NANOAEROSOLS FOR POTENTIAL PULMONARY DELIVERY OF CRITICAL THERAPEUTICS

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DEDICATION

I dedicate this work to my adoring husband, Joni, and my amazing children, Connor and Maya, whose sacrifices made it possible for me to complete this journey and whose belief in me helped me through the most difficult time of my life.

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LIST OF ABBREVIATIONS

Mass Median Aerodynamic Diameter ..... MMAD
Multiple Path Particle Dosimetry ..... MPPD
Polyvinylpyrrolidone ..... PVP
Scanning Mobility Particle Sizer ..... SMPS

ABSTRACT

NANOAEROSOLS FOR POTENTIAL PULMONARY DELIVERY OF CRITICAL THERAPEUTICS

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New approaches and therapies to deliver current drugs are always being sought to improve upon conventional techniques. The method of producing drug nanoaerosol particles based on the Electrospray Neutralization technique is proposed as a new approach in the treatment of lung diseases by delivering drugs deep into lung tissue, increasing their bioavailability as a result of bypassing first-pass metabolism in the liver, and reducing the toxicity and effective dose over oral administration. The goal of this work is to test the applicability of the Electrospray Neutralization technique to generate nanoaerosols from different drug substances and to evaluate how solubility, ionic state, and other physical and chemical properties of drugs affect nanoaerosol generation. Size and mass spectra of generated nanoaerosol particles were taken for fourteen drugs and potential inhaled doses were estimated for humans and mice. It was found that the generator produced nanoaerosol particles with an average geometric size of 30-40 nm for most drugs electrosprayed from 0.1 % solutions in water. Multiple Path Particle

Dosimetry (MPPD) modeling of the total and regional lung deposition predict that humans will get doses of approximately 1 μg/kg/hour and mice will receive approximately 30 μg/kg/hour.

CHAPTER ONE: BACKGROUND

One of the primary goals of drug treatment is to deliver the lowest possible effective dose to mitigate any potential side effects. One such method is delivery directly to the site of infection or treatment area. In the case of lung infections and conditions, the use of aerosolized medication allows for such targeted delivery, and is an increasingly important method of direct drug delivery [1]. 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 [2], mainly from pneumonia, influenza, respiratory syncytial virus, and tuberculosis, but lethal acute respiratory infections can occur from any number of viruses, bacteria, and fungi. These diseases disproportionately affect low- and middle-income countries where they are often the number one cause of death. They are often serious, difficult to treat, spread rapidly, and require just a few particles to infect. The medical community has tools available to treat many cases of these respiratory infections but new therapies that can improve upon current ones are needed, especially for the treatment of resistant cases and when the current method of treatment is not well tolerated. This work improves upon the current method of aerosol delivery, nebulization, by targeting delivery deep into the lung. The deep lung delivery provides the opportunity for targeted treatment directly to the site of alveolar infections and

diseases, and it also enables delivery of a smaller treatment dose for administration of drugs that are not well tolerated due to adverse side effects from larger doses.

Section One: Aerosolized Therapeutics

In the treatment of respiratory infections, aerosolized therapeutics offer an advantage to traditional delivery methods due to their ease of administration, access to the large lung surface area, and limited systemic distribution [3]. 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 especially beneficial [4]. Nebulizers have provided a way to deliver drugs directly to the large lung surface area and effectively reduce the necessary dose and the consequential side effects, but the relatively large particles produced by such a device, 1-5 μm [5], are deposited primarily in the upper portions of the respiratory tract. In addition to the excessive size of generated particles, nebulizers often destroy the activity of fragile drugs and biologically active compounds, due to shear stress or chemical effects from cavitation [6]. Being able to produce smaller particles that can retain their activity would allow for therapeutics to reach the lower parts of the lungs but need special devices for generation. One such device that expands upon the existing nebulizer treatment to produce smaller particles is the nanoaerosol generator; however, most devices lack the ability to nanoaerosolize biologicals.

Section Two: Nanoaerosols

Drug particle size has been shown to be important in the treatment of lung diseases primarily because where the drug is deposited in the respiratory tract affects the ability of the drug to reach the location of the disease being treated. But particle size has also been shown to have an effect on the ability to reach the appropriate drug receptors, for example the bronchodialtors atrovent and albuterol. In the case of atrovent, the receptors are primarily located in the conducting airways, while the receptors for albuterol are mainly found in the medium and small airways. There was no difference in effectiveness for albuterol by using either 3.3 μm or 7.7 μm particles. However, atrovent had an increase in effectiveness with the smaller sized particles, suggesting that targeting the particle size to the receptor location does increase effectiveness [3]. Nanoaerosol generators produce aerosols of 10-2000 nm with the majority of aerosols being less than 200 nm in diameter, which are much smaller than those produced by traditional nebulizers, 1-5 μm [7]. This targeted treatment also has the added benefit of potentially further reducing the necessary treatment dose through bypassing digestive destruction and first-pass metabolism in the liver where it has been documented that a significant amount of drug loss occurs [8]. Delivery directly to the aveolar spaces also relieves drug particles from mucociliary clearance [9].

At present only three basic techniques are known for generation of nanoaerosols. The first uses an add-on heating device with a traditional nebulizer to produce a dried mist of sub-micrometer and nanometer droplets but is only able to achieve that size range

with a very dilute solution [10]. Unfortunately, this technique has all the limitations and drawbacks of the nebulization procedure, including damage to fragile biomolecules and drugs. The second technique is based on evaporation-condensation of a drug vapor. The material is first heated to form a saturated vapor, which becomes supersaturated upon cooling, and forms nanoaerosols through the process of homogenous vapor nucleation [11]. Such a technique is only applicable for those organic compounds that could be evaporated upon boiling or sublimation and thus, it cannot be used to atomize many valuable drugs like proteins, polysaccharides, liposomes and others where heating is detrimental to biological products and therapeutics.

Recently a new technique with broad applications as a potential new therapeutic delivery device has been developed in the Institute of Theoretical and Experimental Biophysics in Moscow [6, 12, 13]. This technique does not employ heat, and thus is suitable for nanoaerosolizing any water- or alcohol-soluble biological. The technique is based on the electrohydrodynamic atomization of a drug solution followed by gas-phase neutralization of the electrospray–generated ions and nanoclusters with oppositely charged ions generated via the same technique. It is unique in that it allows for the formation of a variety of nanoproducts formed by the interaction between the oppositely charged particles, whereas the collisions between similarly charged particles are inhibited. This Electrospray Neutralization technique has been demonstrated to retain almost complete activity of atomized biologicals [13] while other methods of neutralization [14-18] can cause damage to the sprayed material.

Section Three: Advantages of Electrospray Neutralization

Electrospray neutralization is a method that is suitable for producing nanoaerosols for use with a wide variety of biologics and therapeutics. Electrospray Neutralization as a method of nanoaerosol generation has the following advantages: (i) it is applicable to many soluble drugs, (ii) the particle size and concentration can be regulated 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, and (iv) it is gentle on sprayed material allowing retention of biological function.

Recently, the Electrospray Neutralization generator was used to create nanoaerosolized liposome-encapsulated levofloxacin to rescue mice infected with a pulmonary form of tularemia caused by *F. tularensis* subsp. *novicida* [19]. In this study, it was found that nanoaerosolized lipsome-encapsulated levofloxacin results in an 8-fold dose reduction compared to the intraperitoneal delivery method and a 94-fold dose reduction compared to the oral delivery method. Additionally, it was shown that nanoaerosol production required 40 times less initial volume to spray than a 3-jet collision nebulizer and the nanoaerosol generator decreased the dose of levofloxacin required to rescue mice by approximately two-fold as compared to the standard 3-jet collision nebulizer. Pathology results showed that the delivery of nanoaerosolized liposome-encapsulated levofloxacin to the lung did not cause tissue damage [19].

These results provide encouragement to pursue the further development of

nanoaerosol-based therapeutic delivery, especially for its ability to achieve a therapeutic resolution of an infection with a significantly reduced dose and because of the small net amount of therapeutic used. Our previous studies have shown the ability of this device to deliver biological and biologically active substances [6, 13] without causing damage to the lung [19]. The ability of this device to deliver therapeutics directly to the lower lung allows for direct delivery to the site of infection resulting in a reduction in the effective dose and by extension a reduction in the side effects.

Section Four: Research Objectives

It was revealed in the previous studies that successful nanoaerosol generation and size of nanoaerosol particles depends on a variety of factors, such as drug concentration in the sprayed solution, solution conductivity, surface tension of solvent used (water or ethanol) and others. Individual physical (surface activity, solubility limit, ability to hold charges) and chemical properties (red-ox potential, ability to form radicals on electrode) are also capable of strongly affecting the electrospray process. These arguments show a necessity to test the nanoaerosol generator with a number of different types of drugs with a potential for lung therapy.

Section Five: Experimental Goal

Here we undertook a study of how various pharmaceuticals (antibacterials, antivirals, antifungals, peptides, and antibodies) behave upon electrospray neutralization

to outline the application areas and to help researchers and other potential users of the new technology to study the delivery of these nanodrugs.

CHAPTER TWO: EXPERIMENTAL METHODOLOGY

Section One: Apparatus and Materials

Subsection One: Nanoaerosol Generator

The nanoaerosol generator consists of the electrospray neutralization chamber, the high voltage power supply, and the electronic control panel (**Figure 1**). The generator chamber is made up of a 250 mL plastic chamber with holes for the delivery of solutions from the electrospray capillaries as well as for pumping air in and nanoaerosols out. The positive capillary contains the solution to be nanoaerosolized and the negative capillary holds the volatile solvent, which is used to neutralize the highly charged nanoaerosols. The capillaries consist of a glass capillary tip of approximately 3-5 mm long, which is fused to a 200 μL polypropylene pipette tip. The current is delivered to the capillary from the high voltage power supply via a platinum wire. The electronic control panel allows for the control of the spray parameters, including the current, pressure to the capillaries, and airflow.

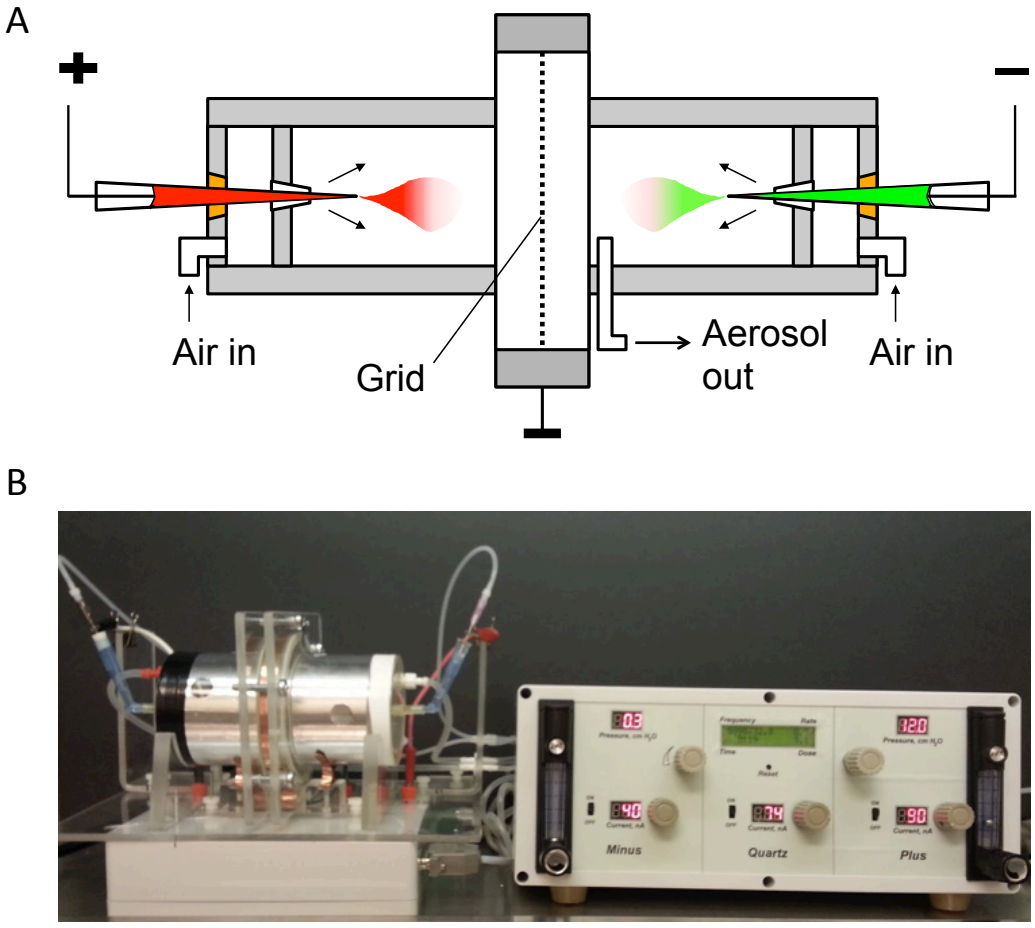

Figure 1: Nanoaerosol Generator (A) Schematic of the electrospray neutralization chamber and (B) photo of nanoaerosol generator with the electrospray neutralization chamber on top of the high voltage power supply (on left) and the electronic control panel (right).

Subsection Two: Selected Therapeutics

To demonstrate that this device had further utility to deliver a wide range of therapeutics from several different classes of antibiotics and therapeutics that are relevant in the treatment of lung diseases and therefore likely applications for this delivery technique, we have chosen a number of drugs to generate nanoaerosol sprays (listed below and **Table 1**). **Table 1** also shows the oral bioavailability and therapeutic blood concentration of the compounds, which is necessary to determine whether achieving therapeutic dosing via nanoaerosol is feasible.

Some of these compounds cannot be given orally either because of low bioavailability or due to toxicity in large doses, but could benefit from an alternative delivery method directly to the site of the infection. These drugs are potentially the most interesting candidates to be tested with the nanoaerosol device as nanoaerosol administration provides a way to overcome this limitation.

In particular we chose the following therapeutic agents to be sprayed in the nanoaerosol generator.

I. Antibiotics:

   a. Ampicillin (RPI Corp. A40040-5.0) is a beta-lactam antibiotic used to treat both gram-positive and gram-negative bacterial infections, including many common respiratory infections, with a bioavailability of about 30-40% [20].

   b. Ceftazidime (Sigma C3809) is a cephalosporin antibiotic useful for the treatment of many bacterial infections especially melioidosis, as well as lower

respiratory tract infections, however it is only given intravenously or intramuscularly [21].

c. Ciprofloxacin (GenHunter Q901) and levofloxacin (TCI L0193) are both fluoroquinolone antibiotics, used to treat many bacterial infections including respiratory tract infections and have good bioavailability [22, 23].

d. Kanamycin (Fisher BP906-5) is an aminoglycoside antibiotic used to treat gram-negative bacterial infections, notably tuberculosis. It has very low oral bioavailability (0.7%) and can have very serious side effects [24, 25].

e. Rifampicin (RPI Corp. R64000-1.0) is a rifamycin antibiotic used to treat tuberculosis and has a bioavailability of 93% [26].

f. Streptomycin (Gibco 11860-038) is an aminoglycoside used to treat tuberculosis and cannot be given orally [27]. Since streptomycin cannot be given orally, a new administration route especially for the treatment for such deep lung diseases as tuberculosis could be very advantageous.

g. Tetracycline (Sigma T-3383) is a polyketide antibiotic used against gram-positive and gram-negative bacteria and has an oral bioavailability of about 75% [28, 29].

II. Antibody (FB11): anti-Francisella LPS mouse monoclonal antibody (Thermo Fisher MA1-21690) have been shown to be at least partially effective as a form of immunotherapy [30].

III. Anticancer Agent: Doxorubicin HCl (RPI Corp. 25316-40-9) is used as a cancer chemotherapy treatment for many different types of cancers although its

cardiotoxicity limits the dosing [31]. It is also a naturally fluorescent dye, which is useful for characterizing its concentration.

IV. Antifungal drug: Amphotericin B (Sigma A9528) is a polyene class of antifungal used to treat severe and systemic fungal infections, but has serious side effects. It has very low oral bioavailability, and is often used intravenously [32].

V. Antimicrobial peptide: Apo6 (China Peptides) is an antimicrobial peptide found in alligator blood plasma that has antimicrobial activity against both gram-positive and gram-negative bacteria [33].

VI. Antiviral drug: Ribavirin (Santa Cruz SC-203238) is an antiviral used to treat respiratory syncytial virus, a major cause of lower respiratory tract infections in infants and children [34].

VII. Medical Diagnostic Dye: Indocyanine green, also called cardiogreen (Sigma I2633), is a fluorescent dye that has been used in medical diagnostic testing since the 1950s to measure cardiac and liver function [35]. It is currently delivered intravenously, but could be useful to deliver by pulmonary route especially for detecting individual tuberculosis tubercules.

Table 1: Therapeutic properties of drugs tested in atomization experiments

Drug Tested	Drug Class	Notable Uses	Oral Bioavailability	Therapeutic blood-plasma concentration (mg/L)
Antibiotics:				
Ampicillin	Beta-lactam antibiotic	Many gram-positive and gram-negative bacteria	30-40% **[20]**	0.02-2 **[36]**
Ceftazidime	Cephalosporin antibiotic	Lower respiratory tract infections, including pseudomonas in cystic fibrosis patients	N.G.[a]	20-40 **[36]**
Ciprofloxacin	Fluoroquinolone antibiotic	Many gram-positive and gram-negative bacteria	70% **[23]**	2.5-4 **[36]**
Kanamycin	Aminoglycoside antibiotic	Gram-negative bacteria, tuberculosis	0.7% **[24]**	1-4 **[36]**
Levofloxacin	Fluoroquinolone antibiotic	Respiratory pathogens, including penicillin-resistant S. pneumoniae	99% **[22]**	2.8-5.2 **[22]**
Rifampicin	Rifamycin antibiotic	Tuberculosis	93% **[26]**	0.1-10 **[36]**
Streptomycin	Aminoglycoside antibiotic	Mycobacteria, tuberculosis	N.G. **[27]**	1-5 **[36]**
Tetracycline	Polyketide antibiotic	Many gram-positive and gram-negative bacteria	75% **[29]**	1-5 **[36]**
Antibody:				
FB11	Monoclonal antibody	Anti-francisella LPS	N.D.[b]	N.D.
Anticancer:				
Doxorubicin	Anticancer agent	Cancer chemotherapy for a wide range of cancers, including lung	5%	0.006-0.02 **[36]**
Antifungal:				
Amphotericin B	Polyene class of antifungal	Severe, systemic fungal infections	0.3% **[32]**	0.2-3 **[36]**
Antimicrobial Peptide:				
Apo6	Investigational antimicrobial peptide	Antimicrobial peptide [37]	N.D.[b]	N.D.
Antiviral:				
Ribavirin	Antiviral	RSV, hepatitis C	64%	2.5-4 **[38]**
Medical Diagnostic Dye:				
Indocyanine green	Medically relevant dye	Medical diagnostic dye. Also used in targeted cancer treatment	N.G.	N.G.

Notes to Table 1:
[a] N.G. = not given, [b] N.D. = not determined

Subsection Three: Desalting Method

For larger compounds that are produced or stored in high salt solutions, such as antibodies and peptides, dialysis (Thermo Scientific #88404) against ultrapure water or desalting columns (Thermo Scientific #89882) was used following manufacturer instructions. This reduced the overall salt concentration and therefore reduced the conductivity, which increased the efficiency of electrospraying.

Subsection Four: PVP filter setup and nanoaerosol collection

In addition to the mass calculations and drug concentrations from the spectra presented in Table 2, direct measurements of the mass concentrations were performed for a few drugs for confirmation by using custom-made, water-soluble PVP filters [39-41]. PVP water-soluble nanofilters consisting of 6% PVP and 80% ethanol were produced by electrospray technique. A nanofilter was placed into a specialized device fitted with a rubber gasket and connected directly to the nanoaerosol device with conductive tubing. Nanoaerosol particles were collected from the electrospray chamber for one hour on the PVP filter.

Subsection Five: Method of detection of PVP samples

After one hour of nanoaerosol collection, the PVP filter was dissolved in 100 μL of milli-Q water and the optical density was measured on a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The absorbance maxima was

then compared to a standard curve to calculate the total collected drug mass. To account for the effects of the dissolved PVP nanofilter on the absorbance spectra, a PVP nanofilter was added to calibration solutions.

Section Two: Experimental Procedure

Subsection One: Nanoaerosol Generation method and Electrospray Neutralization

The nanoaerosol generator used in this study has been described previously [6, 13] and is shown in **Figure 1**. Briefly, the drugs or therapeutics form nano-sized particles through the application of a positive electric current, delivered from a high voltage power supply via a platinum wire electrode, and pressure. The charged drug is sprayed through a glass capillary to produce a cloud of positively charged micro droplets. To stabilize these highly charged droplets, counter-ions are electrosprayed in the same manner but from a negative potential, resulting in net neutral monodisperse nanoaerosols. A volatile solvent is used to produce the negatively charged counter ions because the positively charged nanoaerosol needs to disintegrate before neutralization occurs, which allows for smaller particle formation. Validation of spraying is confirmed visually by shining a laser beam next to the capillary to observe the plume as well as through observation of mass accumulation on the quartz microbalance.

Subsection Two: Nanoaerosol generator device setup

The therapeutic drug solutions were placed into a capillary and sprayed from the positive potential current. Ethanol (100%) was placed into the opposing capillary and sprayed at a negative potential to provide counter ions to the nanoaerosols produced from the therapeutics. A splitter and conductive tubing was used to connect the nanoaerosol device to the Scanning Mobility Particle Sizer, a quartz microbalance to monitor the spraying performance, and the PVP nanofilter. Each drug was sprayed with the nanoaerosol device for a minimum of 30 minutes to collect representative sizing and concentration data.

Subsection Three: Preparation of solutions for spraying

All solutions were prepared with milli-Q water to a concentration of 0.1% (1mg/mL) and a volume of 1mL. Electrical conductivity of each solution was measured with a the CON 11 conductivity meter from Oakton Instruments, (Vernon Hills, IL) or with the S3 conductivity meter from Mettler Toledo, (Schwerzenbach, Switzerland).

Subsection Four: Electrospray Neutralization settings

The positive current was set to 90 nA with an applied pressure of 7-15 cm H_2O to enhance spraying rate. The negative current was set to 40 nA with no additional pressure. The nanoaerosol output rate was adjusted to 2 liters per minute.

Subsection Five: SMPS settings

Aerosol Instrument Manager SMPS software was used with the following devices settings for analysis of the nanoaerosols. The DMA sheath flow rate and the aerosol flow rate were set at 3.0 L/min and 0.71 L/min, respectively.

Subsection Six: Aerosol Sizing

The spectra showing particle size distribution and counts were collected using a Scanning Mobility Particle Sizer (SMPS, TSI Incorporated, Shoreview, MN) consisting of a water-based Condensation Particle Counter (CPA, model 3786), an Electrostatic Classifier (model 3080), and a Differential Mobility Analyzer (model 308). The SMPS provides high-resolution particle size classification and measured distribution of nanoaerosol particle sizes between 20 to 1000 nm.

Subsection Seven: Aerosol Sampling

Custom-made polyvinylpyrrolidone (PVP) nanofilters [39] were used for the collection of nanoparticles because they are chemically inert and have high capturing efficiency (not less than and 90% for nanoaerosol particles, 50 nm and 100 nm in size, respectively) [13, 41]. The filters were produced by electrospray-neutralization technique [39, 41].

Subsection Eight: Estimation of Inhaled Doses

Predicted doses were made based on calculations using the MPPD model, version 3.04 (Chemical Industry Institute of Toxicology, Research Triangle Park, NC) working in the particle size range between 10 nm and 1μm. In calculations of mice doses, the breathing frequency was 273.1 min^{-1} and the tidal volume 0.219 ml. In calculating the estimated human inhaled doses the breathing frequency was 12 min^{-1} and the tidal volume 625 ml. The probability of deposition for each drug was determined in the MPPD calculations as those corresponding to the geometric mean diameter of nanoaerosol particles from its spectrum. The total deposited dose was estimated by summing the fractions of the particle mass in the size range 10 – 1,000 nm, taking into account the average hourly volume passed through the lungs and the deposition probablilty calculated from MPPD.

CHAPTER THREE: RESULTS

Section One: Generation of Nanoaerosols and Characterization of Spectra

We sought to establish whether our nanoaerosol device has the utility to deliver a wide range of therapeutics from several different classes of antibiotics and therapeutics that are relevant in the treatment of lung diseases and therefore likely applications for this delivery technique. In order to demonstrate this, we have chosen a number of drugs to generate nanoaerosol sprays with, listed in **Table 1**. Some of these compounds cannot be given orally either because of low bioavailability or due to toxicity in large doses, but could provide a benefit from an alternative delivery method directly to the site of the infection. These drugs are potentially the most interesting candidates to be tested with the nanoaerosol device as nanoaerosol administration provides a way to overcome this limitation.

Each substance to be atomized was prepared for spray by removing all the salt ingredients and impurities, which substantially affect the electrospray process, by desalting columns, by dialysis, or by choosing a non-ionic drug form for atomization, if possible. It is also important to properly choose solvents, for inhalation only water and ethanol could be safely used either alone or as a mixture.

It was found that all fourteen drugs studied here could be atomized to nanosized aerosol particles from 0.1% solutions in water. All drugs, except ribavirin, which formed

larger sub-micron particles, had geometric mean particle diameters, which were used to normalize the average, of less than 100 nm, **Table 2**, **Figure 2.** The mass median aerodynamic diameter (MMAD), the diameter that divides the particles in half based on mass is also listed for reference. As seen from series of spectra in **Figure 2**, distribution of nanoaerosol particle sizes varied substantially from substance to substance. The peak size is the largest peak seen in the spectra, which for most of the drugs is just one peak. Amphotericin B, ampicillin, Apo6 peptide, ceftazidime, ciprofloxacin, doxorubicin, indocyanine green, kanamycin and levofloxacin displayed spectra with a single maximum, indicating a monodisperse aerosol. The spectra of FB11 antibody, rifampicin, streptomycin and tetracycline consisted of two peaks, while that of ribavirin included three peaks, showing more of a polydisperse aerosol. And while the spectra capture particle sizes from 10 nm to approximately 800 nm this does not mean that there are not larger particles/aggregates in the aerosol, but that they are not observed by this method. Some aspect of this issue can be observed when the mass of the compounds is plotted rather than the number of particles (**Figure 3**).

In addition to variations in the number and position of peaks, their intensities (concentration at maxima) varied from 1.5×10^7 cm^{-3} to 1×10^6 cm^{-3} for most drugs except rifampicin, which showed the lowest concentration of 4.5×10^5 cm^{-3}. This also resulted in differences in the concentration of the number of particles and the mass concentration of the spectra (see **Table 2**), which indicate the number of particles per liter of air and the mass per liter of air, respectively. We speculate that the differences in the spectra reflect variations in the solutions' physical characteristics: molecular weight, solubility, and

conductivity (see **Table 2**) as well as differences in surface activity and other physical properties of the drug compounds studied.