of antibody through the lungs is a relatively new concept. The IgG2A, FB11, is capable of rescuing mice from a lethal pulmonary *F. LVS* infection when delivered intranasally. At 500LD_{50}, 1 μg of FB11 delivered intranasally once daily for five days is sufficient to rescue 80% of mice. Organ burden studies show that the antibody does not prevent infection but lessens the bacterial burden and shortens the disease course. A single treatment with 10 μg against 1500LD_{50} results in an 80% rescue of the mice, which suggests that the first dose, given three hours post infection, is the most important to survival. Post exposure experiments show that starting an otherwise proven treatment 24 hours late results in complete survival against 10LD_{50} *F. LVS* but only an increased mean time to death against 500 and 1500LD_{50} *F. LVS*, whereas delaying treatment 48 or 72 hours against the higher infectious doses results in no effect. These results support that pulmonary delivery of antibody is valid against early pulmonary *Francisella* infections and should perhaps be combined with current treatment methods. Despite these results with *F. LVS*, FB11 was found to not be an effective treatment against *F. tularensis SchuS4* regardless of delivery method. Future experiments should use the ESN nanoaerosol generator to deliver antibody to the lungs and examine pulmonary delivery of antibody against other, non-intracellular, respiratory pathogens.

Continued improvements to the lung delivery system and expanding upon the types of treatments delivered to the lungs can vastly improve the future treatment of various types of pulmonary infections so sustained research efforts are needed in this field.

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BIOGRAPHY

Crystal Propst graduated from Park View High School, Sterling, Virginia, in 2007. She received her Bachelor of Science in Biology from George Mason University in 2010 and, while employed as an environmental scientist at the Computer Sciences Corporation, her Master of Science in Biotechnology from Johns Hopkins University in 2011. During her doctoral studies she completed two internships at the J. Craig Venter Institute in infectious disease and synthetic biology, taught undergraduate microbiology laboratory classes, and received an IIAD Career Development Program and a CEEZAD Transboundary Animal Disease Summer Program fellowship. She completed her Doctor of Philosophy in Biosciences, with a concentration in Microbiology and Infectious Disease, at George Mason University in 2015.