Electrohydrodynamic Atomization for Improved Macromolecular Drug Delivery

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Electrohydrodynamic Atomization for Improved Macromolecular Drug Delivery

A Dissertation
Presented to
the faculty of Engineering and Computer Science
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Michelle Zeles-Hahn
June 2010

Advisor: Corinne Lengsfeld
ABSTRACT

With advances in drug research, the use of biological therapeutics is becoming a reality. Unfortunately, methods for processing and delivering these fragile macromolecules often limit their therapeutic potential. For this dissertation, we explore the aerosolization of macromolecules by way of electrohydrodynamic atomization (EHDA) and how this method can be used to process and deliver therapeutics. EHDA employs a high voltage to break a column of liquid into drops. It was unknown if or how the residual charge left of the resulting droplets would affect lung cells. An in vitro experiment was conducted to spray aerosolized DNA, by way of EHDA, onto human derived lungs cells to test for immunogenic and toxic effects. The lung cells displayed no immunogenic or toxic response to the DNA or high voltage. Previous researchers have used EHDA to aerosolize proteins with mixed results. This work sets forth a simplified thermodynamic theory and provides recommendations to pharmaceutical companies on how to design more stable protein formulations for aerosol processing or delivery. Finally, a new method of producing liposomes was created. It constructs the liposome one layer at a time. The inside of the liposome is sprayed by EHDA, with the lipid and drug in solution together. As the sprayed monolayer passes through a pool containing a solution of lipid in water, the second part of the bilayer attaches to the inner layer creating a complete bilayer liposome.
Acknowledgements

“I have seen that in any great undertaking it is not enough for a man to depend simply upon himself” – Teton Sioux

This accomplishment has been a long time and coming. There are many people, without whom this wouldn’t be possible. As I am not very eloquent with words, I am going to use the words of others to express my heart-felt thanks.

Corinne Lengsfeld  “The best advisors ... give us, out of themselves, the ardent spirit and desire to act right, and leave us then, even through many blunders, to find out what our own form of right action is”
                      – Anonymous

Tom Anchrodoquy  “The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires.”
                      -William A. Ward

Andrew Hahn     “And I am standing here because the man I share my life with has taught me and demonstrates for me every day just what love is.”

Mom and Dad      “If you raise your children to feel that they can accomplish any goal or task they decide upon, you will have succeeded as a parent and you will have given your children the greatest of all blessings.”
                      - Brian Tracy

Rachel Fulstone and Kristil Krug  “An Editor becomes kind of your mother. You expect love and encouragement from an Editor” -Jackie Kennedy

Nicole Payton   “Good friends are like stars...you don't always see them, but you know they're always there.” – Anonymous

Kristil and Dan Krug   “In the cookies of life, friends are the chocolate chips.”
                      – Anonymous

Chris Sponheimer  “It destroys one's nerves to be amiable everyday to the same human being.” - Euripides

Matt Opgenorth  “If you can laugh together, you can work together.”
                      -Robert Orben

Obe, Puck and Sadie  “The greatest pleasure of a dog is that you may make a fool of yourself with him and not only will he not scold you, but he will make a fool of himself too.” - Samuel Butler

Committee Members, National Science Foundation  “I can no other answer make, but, thanks, and thanks.”
                      -William Shakespeare

BES-0433810/BES-0433811
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1 Introduction

Significant progress has been made toward uncovering cellular pathways that cause disease and illness. In response, many potential cures or treatments focus on using biological macromolecules as therapeutics (such as DNA, RNA, or proteins). These macromolecules can be extremely fragile and exceedingly expensive. As a result, many methods used for delivering small molecule therapeutics are not suitable for all macromolecules. One noninvasive method for delivering these molecules to the body is via inhalation, which has the added benefit of avoiding the first pass metabolism. Electrohydrodynamic atomization (EHDA) is a technique that has recently been introduced to the field of pharmacology. Through the process of atomization, liquids are turned into small mono-disperse drops that can be inhaled. Although there is some literature examining EHDA and its use in pharmaceutical delivery, there are many questions that need to be addressed before this technique can be ready for commercial use. For example:

- Will the residual charge on the resulting droplets exasperate compromised tissue?
- Does the EHDA protect proteins like it appears to protect plasmid DNA?
- Can the laminar flow of the EHDA system be manipulated to add additional benefits over traditional aerosolization techniques, i.e., can we make liposomes on demand?
1.1 Macromolecule Fragility

Biological molecules are of increasing interest to the pharmaceutical industry due to their high specificity. They can target or interact with very specific molecules leading to fewer possible side effects and higher efficacy. In addition, integrating therapeutic DNA into the cell has the potential to cure genetic diseases. DNA, RNA, and proteins are known as macromolecules because of their large molecular weight that can range from a few thousand to many million Daltons. Due to their large size and structure-dependant weak hydrogen bonding, these molecules can be easily degraded both physically and chemically during manufacture or delivery. Therefore, it is imperative that the macromolecules be protected and stabilized as much as possible.

1.2 Electrohydrodynamic Atomization

EHDA is a technique that applies a high voltage to a liquid exiting a small capillary tube. The resulting electrical field, between the capillary and the ground ring, generates a potential force on the fluid column that is aligned with the axial direction of the capillary tube. When this electrical potential is sufficiently large to dominate the fluid dynamics of the jet or large enough to overcome surface tension forces in the liquid, classical dripping modes of jet breakup are transformed into thin threads of liquid jets emanating from the liquid-gas interface. It is believed classical Rayleigh breakup drives the division of these thin filaments into droplets. Unless neutralized, these
tiny droplets retain a residual charge from the process and are attracted to any oppositely charged surface.

Figure 1.1 - Diagram of EHDA

The EHD spray is capable of producing drops ranging from fractions of millimeters down to nanometers [1]. It is currently used in a variety of different industries such as: combustion, paint and coating applications, propulsion, agricultural pesticide application, chemical composition in mass spectrometry, and, most recently, drug delivery. As the liquid leaves the nozzle, the shape of the liquid jet and its behavior (also known as “mode”) is dependent upon: the liquid’s properties, the flow rate of the liquid, and the applied voltage [2, 3]. The most stable shape that produces the uniform drop size is known as the Taylor cone and was first described by Sir Geoffrey Ingram Taylor in 1964 [4]. The properties that influence drop size have been debated throughout the years. Recently, Ganan-Calvo et al. discovered a relationship between drop size and the Weber number (Equation 1) [5]. The important properties that determine the radius of the drop ($R_d$) are: density ($\rho$), flow rate ($Q$), radius of the jet ($R$), surface tension ($\sigma$), permittivity of vacuum ($\varepsilon_0$), and electrical conductivity ($K$). To solve for the radius of the drop, three intermediate parameters must be solved for include the Weber number ($We$), pressure difference ($\Delta P$), and jet radius ($R_o$).
Equation 1.1 - From Ganan-Calvo et al. [5], the equations below determine the size of a drop created using EHDA or flow focusing.

\[
\frac{R_d}{R_o} = We
\]

\[
We = \frac{\rho \cdot Q^2}{\pi^2 \cdot R^3 \cdot \sigma}
\]

\[
R_o = \frac{\sigma}{\Delta P}
\]

\[
\Delta P = \left(\frac{\sigma^2 \cdot K^2 \cdot \rho}{\varepsilon_o^2}\right)^{\frac{1}{3}}
\]

1.3 Using EHDA for DNA atomization

There are many different devices available for atomizing macromolecular therapeutics. FDA approved delivery devices include jet nebulizers, ultrasonic nebulizers, metered-dose inhalers (MDI), and dry powder inhalers (DPI). These devices have many drawbacks including creating polydisperse drops that are ingested rather than deposited in the lungs, drug deposition dependency on patient inspiration rate, high waste or residual volume, and overall poor drug deposition in the lungs (less than 50% for nebulizers, less than 25% for MDI, and less than 17% for DPI) [6, 7]. This has sparked a need for new drug atomization methods. EHDA avoids some of the shortcomings of the other aerosol techniques. It creates monodisperse drops one at a time minimizing the residual volume of drug in the device. Since the size of the drop can easily be manipulated, it can be tailored to the best possible size for lung deposition. Finally, studies have been conducted atomizing plasmid DNA that concluded that EHDA was the most effective and structurally safest way to atomize DNA [8].
Although DNA retains structural integrity during atomization with EHDA, an unanswered question still remains. Will these highly charged drops affect cells? The study in Chapter 2 was designed to answer this question by assessing toxicity, inflammatory response, and transfection efficiency of naked and complexed plasmid DNA delivered via EHDA to normal human bronchial/tracheal epithelial cells \textit{in vitro}.

### 1.4 Protein Stability During Atomization

Although proteins are significantly smaller in size than DNA, proteins can pose a potentially more complicated problem in terms of drug delivery. Proteins have tertiary and quaternary structures that are vital for stability and function. Early observations by Maa et al. [9] associated protein stability with surface area to volume ratios. They noted a linear relationship between droplet diameter and protein aggregation. In addition, they believed that over time more protein would be drawn to the interface of the drop causing greater protein denaturation and aggregation, hence stating that degradation is a time dependant process. As a result, we wondered if the residual charge on an EHDA droplet could be used to push proteins off the interface into the bulk solution slowing the time dependent degradation process.

There are few published works using EHDA to atomize proteins [10-12]. Moreover, the results from such studies are conflicting. Some proteins retain 100% activity after spraying, while others lose activity depending on flow rates or concentration. The cause for these discrepancies is currently unknown. Attempts to correlate protein properties that could affect stability such as: size, isoelectric point, type of protein, buffer pH, melting temperature, and percent hydrophobic provided little
insight into the problem. However, crude studies in our lab using a static droplet show no time dependant degradation behavior, putting into question Maa’s original hypothesis. Randolph et al. expanded on Maa’s original theory stating that in order for a protein to remain stable, its change in surface tension divided by the change in surface area must be greater than zero, else the protein unfolds [13]. This statement removes time dependence by removing the concept that the protein must be at the interface to unfold. Delving further into these observations, melting enthalpy is directly correlated to melting temperature and surface tension multiplied by surface area. Enthalpy is an energy; this understanding led to the thought that protein stability during EHDA is a thermodynamic issue. Based on this, Chapter 3 will explore the idea that the energy input into the system necessary to create the aerosol must be less than the energy within the protein in order for the molecule to remain in its native state. Our goal for this chapter is to develop a universal transition criterion for protein stability during aerosolization based on three easily measurable quantities: surface tension, droplet diameter, and melting enthalpy. As such, protein engineers can manipulate formulation to sustain activity using a clear engineering method rather than the current trial and error process.

1.5 Liposome Encapsulation

One modern approach to adding efficacy to therapeutic drugs is by enhancing protection and targeting through encapsulation. Encapsulation for drug delivery consists of a protective coating on the surface of the drop that is typically composed of biocompatible polymers or lipids. Current methods of encapsulation include double emulsion, solvent evaporation/extraction technique, swelling, extrusion, electroformation,
electroinjection, and spray drying. However, these techniques typically suffer from low encapsulation efficiencies and polydisperse size distribution. In addition, these methods are not always capable of fully encapsulating the drug, i.e. a small section from a large molecule can protrude outside the shell.

The laminar flow field inherent to the EHDA process makes it ideal for encapsulation processes whereby one material can be co-extruded concentrically over the other [14, 15]. However, the EHDA process can be used for encapsulation in another way. The small droplet diameters of the process also make it ideal for developing less complicated and more reliable strategies using miscible fluids and molecular diffusion to self-assemble the protective layer on the liquid-gas interface.

EHDA is used as a single step encapsulation process that can be divided into two critical stages. The first stage includes spraying the encapsulation solution that contains the macromolecule and lipid. As the droplet falls towards a catch basin, the hydrophobic tails of the lipid molecules will be drawn to the liquid-gas interface of the drop due to their inherent attraction to hydrophobic/hydrophilic interfaces. The lipids on the surface will have their hydrophobic tails pointing outward of the drop. The second stage occurs when the drop comes into contact with the catch pool. The catch pool is comprised of an aqueous solution with lipids floating on the surface. As the drop passes into the pool, the lipid layer of the drop pairs with the lipid layer of the surface of the pool creating a bilayer. The drop then travels deeper into the pool where the bilayer pinches off, creating a liposome. The critical parameters necessary for the success of this technique include the density and solubility of the lipid in the sprayed solution. Chapter 4 is focused on
using dense salt solutions that are miscible with lipid-isopropanol mixtures as the spray solution in order to create stable liposomes.

1.6 Summary of Aims

The three aims of this research project are to provide a better understanding of EHDA and its possible use in therapeutic drug delivery. Each of these aims build upon one another and need to be addressed before EHDA can be used safely and effectively in the pharmaceutical industry. The following chapters will explore in depth each of the topics in turn. Each chapter is presented as an individual, stand alone paper with the intent to publish each in separate journals.
2 Effect of Electrospray on Human Pulmonary Epithelial Cells

2.1 Introduction

Interest in utilizing DNA-based pharmaceuticals to treat pulmonary diseases such as acute respiratory distress syndrome (ARDS), cancer, asthma, emphysema, and cystic fibrosis has increased in recent years [16-21]. In fact, approximately 8% of all current clinical trials worldwide focus on treating pulmonary diseases (http://www.clinicaltrials.gov). Delivery to this region of the body can be undertaken by three distinct routes: direct injection, systemic delivery through intravenous injection, or topical administration by instillation or aerosolization [17]. Of these three methods, topical administration, specifically aerosolization, is the most popular option due to its ability to directly and non-invasively target the diseased airway cells as well as reducing toxic effects on other organs in the body [22]. Unfortunately, poor in vivo results were encountered when naked plasmid DNA ranging in size from 4.7 to 7.2 kilobase pairs (kbp) was delivered with the two most clinically utilized atomization devices: jet and ultrasonic nebulizers [23, 24]. It has been suggested that loss in DNA molecular integrity, as a direct result of the aerosolization process, may be responsible for these poor results [23-32]. Under these circumstances, a decrease in molecular integrity would be manifested as a strand break in the plasmid DNA resulting in the transition of the native supercoiled conformation into an open circle form. Subsequent strand breaks at the same location on the opposite strand of the plasmid would result in a double strand
break and conversion of the open circle form to a linear structure. Additional double strand breaks then reduce the full-length linear molecule into smaller sized fragments (i.e., ‘fragmented DNA’). To achieve robust gene expression, either the supercoiled or open circle form of plasmid DNA must be preserved [33].

Studies to improve the delivery and expression of non-viral gene therapies via aerosolization are focused in two primary areas. First, in order to improve tidal volume and breathing frequency, carbon dioxide has been incorporated into the surrounding environment [25, 34]. While this method demonstrated a significant increase in therapeutic expression, it is not clinically desirable. Second, cationic lipids (e.g., 1,2-dioleoyl-3-trimethylammonium propane; DOTAP) and polymers (e.g., polyethyleneimine; PEI, poly-L-lysine; PLL) complexed to DNA molecules increase in vivo transfection after aerosolization by both jet and ultrasonic nebulizers [23-32]. These agents have the ability to protect the DNA from nuclease degradation, decrease the molecule’s overall hydrodynamic diameter, and assist in transporting DNA across both cellular and nuclear membranes [35-41]. Unfortunately, in some cases, the cationic agent can fail to dissociate from the DNA molecule within the intracellular environment, thereby preventing gene expression [42-44]. Additionally, some investigators have observed an increase in immune response when DNA complexed to these agents has been delivered via the pulmonary route [45-50].

To overcome the observed loss in plasmid DNA’s molecular integrity during aerosolization, most investigations to date have focused on modifying the therapeutic formulation via the addition of complexing agents as opposed to examining the aerosolization method itself. Our previous studies assessed the effects of various
aerosolization devices on an assortment of sizes (5 kbp to 37 kbp) of naked (non-complexed) plasmid and cosmid DNA [51]. We found electrohydrodynamic atomization (EHDA) sprays to have a high potential for delivery of DNA to the lungs since damage to molecular integrity was minimal for all investigated DNA sizes.

Operating at relatively low flow rates, EHDA sprays (also known as electrosprays) apply high voltages to a fluid overcoming the fluidic surface tension forces to form a fine aerosol [52]. Briefly, a potential force is created between a charged fluid, developed when the fluid flows through an electrically conducting capillary to which the voltage is applied, and a ground plate. The generation of an aerosol occurs at the capillary exit. Interestingly, a sequence of spray modes is observed that is known to be dependent upon a number of factors including: volumetric flow rate, applied voltage, setup of the device (e.g. distance between capillary and plate or capillary radius), and properties of the fluid (e.g., surface tension, density, electrical conductivity) [53-55]. The physical basis for these spray modes is not known, but the first observed mode is the dripping mode. In the absence of an electric field, the liquid drips from the capillary as large droplets. An increase in applied voltage results in an increase in the frequency at which the dripping occurs in conjunction with a decrease in droplet diameter. The reduction in droplet diameter is thought to be a result of a decrease in surface tension due to the accumulation of charges on the fluid surface [54]. A further increase in applied voltage results in the next displayed mode, the cone-jet mode. This mode consists of an axially stable cone meniscus from which a simple, straight linear jet with some varicose instability is emitted. The cone-jet mode is perhaps the most investigated and well-known EHDA spray mode since it produces a fine, nearly mono-disperse aerosol [56,
Destabilization of the cone-jet mode occurs with additional increases in applied voltage and results in the formation of a whipping cone, followed by a multi-jet spray. The whipping cone mode displays an irregular meniscus from which droplets are produced in unpredictable directions, while the multi-jet spray consists of a flat, stable meniscus with multiple small cones extending from the rim of the capillary.

Investigations exploring the use of EHDA sprays as a method for non-pulmonary and pulmonary delivery are limited [58-60]. To our knowledge, only one in vivo study examining the effects of EHDA sprays on respiratory epithelium has been undertaken [61]. Although this study did administer DNA to living mice using EHDA, the study ignored any toxic or immunologic effects and recovered very little of the starting material. Since significant voltages are required to create suitably sized droplets, the potential harm to the respiratory epithelium by these charged droplets must be evaluated before EHDA sprays can be considered a viable method for delivery via the respiratory tract. Also, in vitro assessment of EHDA sprays would benefit from epithelial cell culture models that resemble in vivo conditions as closely as possible [62].

In selecting a lung epithelial cell culture system for assessing the effects of EHDA sprays, the technique by which cells are cultured becomes critical. Conventional liquid-covered culture (LCC) completely immerses the monolayer of cells in growth media, which does not allow direct exposure to the sprayed material. In contrast, cells cultured at an air-liquid interface (ALI) are continually exposed to air and therefore, better simulate the in vivo situation. These cells are grown on a semi-permeable support coated with collagen and are maintained with culture media from the basolateral side only. ALI cultured cells have been shown to develop and retain epithelial cell features
including bioelectric properties, mucin secretion, and the development of cilia and tight junctions [63]. Additionally, cells cultured by this method are still capable of responding to stresses arising from the environment [64, 65]. Previous researchers have compared EpiAirway™ ALI cells to in vivo experiments (in humans and rats) and found that the in vitro permeability correlates directly to in vivo bioavailability, and that EpiAirway™ cells have statistically similar repair kinetics [66-68]. Utilizing commercially available ALI cultured bronchial/tracheal epithelial cells, we assessed the effects of delivering naked plasmid DNA, lipid/DNA complexes, polyethyleneimine (PEI)/DNA complexes, and poly L-lysine (PLL)/DNA complexes by electrospray. Specifically, we examined toxicity and cytokine response as well as monitored transfection efficiency.

2.2 Materials

Luciferase plasmid DNA (5.9 kbp) was provided by Valentis, Inc. (Burlingame, CA); Trizma® hydrochloride (Tris-HCl), agarose, polyaspartic acid, ethidium bromide, and Poly L-lysine, lipopolysaccharide 055:B5 (LPS) were obtained from Sigma-Aldrich (St. Louis, MO); diethylenetriaminepentaacetic acid (DTPA) was purchased from Acros Organics (Fisher Scientific, Hampton, NH); and 5X Receptor Lysis Buffer was acquired from Promega Corp. (Madison, WI); 50X Tris/Acetate/EDTA (TAE) buffer came from Bio-Rad Laboratories (Hercules, CA). Linear PEI (MW 25,000) was obtained from Polysciences, Inc. (Warrington, PA) and used in aqueous solution. DNA was dissolved to a final concentration of 0.02 mg/ml in buffer (10 mM Tris-HCl, 200 µM DTPA, pH 8.5). The lipid, 1,2-Dioleoyl-3-Trimethylmmonium-Propane (DOTAP) was acquired from Avanti Polar Lipids (Alabaster, AL). The modified PEI for low toxicity in the
complexes was a generous gift from Dr. Manfred Ogris of the Ludwig Maximilians Universitat München. The modification of the PEI are found in detail in Russ et al. (2008) and described as pseudodendrimer HD O [69].

2.3 Methods

Preparation and Analysis of DNA Complexes

PEI/DNA complexes were prepared at two different ratios; a 6:1 and 10:1 nitrogen to phosphate ratio. The 10:1 ratio was prepared by mixing 2.7 µg PEI with 2 µg plasmid DNA in 10 mM Tris-HCl and 200 µM DTPA buffer to a total volume of 2 ml in polypropylene centrifuge tubes and incubated for 10 minutes at room temperature. The 6:1 ratio of PEI/DNA was prepared similarly, as well as the modified PEI/DNA complex (also mixed at a 6:1 ratio). The modified PEI was used in addition to the linear PEI because it has been previously described as less toxic [69].

The PLL/DNA complexes were created at a ratio of 6:1 nitrogen to phosphate. After mixing, the complexes were incubated at room temperature for 15 minutes before use. The lipid/DNA complexes were made using lipid mixture of 1:1 DOTAP to cholesterol. A volume of 1.5 ml of 0.04 mg/ml DNA in 10 mM Tris-HCl (without DTPA) was added to 1.5 ml of lipid mixture (containing 0.487 mM DOTAP). The mixture was incubated at room temperature for 15 minutes before spraying.

Electrohydrodynamic Spray

Solutions (3 ml) containing naked DNA or any of the suspensions containing complexes were pumped through a stainless steel capillary tube (0.56 mm inner diameter, 1.07 mm outer diameter) at a flow rate of 0.2 ml/min using a New Era Pump Systems
Model NE-1000 syringe pump (Wantagh, NY). The system, with a needle-to-ground distance of 2.5 cm, was run at either a voltage of 0 kV or -6 kV. A dripping profile was observed at an applied voltage of 0 kV while the applied voltage of -6 kV exhibited a stable cone-jet.

**Cell Culture**

EpiAirway™ tissues, cultured on collagen supports under air-liquid interface conditions, were obtained from MatTek Corp. (Ashland, MA; Figure 2.1). These tissues consisted of normal, human derived tracheal/bronchial epithelial cells that were highly differentiated (e.g., cilia, tight junctions, sodium and chloride channels) and retain properties of normal respiratory epithelial tissue (e.g., actively secrete mucus, electrogenic). The tissues had an average trans epithelial electric resistance (TEER) of 391.2 ± 50.2 ohms/cm². Upon delivery, the tissue inserts were processed according to the supplier’s protocol. Briefly, each tissue insert was transferred to a well in a 6-well plate pre-filled with 900 µl pre-warmed serum free media (AIR-100-MM, MatTek Corp.) and incubated at 37°C in 5% CO₂ overnight (16-18 hours) prior to *in vitro* investigations.

*Figure 2.1 - Schematic of the air-liquid interface tissue technique. The apical surface is exposed to air while the basolateral side absorbs nutrients from the liquid media. Diagram used with permission of MatTek Corp. All rights reserved.*
In Vitro Investigations

At the end of the incubation period, each tissue insert was transferred to a well in a 24-well plate containing 1000 µl pre-warmed AIR-100-MM media. A total of 100 µl (the supplier’s recommended maximum applied volume) containing either naked DNA or one of the complexes in buffer was atomized either directly onto the apical tissue surface for 32 seconds, or into a small cell culture dish with subsequent application to the cell surface. Thus, a total of 2 µg of DNA was applied to each tissue sample. At specific post-application time points from 0 to 72 hours, 1000 µl of underlying culture media was removed from each well and replaced with fresh, pre-warmed AIR-100-MM media. Of this removed culture media, 500 µl was stored at -80°C for evaluation of cellular toxicity while the remaining volume was stored at -20°C for analysis of cytokine response. A 72-hour time study demonstrated that these storage conditions sustained LDH and IL-8 levels comparable to fresh controls. The reported values for cellular toxicity and cytokine response are cumulative for each time point. Control conditions included the incubation of cells in AIR-100 MM media (untreated); cell exposure to the maximum electric field of 2.8 kV/cm (electric field); treatment with plasmid DNA or PEI/DNA complexes that had not been processed through the EHDA spray system (pipette); applying the Tris-HCl buffer (without DNA) directly onto the cells using the EHDA spray system at two different voltages (spray buffer); and treatment of LPS (positive control). ‘Untreated’ tissues were not exposed to an electric field nor were they subjected to DNA application. To expose the tissues to the maximum electric field, the tissue inserts were placed between a dry capillary tip and ground for a total of 32 seconds at a maximum voltage of -7 kV. Controls for the unprocessed DNA, liposome/DNA, and
PEI/DNA complexes were done by pipetting 100 µl of the desired DNA solution directly on top of the tissue. The LPS was used as a positive control because it is known to elicit an IL-8 response in EpiAirway™ cells (as per supplier recommendation). One hundred microliters of 0.1 µg/µl LPS solution was pipetted directly on top of the cells. All of the tissues were maintained at 37°C in 5% CO₂ between time points.

**Evaluation of Cellular Toxicity**

Lactate dehydrogenase (LDH) levels in culture medium were measured using the CytoTox 96 Cytotoxicity Assay Kit (Promega Corp., Madison, WI) following the manufacturer’s protocol. Released LDH in culture medium was measured with a 30-minute coupled enzymatic assay, which converted 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) into a red formazan product that could be detected with UV spectrophotometry. Briefly, 50 µl of each sample was loaded into 96-well plates and fresh AIR-100-MM media was loaded as a blank. Next, 50 µl of substrate solution was added to each well and the plates were incubated for 30 minutes at room temperature in the dark to avoid photobleaching. At the conclusion of the incubation period, 50 µl of stop solution was added to each well. Absorbance values for the samples were then analyzed within 30 minutes of adding the stop solution at the specified wavelength of 490 nm using a SPECTRAmax 190 Spectrophotometer with SoftMax® Pro software (Molecular Dynamics Corporation, Sunnyvale, CA). The background values were subtracted from each sample and the LDH concentrations were calculated by interpolation of a standard calibration curve.
**Evaluation of Inflammatory Response**

The concentration of interleukin 8 (IL-8) in culture medium was measured according to the instructions provided by the manufacturer using a Quantikine enzyme-linked immunoassay (ELISA) kit from R & D Systems (Ann Arbor, MI). The kit included a microplate coated with a monoclonal antibody specific for IL-8. One hundred microliters of assay diluent followed by 50 µl of sample medium was added to each well in the provided plate, then incubated for 2 hours at room temperature. After washing each well, 100 µl of anti-IL8 conjugate was added to each well and the plate incubated for 1 hour at room temperature. The wells were washed to remove unbound conjugate solution and 200 µl of substrate solution was added to each well. The plate was then incubated in the dark for 20 minutes. Finally, 50 µl of stop solution was added to each well and absorbance values were measured within 30 minutes at 450 nm (with a wavelength correction of 540 nm) using a SPECTRAmax 190 Spectrophotometer with SoftMax® Pro software. The concentration of IL-8 was calculated by interpolation of a standard calibration curve.

**Evaluation of Transfection Efficiency**

Seventy-two hours after the application of naked DNA or PEI/DNA complexes, the tissues were washed by gently immersing the collagen support in phosphate buffered saline solution (PBS; MatTek Corp.). Next, 300 µl of 1X Receptor Lysis Buffer was added to the apical tissue surface. The lysates were stored at -80°C until further analysis was undertaken. The lysates were thawed at room temperature then centrifuged for 15 minutes at 4°C and 2000 rpm using a Thermo IEC, Savant Refrigerated Microcentrifuge model SFR13K (Needham Heights, MA). Luciferase activity was assessed using 50 µl
supernatant with 100 µl Luciferase Assay Reagent (Promega Corp., Madison, WI) using a Los Alamos Diagnostics model 535 luminometer (Turner Designs, Inc., Mountain View, CA). Total protein was assessed using a Bio-Rad protein assay kit (Hercules, CA) according to the manufacturer’s protocol. The absorbance was measured at 595 nm using a THERMOMax microplate reader (Molecular Devices, Sunnyvale, CA).

DNA Quantification and Analysis

Control and atomized DNA samples (300 ng) were loaded onto 0.5% agarose gels containing 0.5 mg/ml ethidium bromide and run for 4 hours at 70 V in 1X TAE buffer. Gels containing plasmid DNA were scanned and analyzed for supercoiled, linear, open circle, and fragmented DNA structures using a Fluor-S MultiImager with Quality One® software (Bio-Rad Laboratories, Hercules, CA). The intensity of the bands associated with each structure corresponded to the number of DNA molecules, however the intensity at which ethidium bromide stains has been shown to be dependent upon DNA topology [70]. Therefore, a correction factor of 1.4 was applied to the supercoiled DNA values in order to account for the reduced amount of ethidium bromide that binds to this form of DNA [70]. Percentages of each DNA structure were then calculated by dividing the intensity of each stained band by the total staining intensity (sum of supercoiled, linear, open circle, and fragmented DNA). To dissociate DNA from PEI, polyaspartic acid (25 mg/ml) was incubated with PEI/DNA complexes for 1 hour at 37°C, and the separated DNA was analyzed using agarose gel electrophoresis as described above.

Statistical Analysis

Experimentally determined values for supercoiled, linear, open circle, and fragmented DNA were compared within a range of applied voltages using a one-way
ANOVA. Additionally, comparisons between application methods as well as applied voltages at specific time points were assessed using an ANOVA in conjunction with Tukey’s and Fisher’s multiple comparison tests. Samples having $p$ values less than 0.05 were judged to be significantly different. All experiments were performed on triplicate cell samples.

2.4 Results

Prior to investigations with cells, the effect of varying applied voltages on supercoiled, open circle, and fragmented structures for both naked and PEI/DNA complexes (ratio 1:10) was examined (Figure 2.2A and 2.2B). The lower applied voltages, 0 kV and -3 kV, display dripping profiles while the higher applied voltages, -6 kV and -7 kV, present stable cone-jet and whipping cone profiles, respectively. A slight decrease in supercoiled DNA resulted in a small increase in both the open circle and fragmented forms at the two highest applied voltages (Figure 2.2A). However, the total amount of fragmented DNA remained below 10% for both of these conditions. In contrast, supercoiled, open circle, and fragmented DNA structures remained constant for all applied voltages when DNA was complexed with PEI (Figure 2.2B). These results are similar to our previous findings [71].
Figure 2.2 – Percent of supercoiled (triangle, solid line), open circle (circle, dashed line), and fragmented (square, dotted line) structure for naked plasmid DNA (A) or PEI/DNA complexes (B) at varying applied voltages. The amount of supercoiled, open circle, and fragmented DNA was assessed with agarose gel electrophoresis. Each bar represents the mean ± one standard deviation of DNA structure in triplicate atomization runs.
ALI cultured bronchial/tracheal epithelial cells were utilized to assess the effects of delivering naked plasmid and complexed DNA by electrosprays on respiratory epithelial cells. To evaluate toxicity and inflammatory response, we monitored the release of the cytoplasmic enzyme LDH and the cytokine IL-8 in the culture medium. The extracellular appearances of LDH and IL-8 are commonly used to detect cell damage/death and inflammatory response, respectively, in pulmonary gene delivery studies [72-78]. Cellular transfection rates were also monitored by measuring the expression of luciferase enzyme, which is encoded by the plasmid DNA.

Our toxicity results indicate that samples exposed to the applied electrical field exhibit a slightly increased level of LDH release (Figure 2.3A) when normalized with the untreated tissues (a value of 1 indicates levels equivalent to untreated cells). However, this effect is short-lived and the extracellar release of LDH drops back to the control level after 8 hours. It appears that no permanent damage to the cells is caused by the exposure of the electric field. The electrospray’s applied electrical field and exposure time were 14 and 80 times higher than that of traditional electroporation methods, indicating that cells are not affected by these relatively high electric fields [79]. In addition, there is little effect on the tissues when applying the Tris-HCl buffer on top of the cells, either by pipetting or by processing through the electrospray system at -6 kV (Figure 2.3A). Although a slight increase is seen for the sprayed buffer, the difference is not statistically significant. The positive control for this experiment was completed by lysing the tissue to determine the total amount of LDH available for release from the cell.
The inflammation results for IL-8 are seen in Figure 2.3B. The difference between the control and the buffer (both sprayed and pipetted) for the cytokine release is statistically different for early time points but becomes statistically the same once 12 hours have elapsed (Tukey comparison test). It is not surprising that the application of a fluid to lung cells should elicit a mild cellular response. The positive control for IL-8 consisted of the application of one hundred microliters of 0.1 mg/ml LPS. When compared to the positive control that elicited a 3-fold greater response, the effect of buffer application appears very mild.
Figure 2.3 – Induction of LDH and IL-8 in human tracheal/bronchial epithelial cells after exposure to the control conditions. (A) Normalized (divided by LDH levels in untreated cells) release of LDH for three control conditions: tissues exposed to a maximum electrical field of 7 kV (diamond), tissues with buffer pipetted on top of the cells (square), indirectly sprayed buffer applied to tissues after spraying (triangle), tissues subjected to sprayed buffer at 6 kV (circle), and, the positive control, lysing of the cells (dash). (B) Release of IL-8 in untreated tissues (diamond), tissues with buffer pipetted on top of the cells (square), indirectly sprayed buffer applied to tissues after spraying (triangle), tissues subjected to sprayed buffer at 6 kV (circle), and, the positive control, 0.1 mg/ml LPS was applied (dash). From these graphs, it is easy to see that each condition does not deviate significantly from the untreated tissues, especially when compared to the positive controls. Each error bar represents a 95% confidence interval of released LDH or IL-8 in triplicate tissue samples.

Figure 2.4 depicts the cellular response of the lung cells after the application of plasmid DNA. The results in Figure 2.4A show little change in LDH release with the different methods of DNA application. Similar results are seen for the IL-8 release, with the majority of all samples having statistically similar mean values. The LPS positive control is the only sample with a statistically different mean value. Comparable results were seen at other voltages (-3 kV and -7 kV), suggesting that cellular toxicity and
inflammation are not affected by droplet charge density and size (data not shown). Finally, we did not detect luciferase activity for any condition, indicating that naked DNA was not able to effectively transfect the cells.
Figure 2.4 Induction of LDH and IL-8 in human tracheal/bronchial epithelial cells after administration of naked plasmid DNA. Release of the enzymes were monitored in untreated tissues (diamond), DNA not processed through the system but pipetted directly onto the tissues (square), indirect application of aerosolized DNA (triangle), and direct spray application of DNA (star for 0 kV and circle for 6 kV). (A) Normalized results for LDH release. (B) Results for the IL-8 release. The positive control used for the both graphs was the same as in Figure 2.3 but left off for clarity. Each error bar represents a 95% confidence interval of released LDH or IL-8 in triplicate tissue samples.

Previous studies attempting to deliver DNA to the lungs observed enhanced gene expression when DNA was condensed through complexation with other polymers and lipids [25, 29, 32, 34, 80-83]. Figure 2.5 displays cumulative LDH and IL-8 release after administration of PEI/DNA complexes. A significantly different response is evident when complexes are applied at different PEI/DNA ratios, regardless of whether the complex was sprayed via EHDA. It appears that a 10:1 ratio of PEI to DNA is toxic to the cells while a ratio of 6:1 is not (this includes the PEI alone; the amount of PEI used is the same as in the 6:1 ratio). Despite complexing the DNA with PEI, we did not detect luciferase expression for any of the formulations tested. Since the main objectives of this entire study were to evaluate toxicity and inflammatory response, we did not conduct further experiments to optimize transfection conditions.
Figure 2.5 – Induction of LDH and IL-8 in human tracheal/bronchial epithelial cells after EHDA spray administration of various PEI/DNA complexes at an applied voltage of 6 kV. Release of the cytoplasmic enzymes were monitored in untreated tissues (diamond),
tissues subjected to sprayed DNA (circle), tissues exposed to PEI/DNA complexes not processed through the system (square t), tissues exposed to EHDA PEI/DNA complexes with direct spray application with a ratio of 1:10 (star) and 1:6 (square), and tissue subjected to sprayed PEI alone (triangle). (A) Normalized results for LDH release. The positive control was left off for clarity. (B) Results for the IL-8 release. The positive control used for the IL-8 experiments was 0.1 mg/ml LPS (dash). Each error bar represents a 95% confidence interval of released LDH or IL-8 in triplicate tissue samples.

In the interest of further exploring the effects of agents commonly used to deliver DNA, other polymers and lipids were used to complex DNA. Additionally, the toxic effects of the PEI complexes were investigated more thoroughly. In Figure 2.6, the results for toxic and immune responses to various DNA complexes are shown. It appears that all of the complexes have a negligible effect on the tissues with the exception of the modified PEI when applied alone. In contrast to application of the regular linear PEI (Figure 2.5B), the modified PEI elicited a small immune response when administered alone (slight increase in IL-8 release, Figure 2.6B). The reason for this is unclear, but it appears to be specific to the modified PEI and is not observed in complexes containing this agent.
Figure 2.6 – Induction of LDH and IL-8 in human tracheal/bronchial epithelial cells after EHDA spray administration of various DNA complexes at an applied voltage of 6 kV. Release of the cytokines was monitored in untreated tissues (diamond), tissues exposed to naked plasmid DNA processed through the system (circle), tissues exposed to EHDA Lipid/DNA complexes with direct spray application (square), application of PLL/DNA
complexes sprayed using EHDA directly onto the tissues (triangle), tissues directly sprayed with modified PEI/DNA complexes (star), and tissue subjected to sprayed modified PEI alone (square t). (A) Normalized results for LDH release. The positive control for the LDH was left off this graph for clarity (values can be seen in Figure 2.3). (B) Results for the IL-8 release. The positive control was left off of the graph for clarity. Each error bar represents a 95% confidence interval of released LDH or IL-8 in triplicate tissue samples. The * represents a statistically significant difference between the modified PEI and the other treatments.

2.5 Discussion

By applying significant voltages to a fluid stream, EHDA sprays can produce droplets when the electrical forces are great enough overcome the fluid’s surface tension. This aerosolization method has a wide range of uses from chemical analysis via mass spectrometry to the encapsulation of immiscible liquids on a micro/nano-scale to delivering agricultural pesticides [84, 85]. Yet, the use of EHDA sprays for pulmonary drug delivery remains in its infancy. When not complexed to a cationic agent, native DNA is degraded upon atomization by current aerosolization devices such as jet and ultrasonic nebulizers [23, 24, 71, 86, 87]. In contrast, we have previously shown EHDA sprays to be a gentle method by which naked plasmid DNA can easily be aerosolized without substantial losses in native DNA structure (Figure 2.2A) [71]. Furthermore, complexing DNA with PEI resulted in complete maintenance of DNA conformation (Figure 2.2B). Considering the ability of these methods to maintain DNA integrity, the goal was to test the effect of EHDA sprays on respiratory epithelial cells.

The development of epithelial cell culture models that resemble in vivo conditions has shown great promise [88-90]. Advantages of using these cell culture models include: small amounts of therapeutics are required, they are easier and more economical than in vivo studies, reducing the need for animal testing, and environmental conditions such as
pH and temperature can easily be controlled [62]. A highly differentiated, three-dimensional \textit{in vitro} model that forms tight cellular monolayers with mixed cellular phenotypes (e.g., ciliated and non-ciliated cells) capable of secreting mucus or surfactant is highly desirable since it more closely mimics \textit{in vivo} airway tissue. To this end, protocols for the isolation of primary respiratory epithelial cells originating from both humans and animals have been developed [91-94]. However, a limited number of primary cells can be freshly isolated from their donor and, depending upon the culture technique, morphological and functional changes can occur [95]. Cancer cell lines have also been examined for their suitability to serve as cell culture models [96-100]. Unfortunately, these cancer cell lines can exhibit several abnormal characteristics in addition to their immortality. In this investigation, we utilized commercially available ALI cultured bronchial/tracheal epithelial cells to assess the toxicity, cytokine response, and delivery of naked plasmid and complexed DNA by electrosprays.

Our results showed that the toxicity and inflammatory responses after aerosolization of naked DNA and the DNA complexes were similar over the investigated time span. Furthermore, the results indicate that cells subjected to the applied electrical field do not exhibit a prolonged release of LDH (Figure 2.3A), and cells are able to recover quickly from the initial exposure. Regardless of the condition, the release of extracellular IL-8 is consistent (except for the positive LPS control). An unexpected finding was that the results for the PEI complexes show a PEI/DNA ratio of 10:1 was extremely toxic and immunogenic, whereas the 6:1 ratio was statistically similar to the control. Other studies have also seen the same rise in toxicity with an increase in PEI ratio \textit{in vivo} [101]. Consistent with the studies by Russ et al. (2008), the modified PEI
exhibits little toxicity when complexed with DNA [69]. However, we find that the modified PEI is toxic when delivered alone, similar to that observed for free linear PEI [69, 102].

The primary barrier for efficient delivery of therapeutics to the lungs is the epithelial lining. The apical surface of the epithelium displays a number of features that present barriers to the uptake of foreign material. The cell monolayers are comprised of polarized cells that contain tight junctions that prevent the passage of everything but very small polar molecules. Also, the endocytic capacity of these cells is limited. In addition, goblet cells within the epithelium secrete mucus that inhibits diffusion of foreign particles, thereby reducing access to the plasma membrane. In the bronchial regions of the lung, the apical surface is coated with cilia that beat continually in order to move foreign materials away from the lung. In the deeper regions of the respiratory tract, alveolar macrophages remove the foreign material, typically via phagocytosis [17, 62]. While delivery to the cultured ALI cell system is clearly different from delivery to the lung (e.g., shorter distance between electrospray and apical cell surface), the cells mimic the lungs by being fully differentiated and able to produce mucus. Consistent with our inability to detect expression of the luciferase reporter gene, numerous studies have demonstrated the difficulties associated with transfecting differentiated epithelial cells found in the lungs [103-108]. In addition to the barriers presented by secreted mucus and the presence of tight junctions preventing the passage of foreign materials to the plasma membrane, differentiated cells divide relatively slowly which reduces access to the nucleus [109].
Considering the minimal effect on toxicity and inflammatory response observed despite a very large range in droplet charge (calculated droplet charge ranged from 0 C/m$^2$ at 0 kV up to $2.2 \times 10^{-5}$ C/m$^2$ at -7 kV), the results demonstrate that exposure of the EpiAirway™ cells to electric fields and charged droplets during electrospray administration do not have detectable adverse effects. Although our experimental conditions did not result in successful transfection, the maintenance of DNA integrity combined with the lack of toxicity and minimal inflammation suggest that electrosprays are a potentially safe and effective method for the pulmonary delivery of genetic therapeutics. Additional experiments would need to be conducted in order to optimize transfection efficiency and assess the applicability of this approach in vivo. In addition, the ability of EHDA to accurately vary droplet size, and thereby target different regions of the lung, offers significant advantages over conventional nebulizers.

2.6 Conclusion

Our investigation shows electrosprays to be a gentle method by which plasmid DNA can be aerosolized without a significant loss in native DNA structure. This investigation explored the effects of aerosolization of naked and complexed plasmid via an electrospray on EpiAirway™ cells that had been cultured at an air-liquid interface. LDH release remained low for both the naked and complexed formulations indicating minimal cell toxicity (except for the 10:1 PEI/DNA complex). In addition, cytokine levels appeared nominal and comparable for all conditions, independent of electrospray application. The mild toxicity observed when the tissues were placed in the applied electric field was temporary, and the tissues returned to normal within a few hours after application. Our experiments were designed to assess the toxicity associated with EHDA
sprays; however future studies would be needed to optimize this approach for maximizing transfection. While future *in vivo* experiments are required, our results show that electrosprays present a promising method for administering DNA-based therapeutics to the pulmonary epithelium.
3 Protein Stability During Aerosolization

3.1 Introduction

Since its emergence in the early 1980s, molecular biotechnology, especially the promise of protein therapies, has not reached its full potential. This is due, in part, to the cost of the therapies but also to the unpredictable nature of these macromolecules during processing and delivery. There are currently 85 protein-based therapeutics marketed for pulmonary delivery, with at least 350 more in clinical trials [110]. In addition to those already targeted for aerosol delivery, a majority of proteins are spray dried to increase shelf life. The ability of a protein to sustain activity or native structure after aerosolization has largely been a mystery for process and formulation designers.

In 1997, Maa et al. found a correlation that associated protein stability with surface area to volume ratios [9]. They noted a linear relationship between droplet diameter and protein aggregation. Furthermore, they believed that over time more proteins would be drawn to the interface causing greater protein denaturation and aggregation. The implication of their statement is that protein degradation by aerosolization is a time dependant process. However, studies in our lab showed no change in degradation of a protein within stationary droplets; suggesting that time is not an influencing parameter but instead drop size might be a factor.

Significant literature exists on the aerosolization or atomization of proteins using EHDA. However, a large portion of published research has failed to observe protein
functionality directly after spraying [111-116]. Of those that did monitor protein activity, findings are inconsistent. For example, Gomez et al. examined insulin and found no change in activity between the sprayed versus non-sprayed samples [10]. A few studies examined the effects of spraying bovine serum albumin (BSA). Pareta et al. investigated the secondary structure of BSA using circular dichroism and reported a slight but insignificant change in structure [12]. The paper pointed out that increased protein concentrations correlated to less structural degradation of BSA. Similarly, Xie et al. electrosprayed BSA in tandem with a polymer to study encapsulation [117, 118]. They found that BSA retained more than 80% of its original activity. For lactate dehydrogenase and pyruvate kinase, parameters such as voltage and co-solvent concentration had to be manipulated in order to maximize protein stability [119]. Gowadia and Dunn-Rankin [120] implemented an assay to determine the activity of a lipase enzyme after spraying. They found that the activity of the protein dropped from 90% of the non-sprayed activity to 43% when decreasing the flow rate from 29 cc/hr to 9 cc/hr. This drop in activity is consistent with Maa’s hypothesis because Ganan-Calvo [121] has definitively shown that lowering the flow rate in the EHDA process decreases the drop size. Maa would therefore predict a large reduction in protein activity with the subsequent increase in the surface area to volume ratio associated with this decrease in droplet size.

More recently, Randolph et al. predicted an additional relationship beyond surface area to volume ratio. They recommend for a protein to remain stable its change in surface tension divided by change in surface area must be greater than zero, else the protein unfolds [13]. However, this theory, as well as Maa’s, fails to capture the dramatic
differences in fragility between different proteins or even protein batches. As such, a universal theory must observe energy input to the system to create the aerosol relative to the activation energy required by the protein to jump to a new energy well within the potential landscape and subsequently unfold.

Undoubtedly, the pharmaceutical protein industry would greatly benefit from a simple correlation between easily measurable formulation quantities and the smallest active droplet size for sustained protein stability. The work outlined in this chapter exploits thermodynamic relations utilizing several simplifying assumptions to develop a universal correlation between surface tension, melt enthalpy in solution, and droplet size for predicting protein stability during aerosolization. We present a series of experimental data that validates this correlation and defines a transition region.

3.2 Thermodynamic Theory and Derivation

Proteins remain in their native, folded form until sufficient energy is added to the system, which subsequently drives the proteins into a new energy potential well enabling them to unfold. The amount of energy required to unfold a protein can be measured using a differential scanning calorimeter (DSC). The DSC applies a constant heating rate to both a blank cell (consisting of the buffer solution) and sample cell. As the protein unfolds, the energy applied to the sample cell is used for unfolding and not to heat the cell. It is at this juncture that a temperature difference between the two cells occurs and requires additional heating power to bring the sample cell to the temperature of the blank. The heating power is directly related to the heat capacity ($c_p$) of the sample. The temperature at which the protein unfolds is known as the melting temperature ($T_m$). As
the scan progresses, the heat capacity and temperature of the sample are recorded and can be manipulated to give the melting enthalpy ($dH_m$) of the sample.

$$dH_m = c_p \cdot dT$$  \hspace{1cm} (3.1)

In order to use this relation, the protein must be reversible, meaning that the protein must refold to its original form upon cooling. This assumption is required to relate heating power to heat capacity.

The aerosolization of a protein solution requires energy to break the initial liquid bulk or column into individual droplets. This energy includes disrupting the surface tension of the liquid and increasing the surface area. The total enthalpy ($H_T$) of the closed aerosolization process or system can be explained by breaking the enthalpy of the system into three parts: the air ($H_A$), the surface of the drop ($H_\sigma$), and the drop bulk ($H_B$).

$$dH_T = dH_A + dH_\sigma + dH_B$$  
$$dH_A = TdS + VdP + \Sigma \mu d\xi_i$$  
$$dH_B = TdS + VdP + \Sigma \mu d\xi_i$$  
$$dH_\sigma = TdS + \sigma dA + \Sigma \mu d\xi_i$$  \hspace{1cm} (3.2)

Where the enthalpy depends on temperature ($T$), entropy ($S$), volume ($V$), pressure ($P$), surface tension ($\sigma$), surface area ($A_\sigma$), number of molecules ($n_i$), and chemical potential ($\mu$). Assumptions can be made to simplify these equations. In the part of the system comprised only of air, no heat is transferred and pressure remains constant. If evaporation is negligible from the droplet, the number of air molecules and composition of the air does not change, thus having no change in enthalpy for the air.

$$dH_A = 0$$  \hspace{1cm} (3.3)

For the bulk solution in the drop, it can be assumed that no energy in the form of heat is transferred in the system, thus the $TdS$ term is assumed negligible. Note this would not
necessarily be true in spray drying when evaporation is important, but it is a correct assumption for inhalation applications.

\[
dH_B = VdP + (\Sigma \mu dn_i)_B \quad (3.4)
\]

At the interface of the drop, there is no heat transfer (evaporation is insignificant on this time scale). Again, the \( TdS \) term is neglected.

\[
dH_\alpha = \alpha dA_S + (\Sigma \mu dn_i)_\alpha \quad (3.5)
\]

Combing the three simplified equations, the total enthalpy of the system becomes:

\[
dH_T = \alpha dA_S + VdP + (\Sigma \mu dn_i)_T \quad (3.6)
\]

Relatively low pressures are used to generate the aerosol for this study, as is true for most inhalation and spray dryers, the greatest pressure extending to about 100 psi. Research concerning protein denaturation due to pressure is seen at high pressures, greater than 2,000 kbar (see Table 3.1) \[122-124\]. For this reason, enthalpy changes due to the small pressure changes in our system are also assumed to be negligible. This leaves a final equation that is dependent on surface tension, change in surface area, and change in the number of proteins at the surface and bulk. It is important to realize that the chemical potential of the proteins at the surface is different than the bulk not only because they might be denatured but also because they will partially unfold to expose hydrophobic regions to the gas phase.

\[
dH_T = \alpha dA_S + (\Sigma \mu dn_i)_T \quad (3.7)
\]

Table 3.1 - Common proteins and the pressures at which they denature.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Pressure to Denature at 20°C (kbar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNase</td>
<td>2,000</td>
</tr>
<tr>
<td>α – Chymotrypsin</td>
<td>4,000</td>
</tr>
<tr>
<td>RNase A</td>
<td>6,000</td>
</tr>
</tbody>
</table>
It is assuring that this new relationship is consistent with Randolph’s realization that there is a direct relationship between surface tension and drop size, which affects the stability of a protein [13, 125, 126]. Moreover, this new relationship acknowledges the difference in melt enthalpy from one protein to another. However, it is important to recognize that there is an inherent dependence of the chemical potential term on the surface area term in Equation 3.7. Decreasing the droplet diameter increases the surface area to volume ratio thus impacting the chemical potential term simply though changing the number of protein molecules in the bulk compared to the surface. As chemical potentials are difficult to accurately and simply measure, we will assume a proportional relationship and condense the expression into a single function with C as the proportionality constant. Experimental data is needed to determine the value of C.

\[
(\sum \mu_i n_i)_{f} = f(\alpha dA_s) \\
dH_f = C \alpha dA_s
\]  

(3.8)

Coupling the DSC system and Equation 3.1 with the aerosolization system and Equation 3.8, we propose that if the total energy of the drop is greater than that of the melting enthalpy, the protein will denature.

\[
dH_M < C \alpha dA_s
\]  

(3.9)

### 3.3 Materials

All of the chemical and biological materials were purchased from Sigma-Aldrich (St. Louis, MO) and include: choline oxidase (Alcalignes), potassium chloride [KCl], ethylenediaminetetraacetic acid [EDTA], choline chloride, phenol, 4-aminoantipyrine,
peroxidase (horseradish type II), Trizma® base [Tris], Trizma® hydrochloride [Tris HCl], potassium phosphate (mono- and di-basic) [KPO4], o-nitrophenyl β-d-galactopyranoside, magnesium chloride [MgCl], 2-mercaptoethanol, β-galactosidase (E. coli), methanol, n-(2-hydroxyethyl) piperazine-n (3-propanesulfonic acid) [EPPS], n-benzoyl-l-tyrosine ethylester [BTEE], methanol [MeOH], calcium chloride, α-chymotrypsin (bovine pancreas type II), citrate synthase (porcine heart), oxaloacetic acid, acetyl coenzyme A, and 5,5′-Dithiobis (2-nitrobenzoic acid) [DTNB]. All solutions were prepared with deionized water. For each enzyme, the buffer solution was made using a variety of different pH values (Table 3.2). The purpose of varying the pH was to create protein solutions possessing different melt enthalpies and surface tensions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration of Buffer</th>
<th>pH Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline Oxidase</td>
<td>100 mM Potassium Phosphate</td>
<td>6.7, 7.2, 7.7</td>
</tr>
<tr>
<td></td>
<td>10 mM Tris HCl, 2mM EDTA, 134 mM KCl</td>
<td>7, 8, 8.5</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>100 mM Potassium Phosphate</td>
<td>7.2</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>10 mM EPPS</td>
<td>7.2, 7.6, 7.8, 8, 8.4</td>
</tr>
<tr>
<td></td>
<td>80 mM Tris HCl</td>
<td>7.5, 8, 8.5</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>Deionized water</td>
<td>NA</td>
</tr>
</tbody>
</table>

3.4 Methods

_Drop Generator_

The device used to create the drops for this investigation was a piezoelectric droplet generator (Figure 3.1). The fluid was pumped through a syringe pump (Model 260D, ISCO Inc.) at a constant velocity of 1 ml/min and the fluid was passed through a vibration orifice. A piezoelectric ceramic, similar setup to Berglund _et al._ [127], vibrates creating perturbations in column momentum leading to surface waves that are amplified.
by aerodynamic interaction. This in turn pinches off uniformly sized drops from the liquid jet. The size of the drop is controlled by both the frequency of the piezoelectric and the diameter of the vibrating orifice, both of which are easily manipulated by either changing the frequency or by replacing the orifice disk. For these experiments, the frequency was kept at approximately 20 kHz because each piezoelectric ceramic exhibits a natural frequency leading to an optimal performance octave frequency. Therefore to manipulate the final term in our relationship we employed three orifice disks with diameters of 25, 50, and 75 microns. The drop generator had a tendency to become obstructed, so a 7 micron stainless steel Swaglok® inline filter was placed between the pumps and the piezoelectric.

![Diagram of the drop generator](image)

Figure 3.1 - Diagram of the drop generator, which uses a piezoelectric to create monodisperse drops. Image was modified from Berglund et al.

*Particle Sizing by Imagery*

The drops were imaged using a CCD camera (Model DT100) and software (CameraLink v1.02h) by DuncanTech with a field of view pixel size of 1024 x 1024. As drops were being generated, a strobe light was flashed behind the drops and still images
were taken. In addition, pictures of a sub-millimeter ruler were taken. The drop images were sized by pixel using an onscreen pixel ruler (Pixel Ruler v3.1, Mioplanet Technologies Inc.) and converted to millimeters using the ruler images.

Activity Assays

Enzymatic assays were used to determine the structural stability and activity of each of the proteins before and after aerosolization. The assays were colorimetric in nature and were analyzed using a UV spectrophotometer (Model USB 2000+, Ocean Optics). By observing the rate of product formation, the assay was able to show the activity of the enzyme and thus the structural stability. The rate of activity of the aerosolized sample was compared with the control to give a percent activity.

\[
\% \text{Activity} = \frac{\text{Sample Rate}}{\text{Positive Control Rate}} \times 100
\]  

(3.10)

The activity for each condition was monitored at room temperature (approximately 25°C) and measured in triplicate.

The assay for β-galactosidase was found in [128] and briefly described below. To monitor the activity of β-galactosidase, the following solutions were mixed into a cuvette:

- 0.175 ml of 100 mM KPO4
- 0.033 ml of 30mM MgCl
- 0.033 ml of 3.36 M 2-mercaptoethanol

After the β-galactosidase had been processed, 0.033 ml of the 4 units/ml enzyme solution were added to the cuvette. At time zero 0.033 ml of 68 mM o-nitrophenyl β-d-
galactopyranoside was added and the absorbance of the reaction was recorded at 410 nm for 5 minutes.

The reaction for choline oxidase was described in detail by Sigma-Aldrich [129, 130]. The first step in monitoring the activity of the protein was to create the following reaction cocktail.

$$24.25 \text{ ml of } 2.1\% \text{ w/v choline chloride}$$
$$0.25 \text{ ml of } 1\% \text{ w/v 4-aminoantipyrine}$$
$$0.5 \text{ ml of phenol}$$
$$\sim 1 \text{ mg of Peroxidase}$$

The final solution was light sensitive and needed to be stored appropriately, i.e., wrapped in aluminum foil until used. When ready, 0.988 ml of the cocktail was added into a cuvette and placed in the spectrophotometer with the absorbance reading set to 500 nm. After the choline oxidase had been aerosolized, 0.025 ml of the 0.3 unit/ml solution was collected and placed in the cuvette. The absorbance was recorded at 500 nm for 5 minutes with readings collected at least every 10 seconds.

The UV spectrophotometric reaction for $\alpha$-chymotrypsin was first discovered by Wirnt [131] but later improved by Rafiq and Bailey [132]. The assay for $\alpha$-chymotrypsin required the following solutions:

$$1.07 \text{ mM BTEE in } 4.2\% \text{ v/v MeOH}$$
$$0.08 \text{ M Tris HCl with } 0.1\text{M CaCl}$$

After the solutions were prepared, 0.467 ml of the BTEE solution was placed in a cuvette with 0.5 ml of the Tris HCl solution. The cuvette was placed in the UV spectrophotometer and the absorbance was set to 256 nm. Sprayed chymotrypsin was
collected and 0.03 ml of the 1 mg/ml enzyme was added to the cuvette. The absorbance was recorded at 256 nm for 5 minutes, with readings collected at least every 10 seconds.

The assay used in the UV spectrophotometer for citrate synthase contained:

- 1 mM DTNB in 1M Tris HCl (pH 8)
- 10 mM oxaloacetic acid in 0.1 M Tris HCl
- 10 mM acetyl coenzyme A

The assay was first discovered by Srere in 1966 [133, 134] and requires 0.05 ml of 0.4 units/ml sprayed citrate synthase, 0.1 ml of DTNB, 0.03 ml of acetyl coenzyme A, 0.77 ml of deionized water, and the last solution added at time zero was 0.05 ml of oxaloacetic acid. The peak absorbance was at a wavelength of 412 nm and absorbance readings were collected for 5 minutes at 10 seconds intervals.

**Calorimetry**

The calorimetry measurements were determined using a differential scanning calorimeter (model VP DSC, MicroCal) operating with Origin 7.0 software (MicroCal Piscataway, NJ). The volume for each cell was 0.51 ml. The blank cell was always filled with the same buffer as that of the protein under examination to provide a blank signature for reference. Each run was typically scanned from 10°C to 100°C at a rate of 90°C/hour and at a constant pressure of about 36 psi. The reference buffer scan was subtracted from each enzyme sample to create a stable baseline. The activity assays were more sensitive to concentration than the calorimeter; therefore greater concentrations of proteins were used in the calorimetry experiments (Table 3.3).

<table>
<thead>
<tr>
<th>Table 3.3 - Concentration of proteins used for DSC experiments.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase</td>
<td>0.000564</td>
</tr>
<tr>
<td>Choline Oxidase</td>
<td>0.000556</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>0.04</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

**Surface Tension**

Surface tension was measured with a tensiometer (model Sigma 701, KSV Instruments Monroe, CT) using the ring method (DuNouy). As the ring lifts out of a liquid, there is a force pulling on the ring from the liquid. A maximum force is exerted on the ring just before the liquid breaks, which is a measured by the tensiometer, and is equal to the surface tension of the liquid. The protein solutions used for this measurement were identical to those processed through the drop generator. Measurements were taken in triplicate for each condition and then averaged.

**Surface Area**

The change in surface area was calculated between the unprocessed protein solution in the drop generator and the droplets formed. For the total enthalpy calculation proposed, it is important to maintain the same volume or mass on each side of the equation. Thus the volume for the aerosolization calculation (Equation 3.8) was the same as what used in the DSC (0.51 ml) calculation in Equation 3.1. The mass must be maintained so that the two total enthalpies can be set equal. This is equivalent to rewriting the equations in the enthalpy per mass form. The surface area of the drop generator that corresponded to the volume used in the DSC was 2.8 cm$^2$.

**Data Analysis**

At a minimum, each experiment was conducted in triplicate. All error bars represent one standard deviation in the data.
3.5 Results

In order to fully test the theory presented in Equation 3.8, all three of the parameters were tested. Each parameter (surface tension, drop size, and melt enthalpy) was manipulated in different ways by altering the buffer, pH, protein, and orifice size. The values obtained for all experiments are shown in Table 3.4.
Table 3.4 – Experimental data obtained for all techniques. Errors reported are equal to one standard deviation.

<table>
<thead>
<tr>
<th>Protein (Buffer)</th>
<th>Concentration (mg/ml)</th>
<th>pH</th>
<th>Drop Size (um)</th>
<th>Activity</th>
<th>Melting Enthalpy (J)</th>
<th>Surface Tension (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β - Galactosidase (Phosphate)</td>
<td>0.005</td>
<td>7.2</td>
<td>96 ± 4</td>
<td>60% ± 9</td>
<td>0.29 ± 0.04</td>
<td>70 ± 0.4</td>
</tr>
<tr>
<td>Choline Oxidase (Phosphate)</td>
<td>0.083</td>
<td>7.2</td>
<td>87 ± 19</td>
<td>77% ± 8</td>
<td>0.27 ± 0.03</td>
<td>64.9 ± 0.08</td>
</tr>
<tr>
<td>Choline Oxidase (Tris)</td>
<td>0.083</td>
<td>7</td>
<td>103 ± 10</td>
<td>65% ± 16</td>
<td>0.14 ± 0.02</td>
<td>59.3 ± 2.7</td>
</tr>
<tr>
<td>Choline Oxidase (Tris)</td>
<td>0.083</td>
<td>8.5</td>
<td>120 ± 9</td>
<td>68% ± 11</td>
<td>0.33 ± 0.02</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>Choline Oxidase (Tris)</td>
<td>0.083</td>
<td>8</td>
<td>129 ± 20</td>
<td>38% ± 16</td>
<td>0.22 ± 0.02</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>Choline Oxidase (Tris)</td>
<td>0.083</td>
<td>7</td>
<td>125 ± 19</td>
<td>75% ± 15</td>
<td>0.14 ± 0.04</td>
<td>70 ± 1</td>
</tr>
<tr>
<td>Chymotrypsin (EPPS)</td>
<td>1</td>
<td>8.4</td>
<td>140 ± 20</td>
<td>92%</td>
<td>8.7 ± 0.5</td>
<td>55.3 ± 1.2</td>
</tr>
<tr>
<td>Chymotrypsin (EPPS)</td>
<td>1</td>
<td>7.6</td>
<td>135 ± 19</td>
<td>80%</td>
<td>10.5 ± 0.6</td>
<td>56.7 ± 1.2</td>
</tr>
<tr>
<td>Chymotrypsin (EPPS)</td>
<td>1</td>
<td>7.15</td>
<td>132 ± 22</td>
<td>93% ± 10</td>
<td>10.75 ± 0.6</td>
<td>57.0 ± 1.2</td>
</tr>
<tr>
<td>Chymotrypsin (Tris)</td>
<td>0.5</td>
<td>8</td>
<td>121 ± 29</td>
<td>63% ± 12</td>
<td>5.4 ± 0.5</td>
<td>61.5 ± 1.2</td>
</tr>
<tr>
<td>Chymotrypsin (Tris)</td>
<td>0.8</td>
<td>7.8</td>
<td>94 ± 13</td>
<td>95% ± 20</td>
<td>6.9 ± 0.2</td>
<td>59.3 ± 1.9</td>
</tr>
<tr>
<td>Chymotrypsin (Tris)</td>
<td>1</td>
<td>8</td>
<td>140 ± 29</td>
<td>80% ± 10</td>
<td>6.2 ± 0.5</td>
<td>59 ± 1.9</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>0.096</td>
<td>NA</td>
<td>67 ± 13</td>
<td>2 ± 0.6</td>
<td>0.78 ± 0.1</td>
<td>66.9 ± 2.8</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>0.096</td>
<td>5</td>
<td>54 ± 5</td>
<td>8.5 ± 2.7</td>
<td>0.78 ± 0.1</td>
<td>66.9 ± 2.8</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>0.096</td>
<td>15</td>
<td>71 ± 15</td>
<td>14.2 ± 3.6</td>
<td>0.78 ± 0.1</td>
<td>66.9 ± 2.8</td>
</tr>
</tbody>
</table>
The three orifice disks created drops about twice the size of the diameter used. The 75 micron orifice disk routinely produced drops in the 140 micron range; while the 50 micron disk created drops around 100 microns. The 25 micron disk made drops around 60 microns.

The enzymatic assays were not precise in nature and the majority of them gave an error in the range of 10%. Although the error seems large, a trend in stability is consistently seen in the data. Table 3.4 shows the activity of each protein after processing through the drop generator, where the percentage refers to amount of activity retained compared to the positive control.

Enthalpy data was obtained from the DSC scans. Each scan contained heat capacity versus temperature information. As the protein unfolded, a peak was seen in the heat capacity data. The melting enthalpy was calculated at the peak by integrating the area under the curve. The enthalpy results are enumerated in Table 3.4.

Surface tension measurements were taken directly after adding the protein into the solution. The reported numbers are instantaneous surface tension, also known as dynamic surface tension. This was measured in place of static surface tension; a value averaged over a long period. When the aerosol system creates a drop, it is assumed that the concentration of protein is equal throughout the solution. As the surface is created, protein is drawn to the surface to reduce the free energy of the system. In order to fit with our theory, it is therefore important to measure the surface tension as soon as the solution is created. Values for surface tension are reported in Table 3.4.

All the data presented in Table 3.4 was plotted using the relationship in Equation 3.8. If the hypothesis is correct, then all the data should collapse onto a single trend line.
that has full activity above a transition region and no activity after the transition value. Since the theory has no time dependent term, the transition point should be rather sharp with large variability of the data along that transition line.

There is significant variability in protein activity in the fully active region. We know from the experiments with β-Galactosidase that some protein degradation occurs due to interaction with the materials in the fluid handling system, most likely with the high surface area sintered metal filter. Thus the 100% activity label on the Figure 3.2 is misleading as it is for completely unprocessed protein.

From the Figure 3.2, a clear and rapid decline in activity occurs as predicted. This is the transition region between the stable and unstable protein form and represents the value of the constant C we were trying to obtain (Equation 3.9). The transition is found in a region centered around a value 100. The broadness of the transition value is not surprising since we made a rather large assumption regarding the functionality of the chemical potential term. However, the transition appears fairly uniform and does not appear to be protein dependent. This finding is quite unexpected though very exciting.
Figure 3.2 – Relationship between melting enthalpy, surface tension, drop size and a protein’s activity after aerosolization. A transition is seen around 100.

The majority of the points fit the transition line and supports the trend. There is one data point that resides in the stable region but is suspiciously low. It contained chymotrypsin in EPPS buffer at a pH of 8 and a protein concentration of 0.5 mg/ml. Every possible weakness was examined to find a potential flaw in the data point, including: pH, flow rate, and pressure of the spray system; contamination of the buffer or protein; consistency in all data points; and correlation between preparation and run time. No inconsistencies in the data were found. However, given that the other 17 data points follow the trend, we feel that another unidentified mechanism must be driving the degradation.
The value for the constant C in Equation 3.9 appears to be centered about 100. It was known that the value of the proportionality constant would be greater than one since the chemical potential term was incorporated into the surface tension term. The transition for all proteins used is fairly uniform; therefore the value of C does not appear to be overly protein dependent.

Applying Maa’s data [9] to the theory described in Equation 3.9 provides further validation that this transition value is not protein dependent (Figure 3.3). In using this data, three assumptions were made 1) percent activity equals one minus percent aggregation, 2) surface tension does not vary significantly with concentration, and 3) melting enthalpy does not vary with concentration. A protein can denature without aggregation and aggregation occurs at a slower rate than loss in activity, leading to a lower transition value and a gradual decline rather than a rapid transition line. Furthermore, surface tension does decrease with increasing concentration, thus Maa’s data will shift slightly to higher values. Therefore the first sign of aggregation in Maa’s data should be identified as the transition criteria consistent with the data in Figure 3.4. Astonishingly, the value of C from Maa’s data also falls very near 100.
Figure 3.3 - Graph of protein data from Maa et al [9]. The plot shows that a decrease in melting enthalpy of the protein correlated to a decrease in protein activity.

3.6 Conclusions

The theory presented in this paper will be incredibly useful for the pharmaceutical industry. Formulation and process designers can now take early stage proteins that show therapeutic benefits and conduct two simple tests to determine spray-ability. From those tests, the company will know the smallest, therapeutically active drop they can create with the solution without denaturing the protein. Furthermore, we have identified two easy parameters on the formulation “worksheet” to increase protein stability at smaller droplet sizes: the addition of excipients and modification of buffers. To increase confidence in stability, we recommend that any protein formulation designer stay sufficiently above the transition value of 100 because of the variability seen in the region.
4 Liposome Creation Using EHDA

4.1 Introduction

Biological macromolecules represent a new class of mechanism-based-therapeutic agents. Successful development of this new class of therapeutic agents is reliant on process and delivery issues that are dramatically different than traditional small molecules. One successful strategy utilizes a protective coating to increase efficacy and enhanced specificity and targeting. The range of encapsulation or entrapment techniques is enormous, spanning from existing biological systems (i.e., viral vectors) to synthetic vectors (i.e., liposomes, polymer micro particles etc.). Specifically for the delivery of DNA, two strategies have risen above the rest. Delivering naked DNA is difficult in that it not only needs to be protected and tends to elicit an immune response when exterior to the cell, but the large size of DNA requires condensation in order to facilitate transport to and across the cell and nuclear membranes. The original successful gene therapies used viral vectors to achieve these goals. Adenoviruses were able to successfully transfect cells, but induced inflammatory and antibody responses [135-138]. Other virus vectors have been used, including adeno-associated virus and lentivirus, but because of their small size have limited encapsulation capacity. Another approach has been to utilize non-viral vectors. These are synthesized vectors using biocompatible polymers or lipids, which do not exhibit mutagenesis or elicit inflammatory responses. One technique is to use Polyethylenimine (PEI) or other cationic molecules that easily bind (i.e., complex) to
DNA causing a physical condensing of the DNA size into small, hard particles [35-41]. However, PEI can be toxic depending on the concentration used [45-50]. More importantly, the subsequent unbinding of DNA from the cationic molecule is difficult but a prerequisite for transfection to occur. Therefore, it would be worthwhile to create a vesicle that is non-immunogenic, non-toxic, and neutrally charged. The liposome fits this requirement. It is a soft, spherical vesicle consisting of two layers of lipids (a bilayer) and can carry no charge if neutral lipids, known as zwitterionic, are used. Liposomes are not new constructs. There are numerous techniques for their production, all carrying unique process and delivery problems.

*Liposome Creation Methods*

Although liposomes are rather simple in their components, they can be difficult to create and even more difficult to efficiently encapsulate material. Many techniques are currently used to produce encapsulated liposomes. However, the major techniques fit into four distinct types: dry lipid films or emulsion, micelle-forming detergents, and solvent injection.

Starting with a dry lipid film coating the bottom of a flask, lipids can be hydrated with the aqueous drug solution to be encapsulated. After the addition of the aqueous phase, the flask is agitated for several minutes up to many hours. These film-hydration methods typically create multilamellar vesicles with high encapsulation efficiency. However, they have great variations in size, size distribution, and lamellarity.

First introduced in 1978 by Szoka and Papahadjopoulos [139], reverse phase evaporation works by suspending small drug-containing aqueous drops in excess organic solvent. A phospholipid monolayer accumulates at the interface and stabilizes the
aqueous drops. The organic solvent is slowly evaporated and the micelles transform into a viscous gel-like state and then eventually to liposomes. Encapsulation efficiency of this system is dependant on the lipids and organic solvents used as well as their ratio. Typically the encapsulation efficiency ranges around 30 - 45 % for proteins [140]. The major drawback is the exposure of the material to organic solvents, which can lead to denaturation of proteins.

Detergent depletion works by mixing a detergent in with the lipids in the dry state. Once an aqueous solution is added, micelles form. The solution is put through a dialysis that slowly removes the detergent over time. The detergent helps create vesicles that are uniform and small in size. However, this method is very time consuming and costly due to the detergent [141].

Solvent injection methods typically inject an alcohol solution, containing lipids, very slowly into an aqueous phase. This leads to the formation of unilamellar vesicles. The alcohol must then be removed via dialysis against water. These liposomes have a small size distribution and very low encapsulation efficiencies (25-27%) [142].

Double emulsion techniques are a two-step emulsion process that begins similarly to the reverse phase evaporation. Aqueous drops are stabilized by lipids in an organic solution. The mixture is then added into a new aqueous phase creating a double encapsulation. The issues with this technique include the inability to control liposome size, morphology, and distribution, in addition to working for only a select set of solvents.
Critical Properties

Some of the most important properties for creating liposomes for drug delivery include membrane unilamellarity, vesicle size, lipid to drug ratio, and encapsulation efficiency [143]. The size of the liposome can play a crucial role in the rate of cellular uptake [144]. Vesicle size can be critical when trying to encapsulate large proteins or DNA. A higher encapsulation efficiency corresponds with less waste of the drug product and the greater the potential for efficacy. Current encapsulation efficiencies are at most 60%, thus finding techniques that achieve values higher than this would be highly desirable. An additional property that is important is especially for large macromolecules like DNA is complete encapsulation within the vesicle. If small portions of the molecules are visible from external fluid, they are likely to be damaged and no longer therapeutically effective. As the molecule increases in size and vesicle size decreases, the task becomes exponentially more difficult.

Electrohydrodynamic Atomization

It is believed that EHDA can be utilized to create liposomes with DNA or protein in a self-assembly process, possessing small diameters with narrow size distribution. The benefit is three-fold: (1) EHDA is known to work especially well for DNA; (2) EHDA has been demonstrated to work over a large droplet size range even down to the nanometer scale and (3) EHDA can be operated at conditions known to yield near monodisperse size distributions. These last two traits are especially useful attributes of the process, as some of the other techniques have little control over the size produced and therefore, a limited applicable size range for pharmaceutical production.
4.2 Materials

The solvents used included 1-propanol, ethanol, triacetin, Triton® X-100, and sodium chloride [NaCl] that were purchased from Sigma-Aldrich (St. Louis, MI). Egg phosphatidylcholine [PC] in a chloroform solution was obtained from Avanti Polar Lipids (Alabaster, AL). DNA plasmid (4kbp) was a generous gift from Valentis, Inc. (Burlingame, CA). The Qunat-iT™ PicoGreen® dye kit was purchased from Molecular Probes (Carlsbad, CA). All solutions were prepared with deionized water.

4.3 Methods

Theory

The process envisioned occurs in a single step, creating the lipid bilayer in two separate stages. The first stage includes spraying the encapsulation solution, consisting of the macromolecule and lipid, through EHDA. As the droplet falls towards the catch basin, the lipid molecules will self-assemble at the surface of the drop with the hydrophobic tails pointing outside of the drop. The second stage occurs when the drop comes in contact with the catch pool. The catch pool consists of an aqueous solution with lipids self-assembled on the surface. As the drop passes through the surface into the pool, a bilayer is created when the lipid layer of the drop pairs with the lipid layer of the surface of the pool. As the drop travels deeper into the pool, the bilayer pinches off creating a liposome. Pautot et al. has published a technique that use a similar idea with creating each lipid layer one at a time [145]. However, their technique is done using a completely separate method. They begin by creating a stable inverted emulsion, using lipid as a surfactant. In a second vial, they place a phase-separated fluid with water on
the bottom and oil saturated with lipid on top. At the interface separating the two phases is a layer of lipids. As the first vial is slowly poured on top of the second vial, the denser inverted drops will pass through the oil phase and the interface coming to rest in the water phase. As the drop passes through the interface, the second half of the bilayer is paired up with the lipid on the inverted drop. There are many limitations to this process that include: creating a stable inverted emulsion, having the drop pass through the lipid interface, long diffusion times to set up the lipid at the two phase interface, and the tension at the interface is such that only one size distribution of the drop is stable. Our envisioned process avoids these problems by using EHDA to create the primary drop at any desirable size, then using velocity and density of the drop to penetrate the lipid surface. Finally, using a catch pool consisting of only water and lipids thus avoiding long diffusion times by driving the hydrophobic lipid to the surface of the pool.

Another lipid encapsulation method using EHDA is Co-extrusion. This technique has the potential to yield similar results [146], but the technique is highly sensitive to process variables and conditions. In contrast, the self-assembly technique presented here will be highly robust.

*Phase Diagrams*

The stability of the vesicles formed is highly dependent on the phase stability for the initial and final solutions. Our proof-of-concept study investigated two different phase diagrams; the first exploits two fluids and the second exploits a single fluid. The first phase diagram used triacetin, ethanol, water, and the surfactant Tween 80® (Figure 4.1) [147]. Tween 80® is a nonionic surfactant possessing a hydrophilic head and a hydrophobic tail. Since phosphatidylcholine is a zwitterionic surface-active agent, it was
assumed to be chemically similar enough to Tween 80® and the phase diagram would vary only slightly with the replacement of the lipid.

Figure 4.1 - Tertiary and tetrahedral phase diagrams, consisting of triacetin, water, Tween 80®, and ethanol. Diagram reproduced from [147].

The second proof-of-concept study utilizes PC in different salt solutions. Exploiting these new phase diagrams has helped in the composition of better solutions. Figure 4.2 includes two phase diagrams of PC, buffer, and propanol [148, 149].

Figure 4.2 - Tertiary phase diagrams of 1-propanol and PC. I) includes 0.1 M NaCl solution and II) includes water. The symbols represent: (L) Liposome suspension region, (G) viscous gel phase region, (C) coacervating region, and (S) lipid solution region. Images reproduced from [148] and [149], respectfully.
Using Figure 4.2, the entire two-stage process must traverse from a lipid soluble region (of sprayed solution) to a liposome stable solution (once deposited in the catch pool). The phase diagram is set up in such a way that the PC concentration is based on the total amount of 1-propanol in the system, not the total mass of the system as would be expected. It is also important to note that the objective is to land in the liposome suspension region and not the coacervation region. Coacervation is defined as “the separation of a colloidal systems into two liquid phases” [150]. The desire is to end with a primarily aqueous solution that would support DNA or protein formulations.

Procedure

The first step in creating either the spray or catch solutions was to evaporate the chloroform out of lipid solution. Once the evaporated, alcohol was added into the glass vial contained the solidified lipid. After the lipid is fully soluble in the alcohol solution, it can be used to create either the spray or catch solutions. The spray solution is placed in a stainless steel syringe connected by stainless steel tubing to the EHDA. The EHDA was custom built and is comprised of a New Era Pump Model NE-1000 (Farmingdale, NY) and a power supply from Bertan Series 225-30R (Hauppauge, NY). The nozzle is a stainless steel capillary with an inner diameter of 1.07 mm and an outer diameter of 1.27 mm. The solution was pumped through the system at a constant flow rate with an applied negative voltage. Drops produced by the EHDA landed in a small glass beaker consisting of the catch solution a distance of 7.5 cm below the capillary nozzle. The final solution was then analyzed for size of resulting lipid drops and encapsulation efficacy.
Triacetin Solutions

For the triacetin experiments, the composition of the two solutions is tabulated in Table 4.1.

Table 4.1 – Solution composition for triacetin experiments.

<table>
<thead>
<tr>
<th>Catch Pool Solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PC Lipid</td>
<td>10 mg</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Spray Solution</td>
<td></td>
</tr>
<tr>
<td>PC Lipid</td>
<td>1.25 mg</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.181 ml</td>
</tr>
<tr>
<td>Triacetin</td>
<td>0.728 ml</td>
</tr>
<tr>
<td>Water</td>
<td>0.09 ml</td>
</tr>
</tbody>
</table>

The spray solution was placed in the syringe and the flow rate of the pump was set to 0.04 ml/min. The voltage applied to the system was 2.8 kV in order to obtain a stable Taylor Cone flow. The drops were caught in a small beaker containing 10 ml of catch pool solution.

Salt Solutions

Creating the spray solution using the salt buffer, propanol, water, and DNA was difficult. Precipitation of the DNA or lipid occurred when the right balance of components was not met. The most stable solution is found in Table 4.2.

Table 4.2 - Solution composition for salt buffer experiments.

<table>
<thead>
<tr>
<th>Catch Pool Solution</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PC Lipid</td>
<td>0.38 mg</td>
</tr>
<tr>
<td>Propanol</td>
<td>1.08 ml</td>
</tr>
<tr>
<td>Water</td>
<td>144 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.0134 g</td>
</tr>
<tr>
<td>Spray Solution</td>
<td></td>
</tr>
<tr>
<td>PC Lipid</td>
<td>48 mg</td>
</tr>
<tr>
<td>Propanol</td>
<td>0.35 ml</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5 mg</td>
</tr>
</tbody>
</table>
The spray solution was sprayed at different rates in order to create different drop sizes. Flow rates ranged from 0.02 ml/min to 0.08 ml/ml. The voltage applied to the system remained constant at 2.3 kV. The 20 µl of sprayed drops were landed in 10 ml of the catch solution. The catch solution was placed in a small glass beaker, with a stir bar to prevent drops from landing on top of each other. After spraying the solution, samples were taken from the catch basin to examine for encapsulation efficiency and liposome size.

Dynamic Light Scattering

Lipid particles were sized using dynamic light scattering in a Zeta Sizer (model Nano+ ZS, Malvern) with Zetasizer Software v 6.01. The instrument is able to size particles ranging from 1 nanometer to 6 microns. A 2 ml sample from the solution was placed in a plastic cuvette inside the sizer and analyzed for 20 minutes. Both the starting catch and spray solution were tested before spraying to ensure there were no particles were in the initial solutions.

Fluorescent Assay

The quantification of encapsulated DNA was determined by a fluorescent assay. The assay uses a fluorescent dye, PicoGreen ®, that when bound to double stranded DNA strongly fluoresces with an excitation wavelength of 480 nm and an emission wavelength of 538 nm. The Quant-iT™ PicoGreen® kit was purchased and used following the manufacturer’s protocol. Briefly, 100 µl of sample was loaded into a 96 well plate, followed by 100 µl of PicoGreen® reagent in TE Buffer. The sample was incubated for
5 minutes at room temperature, in the dark to avoid photobleaching. Fluorescence values for the samples were then analyzed in a SpectraMax Gemini EM spectrofluorometer using SoftMax Pro 4.8 software (Molecular Devices, Sunnyvale, CA). The percent of encapsulation was determined by comparing the amount of DNA not encapsulated with the total amount of DNA exposed after breaking open the liposomes. Triton X-100 was mixed with the liposome/DNA solution to a concentration of 0.05 % by volume and incubated at room temperature for 10 minutes to break open the liposomes.

4.4 Results

Producing stable triacetin drops is easy, reliable, and robust. This is due to the immiscibility of water in triacetin creating stratified suspensions that are stable over long periods of time. Although triacetin drops are easily formed without the addition of lipid, they quickly agglomerate upon settling on the bottom of the beaker. Using the phase diagram, the most stable drops were made in the region that contained a large amount of triacetin and a lipid, with a small amount of water (Figure 4.3). After spraying, the triacetin drops slowly decreased in size and were completely dissolved within 12 hours.
Figure 4.3 - Solubility and stability results using phosphatidylcholine instead of Tween 80® on the phase diagram. The dots represent locations of solution solubility. The cross shows locations were the solution is not miscible. The circled region is the only region where it was possible to create stable drops.

Triacetin proved to be a wonderful proof-of-concept material; unfortunately it has limited use in DNA delivery. Macromolecules, such as DNA and proteins, are not soluble in triacetin. Additionally, it is unknown if this process makes liposomes or lipid stabilized emulsions because triacetin is not soluble in water. Therefore, an aqueous based solution system was prototyped.

Recognizing that salt buffers solve the macromolecule solubility and stability problems, our focus shifted to classical buffer constructs. However, electrospraying is difficult and unreliable with purely aqueous solutions. Adding organic solvents not only helps solubilize the lipid, but also can substantially improve the electrospray profile. Using up to 40% propanol does not affect DNA stability in solution [151]. It is important to notice the PC concentration on the phase diagram in Figure 4.2 II. Using a PC concentration of 5% or below will be soluble in the spray solution, but landing in the catch pool will result in coacervation. For this reason, a 10% or greater concentration of
PC has to be used. During the spray process, the drops were not heavy enough to fully penetrate the surface of the catch solution. Penetration requirements are not well understood in literature, but one can imagine that the kinetic energy of the droplet must exceed the surface energy of the surface and additionally that the drop be neutrally buoyant in the catch pool. For the sprayed drop, that impact velocity, droplet size, and density of the droplet are important. In addition, the surface tension and density are of the catch pool are also critical parameters. A stir bar was used to lower the surface tension of the catch pool and imposed a dynamic condition, which helped the drops circulate on the surface and eventually be pulled into the solution.

The drop size and encapsulation results are shown in Table 4.3 for the three flow rates investigated. The primary drop size does appear to scale with flow rate. As the flow rate increases, so does the drop size. This fits with the EHDA relation developed by Ganan-Calvo [5]. It is interesting to note that the encapsulation efficiency is not dependant on drop size and for all velocities is just under 30%.

<table>
<thead>
<tr>
<th></th>
<th>0.08 ml/min</th>
<th>0.04 ml/min</th>
<th>0.02 ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulation</td>
<td>28% ± 10</td>
<td>26% ± 0.4</td>
<td>29% ± 7.7</td>
</tr>
<tr>
<td>Primary Drop</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size (nm)</td>
<td>1188 ± 1067</td>
<td>973 ± 765</td>
<td>794 ± 182</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drop Size (nm)</td>
<td>266 ± 365</td>
<td>92 ± 39</td>
<td>70 ± 33</td>
</tr>
</tbody>
</table>

Table 4.3 – Results from the salt buffer experiments, drop size and encapsulation efficiency. The error represents one standard deviation.
The liposomes produced had two distinct size distributions. The primary drop is the true size produced by the electrospray. The secondary drop is likely formed from the division of the larger vesicles from the surface shear flow created by the stir bar [153].

The results in Table 4.3 provide the proof-of-concept data required to demonstrate the process as a success. However, a narrow size distribution and increased encapsulation efficiency is required to advance the method beyond current limitations. We hypothesize that the wide size distribution and low encapsulation efficiency is linked to the poor penetration and high shear rates at the catch pool surface. Thus, a more dense solution that fully penetrates the catch solution surface and decreasing the surface tension of the catch pool might increase drop penetration. We are currently exploring methods to decrease the catch pool surface tension that include raising the pool temperature to 40°C. Final results from these improvements are still pending and potentially outside the scope of the work.

4.5 Conclusions

Our investigation demonstrates that liposomes can be created using an EHDA self-assembly method. Stable drops are easier to produce when using two immiscible fluids. However, liposomes were created using the salt buffer solution. The goal of a high encapsulation rate was not met; nonetheless a constant encapsulation percentage regardless of the liposome size produced is an improvement over other liposome techniques.
5 Conclusion

The purpose of this study was to examine EHDA and its potential use in pharmaceutical applications. Each of these chapters built upon one another filling the void of information in this area. EHDA was used on human derived lung cells to test for toxicity and immunogenic effects. Protein atomization was examined and factors correlating stability were established. Finally, a novel method of creating liposomes was invented.

**EHDA on Human Pulmonary Epithelial Cells**

Our investigations using EHDA on human derived lung cells show electrosprays to be a gentle method by which plasmid DNA can be aerosolized without a significant loss in native DNA structure. This study explored the effects of aerosolization of naked and complexed plasmid via an electrospray on EpiAirway™ cells that had been cultured at an air-liquid interface. Lactate dehydrogenase released from the cells remained low for both the naked and complexed formulations indicating minimal cell toxicity (except for the 10:1 PEI/DNA complex). In addition, cytokine levels (release of interleukin 8) appeared nominal and comparable for all conditions, independent of electrospray application. The mild toxicity observed when the tissues were placed in the applied electric field was temporary, and the tissues returned to normal within a few hours after application. The experiments were designed to assess the toxicity associated with EHDA
sprays; however, future studies would be needed to optimize this approach for maximizing transfection. While future in vivo experiments are required, our results show that electrosprays present a promising method for administering DNA-based therapeutics to the pulmonary epithelium.

*Protein Stability During Aerosolization*

The aerosolization of proteins has had a wide variety of outcomes. It appears that the method of aerosolization is not as important as the properties of the solution and the drop size created. The theory presented in Chapter 3 details how, if the total energy of the drop is greater than that of the melting enthalpy, the protein will denature. The experimentally determined transition constant for a variety of proteins was found to be around 100.

\[ dH_{ml} < 100 \times \alpha dA_s \]

This relation will be incredibly useful for the pharmaceutical industry. Formulation and process designers can now take early stage proteins that show therapeutic benefits and conduct two simple tests to determine spray-ability. From those tests, the company will know the smallest, therapeutically active drop they can create with the solution without denaturing the protein. To increase confidence in stability, we recommend that any protein formulation designer stay sufficiently above the transition value of 100 because of the variability seen in the region.
**Liposome Creation Using EHDA**

Although there are numbers of methods available for creating liposomes, for use in gene therapy each method carries unique process and delivery problems. Chapter 4 describes a novel process and presents the necessary the proof-of-concept data to demonstrate functionality.

Our investigations demonstrated that liposomes can be created using an EHDA self-assembly method. Stable drops are easier to produce when using two immiscible fluids. However, liposomes were created using the salt buffer solution. The goal of a high encapsulation rate was not met; nonetheless a consistent encapsulation rate of 20% regardless of liposome size is an improvement over some other liposome techniques.
References


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