Exogenous Surfactant as a Delivery Vehicle for Intrapulmonary Therapeutics

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology and Pharmacology

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Abstract

As an organ system, the lung has unique advantages and disadvantages for direct drug delivery. Its contact with the external environment allows for the airways to be easily accessible to intrapulmonary delivery. However, its complex structure, which divides into more narrow airways with each branch, can make direct delivery to the remote alveoli challenging. The objective of this thesis was to overcome this issue by using exogenous surfactant, a lipoprotein complex used to treat neonatal respiratory distress syndrome, as a carrier for pulmonary therapeutics. It was hypothesized that therapeutics administered with a surfactant vehicle would display enhanced delivery to the deeper regions of the lung. Acute respiratory distress syndrome and bacterial pneumonia were selected as prototypical examples of pulmonary conditions in which surfactant-drug combinations may be beneficial. Consequently, the pharmaceuticals utilized were those with antibacterial or anti-inflammatory activities.

To test this hypothesis, the wet bridge transfer system was developed in Chapter 2 as a novel in vitro screening tool for surfactant-based therapeutics. Several antibiotic and anti-inflammatory medications combined with a commercially available exogenous surfactant were screened based on 1) surfactant spreading and 2) the biological efficacy of the transported drug at a remote site. In Chapter 3 this platform, in combination with other in vitro techniques, were utilized to gain the mechanistic insight required for optimizing surfactant vehicle prior to animal studies. Specifically, through these experiments a synthetic surfactant was designed, such that, the antibacterial activity of cathelicidins, a family of potent antimicrobial peptides, was retained when transported to a remote site. Finally, Chapter 4 used a rat model of lung inflammation, to assess the
efficacy of this delivery approach for a mainstay anti-inflammatory. Surfactant based delivery was found to downregulate of a wide variety of inflammatory markers across both sexes.

To conclude, surfactant-based delivery of antimicrobial and anti-inflammatory therapeutics was found to enhance drug delivery and efficacy at remote sites in vitro as well as in vivo. Based on these findings, it is also suggested that future research expand on the optimization process of this thesis for other surfactant-drug preparations and assess those combinations in clinically relevant animal models.

Keywords

Exogenous pulmonary surfactant; synthetic surfactant; intrapulmonary drug delivery; bacterial lung infection; acute respiratory distress syndrome; cathelicidins; glucocorticoids; Pseudomonas aeruginosa.
Summary for Lay Audience

The branching structure of the lung makes direct drug delivery to its more remote regions challenging. The objective of this thesis was to overcome this delivery issue by using exogenous surfactant as a carrier for drugs targeting the lung. It was hypothesized that drugs administered with a surfactant would show enhanced delivery to the more remote regions of the lung. Although many lung conditions could benefit from this approach, this thesis focused on: acute respiratory distress syndrome (Lung Inflammation) and bacterial pneumonia (Lung Infection). Consequently, the drugs explored were those with the ability to kill bacteria and downregulate inflammation.

To test this hypothesis, the wet bridge transfer system was developed in Chapter 2 as a new screening tool for surfactant-drug mixtures. It was used to screen several antibiotic and anti-inflammatory medications that were combined with an exogenous surfactant, based on 1) surfactant spreading as well as 2) drug efficacy at a remote site. In Chapter 3, this platform was combined with other techniques to design a better surfactant vehicle for antibacterial peptides, known as cathelicidins. Finally, Chapter 4 used an animal model of lung inflammation to assess the effectiveness an anti-inflammatory medication delivered by a surfactant vehicle.

Together, the findings of this thesis support surfactant as a drug delivery vehicle for anti-bacterial and anti-inflammatory medications. It is also recommended that future research use the methods outlined in this thesis to design and evaluate surfactant vehicles for other types of medication.
Co-Authorship Statement

Chapter 1 was a literature review developed from a manuscript written by Brandon Baer and Ruud Veldhuizen, in collaboration with Lucas Miguel Pereira de Souza and André Silva Pimentel.

Chapters 2, 3, and 4 describe experimental studies performed by Brandon Baer under the supervision of Dr. Cory Yamashita and Dr. Ruud Veldhuizen.

Chapter 5 was a discussion of the thesis and a proposal for future directions written by Brandon Baer. Ruud Veldhuizen provided feedback on content and structure.

Dr. Fred Possmayer provided intellectual contribution to the development of the wet bridge transfer system in Chapter 2.

In Chapter 3, Dr. Edwin Veldhuizen designed and performed the isothermal titration calorimetry experiments.

In Chapter 3, Dr. Shehrazade Jekhmane was responsible for the Nuclear Magnetic Resonance spectroscopy experiments under the supervision of Markus Weingarth.

In Chapter 3, Dr. Natalia Molchanova and Dr. Jennifer Lin were responsible for synthesis and purification of the SP-C peptoid under the supervision of Dr. Håvard Jenssen and Dr. Annelise Barron.

Mrs. Lynda McCaig provided instrumental assistance with animal work described in Chapter 4, including teaching, hands-on technical assistance, and animal protocol ethics development.

Ms. Shannon Seney provided assistance with multiplex assays described in Chapter 4.
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List of Abbreviations

AMR – Antimicrobial resistance
ANOVA – Analysis of variance
ARDS – Acute respiratory distress syndrome
BAL – Bronchoalveolar lavage
BLES – Bovine lipid extract surfactant
BPD – Bronchopulmonary dysplasia
CATH-2 – Chicken cathelicidin-2
CDS – Constrained sessile drop surfactometers
CFU – Colony forming units
CO2 – Carbon dioxide
COVID-19 – Severe acute respiratory syndrome coronavirus 2
CRAMP – Murine cathelicidin
CXCL-2 – Chemokine C-X-C motif ligand 2
D1 – Dish 1
D2 – Dish 2

DMEM – Dulbecco modified eagle medium

DPPC – Dipalmitoylphosphatidylcholine

E. coli – Escherichia coli

eCATH-1 – Equine cathelicidin-1

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal bovine serum

FiO2 – Fraction of inspired oxygen

Fmoc – 9-fluorenylmethoxy carbonyl

G – Gauge

GR – Glucocorticoid receptor

GRO/KC – Human growth-regulated oncogene/Keratinocyte chemoattractant

HKB – Heat-killed bacteria

ICU – Intensive care unit

IL-1β – Interleukin-1β

IL-6 – Interleukin 6

IL-8 – Interleukin-8

ITC – Isothermal titration calorimetry
LL-37 – Human cathelicidin

LPS – Lipopolysaccharide (LPS)

LTA – Lipoteichoic Acid

MIP-2 – Macrophage inflammatory protein 2

Mono-SP-C – SP-C peptoid protein mimic

MPO – Myeloperoxidase

NF-κB – Nuclear Factor Kappa B

NRDS – Neonatal respiratory distress syndrome

O2 – Oxygen

OD – Optical density

P – Probability value

P. aeruginosa – Pseudomonas aeruginosa

PaO2 – Partial pressure of oxygen

PC – Phosphatidylcholine

PEEP – Positive end-expiratory pressure

PG – Phosphatidylglycerol

PMAP-23 – Pig cathelicidin-23

qPCR – Quantitative polymerase chain reaction
S. aureus – Staphylococcus aureus

SD – Standard deviation

SP-B – Surfactant protein B

SP-C – Surfactant protein C

ssNMR – Solid-state nuclear magnetic resonance

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor α

TSA – Tryptic soy agar

TSB – Tryptic soy broth

VAP – Ventilator associated pneumonia
Chapter 1: General Introduction and Literature Review

A version of this chapter has been published:

1.1 **General Overview**

The therapeutic efficacy of any given drug is influenced by its ability to reach its specific target cell, tissue, or organ. For some organs, such as the skin, accessibility is relatively straightforward and topical drug administration can be highly effective with limited side-effects. For other, internal organs, such as the liver or kidneys, drug administration via systemic routes can be effective but can also be strongly affected by drug metabolism and negative side-effects on other organs. Between these two extremes, the lung presents itself as an organ system with distinct advantages and disadvantages for drug delivery. Its direct contact with the external environment allows for potential localized delivery; this, for example, allows for the highly efficient use of inhalers (puffers) to administer bronchodilators and anti-inflammatory medications in asthmatics. However, localized delivery becomes more difficult for therapeutics that are targeting the deeper areas of the lung, in which the extensive branching structure and large surface area provide substantial hurdles to adequate drug delivery. This problem gets exacerbated in certain lung diseases in which edema fluid, lung collapse and/or tissue remodelling may further affect accessibility.

The purpose of this thesis will be to investigate a potential solution to these latter scenarios, namely the use of exogenous surfactant as a vehicle to improve the delivery of pulmonary therapeutics. This first chapter will describe the complex branching anatomy of the lung and features of exogenous surfactant that would make it ideal for enhancing pulmonary drug delivery. It will also outline clinical scenarios, such as bacterial lung infections and acute respiratory distress syndrome (ARDS), in which surfactant-based therapeutics are deemed to have a strong clinical potential. Specifically, we will explore
the mechanism of action and therapeutic potential of cathelicidins for bacterial lung infections and glucocorticoids for ARDS.

1.2 Lung Structure and Function

The lungs are an essential component of the respiratory system, responsible for facilitating gas exchange between the external environment and the bloodstream. Specifically, oxygen and carbon dioxide are exchanged at the alveoli, moving across the respiratory membrane. These molecules move down their concentration gradients, resulting in oxygen diffusing across the epithelia and endothelia into the deoxygenated blood within the surrounding alveolar capillaries [1, 2]. Carbon dioxide moves in the opposite direction from the blood stream into the alveoli and is eventually exhaled out of the body [1].

Located within the thoracic cavity, the lungs are enclosed by the rib cage and diaphragm. The organ begins as the nasopharynx extends into the trachea, which in turn divides into two main bronchi [1, 2]. Figure 1.1A illustrates this complex branching structure of the airways, which continues to divide from the two main bronchi into smaller and smaller sections, eventually forming the terminal bronchioles [1, 2]. From the trachea to the terminal bronchioles, is referred to as the conducting zone [1, 2]. The structures within this region are involved in air flow, but do not participate in gas exchange. Beyond the terminal bronchioles are the respiratory bronchioles, that diverge structurally from their terminal counterparts with the appearance of alveolar sacs [1, 2]. This division begins the respiratory zone, which refers to structures involved in gas exchange [1, 2]. The alveolar sacs are clusters or groupings of multiple alveoli, each
being surrounded by a dense network of capillaries [1, 2]. The unique branching structure of the airways provides the lungs with a massive surface area at the level of the alveoli and therefore facilitates rapid gas exchange [1, 2].

As illustrated in Figure 1.1B, there are two main cell types that cover the alveolar surface, appropriately named alveolar type I and type II cells. The flat alveolar type I cells cover the majority of the alveolar epithelium and facilitate gas exchange [3]. Their large surface area and thin structure allow for the efficient diffusion of gas molecules during normal respiration [3]. In contrast, the small number of type II alveolar cells cover a much smaller portion of the alveolar surface. These cuboidal type II cells contain lamellar bodies and are responsible for the production and secretion of pulmonary surfactant [3–5]. A complex mixture of specialized surfactant proteins and lipids, pulmonary surfactant is essential for normal breathing mechanics [4, 5]. Type II alveolar cells are also responsible for replacing dysfunctional or damaged type I cells as well as contributing to the innate immune defense of the alveolus [3]. Moreover, the alveolus also contains resident macrophages that contribute to surfactant metabolism and immune defense, through the engulfment of inhaled particles and pathogens [3].
Figure 1.1. Schematic representation of A) the branching anatomy of the airways and B) the alveolar space.
1.2.1 Pulmonary ventilation

To promote gas exchange, air needs to be moved in and out of the lung efficiently. This is accomplished by changing the volume of the thoracic cavity, through a process known as ventilation. Specifically, the volume is increased and decreased through the contraction and relaxation of various respiratory muscles, including the diaphragm and external intercostal muscles. As explained by Boyle’s law, increasing or decreasing the volume of a closed container, in this case the thoracic cavity, will change its pressure gradient with the external atmosphere [2]. Inspiration, or the movement of air into the lung occurs through to the coordinated contraction of the diaphragm and external intercostal muscles. These contractions expand the volume of the thoracic cavity such that the air pressure within the lung is lowered below the air pressure of the external environment. At rest, the relaxation of the diaphragm and external intercostal muscles is enough to cause the lung to recoil to its original size, promoting expiration or the movement of air out of the lung. During exercise, the increased rate of exhalation requires the contraction of additional muscles, such as the internal intercostals, oblique and abdominal muscles [2]. These additional contractions further decrease the volume of the thoracic cavity, creating a larger pressure gradient with the external environment and forcing more air out of the lung.

In addition to the contraction and relaxation of respiratory muscles, ventilation is also affected by distensibility [2]. This property, known as lung compliance, refers to the volume to which the lungs expand per unit of air pressure change [2]. Thus, a lung with low compliance requires a greater amount of force or work from the breathing muscles to inflate. There are two major characteristics of the lung that impact lung compliance: 1)
the elastic properties of the lung tissue and 2) the surface tension within the alveoli [2]. The elasticity of the lung tissue is based on the flexibility of its structural components. Specifically, the amount of collagen or elastin fibers that are meshed inside the airway interstitium regulate this aspect of lung compliance [2]. For example, the more collagen the greater the elastic resistance of the lung and therefore the greater the force required to cause the lung to expand. Surface tension within the lung is created at the air–liquid interface by the attractive force between water molecules, in the fluid lining the airways. These water molecules pull towards each other, in an attempt to force air out of the alveoli, creating a resistance force to the expansion of the region [2]. As such, a high surface tension at the alveolar surface results in a low lung compliance. It is the role of pulmonary surfactant secreted into this fluid layer to reduce surface tension at the air–liquid interface (Figure 1.1B) [2, 4, 5]. The surface tension lowering properties of surfactant are essential for normal lung function and influence both lung compliance as well as alveolar stability [4, 5].

1.2.2 The pulmonary surfactant system

As mentioned above, pulmonary surfactant is a lipoprotein mixture produced by the alveolar type II cells [4, 5]. The mixture is stored inside of these alveolar cells in structures known as lamellar bodies, until it is secreted into the fluid lining the airways [4–6]. Pulmonary surfactant then adsorbs through this fluid layer to the air–liquid interface. During respiration, the surfactant film reduces surface tension based on its concentration at this interface [4, 5]. During exhalation, the surfactant film is compressed or becomes more concentrated at the air–liquid interface, allowing the alveolus to achieve
very low surface tensions (near 0mN/m). However, when the film is expanded or
becomes less concentrated during inspiration, surfactant reduces surface tension in the
alveolus to a lesser degree (5-30mN/m) [4, 5]. By reducing surface tension, pulmonary
surfactant promotes normal lung compliance and prevents alveolar collapse during
exhalation [4, 5]. The functional components of pulmonary surfactant, that produce the
surface tension lowering film are collectively known as “large aggregates”. As the surface
film is compressed and expanded, the process also creates small vesicles of surfactant,
which will be either taken up by alveolar type II cells for recycling or alveolar
macrophages for degradation [7]. These vesicles are the inactive components of surfactant
and are collectively referred to as “small aggregates”.

Pulmonary surfactant can be isolated from lung lavage material by differential
centrifugation and the analysis of surfactant from numerous mammalian species has
revealed a well conserved composition [8, 9]. Approximately 85% of the surfactant
consists of phospholipids. Phosphatidylcholine (PC) being the most abundant at ~75% of
the total phospholipid fraction, with more than half this phospholipid being represented
by the saturated species dipalmitoylphosphatidylcholine (DPPC). Phosphatidylglycerol
(PG) is the second most abundant phospholipid at ~10%, with other phospholipids, such
as sphingomyelin and phosphatidylethanolamine, present in lower amounts [4, 8]. In
addition to phospholipids, surfactant also contains ~5-8% neutral lipids, predominantly
cholesterol, as well as 7-10% surfactant associated proteins designated SP-A, SP-B and
SP-C [8, 10]. Of note, a fourth protein, SP-D, has also been identified but is not co-
isolated with the surfactant obtained by centrifugation [11]. The surfactant proteins B and
C, are small hydrophobic proteins that are tightly associated with the lipid fraction of
surfactant [12, 13]. SP-A and SP-D are also large multimeric glycoproteins of the collectin family [14, 15].

The function of pulmonary surfactant can be broadly divided into two parts, its biophysical function [4, 16, 17] and its host defense function [18, 19]. This thesis will focus on the biophysical properties of pulmonary surfactant, however its role in host defense has been reviewed elsewhere and appears to be mainly mediated through SP-A and SP-D [14, 18, 20]. The biophysical role of surfactant consists of forming a surface tension reducing lipid film at the air–liquid interface of the alveolar surface, with further reduction of this surface tension to near zero mN/m during exhalation when the film is compressed as the surface area of the lung decreases [4, 16, 21]. This reduction of surface tension stabilizes the lung (i.e. it prevents alveolar collapse) and allows for inflation with relative ease.

1.2.3 Biophysical functions of pulmonary surfactant

The biophysical properties of pulmonary surfactant are mediated through its phospholipid components and specialized proteins [4, 16, 21–23]. Specifically, DPPC is thought to be mainly responsible for the surface tension lowering properties of pulmonary surfactant, while the other lipids and surfactant proteins play a role in the formation and maintenance of the surfactant film [4, 16, 21–23]. Briefly, to accomplish its biophysical functions, pulmonary surfactant must have enough fluidity to rapidly transfer its surface-active molecules to the air–liquid interface, and film stability, such that, it can tightly pack its components at the surface during compression.
Experimentally, the rapid adsorption (i.e. transfer of surface-active components to the air–liquid interface) and surface tension lowering properties of pulmonary surfactants have been demonstrated in numerous experiments [8, 16]. Often performed using a Langmuir probe to measure surface tension, these experiments have found that functional surfactants can significantly reduce surface tension to equilibrium values (approx. 23 mN/m) [8, 16]. Mechanistically, the surface tension reducing properties of pulmonary surfactant have been shown to be mediated by its phospholipids (most importantly DPPC), which displace water molecules at the air–liquid interface [4, 8, 16, 22, 24, 25]. By occupying this interface, these amphipathic phospholipids seem to displace water molecules and therefore reduce their collapse or pull force away from air. Although monolayer experiments have also shown that liposomes of pure DPPC can reduce surface tension to equilibrium values, these liposomes adsorb very slowly relative to natural or lipid extract surfactants [24, 25]. These findings suggest that other surfactant components are required for the rapid formation of a surfactant film. Notably, SP-B and SP-C have been shown to enhance the adsorption and spreading capabilities of lipid mixtures, including pure DPPC, in vitro [26–28]. Further, experiments utilizing radioactively labelled surfactant lipids have demonstrated the ability of SP-B and SP-C to break stable phospholipid bilayers and promote the insertion of lipids into a surface film [28]. Additionally, other Langmuir-Wilhelmy studies have found that the level of non-DPPC surfactant lipids, including PG, unsaturated PC, and cholesterol, can also affect the ability of pulmonary surfactant to adsorb and spread, presumably by affecting film fluidity [29, 30]. Thus, the rapid adsorption of pulmonary surfactant to equilibrium seems to require both DPPC, as well as various other lipids, and specialized proteins.
The ability of pulmonary surfactants to reduce surface tension to even lower values (near 0mN/m) during compression has been illustrated by experiments utilizing the pulsating, captive, and constrained sessile drop surfactometers (CDS) [16, 31, 32]. This further reduction in surface tension during exhalation is thought to be linked to the surfactant film being reorganized when compressed, such that, it is enriched in the saturated phospholipid DPPC [4, 16, 21–23]. Basically, interfacial compression is theorized to cause the “squeeze out” of the more fluid, unsaturated lipids within surfactant from the interface, leaving the more solid DPPC. Experimentally, this DPPC refinement has been shown to be mediated through SP-B and SP-C, allowing the surfactant film to achieve very low surface tensions because DPPC can be tightly packed without collapsing, presumably due to its desaturated acyl chains [29, 33]. Mass spectrometry has also been used to show the creation of separate DPPC and non-DPPC lipid monolayers during surfactant compression [21]. Briefly, the study demonstrated that unsaturated phospholipids were “squeezed out” of the surfactant film, leaving a monolayer enriched in desaturated phospholipid species [21]. Together, these studies highlight the role of surfactant proteins, in addition to DPPC, for the surface tension lowering properties of pulmonary surfactant during compression.

1.2.4 Exogenous surfactant therapy

The origins of exogenous surfactant therapy lie in the discovery of endogenous surfactant and the clinical finding of surfactant deficiency in preterm infants, a story chronicled in several excellent reviews [34–38]. Briefly, the critical importance of endogenous surfactant for normal lung function is obvious from the severe lung
dysfunction observed in preterm infants with surfactant deficiency [35, 38]. This condition, termed neonatal respiratory distress syndrome (NRDS), was a major cause of infant mortality until the mid-1980’s at which time clinical trials showed the efficacy of exogenous surfactant therapy [38, 39]. Intratracheal administration of a purified version of animal surfactants was found to significantly increase survival in this vulnerable patient population [40–43]. As such, exogenous surfactant is currently standard therapy in neonatal intensive care units (ICU) [44].

The success of exogenous surfactant in NRDS prompted investigations of this therapy in other lung conditions, most notably ARDS [45, 46]. ARDS can be caused by a variety of initiating insults and is defined by severe lung dysfunction [47–49]. This physiological impairment of lung function showed similarities with observations in NRDS. However, whereas NRDS can be diagnosed immediately at the onset of the injury and is due to surfactant deficiency [35, 38], ARDS is diagnosed later in the disease process when injury is well-established and is associated with surfactant impairments [17, 50, 51]. Nevertheless, the similarities prompted extensive research into exogenous surfactant therapy for this disease [52–55]. Unfortunately, the initial clinical trials and subsequent meta-analysis of the data did not indicate an improvement in mortality due to surfactant therapy in ARDS [46, 56–61]. Specifically, these clinical trials found that the benefits of surfactant therapy for ARDS patients were largely limited to improvements in blood oxygenation. However, as stated in a recent review by Veldhuizen et al., the emergence of severe acute respiratory syndrome coronavirus 2 (COVID-19) related ARDS has prompted a reconsideration of this clinical approach [62]. Specifically, researchers have begun to re-examine the negative results of these early trials with respect to the dose, delivery method and timing of administration [62]. Moreover, the therapeutic
role of exogenous surfactant in the treatment of ARDS has also been revaluated to include (among other approaches), its potential as a drug delivery vehicle [62]. These new insights into the potential role of exogenous surfactant for treating ARDS as well as the limitations of previous trials, have led to five ongoing clinical trials of surfactant therapy for COVID-19 patients [62].

1.2.4.1 Animal derived and synthetic exogenous surfactant preparations

Underlying the above information on the clinical utilization of exogenous surfactants is, obviously, the extensive research and development to produce these therapeutics. The general composition of a select number of currently available preparations is shown in Table 1. The most frequently utilized surfactants are the animal derived preparations obtained from lung lavage material from cattle or pigs [63, 64]. These exogenous surfactants undergo a lipid extraction with organic solvents and as such contain the surfactant lipids as well as the two hydrophobic proteins (SP-B & SP-C). A second type of animal derived exogenous surfactant can be created through the extraction of minced lung tissue with supplementation of DPPC and palmitic acid to enhance the spreadability of the material [65–67]. These preparations contain surfactant proteins B and C, although in lower amounts compared to lavage derived surfactants. Additionally, several synthetic surfactant preparations have also been produced. In general, these synthetic surfactants contain DPPC, PG, and some other lipids, as well as components that allow the lipids to spread [68–73]. In the most promising artificial surfactants, these components are analogues of surfactant proteins B and/or C, such as Mini-B or the synthetic peptoids [72, 73]. For example, CHF5633, a newly developed synthetic surfactant, is enriched with SP-B as well as SP-C peptide analogues, and was found to
have similar clinical efficacy for treating NRDS compared to an animal derived surfactant [74]. Thus, the high efficacy of these synthetic analogues have made the most recent artificial surfactants viable, low cost, and highly versatile alternatives to animal derived surfactant replacements [72–74].

Regardless of the preparation method and specific composition, the functional characteristics, as required for the treatment of NRDS, are similar. An exogenous surfactant should be able to adsorb onto, and spread along, an air–liquid interface, reflective of its ability to distribute throughout the lung when instilled as a bolus liquid. During dynamic compression and expansion cycles these surfactants should be able to reach low surface tension of below 5 mN/m, indicative of the ability to reduce the surface tension at the alveolar wall during inhalation and exhalation to facilitate ventilation with minimal effort.
Table 1.1. Compositions of various exogenous surfactant preparations.

<table>
<thead>
<tr>
<th>Surfactant Component</th>
<th>Lavage-derived surfactants</th>
<th>Minced lung surfactants</th>
<th>Synthetic surfactants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovactant Alveofact®</td>
<td>Calfactant Infasurf®</td>
<td>BLES Bles®</td>
</tr>
<tr>
<td>DPPC</td>
<td>32%</td>
<td>31%</td>
<td>33%</td>
</tr>
<tr>
<td>Unsat PC</td>
<td>50%</td>
<td>45%</td>
<td>53%</td>
</tr>
<tr>
<td>PG</td>
<td>9%</td>
<td>6%</td>
<td>11%</td>
</tr>
<tr>
<td>Other phospholipids</td>
<td>9%</td>
<td>18%</td>
<td>3%</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>Cholesterol</td>
<td>Cholesterol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Surfactant Proteins</td>
<td>SP-B</td>
<td>SP-C</td>
<td>SP-B</td>
</tr>
<tr>
<td>Synthetic Additives</td>
<td>Hexadecanol, Tyloxapol</td>
<td>Recombinant SP-C</td>
<td>KL4 peptide</td>
</tr>
</tbody>
</table>

Phospholipids are expressed as reported averages as a percentage of total phospholipids. See text for references. DPPC: dipalmitoyl phosphatidylcholine; Unsat PC: Unsaturated phosphatidylcholine; PG: phosphatidylglycerol; FFA: free fatty acids; SP: surfactant protein. Other phospholipids within these surfactants may include phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin.
1.3 Exogenous Surfactant as a Drug Delivery Vehicle

The successful treatment of inflammatory or infection-based conditions of the lower respiratory tract, require medications to achieve adequate therapeutic concentrations at the alveoli. Unfortunately, this is made particularly difficult by the complex branching structure of the airways (i.e. becoming more narrow with each division) as well as airway collapse associated with these conditions [1, 75–78]. Despite this delivery issue, the administration of therapeutics directly to sites of infection or inflammation within the lung has numerous benefits over systemic dosing, including increased therapeutic concentrations within the lung and reduced risk of systemic toxicity [79–81]. This concept is best illustrated by the low toxicity and high therapeutic efficacy of aerosolized glucocorticoids in the treatment of asthma [82–84]. Unfortunately, along with aerosolized antibiotics, these inhaled medications tend to accumulate in the central airways, limiting their ability to reach peripheral sites of infection or inflammation [76, 81, 83]. Thus, other direct delivery methods need to be explored to enhance bioavailability at peripheral sites, while limiting side effects [85, 86].

The overall concept of exogenous surfactant as a pulmonary drug delivery agent is relatively straightforward and originates from the success of exogenous surfactant therapy as utilized in conditions such as NRDS [40–44]. Briefly, intratracheally administered exogenous surfactants can adsorb and spread at the air–liquid interface of the airways. This allows exogenous surfactants to distribute throughout the lung, reaching and reducing surface tension at the alveoli (see section 1.2.2 The pulmonary surfactant system). Its ability to spread to the alveoli, when administered intratracheally, inspired the idea that exogenous surfactants could also be utilized as delivery vehicles for pulmonary
therapeutics and other compounds [62, 77, 87–89]. The basic premise is that when combined, exogenous surfactant will facilitate the delivery of a compound to the remote areas of the lung.

A few studies have tested this concept experimentally by directly evaluating pulmonary drug distribution. For instance, studies have utilized fluorescently or radioactively label therapeutics to evaluate the distribution of surfactant-drug mixtures in vivo [89, 90]. One such study, utilized excised hamster lungs that were sliced into 3-mm cross sections, to quantify the distribution of a radioactively labelled version of pentamidine following intratracheal instillation with saline or an exogenous surfactant. Utilizing an exogenous surfactant as a carrier for the radioactively labelled compound resulted in radioactivity being detectable in more of the lung slices (93%) compared to saline (72%) [89]. The radioactivity was also detectable over a larger fraction of the lung slices analyzed, when the compound was instilled with surfactant (43%) compared to saline (21%). The study concluded that exogenous surfactant not only resulted in a more uniform distribution of the anti-infective agent, but that it also delivered the drug to a greater portion of the lung [89]. A similar distribution study in rats, used a radioactively labelled version of dexamethasone to quantify drug levels in both the lung and circulation following administration via either intratracheal instillation or intravenous injection [91]. For intratracheal instillation, the use of an exogenous surfactant as a carrier for dexamethasone resulted in significantly greater lobar delivery compared to saline, with radioactivity being associated with the small airways and alveoli of the lung [91]. Together, this published data demonstrates the enhanced pulmonary distribution that occurs for drugs delivered by a surfactant vehicle.
Another characteristic of exogenous surfactant that supports its usage as a pulmonary delivery vehicle, is its safety. The utilization of any therapeutic, including exogenous surfactants, in a patient population requires controlled clinical trials to examine the efficacy and safety of the therapy. Although some care needs to be taken with surfactant preparations to avoid the formation of stable bubbles capable of blocking airways, no major negative side effects have been reported for surfactant administration. This includes not only the evidence from its utilization in NRDS [40–43], but also the data on safety obtained in the various ARDS trials [46, 56–60]. The safety and toxicology of the selected drugs to be utilized with surfactant would vary among all compounds. However, the premise of surfactant-based drug administration is lung targeted delivery, which would further limit potential side effects associated with systemic administration.

Despite its origins for treating NRDS, it should also be noted that the drug carrier approach for exogenous surfactant targets a different clinical outcome than the simple restoration of the surfactant system. As such, there are clear distinctions that need to be considered for this approach. First, it is imperative that the therapeutic that is mixed with the exogenous surfactant associates with the surfactant but does not inhibit its ability to spread throughout the lung. It is also important that the exogenous surfactant’s ability to reduce surface tension is maintained to open areas of the lungs that are collapsed. The opening up of these collapsed lung units would allow these injured areas to become accessible for the delivered drug. Conversely, it is similarly important that the function of the drug being delivered to the lung is maintained in the presence of the exogenous surfactant and that the drug reaches its intended target. The development of a new in vitro technique that can screen surfactant-based therapeutics based on these characteristics is
the basis for our wet bridge transfer system, described in Chapter 2. In addition, the assessment of a surfactant-based therapeutic in vivo is the basis of Chapter 4.

Lastly, based on the additional variables associated with surfactant-drug combinations, these compounds may require the use of newly designed synthetic surfactants. For example, whereas animal derived surfactants are utilized extensively, and successfully, for surfactant treatments for NRDS, these preparations have a defined lipid-protein composition (Table 1.1). Interaction of a potential therapeutic with surfactant can be affected by that composition. As such, there may be scenarios in which synthetic surfactants are more suitable for drug delivery purposes. In general, synthetic surfactants are more amendable to altering the specific composition and could therefore be optimized for surfactant-carrier interactions. This concept of designing a synthetic surfactant for a specific therapeutic is explored in Chapter 3. Further, although each specific therapeutic-surfactant combination will require a targeted research approach, a general overview of the process we explored throughout this thesis is outlined by Figure 1.2.
1.4 Respiratory Conditions that Could Benefit from Surfactant-based Therapeutics

Throughout this literature review, we have explored many preclinical studies that highlighted the ability of exogenous surfactants to facilitate the distribution of antibacterial and anti-inflammatory agents to remote regions within the lung. Moreover, surfactant as a drug carrier has been used clinically, to treat preterm babies at risk for the development of bronchopulmonary dysplasia (BPD) [92–95]. BPD is a chronic lung disease which develops in premature infants, especially those exposed to prolonged ventilation and high oxygen. In two controlled clinical trials, the effect of an exogenous surfactant, Survanta, with budesonide was compared to surfactant alone with respect to its efficacy in preventing the development of BPD in premature infants [92–94]. The data
provided by the two clinical trials has been analyzed in a systematic review and meta-
analysis which concluded that intra-tracheal administration of budesonide-surfactant
mixtures decreased the incidence of BPD [96, 97]. However, considering the limited
number of trials to date, both by the same team of investigators and with the same
surfactant preparation, both papers also indicated the need for additional large clinical
trials prior to recommending this approach as a standard therapy.

Whereas these clinical trials for BPD provide the first proof-of-principle for this
delivery approach in patients, the clinical utilization of a surfactant-drug combination in
other conditions is lagging. Notably, the expansion of promising preclinical data for
therapeutics delivered by a surfactant vehicle in the treatment of bacterial pneumonia and
ARDS.

1.4.1 Bacterial lung infections

Bacterial pneumonia is an infection of the lower respiratory tract and the leading
cause of death due to infection worldwide [98]. The World Health Organization estimates
that pneumonia accounts for approximately 15% of deaths under the age of 5, and 3.2
million deaths annually [98–101]. These statistics encompass a diverse range of
conditions including community-acquired pneumonia, ventilator associated pneumonia
(VAP), hospital-acquired pneumonia, cystic fibrosis, as well as pneumonia resulting from
complications of other diseases [98, 99, 102–105]. The current treatment paradigm for
bacterial lung infections involves the administration of high-dose oral or systemic
antibiotics [105–107], however, additional treatment strategies are required to overcome
current and future therapeutic challenges.
The rationale for using exogenous surfactant-based therapies to treat this respiratory condition was outlined, in general, in a previous section (see section 1.3 Exogenous Surfactant as a Drug Delivery Vehicle) and includes the need for localized delivery in the deeper areas of the lung [75]. Simply put, the clearance of a bacterial infection requires adequate antibiotic concentrations at the site of infection. Consequently, the sublethal pulmonary bioavailability from oral, systemic, or aerosolized medications may, in fact, be partly responsible for another hurdle in the treatment of these infections, the increasing incidence of antimicrobial resistance (AMR) [76, 81, 104, 108–111]. Moreover, the problem of AMR is not limited to lung infections as it is considered one of the largest current and future threats to global health in general [112–114]. Complicating treatment further, chronic inflammation associated with lung infections also contributes to the adverse outcomes in bacterial pneumonia [98, 106, 115, 116]. For example, in CF and VAP patients, the bronchial mucus layer and endotracheal tube, respectively, can act as reservoirs for bacteria [103, 106, 107, 117]. Consequently, these reservoirs can prevent the host immune system from fully clearing the infection. This leads to a prolonged bacterial colonization of the lungs, increased incidence of AMR, and excessive inflammation that can damage the respiratory system [103, 106, 107, 117]. Therefore, the treatment of bacterial lung infections, may require immunomodulatory therapeutics, in addition to new antimicrobial agents.

Focusing on AMR infections in the lung, it is estimated that nearly 30% of clinical pneumonia isolates from ICU or nursing home patients are resistant to three or more antibiotics, with pathogens such as Pseudomonas aeruginosa (P. aeruginosa) being particularly problematic [98, 118, 119]. Specifically, this gram-negative bacterium has shown a higher capacity for acquiring resistance mechanisms against mainstay
antibiotics, including biofilm development as well as increased expression of efflux proteins and inactivating enzymes [119, 120]. Additionally, in pneumonia patients susceptible to frequent, spontaneous, or chronic infections, their need for prolonged antibiotic treatment promotes even higher rates of AMR [117, 121–125]. Notably, these spontaneous infections are most commonly caused by *P. aeruginosa* or *Staphylococcus aureus* (*S. aureus*), among other pathogens [98, 120]. In conditions such as CF, the acquisition of AMR bacteria represents a pivotal stage in disease progression that is linked with poor outcomes, due to the limited treatment options available [116, 126]. Despite this rising incidence of AMR, there are very few new antibiotics currently being developed [98, 127–130]. This has pushed many researchers to explore new sources of antimicrobial therapeutics, in addition to new routes of drug delivery [127].

1.4.1.1 Antimicrobial peptides: cathelicidins

Antimicrobial peptides are found in the innate immune system of almost all classes of life [131–133]. Specifically, they represent a diverse group of more than 3000 distinct and endogenously produced peptides [131–133]. Due to their diversity, classification can vary, however antimicrobial peptides are often subdivided based on their source, activity, structural characteristics, and amino acid composition [131, 133]. In general, antimicrobial peptides are relatively small (under 100 amino acids) and positively charged [131, 133]. This thesis will focus on one of the main categories of mammalian antimicrobial peptides, cathelicidins, which were utilized in our studies (Table 1.2)[131].

Cathelicidins are a family of antimicrobial peptides present in the innate immune defense systems of many vertebrates, including humans [134], pigs [135, 136], chickens,
[137, 138], and mice [139] (among many others) [140]. Almost all epithelial or immune cells contain a cathelicidin, with the highest concentrations being found in neutrophils [140–142]. Within immune cells, the N-terminal sequence is responsible for the movement of the inactive peptide into storage units known as secretory granules, at which point the sequence is removed [140, 143, 144]. When immune cells interact with an inflammatory marker, microbe, or pathogen that requires an immune response the secretory vesicles are released [140, 143, 144]. Then a specific protease cleaves the “cathelin” domain, allowing for the mature cathelicidin to elicit its antimicrobial and immunomodulatory functions [140, 143, 144]. This family of peptides derives its name from a 99 to 114 amino acid pro-sequence, referred to as the “cathelin” domain; a region with close homology to cathelin (the cathepsin L inhibitor) [140]. This domain is highly conserved across species, however it is only found in the inactive, pro-peptide [140]. The full-length pro-peptide contains an N-terminal signalling sequence, the “cathelin” domain, and a short antimicrobial domain at the C-terminus [140, 145]. The biologically active cathelicidin will consist of this small (12 to 100 amino acids) C-terminal sequence, which displays very high interspecies and intraspecies diversity [140, 144, 145]. Consequently, mature cathelicidins are quite diverse in their structure, net charge, amino acid sequence length and/or composition, as well as antimicrobial or immunomodulatory activity (Table 1.2) [140, 144–146].
**Table 1.2.** Characteristics of cathelicidins explored in this thesis.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mature Amino Acid Sequence</th>
<th>Length</th>
<th>Charge</th>
<th>Structure</th>
<th>*Antimicrobial Activity</th>
<th>*Anti-inflammatory Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>CRAMP</td>
<td>ISRALAGLLRKGEKIGEKLKIGQIKNFFQKLVPQPE</td>
<td>38</td>
<td>+6</td>
<td>One α-helix</td>
<td>9 hrs</td>
<td>0.5 hrs</td>
</tr>
<tr>
<td>LL-37</td>
<td>LLGFDRKSKEKGFEKRVQRKDFLRNLVPRTES</td>
<td>37</td>
<td>+6</td>
<td>One α-helix</td>
<td>10 µM</td>
<td>0.5 hrs</td>
</tr>
<tr>
<td>CATH-2</td>
<td>RFGRFLRKIRRFRPKVTITIQGSARF-NH2</td>
<td>26</td>
<td>+9</td>
<td>Two α-helices connected by a proline hinge</td>
<td>5 µM</td>
<td>2.5 µM</td>
</tr>
<tr>
<td>PMAP-23</td>
<td>RIIDLLWRVRRPQKPKFVTWVVR</td>
<td>23</td>
<td>+6</td>
<td>Two α-helices connected by a proline hinge</td>
<td>9.5 hrs</td>
<td>20 µM</td>
</tr>
</tbody>
</table>

*Antimicrobial activity refers to the cathelicidin concentration (0.33-20 μM) that significantly delayed bacterial growth (1x10⁶ CFU/ml) of *E. coli* or *S. aureus* for a minimum of 10 hours in Mueller Hinton Broth [147]. If this could not be achieved, hours of growth delay for 20μM was depicted instead. *Anti-inflammatory activity refers to the peptide concentration (0.08-20 μM) that significantly reduced TNF-α expression of murine macrophages, 2 hours after co-incubation with 100 ng/ml lipopolysaccharide (LPS; *E. coli*) or 1 μg/ml lipoteichoic acid (LTA; *S. aureus*) [147]. If this could not be achieved, >20μM was depicted instead.
1.4.1.2 Cathelicidins: mechanisms of action and therapeutic potential

The cationic and amphipathic nature of cathelicidins is at the center of their bactericidal properties [131, 140, 148–150]. These peptides can elicit a multitude of antibacterial mechanisms, however, most can be classified as either 1) damaging the bacterial membrane or 2) disrupting essential intracellular processes [131, 140, 148, 151, 152]. For the former pathway, the high diversity of cathelicidins has led to the creation of several models, including the carpet-, toroidal- and barrel-stave models, that each illustrate their interaction with the cell membrane in a different way [131]. For example, utilizing lipid mixtures that mimic the composition of a bacterial membrane and fluorescence spectroscopy, researchers have demonstrated that the pig cathelicidin (PMAP-23) likely elicits its antibacterial properties by accumulating on or “carpeting” the surface of the membrane [153]. It is speculated that at the core of these different models is the electrostatic binding of the cationic cathelicidins to the negatively charged phospholipids composing the bacterial membrane [131]. Specifically, this interaction has been found to result in the peptides accumulating on or embedding in the cell membrane [149, 154–156]. Consequently, this can cause either a detergent-like destruction of the membrane, the formation of full cathelicidin-lined channels, or simply create holes in the membrane [131, 140, 151, 156]. For example, additional fluorescence microscopy studies have shown that the human cathelicidin (LL-37) “carpets” the bacterial membrane of Escherichia coli, causing a global destruction of the membrane, yet utilizes a more toroidal mechanism to create distinct pores in the membrane of Bacillus subtilis [157, 158]. Regardless of the exact method, the end result of these interactions with the bacterial membrane is usually a loss of membrane integrity and eventually bacterial death [146, 154, 159–163]. For the latter pathway, it has been demonstrated that some
cathelicidins can enter the cytoplasm from the cell membrane and interact with intracellular components, such as RNA, DNA, enzymes, as well as various chaperones [140, 152, 156]. One such cathelicidin is indolicidin, a bovine cathelicidin that has been found to permeabilize the membrane of *E. coli* without causing lysis, instead eliciting its antimicrobial effects by inhibiting DNA synthesis [164]. The binding of these negatively charged intracellular molecules has resulted in cathelicidins preventing aspects of protein synthesis, cell division, DNA replication, nucleic acid biosynthesis, and a number of other processes essential to bacterial survival [151, 152, 156, 165–169]. Through these two main mechanisms, these positively charged peptides have retained antimicrobial activity for millions of years [170].

It should also be noted that this multi-target approach to bacterial killing, makes any single resistance mechanism likely ineffective against cathelicidins [171–174]. Further, avoiding cathelicidin-mediated disruption of the bacterial membrane would require massive changes to its core elements and would likely come at the cost of normal membrane function and structural integrity [140, 151, 173]. Moreover, the strong negative charge of the bacterial membrane has been found to cause cathelicidins to selectively interact with these microbes over the more neutrally charged eukaryotic cells [131, 140, 175]. Together these factors help explain the current efficacy of these peptides against AMR bacteria [169, 176–179] and support their potential as the next generation of antimicrobial therapeutics [131, 172].

Beyond their direct antibacterial effects, cathelicidins have also been shown to exhibit a diversity of immune-related functions that may be beneficial for treating lung infections [141, 177, 180]. The exact effect, whether pro-inflammatory or anti-inflammatory can vary greatly based on the presence of microbial by-products or the
peptide itself. For example, LL-37 and the murine cathelicidin (CRAMP) have both been found to skew macrophage polarization towards a more pro-inflammatory phenotype, yet also reduce pro-inflammatory cytokine production in these macrophages in the presence of bacterial by-products [141]. Additionally, the specific pathways involved in the immunomodulatory effects of cathelicidins, require further study. However, they can be broadly classified into directly or indirectly affecting chemokine production, apoptosis, or chemotaxis of various immune cells, among other mechanisms [141]. Simply put, the ability of these peptides to regulate the immune response goes well beyond their ability to electrostatically bind negatively charged microbial by-products and prevent toll-like receptor (TLR) activation [141, 177, 180]. In fact, in the context of bacterial lung infections, even pro-inflammatory effects may be beneficial to help boost the immune response and ensure proper elimination of bacteria in the lung. In summation, the wide array of immune-related effects elicited by cathelicidins could promote a more balanced rather than excessive immune response during infection, in addition to aiding bacterial clearance.

Overall, the therapeutic potential of cathelicidins is quite diverse. For example, cathelicidins have been shown to promote wound healing [181, 182], help regulate the immune system [141, 177, 180], and combat a wide variety of pathogens, including bacteria [155, 160, 172, 183], fungi [184, 185], viruses [186–188], and parasites [189, 190]. Many of these therapeutic properties could be of significant value for treating bacterial pneumonia. However, this thesis will concentrate on the mechanisms of bacterial killing and immune modulation through which these ancient peptides can help combat AMR infections in the modern day [140, 141, 176, 177, 180, 191–195]. Additionally, despite their therapeutic promise, few cathelicidins or other antimicrobial
peptides have been assessed in clinical trials [196]. Obstacles to development often involve either toxicity towards eukaryotic cells (observed at high doses) or the loss of activity due to environmental conditions in vivo [79, 180, 196–199]. Thus, to make these peptides a clinical reality for pneumonia, preclinical studies are required to address pulmonary delivery and toxicity challenges, as well as inhibitory interactions with environmental factors. Lastly, the selection of an optimal cathelicidin for surfactant delivery requires that the peptide has broad spectrum activity against clinically relevant bacteria, such as P. aeruginosa, and resistance to environmental inhibition.

1.4.1.3 Chicken cathelicidin-2

Based on previous studies, one cathelicidin with properties suggestive of a strong therapeutic potential is chicken cathelicidin-2 (CATH-2) [79, 137, 177, 180, 200, 201]. Originally called CMAP-27, CATH-2 is a 26 amino acid peptide found almost exclusively in immune cells known as heterophils [138, 202]. It is one of four cathelicidins identified in chickens and is cleaved into its active form by a serine protease [137, 146, 202]. In terms of its antibacterial properties, CATH-2 has shown high efficacy against a wide variety of clinically relevant bacteria, including P. aeruginosa and S. aureus, independent of their resistance mechanisms to classical antibiotics [79, 137, 146, 177, 201, 203]. Live imaging studies, utilizing fluorescently labelled versions of CATH-2, have also characterized its ability to rapidly permeabilize the bacterial membrane and enter into the cytoplasm of both Gram-positive and Gram-negative bacteria [152, 156]. In addition, in vitro and in vivo experiments have found that bacteria, such as P. aeruginosa, do not elicit a pro-inflammatory response when killed by CATH-2, unlike other methods of bacterial killing [180]. This immunologically silent killing of bacteria highlights the
dual activity of this peptide for treating bacterial lung infections. Specifically, it has been found to not only kill pathogens that target the airways, but also regulate the subsequent host immune response [146, 177, 180, 192, 204]. Overall, CATH-2 has been shown to have a diverse range of immunomodulatory properties, including its ability to downregulate the recruitment of inflammatory cells and production of pro-inflammatory chemokines in response to bacterial by-products [177, 192, 204]. This broad-spectrum activity against AMR bacteria, in combination with its efficacy for immune regulation, make CATH-2 an ideal candidate for treating bacterial lung infections.

Beyond its potency as an antimicrobial and anti-inflammatory agent, it should also be noted that the activity of CATH-2 has been found to be more resistant to environmental factors than other peptides. For example, at “high” salt concentrations the potent antibacterial effects of many cathelicidins, including LL-37 and CRAMP, have been found to be significantly reduced [199]. This salt sensitivity among these peptides poses a major obstacle in their development as novel therapeutics for lung infections, as similar salt levels are observed in the airways [205–207]. Complicating treatment further, many respiratory conditions linked to chronic infections, such as CF are characterized by even higher salt concentrations in the airways [205, 208]. In contrast, CATH-2-mediated bacterial killing has been shown to be unaffected by “high” salt conditions [137, 146]. Additionally, the antibacterial effects of many cathelicidins, like LL-37 and eCATH-1 (horse) have also been shown to be inhibited by physiological levels of serum proteins [180, 197]. However, under serum conditions that resulted in the complete loss of activity for these other peptides, the potent bacterial killing of CATH-2 was found to be unaffected [180]. This insensitivity to complex environmental factors, could be associated with the unique structure of CATH-2 (Table 1.2). Specifically, the peptide contains a
unique kink region, created by a proline residue at amino acid position 14 [146]. This region has been shown to be essential to the therapeutic properties of CATH-2 and has also been used to explain its greater retention of activity when combined with a surfactant vehicle, compared to other cathelicidins [79, 146, 177]. Together, its resistance to external factors, high potency against AMR bacteria, and various immunomodulatory effects, make CATH-2 an ideal candidate for surfactant-based delivery in the treatment of pneumonia.

### 1.4.1.4 Evaluating surfactant-cathelicidin mixtures

Our research group has previously evaluated surfactant-cathelicidin combinations based on the antimicrobial functions of the suspended peptides. For example, bacterial killing curves against various lab stains and clinical isolates have been utilized to determine surfactant’s effect on the bactericidal properties of these peptides [79, 177]. In general, these studies found that combining a cathelicidin with an exogenous surfactant significantly inhibited its antibacterial activity [79, 177]. Further, despite their potency against AMR bacteria, the antimicrobial effects of all cathelicidins tested, except CATH-2, were found to be completely abolished when suspended in BLES [79, 177]. Although the exact mechanism was not investigated, it was speculated that the reduction in bacterial killing was likely related to the cationic nature of these peptides and their ability to interact with the negatively charged phospholipids of the exogenous surfactant used [79]. This theory has been supported by a subsequent computer modeling study, but not directly tested [209]. Thus, a better understanding for the inhibitory mechanism of exogenous surfactants on cathelicidins, and the structural characteristics of CATH-2 that allowed it to retain more of its antimicrobial activity, is required.
Additionally, it should be noted that although there has not been any indication that surfactant interferes with the anti-inflammatory properties of cathelicidins, there has also been a lack of direct in vitro analysis. Specifically, there are no studies that have directly compared the anti-inflammatory effects of a cathelicidin suspended in surfactant to the peptide alone. In general, studies evaluating the immunomodulatory effects of these peptides in combination with surfactant have been limited to overall efficacy in vivo [177]. Therefore, better insight into the effects of surfactant on the immunomodulatory properties of these peptides is needed.

Using various biophysical techniques, our research group has also performed studies to evaluate the functionality of surfactant in surfactant-cathelicidin mixtures. One such study, utilized the Langmuir probe and atomic force microscopy to demonstrate that combining cathelicidins with bovine lipid extract surfactant (BLES) enhanced surfactant spreading, through their ability to incorporate into the surfactant film [79]. However, in the same study, biophysical analysis on a CDS revealed that this incorporation by some cathelicidins, including CRAMP and LL-37, interfered with the ability of BLES to achieve low surface tensions during cyclic compressions, whereas other peptides, such as CATH-2 did not [79]. These findings have been further supported by a recent computer modeling study, that utilized molecular dynamic simulations to reveal that antimicrobial peptides, including LL-37 and CATH-2, can quickly penetrate the pulmonary surfactant film [209]. These biophysical techniques were expanded upon in Chapter 2 to develop a new in vitro screening tool for surfactant-based therapeutics, including surfactant-cathelicidin preparations.
1.4.2 Acute respiratory distress syndrome

In addition to bacterial pneumonia, a second respiratory condition that may benefit from treatment with a surfactant-based therapeutic is ARDS. As touched on previously (see section 1.2.4 Exogenous surfactant therapy), ARDS is defined by the physiological criteria of severe lung dysfunction, that most commonly occurs after initiating insults, such as sepsis or pneumonia, to the lung [47–49, 210]. The Berlin definition for ARDS established specific diagnostic criteria for the syndrome, including a partial pressure of oxygen/fraction of inspired oxygen (PaO\(_2\)/FIO\(_2\)) ratio of less than 300 mmHg with a minimum of 5 cmH\(_2\)O for positive end-expiratory pressure (PEEP) during mechanical ventilation [47–49, 210]. It also created three different categories of ARDS (mild, moderate, and severe) based on the severity of a patient’s hypoxemia [47–49, 210]. Overall, ARDS is a condition with a 30-50% mortality rate, whose treatment relies heavily on mechanical ventilation strategies to improve survival [211–213]. Even before the COVID-19 pandemic, it was the most common cause of death in the ICU and a large burden on the health care system [210, 214–216]. Furthermore, to combat COVID-19 related ARDS, many researchers have begun to reconsider direct surfactant therapy and its potential as a drug delivery vehicle [62].

Despite extensive research into the pathophysiological processes affecting the initiation, development, and severity of ARDS, targeting these processes by pharmacological intervention has been largely unsuccessful [214, 217–220]. Specifically, there have been many drugs tested in clinical trials for ARDS that failed to demonstrate a significant decrease in mortality, despite strong scientific evidence rationalizing their potential benefits for treating the common processes associated with the disease [214,
include, among many others, β2 agonist (to reduce edema), heparin (to reduce fibrin deposition), antioxidants (to mitigate oxidative stress), exogenous surfactant (to restore the surfactant system), and glucocorticoids (to downregulate the overwhelming inflammation). Although a variety of aspects may have contributed to the lack of pharmacological efficacies observed, drug deposition in the areas required for clinical efficacy is certainly one important aspect. For example, many of the ARDS trials mentioned utilized aerosolization as a drug delivery technique in which, as mentioned above (see section 1.3 Exogenous Surfactant as a Drug Delivery Vehicle), results in drug deposition mainly in the central airways rather than the alveoli [214, 217–220]. Moreover, drug distribution for inhaled therapeutics is also dependent on airflow. As such, inhaled drugs will accumulate mainly in the inflated areas of the lung, whereas the pathophysiological processes targeted by the drug occur in areas with edema which does not receive airflow. These, as well as the added aspects of overcoming the endogenous surfactant impairment and positive outcomes in ongoing clinical trials for surfactant therapy, provide a strong rationale for utilizing a surfactant vehicle when testing therapeutics for ARDS [62].

Further complicating treatment, ARDS is a complex and heterogeneous disease, whose pathological progression can vary greatly across patients [48, 210, 221–223]. For example, ARDS from gastric acid aspiration may start with a chemical injury to the epithelium and endothelium, leading to edema and surfactant dysfunction, followed by pulmonary inflammation [210, 223–225]. In contrast, COVID-19 related ARDS likely begins due to the infection of type II alveolar cell, whereas sepsis-induced ARDS is initiated by systemic inflammation [213, 216]. Although the chronological order of these pathological events is not always clear, there is a common final pathology, observed at
the alveoli, in most forms of ARDS (Figure 1.3). Further, regardless of the underlying processes or initiating events, it seems that overwhelming and maladaptive inflammation in the lung is a main contributor to the disease [48, 221–223, 225, 226]. This dysregulated pulmonary inflammation is manifested by inflammatory cell infiltration (predominately neutrophils), secretion of proteases, inflammatory mediators, and reactive oxygen species [48, 221, 222, 226, 227]. Additionally, this accumulation of activated leukocytes and inflammatory mediators can cause damage to the respiratory units of the lung, as well as alter the permeability of the alveolar-capillary barrier [223, 228, 229]. This could then lead to edema and alterations to the endogenous pulmonary surfactant system, further contributing to ARDS development [50, 51, 230]. Therefore, new therapeutic approaches that can treat alveolar inflammation in ARDS patients would be of substantial therapeutic value, regardless of the different pathophysiological pathways that could have led to the condition.
Figure 1.3. Schematic model of intratracheally administered drugs traveling through the airways to arrive at the alveolar surface of a A) healthy or B) ARDS patient. In addition to airway collapse prior to reaching the alveoli, the drug will also encounter various pathologies at the alveoli of the ARDS patient, such as edema fluid, impaired surfactant layer, impaired gas exchange, and inflammation. Inflammation includes neutrophil infiltration and the release of inflammatory mediators.
1.4.2.1 Glucocorticoids

A subclass of corticosteroids, glucocorticoids are found in mammalian species and play an important role in many physiologic processes [231, 232]. Endogenously, these steroid hormones are derived from cholesterol at the cortex of the adrenal gland and regulated by the hypothalamic-pituitary-adrenal axis, in response to physiological stress or the natural circadian rhythm [231, 232]. At the cellular level, their effects are mediated through their binding with the glucocorticoid receptor (GR), found in the cytoplasm of almost all human cells [232, 233]. The GR contains various structural domains important for glucocorticoid binding, translocation to the nucleus, and DNA binding [234]. In the absence of its “ligand”, it is kept in the cytoplasm as a multiprotein complex, where other molecules cover its nuclear translocation regions [235]. As such, the binding of a glucocorticoid causes the GR to dissociate from this multiprotein complex and translocate into the nucleus of the cell [235, 236]. Once inside the nucleus, the activated glucocorticoid receptor functions as a ligand-dependent transcription factor. Functionally, glucocorticoids derive their name from their ability to increase serum glucose and as such, regulate the metabolism of various macromolecules [231, 232]. However, they have also been found to play an essential role in immune modulation, reproduction, water homeostasis, general growth, as well as normal cardiovascular and cognitive function [231, 232]. Although different glucocorticoids are produced across species, the main physiologic glucocorticoid in humans is cortisol [231, 232]. As such, synthetic glucocorticoids have been designed to structurally and functionally mimic cortisol [237]. This thesis will focus on the anti-inflammatory effects of pharmacologic glucocorticoids.
1.4.2.2 Glucocorticoids: mechanisms of action and therapeutic potential

Clinically, the main therapeutic success of glucocorticoids has been in the treatment of inflammatory diseases, such as asthma, arthritis, Crohn’s disease, and ulcerative colitis (in addition to many others) [82, 237–240]. Specifically, the anti-inflammatory effects of pharmacologic glucocorticoids are mostly attributable to their ability to change the transcription state of genes in immune cells [238]. Briefly, the glucocorticoid bound GR can affect gene expression directly, by binding DNA or indirectly, through protein-protein interactions with other transcription factors [232, 233, 236, 238]. For the direct pathway, glucocorticoids can affect transcription by increasing or decreasing the accessibility of DNA through chromatin remodeling, epigenetic modifications, and the recruitment of coactivator complexes [241–245]. For example, it has been shown that the binding of this activated receptor to the promoter region of a gene can promote [243, 246–248] or downregulate [249, 250] transcription by altering the recruitment of transcription proteins, including RNA polymerase. For the indirect pathway, glucocorticoids can affect gene expression by interacting with various proteins and transcription factors to regulate signaling cascades [232, 243]. For example, dexamethasone has been shown to elicit its anti-inflammatory effects by binding NF-kB, Activator Protein-1 and Protein S (among others) [251–255]. Specifically, these studies found that a therapeutic dose of dexamethasone suppressed the transcription for genes encoding pro-inflammatory cytokines, chemokines, cell adhesion molecules, and various enzymes involved in the inflammatory response [251–255]. Overall, these effects were found to result in fewer leukocytes, such as neutrophils, emigrating into the site of inflammation and promoted their clearance [251–255]. This potent efficacy for regulating various aspects of the inflammatory response, combined with their relatively low cost and
straightforward administration have lead to glucocorticoids becoming one of the most prescribed medications in the world for treating inflammation [256–259].

Unfortunately, these highly effective drugs are not without their adverse side effects [260]. Their potential for “off target” exposure is associated with their inherent ability to pass through biological membranes and the universal expression of the glucocorticoid receptor [232, 233]. Although not an exhaustive list, treatment with systemic glucocorticoids has been associated with an increased risk of diabetes mellitus [261, 262], serious infection [263–265], fractures [266, 267], and osteoporosis [268]. The risk of these adverse effects was also found to increase in a dose and duration dependent manner. However, glucocorticoid treatment strategies utilizing more targeted administration methods have been demonstrated to increase efficacy and decrease rates of adverse effects [82, 269–272]. Due to these advantages, it makes intuitive sense to explore methods of direct delivery for glucocorticoids treating respiratory condition such as ARDS.

The rationale for using surfactant-delivered glucocorticoids as a therapy for ARDS was outlined, in general, in previous sections (see sections 1.3 Exogenous Surfactant as a Drug Delivery Vehicle & 1.4.2 acute respiratory distress syndrome) and includes the need for downregulating inflammation in the deeper regions of the lung. Briefly, the severe lung dysfunction characterizing ARDS is thought to be a consequence of overwhelming inflammation, and the subsequent pulmonary surfactant dysfunction, as well as edema at the alveoli [48, 221–223, 225, 226]. Despite this strong rationale for anti-inflammatory therapies in ARDS, glucocorticoids are not currently recommended for the disease [273, 274]. As shown in Table 1.3, this could presumably be due to most clinical trials with glucocorticoids failing to reduce mortality rates among ARDS patients. However, it
should be noted, that these studies used suboptimal methods to deliver glucocorticoids. For example, the most widely used technique to administer glucocorticoids, systemic administration, is affected by hepatic drug metabolism, renal clearance, and off-target effects, which would all reduce efficacy [275]. Additionally, direct administration of glucocorticoids via aerosol-based strategies has been shown to have limited utility in ARDS, as delivery is determined by the airflow and CT-imaging studies in ARDS have demonstrated a lack of lung inflation in injured regions [276, 277]. Thus, one interpretation of these clinical trials is that the effectiveness of glucocorticoids was limited by the lack of drug deposition at the alveoli. Lastly, as mentioned in a previous section (see section 1.2.4 Exogenous surfactant therapy), exogenous surfactant therapy has also been examined for patients with ARDS [46, 56–62]. Although initial meta-analyses of this therapy did not show an improvement in mortality, intratracheal surfactant administration did improve oxygenation and lung compliance [60, 61]. This latter improvement is indicative of its proposed function to reach and recruit collapsed areas of the lung, as also demonstrated by animal studies [278–280]. Thus, in theory, exogenous surfactant could overcome the delivery-hurdle encountered for glucocorticoids in ARDS to allow the combination to become a desperately needed therapy for the disease.
Table 1.3. Selected clinical trials with glucocorticoids for ARDS.

<table>
<thead>
<tr>
<th>Glucocorticoid</th>
<th>Route of Administration</th>
<th>Maximum Dose Administered (per day)</th>
<th>Number of Patients</th>
<th>*Mortality Rate (Length of Study)</th>
<th>Pulmonary Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylprednisolone</td>
<td>Oral</td>
<td>2 mg/kg</td>
<td>24</td>
<td>No Significant Change (32-Days)</td>
<td>Improvements in lung function and injury score</td>
</tr>
<tr>
<td>[281]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increase in PaO₂/FiO₂ ratio</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>2 mg/kg</td>
<td>180</td>
<td>No Significant Change (60-Days)</td>
<td>Increase in the number of ventilator-free days during the first 28 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increase in PaO₂/FiO₂ ratio and lung compliance during the first 28 days</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>1 mg/kg</td>
<td>27</td>
<td>Significantly Reduced (14-Days)</td>
<td>Improvements in PEEP, with earlier extubation from ventilation after 7 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increase in PaO₂/FiO₂ ratio after 7 days</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Intravenous</td>
<td>200 mg</td>
<td>149</td>
<td>No Significant Change (21-Days)</td>
<td>No significant change in ventilator outcomes</td>
</tr>
<tr>
<td>[284]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No significant change in PaO₂/FiO₂ ratio</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>Intravenous</td>
<td>200 mg</td>
<td>197</td>
<td>No Significant Change (28-Days)</td>
<td>No significant change in ventilator outcomes</td>
</tr>
<tr>
<td>[285]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increase in PaO₂/FiO₂ ratio after 7 days</td>
</tr>
<tr>
<td>Dexamethasone [286]</td>
<td>Intravenous</td>
<td>20 mg</td>
<td>277</td>
<td>Significantly Reduced (60-Days)</td>
<td>Increase in the number of ventilator-free days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increase in PaO₂/FiO₂ ratio at day 10</td>
</tr>
<tr>
<td>Dexamethasone [287]</td>
<td>Intravenous</td>
<td>10 mg</td>
<td>299</td>
<td>No Significant Change (28-Days)</td>
<td>Increased number of ventilator-free days</td>
</tr>
<tr>
<td>Budesonide [288]</td>
<td>Inhalation</td>
<td>2 mg</td>
<td>53</td>
<td>No Significant Change (15-Days)</td>
<td>Reduction of all markers of pulmonary fibrosis measured at day 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increase in PaO₂/FiO₂ ratio after 11 days</td>
</tr>
<tr>
<td>Budesonide [289]</td>
<td>Inhalation</td>
<td>2 mg</td>
<td>60</td>
<td>Not measured (3-Days)</td>
<td>Increase in PaO₂/FiO₂ ratio for 1st hour after treatment</td>
</tr>
</tbody>
</table>

*Mortality Rate refers to all-cause mortality at the final timepoint of the study.
1.4.2.3 Glucocorticoids: mechanisms of action and therapeutic potential

A glucocorticoid that has been studied extensively in combination with exogenous surfactants is budesonide [78, 94–96, 290–295]. Budesonide is currently a mainstay treatment for respiratory conditions, like asthma [83, 296, 297] and a variety of inflammatory bowel conditions [298, 299]. It has also shown some clinical success in treating various other obstructive airway diseases [83, 272, 300–302] and in combination with formoterol, preventing ARDS development [303]. Additionally, budesonide delivered by a surfactant vehicle has had success in clinical trials for BPD [92–95] and in various animal models [94, 294, 295]. For example, in a piglet model of meconium aspiration, budesonide combined with a porcine derived exogenous surfactant resulted in improvements to a variety of inflammation-related outcomes, compared to surfactant or budesonide alone [94]. Together, these studies demonstrate the potential for surfactant-delivered budesonide to be an effective treatment option for the alveolar inflammation associated with ARDS.

Further supporting the potential of this mixture to treat ARDS are the specific characteristics of budesonide. A synthetic, nonhalogenated 16,17-acetal steroid molecule, budesonide has been found to have a topical anti-inflammatory potency 1000-fold higher than the endogenous glucocorticoid, cortisol [304]. In fact, it was originally developed to be a better, more selective intrapulmonary therapeutic for asthma [83], with higher local activity and lower systemic bioavailability [83, 299, 304, 305]. Its minimal availability in circulation and local selectivity for the lung, following inhalation, have been shown to be linked to its interaction with lung enzymes and extensive first-pass metabolism [306, 307]. Moreover, although budesonide is moderately lipophilic, its retention and prolonged activity in the airways have been found to be greater than similarly lipophilic
glucocorticoids [83, 306, 308, 309]. Pharmacokinetic studies performed *in vivo* found that these properties are linked to the rapid esterification of budesonide in the airways, by coenzyme A [306–308]. Briefly, as explained by Van Den Brink et al., the subsequent fatty acid conjugates of budesonide form intracellular deposits inside lung tissue [306]. Then, over an extended period the drug is released through the activity of intracellular lipases. Furthermore, being slightly more water soluble than other inhaled glucocorticoids budesonide has also been shown to display faster dissolution in bronchial fluid [84, 310]. Consequently, this has been found to result in a more rapid uptake of the drug into tissue, with minimal removal from various clearance mechanisms in the lung [310]. Lastly, the tendency of budesonide to have fewer off-target effects has been attributed to its rapid metabolism by hepatic CYP3A enzymes and consequently, shorter systemic half-life [83, 308, 311, 312]. Specifically, this extensive first-pass metabolism seems to create inactive metabolites of budesonide, that are easily excreted [83, 308, 311, 312]. Together, these pharmacokinetic features give intratracheally administered budesonide a long duration of local therapeutic effects with minimal systemic exposure [83, 313]. Thus, with a surfactant delivery strategy, intratracheally administered budesonide could reach the peripheral sites of inflammation associated with ARDS and be a long-awaited pharmacological treatment for the disease.

**1.4.2.4 Evaluating surfactant-glucocorticoid mixtures**

As mentioned in the section above, the impact of glucocorticoids, notably budesonide, on the function of various exogenous surfactants has been studied extensively. Specifically, *in vitro* analysis has been used to investigate its incorporation, transport, and overall effect on the surface tension lowering effects of different exogenous
surfactants [78, 290, 291, 293, 314]. For example, utilizing a captive bubble surfactometer, budesonide has been shown to have a concentration-dependent effect on the biophysical properties of the exogenous surfactant, Survanta, but not BLES [290]. The researchers found that high concentrations of budesonide were shown to significantly inhibit the surface tension lowering properties of Survanta during compression and reduced surfactant film stability [290]. Thus, the authors speculated that there was an optimum type of exogenous surfactant and glucocorticoid concentration for this interaction to occur without inhibiting the surface tension lowering properties of surfactant. In a follow up study, utilizing the low cholesterol surfactant, Curosurf, it was found that higher concentrations of budesonide would fluidize the surfactant film, without altering the minimum surface tension achieved during compression [291]. Expanding on the findings of the former study, the researchers inferred that combining glucocorticoids with low cholesterol surfactants, like Curosurf or BLES would allow for larger amounts of the drug to be delivered to the lung without compromising the surface activity of the surfactant [291]. This theory was also supported by a recent wet bridge study, that utilized a fluorescently labeled derivative of glucocorticoids, Beclomethasone Dipropionate to evaluate drug transport by Curosurf [78]. When administered alone to the donor well, the fluorescence of Beclomethasone Dipropionate was not detectable in the recipient well. However, administering the drug with the low cholesterol exogenous surfactant resulted in a significantly larger fluorescent signal for the recipient well [78]. From these results, the researchers concluded that exogenous surfactant was an effective delivery vehicle for corticosteroids, capable of efficiently transporting these drugs over the air–liquid interface. Together, these in vitro studies showcase how the lipid composition of an
exogenous surfactant and the concentration of the suspended glucocorticoid can affect their interaction.

In contrast to the numerous in vitro studies analyzing the effect of glucocorticoids on surfactant function, there has yet to be a study exploring the effects of this interaction on glucocorticoid function. Moreover, animal studies with surfactant-glucocorticoid mixtures have mainly focused on biodistribution within the lung, treating surfactant deficiency, and the efficacy of this mixture as a prophylactic treatments for lung inflammation [91, 293–295]. For example, a recent animal experiment, using a radioactive derivative of the drug, $^{18}$F-budesonide, demonstrated that intratracheally instilling budesonide with Survanta enhanced its biodistribution within the lung [293]. Further supporting this delivery approach for glucocorticoids, a study using a rabbit model of meconium aspiration assessed the prophylactic efficacy of Curosurf combined with budesonide [294]. The researchers found that prophylactic administration of this combination alleviated lung inflammation more effectively than either the drug or Curosurf alone [294]. Although these studies illustrate the benefits of exogenous surfactants for enhancing the delivery and prophylactic efficacy of glucocorticoids, they also highlight that additional experiments are required. The investigation of the anti-inflammatory effects of a glucocorticoid alone or in combination with surfactant at a distal site is explored in Chapter 2. In addition, Chapter 4 assessed the in vivo efficacy of this combination when administered after an inflammatory insult has occurred in the lung.
1.5 Summary, Objectives, and Hypothesis

The complex branching structure of the airways makes direct delivery of therapeutics to peripheral sites of inflammation and infection particularly difficult. This delivery challenge has prompted the treatment paradigm for lower respiratory tract conditions to move towards systemic dosing regimes. Unfortunately, these systemic treatment strategies have been shown to be ineffective for inflammatory conditions like ARDS and may even promote AMR among pneumonia patients. Moreover, systemic dosing comes with the consequence of adverse side effects, largely avoided by routes of direct delivery. Thus, there is an urgent need for novel delivery strategies, like exogenous surfactant vehicles, that can promote a more localized drug distribution to peripheral sites within the lung.

The overall objective of this thesis was to create a process for screening, designing, and testing exogenous surfactants as delivery vehicles for specific intrapulmonary therapeutics. The overall hypothesis was that exogenous surfactant delivery would enhance the efficacy of antimicrobial cathelicidins and anti-inflammatory glucocorticoids for the treatment of bacterial pneumonia and ARDS, respectively.

To test this hypothesis, Chapter 2 focused on the full characterization and utility of the wet bridge transfer system, as an in vitro screening tool for surfactant-based therapeutics. Chapter 3 implemented this system to further investigate the inhibitory effects of exogenous surfactant on the antimicrobial cathelicidin function, as well as to design a more ideal synthetic surfactant to deliver these peptides. Finally, Chapter 4 utilized a rat model of lung inflammation in both sexes to explore the therapeutic efficacy of budesonide delivered by a surfactant vehicle compared to the drug alone.
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Chapter 2: The Wet Bridge Transfer System: A Novel Tool to Assess Exogenous Surfactant as a Vehicle for Intrapulmonary Drug Delivery

A version of this chapter has been published:

2.1 Introduction

Due to the complex branching structure of the lung, drug delivery to both the proximal and distal regions of this organ remain a significant barrier for the delivery of intrapulmonary therapeutics. Whereas nebulization can be utilized to effectively target airways, it has limited utility for drugs that may require deposition to the distal alveolar regions of the lung [1, 2]. Furthermore, the distribution of nebulized material is largely determined by the flow of the inhaled gases and therefore may not be distributed equally among all airways, in particular those that may be diseased. Clinical success for direct endotracheal instillation of non-nebulized drugs has also been limited [3, 4], with several pre-clinical animal models demonstrating that such delivery is primarily localized to the central airways with little peripheral deposition [5–7]. Consequently, the vast majority of pulmonary diseases are currently treated with high systemic doses of medications which can either lead to suboptimal intrapulmonary efficacy or a high prevalence of systemic side effects [8]. For example, conventional treatment of acute bacterial pneumonia, a common disease of the peripheral airways, includes the empiric use of oral or intravenous antibiotics. Treatment failure for this condition may result from inadequate tissue concentrations, discontinuation due to systemic toxicity, or the induction of antibiotic resistance due to insufficient killing properties. Thus, alternative strategies for targeted pulmonary drug delivery are required.

One proposed strategy to overcome these challenges is through the use of exogenous surfactant as a delivery vehicle, due to its unique biophysical properties. A concept previously reviewed by Haitsma et al., (2001), these surfactants have the unique
ability to efficiently and rapidly distribute throughout both the proximal and distal airways [9]. Additionally, exogenous surfactants represent the current standard of care in premature neonates in the setting of absolute or relative surfactant deficiency [10–13]. Furthermore, it has been shown in preclinical studies that certain therapeutics combined with an exogenous surfactant have a significantly greater pulmonary distribution when injected directly into the trachea of animals compared to the distribution of therapy alone without a surfactant vehicle [14, 15]. Therefore, exogenous surfactants, like bovine lipid extract surfactant (BLES), are promising candidates to facilitate pulmonary drug delivery.

Despite the theoretical advantages and promising preliminary animal studies, surfactant-mediated drug delivery remains underutilized in clinical practice. The in vivo data on surfactant as a carrier for agents instilled directly to the lung is limited, with few in vitro experiments investigating the interactions between surfactant and drugs [16–20]. Moreover, the translation of exogenous surfactant into a multipurpose, clinically used drug vehicle is hampered by the current testing methodology in which multiple permutations of surfactant and drugs require expensive and laborious animal studies for pre-clinical testing [9, 16, 17, 21].

Based on the above observations, the objective of this study was to develop and test an in vitro technique to rapidly assess, screen and optimize surfactant-based therapies prior to animal studies. This technique is based on methodology utilized in the study of biophysics and consisted of a wet bridge transfer system. In the current study we utilize this system to characterize surfactant spreading at different concentrations, volumes, and temperature, as well as examining the effectiveness of surfactant as a carrier for antibiotics and anti-inflammatory agents.
2.2 Materials and Methods:

Reagents:

*Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853 (ATCC, Manassas, VA, USA) was obtained from Sigma-Aldrich. Gentamicin, colistin, and ciprofloxacin solutions were all obtained from Sigma-Aldrich. Budesonide 0.5 mg/ml (AstraZeneca Södertälje, Sweden) is a commercially available clinical preparation suspended in deionized water. BLES 27 mg/ml phospholipid concentration (BLES Biochemicals, London, ON, Canada) is a commercially available clinical surfactant preparation, stored in 1.5 mM calcium chloride and 100 mM sodium chloride. Chicken cathelicidin CATH-2 was synthesized by Fmoc (9-fluorenlymethoxy carbonyl) solid-phase synthesis chemistry. The peptide was purified to a minimum purity of 95% by reverse phase high-performance liquid chromatography prior to biological testing. The peptide was then suspended in non-buffered sterile saline. For experimental use BLES was re-suspended in sterile saline to a phospholipid concentration of 10 mg/ml, with 100 μM CATH-2, 50 μg/ml budesonide, 100 μg/ml gentamicin, 100 μg/ml colistin, or 100 μg/ml ciprofloxacin.

The Wet Bridge Approach:

The Wet Bridge Transfer System, schematically shown in Figure 2.1A, consisted of a Teflon block with two 20mm diameter wells, a delivery well (Dish 1, D1) and a remote well (Dish 2, D2). Each well has a depth of 1mm and was separated by a 0.2mm high raised Teflon bridge. To measure surfactant mediated transfer, each well was filled with 1 mL 150mM NaCl, 1.5mM CaCl₂, and 5mM Hepes (pH 7.4) solution. Then a continuous liquid interface between the two dishes was created using a 2cm x 0.5cm
wetted piece of ashless filter paper placed over the Teflon bridge (Figure 2.1A). In this system, therapeutics or other compounds can be injected into the delivery well, with subsequent measurements of transfer in the remote well. The principle of the methodology is that surfactant injected into the delivery well will be able to adsorb to the air-liquid interface, and through its spreading capabilities, transfer over the wet bridge to the remote well (Figure 2.1B). In contrast, drugs administered to the delivery well that are not able to adsorb to the surface or spread across the wet bridge will therefore remain in delivery well (Figure 2.1C). If, however the drugs are mixed with surfactant they will then be carried by the surfactant to the surface of the delivery well and transported over the wet bridge to the remote well (Figure 2.1D).

Unless otherwise noted, our experiments were performed at room temperature (24°C) using 200 µL of BLES (10 mg/ml) combined with various drugs/compounds: colistin (100 µg/ml), gentamicin (100 µg/ml), ciprofloxacin (100 µg/ml), CATH-2 (100 µM), or budesonide (50 µg/ml). To assess the effect of temperature, the wet bridge system was either put into a cold chamber (4°C), kept at room temperature, or placed into an infant incubator (37°C).
Figure 2.1. Schematic representation of the A) Wet Bridge Transfer System when a B) surfactant, C) drug, or D) surfactant/drug mixture is administered to the delivery dish.

Functional outcomes on the Wet Bridge: Spreading:

Surface tension measurement using the Langmuir probe and FilmWare 2.51 software of the Langmuir balance was used to assess spreading of surfactant. The Langmuir probe was dipped into the surface of D1 or D2 to monitor surface tension. The surfactant samples were then administered to the delivery well and surface tension was recorded over the subsequent 480 seconds.

Functional outcomes on the Wet Bridge: Bacterial Killing:

For bactericidal and anti-inflammatory experiments an overnight culture of *P. aeruginosa* ATCC 27853 was grown in tryptic soy broth (TSB). Using measurements of optical density (OD), 2x10^6 colony forming units (CFU) of bacteria were then either stored in saline or seeded to the remote dish of the Wet Bridge Transfer system. Both the delivery dish and remote dish were filled with 1mL of sterile saline and 200 µL of various
antibiotics with or without surfactant were administered to the surface of the delivery dish. Eight minutes after administration the wetted piece of filter paper was removed using tweezers and all fluid was collected from both dishes and incubated at 37°C for 1 hour. Then 50 μL of each sample from the remote dish was diluted with 50 μL of saline in a polypropylene coated 96-well plate and subsequently diluted 10-10,000-fold. CFU/ml was determined by spot plating 10 μL of each dilution in triplicate on tryptic soy agar (TSA) plates. These plates were incubated overnight at 37°C and colonies were counted to a detection limit of 10² CFU/ml. Samples from the delivery dish were also spot plated on TSA plates and incubated overnight at 37°C to ensure no bacterial transfer.

Functional outcomes on the Wet Bridge: Anti-inflammatory analysis:

RAW 264.7 murine macrophages were a kind gift from Wei-Yang Lu (Robarts Research Institute, Western University, Canada). Cells were cultured in “complete growth media” consisting of Dulbecco modified eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 5% Penicillin/Streptomycin. Cells were seeded in 12-well plates (3x10⁶ cells/well) and allowed to grow to confluence overnight. They were stimulated with 2x10⁶ CFU/ml of heat-killed bacteria (90°C for 10 minutes) from the overnight culture for 15 minutes prior to the administration of treatments. The ashless filter paper was wetted with complete growth media and placed over the Teflon bridge before the delivery dish and remote dish were filled with 1mL of complete growth media. Then 200 μL of the CATH-2 or budesonide treatments were administered to the delivery dish with or without BLES. Eight minutes after this administration the filter paper wet bridge was removed with tweezers. Fluid from both dishes was collected and administered onto the stimulated macrophages. The 12-well plates were then incubated
for 4 hours at 37°C and 5% CO₂. IL-6 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA), according to the manufacturer’s instructions.

Statistics:

The effect of different phospholipid concentrations on the surface tension reducing properties of BLES was calculated using a two-way measure of analysis of variance (ANOVA), followed by a Tukey-Kramer post hoc test using saline in Dish 1 or Dish 2 as the control group. The influence of different volumes of administration and temperature conditions as well as different therapeutics on surfactant spreading and surface tension lowering properties were calculated using a one-way ANOVA, followed by a Tukey-Kramer post hoc test using BLES at room temperature as the control group. The efficacy of the surfactant/drug mixtures at the remote well was calculated by analyzing CFU/ml or IL-6 content using a one-way ANOVA, followed by a Tukey-Kramer post hoc test, and using saline or BLES in Dish 2 as control groups. GraphPad Prism 6 was used to graph and analyze data with a minimum of three independent experiments per group. Means are reported ± the standard deviation (SD), and values were considered significantly different at a probability value (P) of less than 0.05.

2.3 Results

Spreading/Surface Tension:

Initial experiments characterized surfactant spreading on the wet bridge system by administering different concentrations of BLES to the surface of D1 and measuring
surface tension using a Langmuir probe in either D1 or D2. Figure 2.2AB shows the change in surface tension over time in D1 and D2 following the administration of surfactant to D1. For the first 30 seconds following the administration of surfactant the surface tension in D1 and D2 showed an exponential drop in surface tension for concentration greater than 1 mg/ml. Higher surfactant concentrations showed a further decrease in surface tension during this initial period, but similar linear reductions in surface tension during the remaining 7 minutes for both dishes. Quantification and statistical comparisons of the minimum surface tension achieved during the 8 minutes following administration is shown in Figure 2.2C. The data shows that all concentrations of BLES achieved a significantly lower minimum surface tension compared to the saline control in D1 and D2. Moreover, higher phospholipid concentrations of BLES achieved significantly lower minimum surface tensions than the lower phospholipid concentrations of BLES (Figure 2.2C). This was observed in both D1 and D2.
Figure 2.2. Change in surface tension of A) Dish 1 and B) Dish 2 following administration of BLES to the surface of Dish 1. Arrow indicates time of surfactant administration. C) Minimum surface tension achieved in Dish 1 and Dish 2 over the 480-second period following surfactant administration. Statistical differences were determined by two-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD; n=3.
To assess the effect of delivery volume, an experiment was performed in which equal amounts of surfactant were delivered in different volumes to D1. The data shown by Figure 2.3 illustrates that the minimum surface tension achieved in D1 or D2 following the injection of the different volumes was similar among the groups.

**Figure 2.3.** Minimum surface tension achieved over the 480-second period following the administration of different volumes of surfactant with equivalent phospholipid content to Dish 1. Statistical differences were determined using a one-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD, n=3.

To further characterize the wet bridge transfer system, the effect of three different temperatures on the minimum surface tension achieved in D1 or D2 was tested using various doses of BLES. Administration of surfactant at room temperature achieved significantly lower minimum surface tensions in D1 compared to the low temperature (ice chamber) condition at surfactant concentrations of 1, 5 and 10 mg/ml (Figure 2.4A).
Instilling surfactant at room temperature achieved a significantly lower minimum surface tension in D2 at surfactant concentrations of 1, 5, 10 and 20 mg/ml compared to the low temperature condition (Figure 2.4B). Moreover, administering surfactant at the body temperature (infant incubator) condition resulted in significantly lower minimum surface tension readings compared to room temperature at surfactant concentrations of 1, 5, and 10 mg/ml for D1 (Figure 2.4A) as well as 1, 5, 10, and 20 mg/ml for D2 (Figure 2.4B). The minimum surface tension achieved under the body temperature condition was significantly lower across both dishes for all concentrations of surfactant compared to the low temperature condition (Figure 2.4AB).
Figure 2.4. Minimum surface tension achieved under low, room, and body temperature conditions in (A) Dish 1 and (B) Dish 2 over the 480 second period following surfactant administration. Statistical differences were determined using a one-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD, n=3.
**Proof of principle experiment 1: Surfactant with Antibiotics:**

To assess the influence of different antibiotics on surfactant spreading, the minimum surface tension was calculated for each surfactant/antibiotic mixture in D2 over the 8 minutes following administration to D1 and compared to the saline and BLES controls. All surfactant/antibiotic preparations achieved significantly lower minimum surface tensions compared to the saline control, but not significantly higher than surfactant alone (Figure 2.5). Notably, the addition of colistin to BLES resulted in a significantly lower minimum surface tension compared to the BLES control.

![Image](image.png)

**Figure 2.5.** Minimum surface tension achieved in Dish 1 and Dish 2 over the 480-second period following surfactant/antibiotic administration. Statistical differences were determined using a one-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD, n=3.
To characterize bacterial killing at a distal site, antibiotics were administered alone (solid bar) or in combination with BLES (dashed bar) to D1, and a colony counting assay was performed in D2. Figure 2.6 shows that none of the antibiotics administered alone to the delivery dish had any significant effects on the bacterial growth in D2 compared to either saline or BLES controls. However, BLES/gentamicin and BLES/ciprofloxacin both showed significantly more bacterial killing in D2 compared to the controls as well as gentamicin or ciprofloxacin alone (Figure 2.6). In contrast, colistin showed no significant change in its bacterial killing when combined with BLES compared to the saline, BLES or the antibiotic alone (Figure 2.6).

**Figure 2.6.** Bacterial killing in Dish 2 (2x10⁶ CFU/ml *P. aeruginosa*) one hour following administration of treatment to Dish 1. Statistical differences were determined using a one-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD, n=4.
**Proof of principle experiment 2: Surfactant with Anti-inflammatory drugs:**

To evaluate the influence of different anti-inflammatory drugs on surfactant spreading, the minimum surface tension was calculated for mixtures of surfactant combined with either CATH-2 or budesonide in D2 and compared to saline and BLES controls. The minimum surface tension achieved in the D2 by BLES/CATH-2 or BLES/budesonide was significantly lower than the saline control (Figure 2.7). Additionally, the minimum surface tension achieved by BLES/budesonide in D2 was not significantly different compared to surfactant alone (Figure 2.7). However, the BLES/CATH-2 preparation achieved a significantly lower minimum surface tension compared to the BLES control in D2 (Figure 2.7).

**Figure 2.7.** Minimum surface tension achieved in Dish 1 and Dish 2 over the 480-second period following surfactant/anti-inflammatory administration. Statistical differences were determined using a one-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD, n=3.
To assess the ability of an exogenous surfactant to enhance drug delivery, the anti-inflammatory properties of budesonide or CATH-2 in D2 were determined with either the drug being administered alone (solid bars) or in combination with BLES (dashed bars) to D1. The positive control signals for this experiment were created by stimulating macrophages in D2 with heat-killed bacteria prior to the administration of either saline or BLES to D1, creating a large IL-6 signal compared to unstimulated macrophages (red dashed line). Furthermore, this data reveals that neither budesonide nor CATH-2 administered alone to D1 had any significant effect on the IL-6 content of D2 compared to the positive controls (Figure 2.8). However, co-administration of BLES with either budesonide or CATH-2 into D1 resulted in significantly lower IL-6 concentrations for D2 compared to the controls, budesonide, or CATH-2 alone (Figure 2.8).
Figure 2.8. Anti-inflammatory effects of budesonide or CATH-2 alone or mixed with BLES on stimulated macrophages in Dish 2. RAW264.5 macrophages (1x10⁶ cells/ml) were stimulated with 2x10⁶ CFU/ml of heat-killed *P. aeruginosa* 15 minutes prior to administration of treatments to Dish 1. IL-6 content was measured 4 hours following administration of therapeutic mixtures into Dish 1. Dashed red line indicates the IL-6 content for unstimulated macrophages. Statistical differences were determined using a one-way ANOVA with Tukey-Kramer post hoc test. * p<0.05. Error bars = SD, n=6.
2.4 Discussion

Exogenous surfactant has the potential to be an effective delivery agent for pulmonary drugs, however clinical translation remains unfulfilled. The current study illustrates the potential use of the wet-bridge transfer system as a tool to screen different surfactant/drug preparations based on their ability to spread and elicit therapeutic effects at a distal site. Herein, we demonstrate that spreading within this system was shown to be dependent on surfactant concentration and temperature. Furthermore, utilization of the system was illustrated using surfactant-antibiotic preparations in which surfactant showed differing effects on different antibiotics and anti-inflammatory agents. It was concluded that this technique can be utilized as an effective in vitro tool to screen and optimize various surfactant-drug therapy strategies prior to in vivo testing.

The wet bridge transfer system we developed to test surfactant as a carrier of various molecules for pulmonary delivery was based on previous descriptions of biophysical studies examining surfactant lipid film. For example, the biophysical studies performed by Yu & Possmayer (2003) used a transfer system to investigate surfactant lipid transfer across an air-liquid interface. Additionally, Hidalgo et al., (2017) modified a wet bridge system with a captive bubble surfactometer to determine whether surfactant would be able to achieve and sustain low surface tensions when combined with corticosteroids prior to compression–expansion cycling. Our study takes advantage of the wet bridge system, but rather than solely focusing on biophysical properties, we explored the transfer of clinically relevant therapeutics including antibiotics and anti-inflammatory agents through functional outcomes at the transfer side of the bridge.
The concept of enhancing pulmonary delivery for antibiotics through an exogenous surfactant is appealing and has been previously investigated [2–4, 7, 9, 11, 16, 17, 23, 24]. Our data adds to this information as it illustrated that gentamicin, ciprofloxacin, and colistin did not inhibit the spreading capability of surfactant, and only gentamicin and ciprofloxacin elicited effects in the secondary dish when combined with BLES. Thus, it can be speculated that exogenous surfactant would be a useful pulmonary drug delivery vehicle to improve the distribution of these two antibiotic medications to the more remote areas of the lung. In contrast, the inhibitory effect that surfactant exerted on colistin appears to limit the usefulness of this antibiotic with surfactant, or alternatively illustrates the need to further investigate drug-surfactant interactions to optimize this combination.

Secondly, we investigated the use of the wet bridge transfer system to study the transfer of anti-inflammatory compounds when combined with exogenous surfactant. Budesonide was chosen since it is currently being utilized in combination therapy with surfactant for clinical use and its interaction with surfactant has been studied from a biophysical and biocompatibility perspective [20, 25, 26]. The evidence provided here with the wet bridge transfer system suggest that it can be used to further explore the efficacy of other exogenous surfactants, and optimal dosing, without extensive animal studies.

One of the key features of drug delivery by surfactant will depend on the nature of the interaction between the surfactant and the specific drug being tested. Specifically, it is essential that the drug is transported with the surfactant, but that the interaction does not inhibit the innate function of either the surfactant or the drug itself. Previous studies have indicated that the interactions between exogenous surfactants and therapeutics is
dependent on either the lipid solubility of a drug or its ability to bind specific components of surfactant, such as the phospholipids or surfactant associated proteins [27]. For example, the second of our anti-inflammatory agents, CATH-2, is a positively charged peptide which likely interacts with the negatively charged lipids of surfactant. This has previously been shown to have a slight inhibitory effect on this peptide when testing its other function, the ability to kill bacteria [18, 19]. It is feasible that the wet bridge system could be used to develop a surfactant with a different lipid composition to optimize the transfer and function of the surfactant-CATH-2 preparation.

Overall, our system for assessing surfactant as a carrier has several advantages and potential usages. For example, any other drug that requires delivery to the distal alveolar regions of the lung may be suitable for testing on this system, such as combination of surfactant with drugs for pulmonary fibrosis, pulmonary hypertension or primary lung cancers may have some therapeutic potential. In addition, delivery of other compounds, like contrast agents for imaging studies, could also be investigated. Furthermore, whereas our studies were performed using a sterile wet bridge and delivery dish, other relevant in vivo conditions could be easily incorporated in this proposed set up. Inclusion of serum proteins, to mimic edema, or proteases and lipases, to mimic inflammatory environments, may be utilized to provide further insight into the properties, interaction, and efficacy of surfactant drug combinations.

Despite the advantages of our proposed methodology, several limitations exist which may be relevant. For example, one limitation of the technique is that the volume and surface area of the two dishes does not reflect that of the extremely thin hypo-phase of the lung. This requires therapeutics within our experimental setup to cross a much larger distance through the hypo-phase within D2 to exert their therapeutic effects on the
bacteria or macrophages. It would be possible, however, to build wet bridge transfer systems with different size and depth for the delivery dish. The system is also limited by the non-alveolar macrophages used in experimentation and the very simplified unbranching path from “D1 to D2”. However, these limitations could easily be overcome through the harvesting of alveolar macrophages from bronchoalveolar lavage (BAL) and the construction of a wet bridge transfer system with multiple secondary dishes branching from the delivery dish. It is also feasible to use the wet bridge and simply collect the transferred surfactant-drug combination for further analysis of the experimental system.

Preliminary experiments for the current study were performed in this fashion and provided a confirmatory series of experiments for the antimicrobial and anti-inflammatory effects. The collection of transferred material in these experiments may also allow for future compositional assessment. A second, more substantial limitation is that the in vivo environment is much more complex than those environments tested with our wet bridge. The wet bridge system detects the ability of surfactant to spread over a relatively short distance whereas in vivo surfactant distribution is affected by more than its spreading ability alone. Aspects like gravity, delivery volume, delivery technique, and mechanical ventilation strategy (if employed) would all have an impact on the surfactant-based drug delivery. Certainly, the wet bridge transfer technique cannot eliminate in vivo experimentation, however it does offer a relatively inexpensive approach to rapidly screen surfactant/drug mixtures prior to animal, and ultimately clinical studies.

**Conclusion:**

From the data shown in the current study it can be concluded that the wet bridge transfer system can effectively screen surfactant-based therapies through their spreading
and efficacy at a remote site. Although, the system will always be limited by its ability to imitate the physiological characteristics of an *in vivo* system, it offers the best method for rapidly and accurately assessing surfactant based therapies *in vitro* [28, 29]. However, future animal studies are still needed to validate the findings of the wet bridge system and to further investigate the distribution patterns and metabolism of these preparations *in vivo*. Exogenous surfactants are promising delivering agents for pulmonary therapeutics and are expected to improve drug distribution and drug efficacy for patients with peripheral airway diseases. This direct delivery of medications to the airways, offered by surfactant should increase local effectiveness and reduce systemic toxicity.
References


https://doi.org/10.1159/000244206
Chapter 3: Optimizing Exogenous Surfactant as a

Pulmonary Delivery Vehicle for Chicken Cathelicidin-2

A version of this chapter has been published:

3.1 Introduction

The increasing incidence of antimicrobial resistance (AMR) in bacterial pneumonia has instigated a much-needed search for new therapeutic approaches for these types of infections [1, 2]. One approach, involving the utilization of exogenous surfactant for the delivery of antimicrobial peptides (AMPs) to the infected lungs is supported by a strong theoretical foundation [3, 4]. The AMPs, such as the chicken cathelicidin, CATH-2, can target a wide spectrum of antibiotic resistant bacteria, making them potential novel therapeutics for bacterial pneumonia, which can be delivered to the areas of infection by exogenous surfactant [3, 5–7]. Unfortunately, simply mixing CATH-2 or other AMPs with a commercial exogenous surfactant has been shown to impact a drug’s therapeutic efficacy [5, 8]. For example, we recently demonstrated that an exogenous surfactant with CATH-2 exhibited antimicrobial activity against antibiotic-resistant bacterial isolates from cystic fibrosis patients, albeit with less efficacy than CATH-2 by itself [5]. Therefore, in order to improve this promising approach, it is important to understand how surfactant interferes with cathelicidin function and investigate strategies to minimize this interaction, while maintaining the benefits of surfactant delivery.

The primary benefit of utilizing CATH-2, and other cathelicidins, for therapeutic purposes is their diverse range of pathways to kill bacteria, since their positive charge allows them to interact directly with both the negatively charged lipids of the bacterial cell wall as well as intracellular targets such as DNA or RNA [9–12]. This multi-target approach to killing bacteria has been demonstrated to be effective against a wide spectrum of antibiotic-resistant organisms [5–7]. Unfortunately, the use of AMPs to treat bacterial lung infections has been largely unsuccessful, due to an inability to directly
deliver these peptides to the peripheral sites of infection [13–15]. Therefore, improving the pulmonary delivery of these highly effective antimicrobial agents with an exogenous surfactant would have substantial therapeutic value.

The goal of using exogenous surfactant for drug delivery is to open up collapsed airways and areas with edema, in order for the therapeutic to reach the areas of the lung affected by infection [16, 17]. Most commercially used exogenous surfactants are derived from animal lungs and are complex mixtures of phospholipids (85%), neutral lipids (5-8%) and specialized surfactant proteins, designated SP-B and SP-C (7-10%) [17–19]. Although variations exist among different products, the main lipid components of these exogenous surfactants are saturated (dipalmitoylphosphatidylcholine; DPPC, approx. 40%), unsaturated phosphatidylcholine (PC; approx. 35%), the negatively charged phosphatidylglycerol (PG; approx. 10%), and neutral lipids like cholesterol (5-8%) [3].

These lipids, together with SP-B and SP-C are ultimately responsible for the ability of exogenous surfactant to rapidly adsorb to the air-liquid interface and spread throughout the airways [18, 20, 21].

Together, the above information on surfactant composition and cathelicidin properties indicates that CATH-2 may interact with the negatively charged phospholipids within surfactant. However, this has not been demonstrated directly, nor is there evidence that this interaction interferes with cathelicidin function or if this interaction is crucial for surfactant’s ability to act as a carrier for cathelicidins. It is hypothesized that the PG component of surfactant inhibits CATH-2 function and that an exogenous surfactant, with a reduced PG composition would increase peptide mediated killing at a distal site.
3.2 Materials and Methods

Preparations:

The chicken cathelicidin, CATH-2 was synthesized and purified as described previously [22]. It is comprised of 26 amino acids – RFGRFLRKRFRPKVTITIQGSARF-NH2 and has a positive charge of +9 [23]. The commercially available surfactant, bovine lipid extract surfactant (BLES) was generously provided by BLES Biochemicals (London, ON, Canada). BLES was stored in 100 mM sodium chloride and 1.5 mM calcium chloride with a phospholipid concentration of 27 mg/mL. Lipid enriched preparations of BLES were created through sonication at 37°C for 2 hours. The addition of 11.6 mg or 27 mg of individual lipids (DPPC, POPC, or POPG) per mL of BLES created 30% or 50% lipid enriched versions of BLES respectively. The SP-C peptoid protein mimic (mono-SP-C) utilized in the synthetic surfactants was synthesized and purified (>97%) according to previously published protocols [24]. The lipid and peptoid compositions of the synthetic surfactant preparations are summarized in Table 3.1. All BLES preparations, lipid mixtures, and synthetic surfactants were used at 10 mg/mL phospholipid.

Bacterial killing curves:

Bacterial killing curves were performed as previously reported [5]. Briefly, an overnight culture of Pseudomonas aeruginosa (P. aeruginosa; ATCC 27853), obtained from Sigma-Aldrich (Oakville, ON, Canada), was grown in tryptic soy broth. Using measurements of optical density, 2×10^6 colony forming units (CFU) of bacteria were resuspended in saline. Varying concentrations of cathelicidins 0–20 µM were then mixed
with either saline (No Lipids), BLES, individual lipid components at 1–2 mg/mL phospholipid, lipid enriched BLES or a synthetic surfactant. These mixtures were then incubated with the bacteria for 3 hours at 37°C before being serially diluted 10–10,000-fold, with 10 µl of each dilution being spot plated in triplicate on tryptic soy agar (TSA) plates. The plates were then incubated at 37°C overnight and counted the following morning. No bacterial growth was designated as a bacterial concentration of less than 100 CFU/mL.

Isothermal titration calorimetry (ITC):

For ITC analysis, vesicles of POPC and POPG were generated using the extrusion technique as previously reported [25]. Phospholipid content was determined as inorganic phosphate after treatment with perchloric acid by UV-VIS spectroscopy[26]. POPC and 10%POPG/90% POPC vesicles were diluted to 1.5 mg/mL, while POPG vesicles were further diluted to 0.15 mg/mL. For measurements using BLES, the stock solution was diluted to 1.5 mg/mL. Interactions between CATH-2 and large unilamellar vesicles consisting of POPC and/or POPG, or between CATH-2 and BLES were tested using ITC. All ITC experiments were performed on a Low Volume NanoITC (TA instruments - Waters LLC, New Castle, USA). In each experiment, the ITC cell chamber was filled with 190 µl of vesicles or BLES, and the syringe was filled with a 50 µl solution of 320 µM CATH-2. Titrations were incremental with 2 µl injections at 300 seconds intervals. Experiments were performed at 37°C and data were analyzed with the Nano Analyze software (TA instruments - Waters LLC).
Solid-State nuclear magnetic resonance (ssNMR) spectroscopy:

For ssNMR, DOPG unilamellar vesicles were prepared with 5 mM HEPES pH 7.5 and 50 mM NaCl by the extrusion technique and using filters with a 0.2 µm cut off[27]. Phospholipid concentration was determined as inorganic phosphate after treatment with perchloric acid[28]. CATH-2 was added to DOPG vesicles to a final molar ratio of 1:50 CATH-2/DOPG. The interactions between CATH-2 and unilamellar vesicles of DOPG were assessed using ssNMR. Vesicles were collected after ultracentrifugation and were spun in 3.2 mm rotors. Static $^{31}$P ssNMR spectra were acquired at 500 MHz magnetic field ($^1$H-frequency) and a sample temperature of 295 K. Heteronuclear proton decoupling did not affect the spectra and was switched off for all measurements. The resulting $^{31}$P powder pattern was apodised with 50 Hz exponential line-broadening and baseline corrected.

Bacterial killing and surface tension measurements on the wet bridge transfer system

To analyze surfactant spreading and bactericidal properties, the wet bridge transfer system was set up as previously described[29]. The system consisted of a Teflon block with two 20 mm diameter wells, a delivery well, and a remote well. Each well has a depth of 1 mm and was separated by a 0.2 mm high raised Teflon bridge. A wetted piece of ashless filter paper was placed over the Teflon bridge to join the delivery and remote wells. Both wells were then filled with 1 mL of a 150 mM NaCl, 1.5 mM CaCl$_2$, and 5 mM HEPES (pH 7.4) solution. To determine surfactant spreading, preparations were administered into the delivery well and surface tension was measured in the remote well using a Wilhelmy probe. FilmWare 2.51 software of the Langmuir balance was used over
a period of 480-seconds after 200 µL of surfactant preparations or saline was administered to the delivery well.

For bacterial killing experiments with the lipid enriched versions of BLES, the overnight culture of *P. aeruginosa* was serially diluted to $2 \times 10^5$ CFU and seeded to the remote well. Then 200 µL of cathelicidins (0-100µM) were administered to the surface of the delivery well with saline, BLES or 30% lipid enriched BLES preparations. For bacterial killing experiments with the synthetic surfactant preparations, the wet bridge transfer system was modified through the addition of a lower sucrose layer. Both wells were filled with 800 µL of a 10% sucrose solution (150 mM NaCl, 1.5 mM CaCl$_2$, 5 mM HEPES). Then 100 µL of the same solution without sucrose was added to make a thin upper layer on top of the sucrose layer. For these experiments the overnight culture of *P. aeruginosa* was serially diluted to $2 \times 10^6$ CFU of bacteria and seeded to the remote well above the sucrose layer. For treatments, 100 µL of either saline, BLES or synthetic surfactants with or without CATH-2 (100 µM) were administered to the surface of the delivery well. Eight minutes after administration the wetted piece of filter paper was removed, and all fluid was collected from both dishes to be incubated at 37°C for 3 hours. Then 50 µL of each sample from the remote well was diluted with 50 µL of saline in a polypropylene coated 96-well plate and subsequently diluted 10–10,000-fold. CFU/mL was determined by spot plating 10 µL of each dilution in triplicate on TSA plates. These plates were incubated overnight at 37°C and colonies were counted to a detection limit of 100 CFU/mL. Samples from the delivery dish were also spot plated on TSA plates and incubated overnight at 37°C to ensure no bacterial transfer.
**Statistical Analysis:**

All data points shown represent the average of at least three independent repetitions. Statistical significance was determined by two-way analysis of variance and one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test to determine differences among experimental groups. Results are presented as mean ± the standard deviation and were considered statistically significant with a P-value of less than 0.05.
Table 3.1. Lipid and peptoid compositions for synthetic surfactants.

<table>
<thead>
<tr>
<th>Surfactant Preparation</th>
<th>DPPC %Phospholipids</th>
<th>POPC %Phospholipids</th>
<th>POPG %Phospholipids</th>
<th>Cholesterol %Phospholipids</th>
<th>Mono-SP-C %Weight</th>
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<td>40</td>
<td>20</td>
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<td>2</td>
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<tr>
<td>10% POPG</td>
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<tr>
<td>5% POPG</td>
<td>35</td>
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<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2.5% POPG</td>
<td>35</td>
<td>57.5</td>
<td>2.5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>0% POPG</td>
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<td>60</td>
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<td>5</td>
<td>2</td>
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<tr>
<td>Lipids only</td>
<td>35</td>
<td>50</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>
3.3 Results

*Bacterial killing curves:*

To investigate how exogenous surfactant and its lipid components affect antimicrobial peptide function, bacterial killing curves were performed with CATH-2 combined with 10 mg/mL BLES or 1–2 mg/mL of individual surfactant lipids (Figure 3.1). Shown on each of the panels (Figure 3.1A–D) for comparison purposes, CATH-2 combined with saline (no lipids) exhibited potent bactericidal activity against *P. aeruginosa*, reducing bacterial growth below detectable limits at 5 to 10 μM. In the presence of 10 mg/mL BLES, the antimicrobial properties of CATH-2 were significantly reduced at concentrations of 5 μM or greater (Figure 3.1A). Since BLES contains approximately 10% PG, CATH-2 mediated killing was tested in the presence of 1 mg/mL POPG. Similar to BLES, the presence of POPG at 1 mg/mL phospholipid significantly reduced CATH-2 killing at concentrations of 5 μM or greater compared to the peptide combined with no lipids (Figure 3.1B). Additionally, mixing CATH-2 with 2 mg/mL POPG resulted in a complete loss of its bactericidal properties. In contrast to the effect of POPG, there was no significant difference in the bacterial killing of CATH-2 combined with DPPC or POPC at 1–2 mg/mL phospholipid, compared to the peptide with no lipids (Figure 3.1CD). To explore if the PG effect was specific to CATH-2, killing curves for CRAMP, PMAP-23 and LL-37 were also performed in the presence of BLES or individual surfactant lipids. All three peptides showed complete inhibition of their antimicrobial function when combined with 10 mg/mL BLES or 1–2 mg/mL POPG (Figure 3.2A-C).
Figure 3.1. Killing curves for CATH-2 combined with BLES or individual surfactant lipids. Shown are the bacterial killing curves for CATH-2 suspended in either A) BLES, B) POPG, C) POPC, D) DPPC or no lipids (saline). Colonies were counted to a detection limit of 100 CFU/mL. *p<0.05 vs No Lipids. Error bars = SD; n=3.
Figure 3.2. Bacterial killing for AMPs combined with individual surfactant lipids. Shown are the bacterial killing curves for A) PMAP-23, B) LL-37, or C) CRAMP suspended in saline (No Lipids), BLES, POPG, POPC, or DPPC against $2 \times 10^6$ CFU/mL.
*P. aeruginosa*. Error bars = SD; n=3. Porcine PMAP-23, human LL-37 and mouse CRAMP were all synthesized and purified as described previously [22]. Bacterial colonies were counted to a detection limit of 100 CFU/ml. When combined with no lipids each of the AMPs exhibited potent bactericidal activity. However, in the presence of BLES or 1–2 mg/ml POPG the antimicrobial properties of all three cathelicidins were completely abolished. There was no difference in the bacterial killing of any of the AMPs in the presence of DPPC or POPC at 1–2 mg/mL phospholipid compared to the peptide with no lipids.

To determine the effects of modifying an existing surfactant’s lipid composition on cathelicidin function, bacterial killing curves were performed for CATH-2 mixed with 30–50% lipid enriched preparations of BLES (10 mg/mL). Suspension of CATH-2 in 30% or 50% POPG enriched BLES resulted in a complete loss of the peptide’s antimicrobial properties (Figure 3.3A). However, at concentrations of CATH-2 of 5 μM or greater, the 30% DPPC and POPC enriched BLES preparations were found to kill significantly more bacteria than BLES/CATH-2, resulting in a 2–log reduction in bacterial viability at 20 μM (Figure 3.3BC). At those concentrations of CATH-2, 50% DPPC or POPC diluted BLES also showed significantly more bacterial killing compared to their 30% equivalents.
Figure 3.3. Antimicrobial activity of CATH-2 in lipid enriched preparations of BLES.

Displayed are the bacterial killing curves for CATH-2 suspended in 10 mg/mL of 30-50%
A) POPG, B) POPC, or C) DPPC enriched versions of BLES. Colonies were counted to a
detection limit of 100 CFU/mL. *p<0.05 for BLES + 30% lipid vs BLES, +p<0.05 for
BLES + 30% lipid vs BLES + 50% lipid. Error bars = SD; n=3.
**ITC and ssNMR of CATH-2 mixed with individual surfactant lipids:**

To further examine the interactions between cathelicidins and exogenous surfactant, CATH-2 and large unilamellar vesicles of individual lipids or BLES were tested using ITC. As displayed in Figure 3.4A (bottom panel), there was no, or very little heat production observed when CATH-2 was injected into the POPC sample, with an enthalpy of -3.0 kJ/mol. However, when 10% POPG was added to the vesicles, a large increase in heat production was observed following CATH-2 injection, indicative of exothermic binding (Figure 3.4A; top panel; ΔH = -13.2 kJ/mol). For both BLES and 100% POPG vesicles, the binding of CATH-2 resulted in a 3–fold higher release of heat (Figure 3.4B), with enthalpies of -34.6 and -34.2 kJ/mol respectively.
Figure 3.4. ITC Binding of CATH-2 to vesicles and BLES. Shown are representative thermograms of ITC experiments with titration of 320 μM CATH-2, into 1.5 mg/mL BLES, POPC and 10%POPG vesicles, or 0.15 mg/mL POPG vesicles.
With ssNMR spectroscopy, CATH-2 combined with unilamellar vesicles of DOPC and DOPG were assessed by acquiring $^{31}$P chemical shift anisotropy powder pattern spectra under static conditions. These patterns result solely from the $^{31}$P chemical shift anisotropy and are sensitive to both the lipid headgroup mobility and orientation[30–32]. The addition of CATH-2 to the DOPG vesicles resulted in a modest but clear broadening of the $^{31}$P powder pattern by 1.6 ppm (310 Hz) (Figure 3.5). For BLES the addition of CATH-2 caused a broadening of the powder pattern by 2.6 ppm (470 Hz), similar as in DOPG, demonstrating peptide-surfactant binding and modulation of the surfactant headgroups. Lastly, isotropic signals were not observed for any of the measurements, indicating that CATH-2 does not cause very strong curvature to the membrane or the formation of spherical micelle-like structures (while curvature effects could still modulate the $^{31}$P powder spectrum)[32, 33].
Figure 3.5. ssNMR of CATH-2 mixed with BLES or liposomes. Static $^{31}$P solid-state NMR spectra acquired at 500 MHz magnetic field. (upper panel) Spectra acquired with unilamellar DOPG vesicles in the absence (black lines) or presence of CATH-2 (red lines). The span of the powder pattern at approximately 10% signal height is 42.6 ppm (8610 Hz) and 41 ppm (8300 Hz) in the presence and in the absence of CATH-2, respectively. (lower panel) Spectra were acquired with BLES surfactant in the absence (black lines) and the presence of CATH-2 (magenta lines). The span of the powder pattern at approximately 10% signal height is 59.7 ppm (12090 Hz) and 57.1 ppm (11560 Hz) in the presence and in the absence of CATH-2, respectively. All spectra are normalised.
**Bacterial killing and spreading over the wet bridge for lipid enriched BLES:**

To further examine the effect of PG on CATH-2 responses, BLES preparations were enriched with individual lipids to change the relative percentage of PG within the preparation. POPC and DPPC at 30 and 50% were utilized to decrease the relative PG content, whereas POPG was used to increase the PG content within the BLES preparation. Subsequently, the wet bridge transfer system was utilized to investigate if CATH-2, suspended in lipid enriched preparations of BLES, delivered at a remote site could affect bacterial killing, at a distal site. Suspension in BLES resulted in significantly more bacterial killing by CATH-2 (20–100 µM) in the remote well, compared to the peptide alone (Figure 3.6). CATH-2 (50–100 µM) suspended in 30% POPC enriched BLES resulted in significantly more bacterial killing compared to BLES/CATH-2. When mixed with 30% DPPC enriched BLES, CATH-2 (0–100 µM) displayed no change in bacterial killing, compared to BLES/CATH-2. Additionally, the suspension of CATH-2 (50–100 µM) in 30% POPG enriched BLES resulted in significantly less bacterial killing at the remote dish compared to BLES+CATH-2. Suspending the other AMPs, CRAMP, PMAP-23, or LL-37 at 100 µM with 30% POPC enriched BLES were also shown to significantly improve bacterial killing in the remote well compared to BLES/peptide or the peptide with no lipids (Figure 3.7).
Figure 3.6. Bacterial killing for CATH-2 over the wet bridge transfer system. Presented are the bacterial counts in the remote dish (2x10^5 CFU/mL *P. aeruginosa* seeded) three hours following the administration of CATH-2, suspended in saline (No lipids), BLES, or 10 mg/mL 30% lipid enriched versions of BLES, to the delivery dish. *p<0.05 vs BLES. Error bars = SD, n=3.
Figure 3.7. Bacterial killing for cathelicidins over the wet bridge transfer system. Presented are the bacterial counts in the remote well (2x10^5 CFU/mL *P. aeruginosa* seeded) three hours following administration to the delivery well of either saline (No Lipids), BLES (10 mg/mL) or 30% lipid enriched versions of BLES (10 mg/mL) with or without CRAMP, PMAP-23, or LL-37 at 100µM. *p<0.05 vs BLES. Error bars = SD, n=3. When combined with 30% POPC enriched BLES all three peptides killed significantly more bacteria in the remote well compared to BLES or no lipids.
To examine the spreading characteristics of the 30% lipid enriched preparations of BLES, the surface tension in the remote well of the wet bridge was measured over a 480 second period after instillation into the delivery well. All 30% lipid modified mixtures of BLES achieved surface tensions significantly lower than saline (Figure 3.8). However, the surface tension achieved by 30% DPPC or POPC diluted BLES were significantly higher than BLES alone.

![Bar chart showing surface tension measurements](image)

**Figure 3.8.** Surfactant spreading over the wet bridge transfer system for lipid enriched BLES. Shown are the surface tensions achieved in the remote dish after the 480-second period following administration of saline, BLES (10 mg/mL) or 30% lipid enriched preparations of BLES to the delivery dish. *p<0.05 vs BLES. Error bars = SD; n=4.
Bacterial killing curve for synthetic surfactants:

To further explore the inhibitory effects of PG and the potential of a PG-free surfactant, bacterial killing curves were performed for CATH-2 combined with synthetic surfactants of varying PG content. At CATH-2 concentrations of 5 μM and above, bacterial killing was found to be significantly greater for synthetic surfactants with 5% or less POPG and significantly lower for preparations with 20% POPG compared to BLES (Figure 3.9). The synthetic surfactant with a POPG composition of 10% displayed similar bactericidal properties to BLES when combined with CATH-2 at 0–20 μM. Lastly, combining CATH-2 with PG-free synthetic surfactant (0% POPG) resulted in antimicrobial properties no different than the peptide alone.
Figure 3.9. Bacterial killing curves for CATH-2 combined with synthetic surfactants.

Shown are the bacterial killing curves for CATH-2 suspended in saline (No Lipids), 10 mg/mL of BLES or synthetic surfactant with varying PG content. Colonies were counted to a detection limit of 100 CFU/mL. The composition of all synthetic surfactants is displayed in Table 1. Error bars = SD; n=3.

Bacterial killing and spreading over the wet bridge for synthetic surfactants:

To examine the spreading characteristics of the synthetic surfactants, the surface tension in the remote well of the wet bridge was measured over a 480 second period after instillation into the delivery well. All of the synthetic surfactants tested achieved surface tensions similar to BLES and significantly lower than saline or the lipids alone (Figure 3.10).
Figure 3.10. Spreading over the wet bridge transfer system for synthetic surfactants. Shown are the surface tensions achieved in the remote dish after the 480-second period following administration of saline, lipids only, BLES (10 mg/mL) or a synthetic surfactant preparation to the delivery dish. *p<0.05 vs BLES. Error bars = SD; n=4.

To further evaluate the efficacy of a PG-free synthetic surfactant for delivering AMPs to a distal site, CATH-2 (100µM) was administered to the delivery dish of the wet bridge transfer system with no lipid, BLES or in combination with a synthetic surfactant. Administering CATH-2 with BLES or the 10% PG synthetic surfactant showed similar bacterial killing at the remote well (Figure 3.11). CATH-2 suspended in a PG-free synthetic surfactant resulted in significantly more bacterial killing in the remote well than
all other preparations mixed with the peptide. This data implies that the synthetic surfactant without PG was capable of spreading over the wet bridge and acting as a carrier for CATH-2 to induce killing at a remote location.

Figure 3.11. Bacterial killing over the wet bridge for synthetic surfactants/CATH-2. Presented are the bacterial counts in the remote dish (2x10⁶ CFU/mL P. aeruginosa seeded) three hours following administration to the delivery dish of either saline (No lipids), BLES, or synthetic surfactants with or without CATH-2. a*p<0.05 vs BLES, b*p<0.05 vs 10% PG synthetic surfactant, *p<0.05. Error bars = SD; n=9-11.

3.4 Discussion

The current study tested the hypothesis that the PG component of surfactant inhibits CATH-2 function and that an exogenous surfactant with a reduced PG composition would increase peptide mediated killing at distal sites. Overall, our results supported this hypothesis. Specifically, measurements of bacterial killing as well as both assessments of binding indicated that PG interacts with CATH-2, inhibiting its function. Furthermore, reducing or eliminating the PG content in an exogenous surfactant improved
the ability of CATH-2 to kill bacteria. It was also shown that a PG-free synthetic surfactant was capable of carrying CATH-2 to distal sites to kill bacteria. Based on these observations it is concluded that synthetic PG-free surfactants will enhance the pulmonary delivery of CATH-2 without inhibiting its antimicrobial function.

Clinically, the relevance of this study is associated with the potential positive benefits of treating bacterial lung infections with cathelicidins delivered by a surfactant vehicle to reach the deeper areas of the lung. For many pneumonia patients, the acquisition of antimicrobial resistant bacteria represents a decisive stage in disease progression, towards poor outcomes [34–38]. The ability of CATH-2 and other AMPs to target antibiotic resistant bacteria make these molecules interesting for the development of novel therapeutics [11, 39, 40]. The ability of exogenous surfactants, to re-open collapsed airways and allow antimicrobials access to regions blocked off during the infection will also be essential for combating resistance [41–43]. The direct delivery of antimicrobials will elevate local therapeutic concentrations at the pulmonary sites of infection, improving bacterial clearance and limiting the development of resistance [15, 44]. Together, CATH-2 and exogenous surfactant represent a desperately needed treatment strategy to address the growing threat of multidrug-resistant lung infections.

An important aspect of the current paper was the utilization of a synthetic PG-free surfactant, customized for its ability to maintain CATH-2 activity. Several molecular dynamic simulations have suggested that cathelicidins would electrostatically bind the anionic lipids in bacterial membranes[45–48]. Similarly, NMR studies have also demonstrated that AMPs have a strong tendency to form helical structures that bind negatively charged molecules, such as the lipopolysaccharides of Gram negative bacteria [49, 50]. Altering such electrostatic interactions, through additional anionic lipids, lipid
lysinylation, or changes to the cationic nature of the peptide, have all been shown to alter bacterial killing [51–54]. In support of these observations, our ssNMR, ITC and bacterial killing data provides strong evidence that PG was the inhibitory component of the exogenous surfactant, likely by restricting CATH-2 from directly or indirectly binding negatively charged lipids, DNA or other bacterial components [11, 40, 55]. Moreover, the enhancement in CATH-2 function following the addition of POPC or DPPC to BLES, illustrates the potential benefit for lowering PG content in surfactant to reduce electrostatic interactions with these peptides. As such, we generated a PG-free surfactant using a surfactant protein mimic of SP-C and demonstrated that such a surfactant, mixed with CATH-2, was significantly better at killing bacteria at a remote site compared to PG-containing surfactants. Since AMPs have been shown to form weak hydrophobic interactions with neutral lipid membranes [56–58] it is likely that the PG-free preparation transported CATH-2 via these weaker hydrophobic interactions. Importantly, this weaker binding likely allowed CATH-2 to still interact with the bacteria at the remote site. Thus, we conclude that synthetic PG-free surfactants are optimal delivery vehicles for CATH-2 and that it is worthwhile investigating such therapeutics in future in vivo studies.

The generation of a PG-free surfactant for the delivery of CATH-2 also provided proof of a more general concept, that synthetic surfactants can be customized for drug delivery. The majority of studies exploring surfactant as a carrier of pulmonary antibiotics and other pulmonary therapeutics simply mix the drug with a commercial exogenous surfactant preparation developed for the treatment of surfactant deficient premature infants rather than as a delivery vehicle [3, 16, 29, 41, 59]. Although some success has been obtained with these approaches [3], many of the surfactants utilized were animal derived preparations in which the composition is established by its endogenous source.
The advantage of a synthetic surfactant is that the specific composition can be optimized for the delivery of drugs. We utilized an approach of using surfactant protein mimics, or peptoids, since a recent study demonstrated that these peptoid-based surfactant were not only active in vivo, but equivalent to animal-derived surfactants for improving oxygenation and other physiological outcomes in a model of acute lung injury [60].

Peptoids are structurally based on a polypeptide backbone but with side-chains appended to nitrogen-backbone, they are highly stable to proteolysis and can be made in high yields [24, 61, 62]. Once synthesized, the peptoids can also be easily mixed with a specific lipid mixture optimized to spread throughout the lung and for drug delivery, as illustrated in vitro with our PG-free surfactant. Overall, we propose that the development of peptoids and other synthetic analogs of the hydrophobic surfactant proteins will allow for a more mechanistic approach to developing surfactant-based drug delivery approaches.

Having established the effect of exogenous surfactant on CATH-2, there is also therapeutic value in exploring if similar approaches can be utilized for other cathelicidins, thereby increasing the clinical arsenal of antimicrobial agents. Unfortunately, the presence of exogenous surfactant completely abolished the antimicrobial function of many cathelicidins, including human LL-37, mouse CRAMP and pig PMAP-23 [8]. However, the current study combined each of these peptides with a lipid enriched preparation of surfactant and demonstrated that they could all benefit from delivery by a surfactant with a reduced PG content. This ability to create functional exogenous surfactants, that minimally interact with a variety of AMPs, will substantially increase the treatment options for lung infections. For these reasons, developing exogenous surfactant as a delivery vehicle for multiple AMPs, each with their own unique antibacterial pathway, would create a promising new pipeline of anti-infective therapeutics.
It should also be noted that there are several limitations to our study. First, the bacterial killing was limited to one strain of bacteria. However, it is important to note that our previous study showed a similar pattern of inhibition for CATH-2, by surfactant against several Cystic Fibrosis derived bacterial strains. As such, we anticipate that PG-free surfactant with CATH-2 will likely provide improved killing activity against other strains as well [5]. Secondly, the PG-free synthetic surfactant was only tested in combination with one cathelicidin, CATH-2. However, we did demonstrate that several other cathelicidins were also inhibited by PG and benefited from a lipid enriched surfactant with a reduced PG composition. From these findings, we predict that LL-37, CRAMP, and PMAP-23 would all benefit from delivery by a PG-free synthetic surfactant. It should also be noted that high concentrations of AMPs have been associated some cytotoxic effects towards mammalian cells, potentially limiting their therapeutic potential [8, 63–65]. However, a recent in vivo study showed that co-instillation of CATH-2 with an exogenous surfactant was well tolerated, with no deleterious effects up to 100 µM [8]. Lastly, our synthetic PG-free surfactant was only designed based on minimizing PG content. The preparation contains POPC, DPPC and cholesterol as well as a SP-C peptoid, however, this composition could be further optimized with respect to these remaining components as well as other surfactant components.

In conclusion, this paper has demonstrated how exogenous surfactant can be designed to be a more effective delivery system for CATH-2. Further, we propose that this concept could be applied to other intrapulmonary therapeutics. Direct drug delivery is a major hurdle for many pulmonary conditions and designing exogenous surfactants with specific drug-delivery properties offers an intriguing method to overcome that obstacle.
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Chapter 4: Exogenous Surfactant as a Pulmonary Delivery Vehicle for Budesonide In Vivo

A version of this chapter has been published

Inflammation is associated with many respiratory conditions, including asthma, pneumonia, bronchopulmonary dysplasia (BPD), and Acute respiratory distress syndrome (ARDS). However, the effectiveness of anti-inflammatory medications – such as glucocorticoids – is location-specific for these conditions in terms of airway (bronchi) or airspace (alveolar) involvement [1]. In asthma, for example, inflammation is observed primarily in the small airways, which allows for a more direct delivery of therapeutics, as evidenced by the effectiveness of standard inhalers [1, 2]. On the other hand, in conditions such as ARDS and bacterial pneumonia, inflammation occurs in the more distal, alveolar, regions of the lung [1, 3, 4]. Here, the large surface area, and associated regions of alveolar edema or airway collapse may contribute to an inability of airway-delivered therapies to reach distal lung units, to provide effective anti-inflammatory functions [1, 3, 4]. In these clinical scenarios, alternative strategies are required to deliver therapeutic concentrations of anti-inflammatory medications to these peripheral sites within the lung.

One such approach, is with the use of exogenous surfactant as a delivery vehicle for glucocorticoids such as budesonide. Exogenous surfactant is a complex mixture of lipids and specialized proteins, usually obtained from natural sources such as cows or pigs [5]. The endogenous material, produced by type II alveolar cells in the lung, has been well studied and serves a vital biophysical role in reducing surface tension, thereby stabilizing the alveoli during normal breathing [6, 7]. The discovery of surfactant deficiency in preterm infants led to the development of exogenous surfactant therapy [8]. Given intratracheally, it spreads throughout the lung, improving lung function and has
resulted in significant reductions to infant mortality due to prematurity [8, 9]. It is suggested that the spreading properties of exogenous surfactant could improve glucocorticoid delivery to peripheral sites of inflammation in the lung. In support of this notion, exogenous surfactant has already been shown to enhance the delivery of glucocorticoids to remote sites using in vitro approaches [10–12]. Additionally, it has been shown that through these properties exogenous surfactant can re-open collapsed airways, overcome regions of edema, and efficiently spread to the more remote sites of inflammation in an injured lung [6]. Together, this data highlights the potential for exogenous surfactant to provide locally acting anti-inflammatory drugs access to remote regions of the lung otherwise inaccessible to therapeutics.

When combined with the efficacy of glucocorticoids, the innate biophysical properties of surfactant suggest that utilizing exogenous surfactant as a vehicle for budesonide would improve its effectiveness for treating remote inflammation in the lung. However, this has yet to be demonstrated in vivo. It was therefore hypothesized that fortifying an exogenous surfactant with budesonide would enhance efficacy for treating pulmonary inflammation in vivo.

4.2 Materials and Methods

Reagents

Heat-killed bacteria (HKB) was created from a lab strain of Pseudomonas aeruginosa (P. aeruginosa; ATCC 27853), purchased from Sigma-Aldrich (Oakville, ON, Canada). Using measurements of optical density, the bacteria was diluted in saline to $3 \times 10^6$ colony forming units per ml, before being heated at 90°C for 15 minutes. A commercially
available preparation of budesonide (0.5 mg/ml), suspended in deionized water, was obtained from AstraZeneca (Södertälje, Södermanland, Sweden). Bovine lipid extract surfactant (BLES) at 27 mg/ml phospholipid concentration was obtained from BLES Biochemicals (London, ON, Canada).

**Animal models and treatments**

All animal work was carried out in accordance with guidelines set forth by the Western University Council for Animal Care. For breeding, two adult male and seven adult female Wistar rats (250 g) were purchased from Charles River (St-Constant, QC, Canada). Acclimatization to the animal care facility and breeding were carried out as previously described [13]. Once pregnant, rats were housed individually and received standard chow. Immediately after birth the litters were culled to 10 pups in order to limit the effect of litter size on outcomes.

To initiate pulmonary inflammation, male or female offspring were weighed, anesthetized, and intratracheally instilled with 2 µl of HKB or saline per gram of body weight at 25-35 days of age. In animals randomized to a treatment group, this first instillation was followed by an instillation of either budesonide (50µg/ml) or BLES/budesonide (10mg/ml; 50µg/ml). To minimize the potential effects of any distinct litter, only 1 or 2 animals per litter were randomized to any individual experimental group. Animals were monitored for 6 hours following instillation, before being euthanized by intraperitoneal injection of sodium pentobarbital and exsanguination, by severing the descending aorta. After this, a bronchoalveolar lavage (BAL) was performed as previously described [14], before the lungs were excised, divided into four pieces and snap frozen in liquid nitrogen to be stored at -80°C.
Outcomes

Inflammatory cell counts and differential cell analysis of the lavage were done as previously described [14]. Briefly, lavage volume was recorded and centrifuged at 150 x g for 10 minutes to obtain a cell pellet. This pellet was resuspended and used for cell counting and differential analysis to obtain the number of inflammatory cells and neutrophils in the BAL. Protein content of the BAL was also measured using a Micro BCA protein assay kit from Pierce Biotechnology (Rockford, IL, USA), per manufacturer’s instructions. A multiplexed immunoassay kit was utilized per manufacturer’s instruction (R&D Systems, Minneapolis, MN) to measure the concentrations of TNF-α, IL-6, MIP-2 and GRO/KC. A Bio-Plex 200 readout system was utilized from Bio-Rad (Santa Rosa, CA, USA) and cytokine levels (pg/mL) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad). An aliquot of the 150 x g supernatant was also analyzed through a Duck-Chong phosphorous assay as previously described [15]. Briefly, the total amount of surfactant in the lavage was determined through the measurement of phospholipid-phosphorus [16, 17]. The remainder of the supernatant was then centrifuged at 40,000 x g for 15 min to obtain a pellet of the active form of surfactant, the large aggregates. This resuspended pellet, as well as its supernatant containing the small aggregates, was also analyzed for phospholipid-phosphorus [16, 17].

Frozen lung tissue was utilized for myeloperoxidase (MPO) activity as previously described [18]. Briefly, pieces of frozen lung were weighed and then homogenized in 0.02 M potassium phosphate buffer (pH 6; Sigma-Aldrich) using a PT2100 homogenizer. Lung homogenate was spun at 6000 x g for 20 minutes at 4°C, with the resulting pellet being
resuspended in 1% hexadecyltrimethylammonium hydroxide detergent solution. This mixture was then re-homogenized, before being sonicated at 4°C and 30% amplitude for 10 seconds. The resulting preparation was spun at 13,000 x g for 10 minutes at 4°C. Its supernatant was then aliquoted into a 96-well plate at 2 mg/ml and mixed with an MPO cocktail containing 3,3',5,5'-tetramethyl-benzidine. Hydrogen peroxide was then added to each well and the plate was incubated at 37°C for 5 minutes. Sulfuric acid was used as a stop solution and the plate was read at 450 nm using an iMark plate reader (BioRad). MPO activity (units/mg) was calculated from standard curves.

**Statistical Analysis:**

All data points shown represent one male or female rat. Statistical significance was determined by two-way analysis of variance (ANOVA), followed by a Tukey-Kramer post hoc test to determine differences among experimental groups. Results were considered statistically significant with a P-value of less than 0.05.

**4.3 Results**

Baseline characteristics of the experimental groups are shown in Table 4.1. Prior to the first instillation, body weights were found to be similar among the experimental groups. There were also no significant differences across groups for protein content in the BAL. The phospholipid composition of surfactant, including total surfactant, as well as the large aggregate and small aggregate sub-fractions were significantly higher in male and female rats receiving BLES/budesonide compared to all other treatment groups.
Table 4.1. Baseline characteristics for male or female rats in each treatment group.

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<th>HKB + Budesonide</th>
<th>HKB + BLES/Budesonide</th>
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<td></td>
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<td>Females</td>
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<tr>
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<td>9</td>
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<tr>
<td>Body Weight</td>
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<td>Protein</td>
<td>23.8 ± 10.7</td>
<td>19.6 ± 9.2</td>
<td>19.2 ± 7.7</td>
<td>21.7 ± 5.7</td>
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<td>10.7 ± 1.7</td>
<td>11.5 ± 1.9</td>
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<tr>
<td>Large Aggregates (mg/kg BW)</td>
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<td>6.9 ± 1.2</td>
<td>6.3 ± 1.2</td>
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<tr>
<td>Small Aggregates (mg/kg BW)</td>
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<td>4.7 ± 1.2</td>
<td>3.8 ± 0.9</td>
</tr>
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</table>

*p<0.05 vs saline.

To evaluate the anti-inflammatory effects of delivering budesonide with an exogenous surfactant, standard inflammatory markers were analyzed using BAL and frozen lung tissue. The instillation of HKB resulted in a significantly higher number of inflammatory cells compared to saline (Figure 4.1). The instillation of budesonide, following HKB, did not have a significant effect on the number of inflammatory cells as compared to the saline or HKB groups. Instillation of BLES/budesonide resulted in significantly lower numbers of inflammatory cells compared to HKB and budesonide groups. Differential cell analysis showed that the administration of HKB resulted in a
significantly higher number of neutrophils compared to saline, but that both budesonide and BLES/budesonide had significantly lower neutrophil counts compared to HKB (Figure 4.2A). Additionally, the number of neutrophils was significantly lower in animals administered BLES/budesonide compared to those given budesonide alone. The instillation of HKB or HKB followed by budesonide also resulted in significantly higher MPO activity compared to saline (Figure 4.2B). However, only animals instilled with BLES/budesonide had significantly lower MPO activity compared HKB or budesonide groups. The instillation of HKB or HKB followed by budesonide was also shown to result in significantly higher levels of all pro-inflammatory cytokines tested compared to the saline group (Figure 4.3A-D). Compared to animals administered HKB, those receiving a second instillation of BLES/budesonide showed significantly lower IL-6 and TNF-α concentrations (Figure 4.3A-B). Furthermore, the BLES/budesonide group showed significantly lower concentrations of TNF-α and GRO/KC than the budesonide group (Figure 4.3B-C). Although the BLES/budesonide group showed lower levels of MIP-2 and GRO/KC relative to HKB and budesonide groups, the levels were still significantly higher than the saline group (Figure 4.3C-D).
Figure 4.1. The effect of instilling budesonide or BLES/budesonide on the number of inflammatory cells in BAL for pediatric rats. Solid circles indicate female rats, open circles represent male rats. n = 10-17, *p<0.05 vs saline, †p<0.05.
Figure 4.2. The effect of instilling budesonide or BLES/budesonide on the A) number of neutrophils in the BAL and B) MPO activity in the lung tissue of pediatric rats. Solid circles indicate female rats, open circles represent male rats. n = 10-17, *p<0.05 vs saline, †p<0.05.
Figure 4.3. The effect of instilling budesonide or BLES/budesonide on pro-inflammatory cytokine concentrations in the BAL: 
A) IL-6, B) TNF-α, C) GRO/KC, and D) MIP-2. Solid circles indicate female rats, open circles represent male rats. n = 9-12, *p<0.05 vs saline, +p<0.05.
Figure 4.4. The effect of instilling BLES on A) the number of inflammatory cells, and B) the number of neutrophils in BAL, as well as C) MPO activity in the lung tissue of pediatric rats. Solid circles indicate female rats, open circles represent male rats. n = 3.
4.4 Discussion

This chapter tested the hypothesis that fortifying an exogenous surfactant preparation, BLES, with budesonide would enhance efficacy for treating pulmonary inflammation \textit{in vivo}. Overall, our results supported this hypothesis. Specifically, BLES was shown to enhance the anti-inflammatory effects of budesonide in a rat model of lung inflammation by reducing the number of neutrophils, as well as the concentrations of a several pro-inflammatory mediators in the BAL. Furthermore, combining budesonide with BLES was shown to be beneficial for reducing MPO activity in the lung tissue. It should also be noted that these results were displayed across both sexes. Based on these observations, it is concluded that utilizing exogenous surfactant as a pulmonary vehicle for budesonide enhanced its ability to treat lung inflammation.

To address our hypothesis, we utilized an \textit{in vivo} model, where HKB was instilled into the lungs of young rats. The rationale for using young rats was practical in nature to limit animal usage, as these animals were also utilized for a separate experiment [19]. The HKB solution contains a mixture of bacterial components, including lipopolysaccharides, that caused a rapid inflammatory response as evidenced by the significant increases in neutrophil counts, MPO activity and inflammatory cytokine concentrations as compared to animals not receiving HKB. The experimental treatment tested was BLES/budesonide, for which intratracheal instillation was confirmed via the increased surfactant levels in the BAL fluid following the experiment. It should be noted that our experimental design did not include a BLES only treatment. However, an additional experiment with 6 pups revealed that BLES alone did not impact the inflammatory response induced by the
instillation of HKB (Figure 4.4). Based on these considerations, we deem the experimental approach an appropriate test of our hypothesis.

An important aspect of this chapter was the evaluation of a potential glucocorticoid-based treatment strategy in both males and females. Although our objective was not to understand the underlying pathways leading to potential sex differences, numerous studies have demonstrated the role of sex in patient sensitivity to glucocorticoid treatment [20–24]. Unfortunately, these previous studies have also been inconsistent with respect to their findings. For example, when developing guides to predict responsiveness among asthmatic children, both Wu et al. (2017) and Galant et al. (2014) found that the female sex was associated with a higher likelihood of responsiveness to inhaled glucocorticoids [20, 21]. On the contrary, some clinical trials and epidemiological studies have observed beneficial effects for daily glucocorticoid treatment in males, but not females [22, 23]. For the current model of pulmonary inflammation, the instillation of HKB was found to result in similar inflammatory responses among males and females. Moreover, no sex differences were found for the responsiveness of rats to either of the glucocorticoid treatments. Since sex hormones have been shown to play such an essential role in inflammatory responses, this lack of differences may be related to the young, sexually immature, age of the animals [25].

Despite these findings, the extensive role sex hormones play in modulating inflammatory pathways combined with the variability shown in human studies suggests that sex must be considered when evaluating new glucocorticoid based treatment strategies [26].

From a clinical standpoint, this chapter builds on previous work in the neonatal population. Specifically, it adds to previous clinical studies that explored exogenous surfactant or glucocorticoids as preventative treatments for poor pulmonary outcomes and
respiratory conditions like BPD [27, 28]. For example, there are a number of clinical trials which have found that administering surfactant multiple times or using it as a vehicle for budesonide may reduce the risk of BPD [29, 30]. Similarly, there have been clinical trials that have found intratracheal instillations of budesonide, with a surfactant vehicle helped to prevent the development of chronic lung disease among preterm infants [31]. The current chapter expands these prophylactic approaches in premature lungs, by demonstrating anti-inflammatory effects of this treatment strategy, subsequent to the pulmonary inflammation.

To extrapolate our data to the clinical arena, there are a variety of respiratory conditions that may benefit from an anti-inflammatory exogenous surfactant; however, its potential for treating ARDS is of particular interest. In the first half of 2020, ARDS became a well-known syndrome as the critical pulmonary complication resulting from severe acute respiratory syndrome coronavirus-2 infections, known as COVID-19. However, even before the emergence of COVID-19, ARDS was the most common cause of death in the ICU, with no effective pharmacological therapies available [32–35]. Importantly, it has been shown that disease severity and progression are directly associated with the accumulation of neutrophils into the alveolar space [36, 37], and that many aspects of the ARDS pathophysiology, such as edema formation and surfactant dysfunction, are consequences of excessive inflammation in the lung [3]. This has provided a strong rationale for glucocorticoid-based treatments, as evidenced by numerous clinical trials for ARDS and the ongoing trial for COVID-19 [38–40]. Unfortunately, to date, these highly effective anti-inflammatory medications have failed to prevent ARDS or show mortality benefits [39, 40]. One interpretation of this data is that the efficacy of the glucocorticoids is limited by suboptimal drug delivery. Based on our data, it is tempting to speculate that
exogenous surfactant, as a delivery vehicle, will allow glucocorticoids to become an effective treatment option for ARDS.

It should be noted that there are several limitations to this chapter. First, the experiments within this chapter only explored the benefits of one surfactant-glucocorticoid preparation. The improvements observed for budesonide when administered with BLES, suggest therapeutic value in exploring a similar approach for other glucocorticoids or anti-inflammatory medications. To this end, our lab intends to perform more elaborate in vivo studies with multiple commercially available glucocorticoids, like dexamethasone and hydrocortisone, to further explore the benefits of surfactant delivery. Secondly, our model of pulmonary inflammation did not imitate the edema or airway collapse observed in many respiratory conditions. The current chapter did measure protein content in the BAL, however its unchanging level across treatment groups suggests that a stronger stimulus is required to disrupt the alveolar-capillary barrier. Although there is strong scientific evidence that exogenous surfactant can overcome regions of edema and airway collapse, future studies should still evaluate this treatment strategy under these inhibitory conditions [41].

In conclusion, this chapter demonstrates that the use of exogenous surfactant as a delivery vehicle for budesonide can make it more effective for treating lung inflammation. Further, we propose that this novel treatment strategy can overcome the delivery challenges associated with respiratory conditions like ARDS and treat the neutrophilic inflammation underlying the disease. With no effective pharmacological options currently available for this condition, direct delivery with exogenous surfactant offers an intriguing method for mainstay medications to begin effectively treating this devastating disease.
References


Chapter 5: Summary, Discussion and Future Directions
5.1 General Overview

The objective of this thesis was to develop a process through which exogenous surfactants could be designed into more effective vehicles for intrapulmonary therapeutics. The challenge of direct drug delivery to the alveoli and adverse consequences of the current systemic dosing paradigm in respiratory conditions were the rationale for pursuing this novel delivery strategy [1–7]. The hypothesis of this thesis was that exogenous surfactant delivery would enhance the efficacy of antimicrobial cathelicidins and anti-inflammatory glucocorticoids for the treatment of bacterial pneumonia and ARDS, respectively. The specific pursuit of cathelicidins for surfactant-based delivery was brought on by the high incidence of bacterial pneumonia worldwide, rising rates of AMR infections, and lack of new antimicrobials in development [3, 8–16]. Similarly, the investigation of glucocorticoid-surfactant mixtures was prompted by the long clinical history of effectively treating lung inflammation with glucocorticoids and the desperate need for new therapeutics to treat ARDS [17–23]. This final section will summarize the major findings of each chapter. It will also explore their overall significance to highlight how the chapter fits into the development process for exogenous surfactants as drug vehicles, outlined throughout this thesis (Figure 5.1). Lastly, based on the literature, and some additional limitations to those discussed in the chapters themselves, this final section will propose future experiments to expand on the findings of this thesis.
**Figure 5.1.** Schematic summary of the surfactant-based drug development process explored throughout this thesis.

### 5.2 Summary of Major Findings Chapter 2: The Wet Bridge Transfer System

Chapter 2 began with the development and characterization of the wet bridge transfer system as a model system for screening surfactant-based therapeutics. Briefly, this *in vitro* platform utilized two connected wells, in which drugs were instilled into a delivery well and function was tested in a remote well, allowing the system to mimic the remote areas of the lung where drug activity would be required. The concept behind this methodology was that surfactant injected into the delivery well would adsorb to the surface and, through its spreading capabilities, transfer over the wet bridge to the remote
well. The results of this chapter demonstrated that surfactant spreading across the wet bridge was dependent on surfactant concentration and temperature, but independent of administration volume. The technique was also utilized for screening therapeutics mixed with surfactant, specifically antibiotics, by measuring their ability to kill \textit{P. aeruginosa} bacteria in the remote well, as well as anti-inflammatory agents, using stimulated macrophages in the remote well and IL-6 concentration as an outcome. The basic idea was that a drug combined with surfactant would be detected in the remote dish only if it was transported by surfactant. Through these experiments, it was observed that the potential efficacy of two antibiotics (gentamicin and ciprofloxacin) as well as two anti-inflammatory agents (budesonide and CATH-2) benefited from surfactant delivery, showing increased efficacy in the remote well compared to the drug alone. Based on these findings, it was speculated that exogenous surfactant would be a useful pulmonary drug delivery vehicle to improve the distribution of these medications in the lung. In contrast, the antibiotic, colistin was not found to benefit from surfactant delivery as it was concluded that the inhibitory effects of surfactant on colistin would limit the usefulness of this antibiotic. Alternatively, the utilization of this antibiotic with surfactant would require further optimization. Together, these findings showcased the wet bridge transfer system as a versatile screening tool for surfactant-drug preparations.

Figure 5.2 shows how these findings fit into the overall experimental paradigm of this thesis. Specifically, Chapter 2 highlighted the utility of the wet bridge transfer system as a novel \textit{in vitro} tool for rapidly screening surfactant-based therapeutics, prior to animal studies. The technique expanded on currently available \textit{in vitro} technology, including methods for evaluating surfactant biophysics, to allow surfactant-drug mixtures to be assessed based on their ability to maintain both 1) surfactant function (through its ability
to spread) and 2) therapeutic function (antibacterial or anti-inflammatory properties) at a **distal site** (Figure 5.2). Thus, this relatively inexpensive in vitro screening tool would have the potential to select optimal preparations to test in vivo and therefore reduce the number of animal experiments required.

Beyond the limitations already discussed in Chapter 2, this chapter could be further enhanced by a more rigorous biophysical analysis of surfactant-drug mixtures. Specifically, other techniques, in addition to the wet bridge, could be utilized to assess the effect of a drug on the surface tension lowering properties of an exogenous surfactant during expansion/compression cycling [24–26]. For example, future experiments could utilize the constrained sessile drop surfactometer (CDS) to further evaluate the effects of a drug on surfactant function and design better synthetic surfactants. Through this approach, researchers could put the retention of all aspects of surfactant function at the forefront of the optimization process.
Figure 5.2. Schematic summary highlighting how the new methodology of Chapter 2 (the wet bridge transfer system) fits into the surfactant-based drug development explored throughout this thesis.

5.3 Summary of Major Findings Chapter 3: Surfactant Optimization

Figure 5.3 demonstrates how this data fits into the overall experimental paradigm of this thesis. Specifically, the chapter utilized the wet bridge transfer system, in combination with other in vitro techniques, to further explore the interactions between cathelicidins and exogenous surfactant. The experiments in this chapter expanded upon a previous study from our research group, which found that suspending cathelicidins in BLES significantly impaired their antibacterial function [3]. The authors speculated that the negatively charged PG component of surfactant was inhibiting the function of these positively charged peptides. Thus, the goals of Chapter 3 were to determine if PG was an
inhibitory agent within exogenous surfactant and subsequently design a functional, PG-free synthetic surfactant to increase peptide mediated killing of bacteria at a distal site.

Although many of the experiments described in Chapter 3 were also performed with three additional cathelicidins (LL-37, CRAMP, PMAP-23), optimization was concentrated on CATH-2, the cathelicidin previously shown to best retain its antimicrobial properties when mixed with BLES [3, 14]. To better understand how surfactant lipids interacted with CATH-2 and affected its bactericidal function, isothermal titration calorimetry and solid-state nuclear magnetic resonance spectroscopy, as well as bacterial killing curves against *P. aeruginosa* were utilized. The wet bridge transfer system was also used to evaluate surfactant spreading and peptide transport (Figure 5.3).

Investigation of the interactions between individual surfactant lipids and CATH-2 revealed that PG was the only surfactant lipid to significantly inhibit CATH-2 function, showing a stronger electrostatic interaction with the peptide than other lipids. Furthermore, when the PG content of an existing surfactant was diluted, through the addition of other surfactant lipids, the suspended CATH-2 displayed improved antibacterial function. Based on these findings, it was theorized that the electrostatic interaction between the negatively charged PG and positively charged peptide was likely preventing CATH-2 from binding negatively charged lipids, DNA, or other bacterial components. Lastly, although diluting the PG content in an existing surfactant, through the addition of other lipids, significantly improved peptide function and distal killing, it also reduced surfactant spreading. It was speculated that this loss of surfactant function was caused by a dilution of not only PG, but other surfactant components, including SP-B and SP-C. To overcome this impairment in surfactant spreading, synthetic mimics for SP-B and SP-C were utilized, to develop a synthetic PG-free surfactant. Mixing this
optimized synthetic surfactant with CATH-2 further improved its delivery and function at a remote site. Based on these in vitro experiments, synthetic PG-free surfactants were deemed optimal for delivering cathelicidins to the lung.

Figure 5.3. Schematic summary highlighting how the wet bridge transfer system and new optimization approach of Chapter 3 (synthetic surfactants) fit into the surfactant-based drug development explored throughout this thesis.

The findings within this chapter highlighted two important aspects. First, the chapter demonstrated how synthetic surfactants could be designed to retain both surfactant and antimicrobial peptide function. Specifically, it showcased how the lipid composition of synthetic surfactants could be optimized to overcome their electrostatic interactions with CATH-2. As such, the chapter provided proof that synthetic surfactants could be customized to deliver a specific therapeutic. Second, the chapter illustrated a more mechanistic approach to developing surfactant-based drug vehicles through the use of synthetic surfactants and various in vitro techniques. Importantly, this approach could potentially be expanded to other therapeutics, improving suspended drug functionality, while maintaining the spreading capabilities of surfactant.
Within the chapter itself, several valid limitations were discussed. However, Chapter 3 could have also benefited from a further expansion of its optimization process. For example, with the proper insight into the structural characteristics of functional and non-functional cathelicidins, the chapter could have benefited from the investigation of designer peptides as part of its optimization of surfactant-peptide mixtures [27]. This approach would integrate the optimization of the therapeutic, in addition to the surfactant vehicle, into the optimization process outlined in this chapter. Further, although Chapter 3 thoroughly investigated the antibacterial properties of surfactant-cathelicidin mixtures, it did not directly evaluate the effects of this interaction on the anti-inflammatory properties of these peptides. There have been numerous studies demonstrating the potential of cathelicidins as anti-inflammatory agents [28, 29]. Within our own lab, we have shown the in vivo efficacy of a cathelicidin-surfactant mixture for reducing lung inflammation [14]. However, the direct in vitro analysis of their anti-inflammatory properties alone and in combination with an exogenous surfactant has been notably absent in the literature. Thus, for cathelicidins delivered by a surfactant vehicle to become viable treatment options for pneumonia, it would be recommended that future studies gain a better insight into the effect of exogenous surfactants on their immunomodulatory properties.

5.4 Summary of Major Findings Chapter 4: Relevant Animal Model

To expand on the in vitro findings of Chapter 2, this chapter explored the utility of a surfactant vehicle for budesonide in vivo. Figure 5.3 demonstrates how utilizing a relevant animal model of pulmonary inflammation fits into the overall experimental
paradigm of this thesis. Specifically, an animal model of pulmonary inflammation was created, through an intratracheal instillation of heat-killed *P. aeruginosa* into the lungs of male and female rats. This inflammation was then treated thirty minutes later using a second intratracheal instillation of either saline, budesonide, or BLES combined with budesonide. The therapeutic efficacy of budesonide compared to surfactant delivered budesonide was determined by measuring various markers of inflammation in the BAL and lung tissue. Although budesonide exhibited anti-inflammatory effects when administered alone for downregulating the number of neutrophils in the BAL, delivery with BLES significantly enhanced those effects. Moreover, the BLES/budesonide combination was found to significantly lower several other pro-inflammatory mediators in the BAL and lung tissue compared to the drug alone. With these results being shown across both sexes, it was concluded that utilizing exogenous surfactant to deliver budesonide made it more effective for treating lung inflammation.

Chapter 4 has several significant findings as well as limitations. However, its overall significance was its assessment of this surfactant-glucocorticoid mixture in a relevant animal model (Figure 5.4). Specifically, it demonstrated that this anti-inflammatory surfactant, administered after an inflammatory insult, was more effective for reducing neutrophil-driven lung inflammation than the drug alone for both male and female animals. These *in vivo* findings have clinical significance, as they further support this therapy as a potential treatment for inflammatory conditions, notably ARDS. Based on the chapter’s findings, it is tempting to suggest that a surfactant vehicle would allow glucocorticoids to overcome the delivery challenges associated with ARDS and treat the underlying neutrophilic inflammation. However, the clinical relevance of this animal model to ARDS is a major limitation of Chapter 4. For example, as mentioned in the
chapter itself, this rat model of lung inflammation did not imitate the edema or airway collapse observed in ARDS patients. Further, this model and its outcomes did not fulfill the criteria outlined by the American Thoracic Society guidelines for ARDS animal models [30]. Notably, the guidelines recommend achieving at least three of the following four criteria: 1) evidence of physiological dysfunction, 2) histological evidence of tissue injury, 3) alteration of the alveolar capillary barrier, and 4) the presence of an inflammatory response [30]. Thus, despite the supportive findings in this chapter, truly understanding the therapeutic potential of a surfactant delivered glucocorticoid for ARDS, would require a more clinically relevant animal model of the disease.
Figure 5.4. Schematic summary highlighting how the *in vivo* assessment of BLES/budesonide in Chapter 4 (relevant animal model) fits into the surfactant-based drug development explored throughout this thesis.

### 5.6 Future Directions: Cathelicidins

As mentioned above, most of the research surrounding cathelicidins, as well as their combination with exogenous surfactants has focused on their ability to kill AMR bacteria. However, their ability to regulate the immune system could also be beneficial for treating bacterial pneumonia. Unfortunately, it is unknown if exogenous surfactant inhibits the immunomodulatory properties of these peptides. Specifically, their ability to electrostatically bind negatively charged microbial by-products could be a potential mechanism inhibited by a surfactant vehicle. The wet bridge experiments within Chapter
2 established an *in vitro* model for evaluating the anti-inflammatory properties of therapeutics, including a cathelicidin. As such, this model could be easily modified to directly assess the anti-inflammatory properties of different cathelicidins alone or in combination with an exogenous surfactant. Based on the findings of these initial wet bridge experiments, as well as the extent of surfactant’s inhibitory effects, the next logical step would be optimization. The approach would be similar to Chapter 3, investigating the effect of different lipid components on the anti-inflammatory function of different cathelicidins, with the ultimate goal of developing a more ideal synthetic surfactant. Lastly, optimal surfactant-cathelicidin combinations should also be explored *in vivo*. Specifically, in a relevant animal model for pneumonia with outcomes for both their antimicrobial and anti-inflammatory effects.

The goal of the wet bridge experiments should be to screen different cathelicidins in combination with a surfactant vehicle. However, the *in vitro* model could also be expanded. For example, the wet bridge experiments could utilize primary or alveolar macrophages isolated from animals or even ARDS patients. Further, a more diverse range of inflammatory outcomes could be measured with additional techniques. For example, cells and media could be collected for measuring NF-κB activation, as well as the level of other inflammatory cytokines (TNF-α, IL-1β, and IL-8) by qPCR and ELISA. To gain a better functional measurement of the inflammatory response, media could also be collected for a neutrophil chemotaxis assays, an approach that would require a chemotaxis chamber. By expanding outcomes to include a greater variety of pro-inflammatory mediators, these experiments will further the current understanding of the immunomodulatory properties of cathelicidins in combination with exogenous surfactant.
5.5 Future Directions: Glucocorticoids *In Vitro*

As mentioned previously, the biophysical analysis of synthetic surfactants in combination with various therapeutics could be expanded beyond the wet bridge transfer system. Notably, glucocorticoids, one of which was explored *in vitro* in Chapter 2 and *in vivo* in Chapter 4, have been shown to negatively impact the surface tension lowering properties of exogenous surfactants during expansion/compression cycling [31–33]. Thus, through the use of additional techniques that can assess surfactant function during expansion/compression cycling, the optimization process established in Chapter 3 could be expanded to create ideal synthetic surfactants for glucocorticoids.

A common interpretation of the inhibitory effects of glucocorticoids on surfactant function is that they are associated with the structural similarity between glucocorticoids and cholesterol [31–34]. Briefly, at normal levels within pulmonary surfactant, cholesterol associates with DPPC containing domains and has minimal impact on surfactant function (Figure 5.5). In contrast, elevated cholesterol, beyond levels that will only interact with DPPC, will begin to alter the non-DPPC containing domains [35, 36]. This creates a more fluid structure, which has been shown to impair surfactant function. Therefore, developing a functional cholesterol-free synthetic surfactant, with a high DPPC concentration would be ideal for delivering glucocorticoids like budesonide. The basic idea being to create synthetic surfactants less susceptible to inhibition by the glucocorticoids and/or have the capacity to carry larger amounts of the glucocorticoid to remote sites.
Figure 5.5. A schematic comparison of surfactant films with A) no cholesterol, B) normal amounts of cholesterol, and C) high amounts of cholesterol. Elevated cholesterol leads to surface film collapse and surfactant dysfunction.

To assess this concept experimentally, techniques like the CDS could be utilized in addition to the wet bridge transfer system to evaluate surfactant function [37]. Like the optimization process for cathelicidins (Chapter 3), researchers could develop synthetic surfactant mixtures using individual surfactant lipids and protein mimics, creating varying concentrations of cholesterol and DPPC. These surfactant mixtures would then be evaluated on the wet bridge system for spreading and the CDS for their function during expansion/compression. Once functional synthetic surfactants were established, these biophysical techniques could then be utilized to determine the concentration dependent inhibitory effects of various glucocorticoids compared to cholesterol. Additionally, the anti-inflammatory effects of these glucocorticoids, delivered by cholesterol-free synthetic surfactants, could be evaluated on the wet bridge, utilizing a stimulated macrophage model (Chapter 2; see section 5.6 Future Directions: Cathelicidins). Lastly, to further explore glucocorticoid-surfactant mixtures as a therapy for ARDS, it would be recommended that the specific glucocorticoids, dexamethasone, methylprednisolone, and hydrocortisone, be investigated, in addition to budesonide. These glucocorticoids are all
commercially available and have been previously utilized in clinical trials or animal study
for ARDS (Table 1.3) [38, 39]. Thus, their optimization with a synthetic surfactant
vehicle could result in a new arsenal of anti-inflammatory medications for ARDS to be
tested in animal studies.

5.7 Future Direction: Glucocorticoid In Vivo

Assessing the therapeutic efficacy of glucocorticoids delivered by exogenous
surfactants for ARDS, requires a clinically relevant animal model. ARDS is a complex
and heterogenous disease. However, a sepsis-induced rat model, which incorporates
mechanical ventilation would represent the clinical scenario and could expand upon the
findings of Chapter 4.

Experimentally, male and female rats could be given an intraperitoneal injection
of fecal slurry solution or undergo cecal ligation and puncture surgery to induce sepsis
[40–42]. Then rats would be connected to a rodent ventilator, with the mechanical
ventilation settings being based on protective low tidal volume guidelines currently used
in the clinical setting [18]. For treatments, rats on the mechanical ventilator would receive
an intratracheal bolus of either 1) saline, 2) glucocorticoid, 3) exogenous surfactant, or 4)
surfactant-glucocorticoid. As stated earlier, the American Thoracic Society guidelines
recommends at least three of the following four criteria for an ARDS model: 1) evidence
of physiological dysfunction, 2) histological evidence of tissue injury, 3) alteration of the
alveolar capillary barrier, and 4) the presence of an inflammatory response [30].
Therefore, the outcomes of this experiment should be based on completing these criteria.
To determine evidence of physiological dysfunction, it would be suggested that various physiologic outcomes be monitored during ventilation, including lung compliance and peak inspiratory rate. In addition, mean arterial pressure, heart rate and baseline blood gas measurements, such as arterial oxygenation could also be assessed. Like the *in vivo* experiment performed in Chapter 4, the inflammatory response within the lung could be evaluated by performing a BAL and freezing lung tissue. The BAL could be analyzed to calculate inflammatory cell counts and differentials, as well as the concentrations of various pro-inflammatory cytokines. The lung tissue could be homogenized for an MPO activity assay. However, these inflammatory outcomes could also be expanded by investigating with qPCR, macrophage polarization, or immunocytochemistry. For example, in an additional cohort of animals, lung tissue could be collected and fixed for immunohistochemical analysis of cellular influx (e.g. stain for Ly6B.2 to determine interstitial neutrophil infiltration). For assessing alterations to the alveolar-capillary barrier, a wet/dry ratio of the lung could be determined, in addition to the BAL being analyzed to determine protein levels. Vascular permeability could also be examined by measuring Evan’s blue albumin leak into the pulmonary tissue. For histological evidence of tissue injury, it would be recommended that fixed lung tissue be sectioned and scored by a pulmonary pathologist blinded to experimental groups. In addition, although not part of the recommended guidelines, we would suggest evaluating alterations in endogenous pulmonary surfactant function and pool size, perhaps through the utilization of the CDS, as well as a phosphorous assay. Lastly, there are multiple reasons for the recommendation of rats instead of mice for this model: 1) it would be easier to collect larger quantities of samples, 2) many labs, including our own lab have more experience ventilating rats, and 3) rats show greater physiological stability when placed on a ventilator than mice do [43,
Together, these factors, as well as the discussed outcomes should allow this rat model to fulfill the American Thoracic Society guidelines and assess this novel therapy in a clinically relevant ARDS model.

5.8 Concluding Remarks

Based on the data accumulated throughout this thesis, we conclude that exogenous surfactants can be utilized to enhance the distribution and therefore efficacy of intrapulmonary therapeutics. Its utility as a carrier for pulmonary therapeutics makes intuitive sense and, more importantly, is supported by scientific evidence. Chapter 2 showcased the newly developed wet bridge transfer system and how it could be utilized to study surfactant as a drug carrier, as well as screen surfactant-based therapeutics (Figure 5.2). Chapter 3 demonstrated how customized synthetic surfactant could extend the delivery benefits of a surfactant vehicle to compounds previously shown to have their therapeutic function impaired by exogenous surfactants (Figure 5.3). Further, this chapter demonstrated how a more thorough understanding into the interactions of drug-surfactant preparations, could result in synthetic surfactants specifically designed to retain a drug’s function and surfactant function. Chapter 4 showed how relevant animal models could be utilized to expand upon the currently available in vitro tools for studying surfactant as a drug carrier and assess their in vivo efficacy (Figure 5.4). Together, these chapters outlined a process for surfactant design that integrated both in vitro and in vivo studies, enhanced the understanding of surfactant as a drug carrier, and allowed for continuous feedback to improve on previous experiments (Figure 5.1). It is the opinion of this author,
that this integrative approach will be essential to translate the basic idea of surfactant as a carrier for pulmonary drugs toward clinical trials and ultimately clinical practise.

To illustrate the concepts associated with surfactant-based drug delivery the experiments within this thesis focused on bactericidal compounds (cathelicidins) for pneumonia and anti-inflammatory agents (glucocorticoids) for ARDS. However, the potential drug targets for these conditions are not limited to antimicrobial and anti-inflammatory agents. For example, a β2 agonist to reduce edema formation associated with ARDS has been investigated [45, 46]. Unfortunately, meta-analysis of the data did not show a benefit of the drug and, in fact, indicated the potential of detrimental effects [47]. Although other conclusions are feasible, inadequate drug delivery to the target site may have contributed to these undesirable results. Specifically related to the β2 agonist, delivery of this drug via aerosol will unlikely reach the edematous areas of the lung where the drug is required. Revisiting this drug, and concept of reducing edema in ARDS, by studying its combination with surfactant has the potential for different outcomes. This concept may apply not just to β2 agonists, but also to the many other pharmacological agents that have been unsuccessfully tried in patients with pneumonia or ARDS [19, 48, 49]. Additionally, whereas this thesis primarily focused on bacterial pneumonia and ARDS as potential clinical targets, it should also be noted that other pulmonary diseases may be equally appropriate for the utilization of surfactant-based therapies. For example, drugs for pulmonary fibrosis or emphysema, marked by a thickening and destruction of the alveolar walls respectively, may be potential targets for such investigations. Further, one can also imagine delivery of other components, for example contrast agents for various imaging approaches, to the lung via surfactant-based administration. As such, we
are certain that surfactant as a vehicle for various drugs and other compounds is a promising approach in a variety of circumstances requiring pulmonary delivery.

Lastly, this thesis provided strong in vitro and in vivo data supporting the more targeted use of cathelicidin- and glucocorticoid-fortified surfactants in patients afflicted with bacterial pneumonia or ARDS. For cathelicidin-fortified surfactants, the combination is clearly a bit behind in its development towards clinical trials compared to glucocorticoids. However, based on the strong scientific data presented throughout this thesis, they clearly have potential and should be explored in relevant animal models. For glucocorticoids, the data from our thesis implies that the combinatorial effect of exogenous surfactant with these drugs may overcome the limitations of those observed for each therapy, in previous clinical trials [50–57]. As such, the next logical step for the developmental process presented in this thesis would be to assess the combination in large animal models and design phase 1 or 2 clinical trials. The assessment of this therapeutic combination in clinical trial is also supported by the ongoing trials of surfactant therapy to treat COVID-19 patients and surfactant-glucocorticoid combination in infants [58–62]. Simply put, the glucocorticoid-surfactant approach is a repurposing of two clinically approved drugs that are currently utilized in humans [63, 64]. Thus, the initiation of such trials should be relatively straightforward.
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**Topic:** The Biophysical Alterations to Pulmonary Surfactant during Lung Injury

**RELATED WORK**

**EXPERIENCE:**

Teaching Assistant Physiology 2130 (2018—2021)

The University of Western Ontario, Canada

Teaching Assistant Physiology 3000E (2017)

The University of Western Ontario, Canada.

Teaching Assistant Physiology 3130z (2016)

The University of Western Ontario, Canada.

**PUBLICATIONS:**

**Original Articles:**


2. Veldhuizen R, **Baer B,** McCaig L, Solomon LA, Cameron L, and Hardy DB. (2020)


**Review articles:**


**Published Abstracts:**


### SCHOLARSHIPS & AWARDS:

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<td>05/21</td>
<td>The Lucille &amp; Norton Wolf Trainee Publication Awards London Health Research Day</td>
<td>$1000</td>
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<td>“Optimizing Exogenous Surfactant as a Pulmonary Delivery Vehicle for Chicken Cathelicidin-2” published in Scientific Reports / Nature</td>
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<td>11/20</td>
<td>Clinical and Basic Pharmacology Poster Award</td>
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<td>Physiology and Pharmacology Research Day</td>
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<td>Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST)</td>
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<td>Poster Presentation Award for Advances in Structural and Physiological Treatment of Disease and Therapeutic Intervention</td>
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<td>London Health Research Day “Exogenous Surfactant as a Pulmonary Drug Delivery Vehicle for Budesonide”</td>
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<td>“Using the Wet Bridge Transfer System to Assess Exogenous Surfactant as a Pulmonary Drug Delivery Vehicle”</td>
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<td>“A Novel Surfactant-Based Host Defense Peptide for the Treatment of Antibiotic-Resistant Lung Infections”</td>
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<td>Queen Elizabeth Aiming for the Top Students Scholarship</td>
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**PRESENTATIONS:**

**Podium Presentations**

1. Exogenous surfactant as a drug delivery vehicle for mainstay glucocorticoids in the treatment of ARDS. (Jun. 2021) Canadian Society of Pharmacology and


15. Cath-2 suppression of pulmonary inflammation following the instillation of killed cystic fibrosis pathogens. (Oct. 2016) Student Development and Recognition Program, Lawson Health Research Institute, London, Canada

16. Lung Lavage and Surfactant Replacement for the Ex Vivo Pre-transplant Treatment of Donor Lungs Injured Due to Gastric Acid Aspiration. (September 2015). London-Toronto Lung Research Symposium, St. Joseph’s Hospital, London, Canada
Posters Presentations

4. Using the Wet Bridge Transfer System to Assess Exogenous Surfactant as a Pulmonary Drug Delivery Vehicle. (May 2018) Joint Annual Meeting for Canadian Society of Pharmacology and Therapeutics, Toronto, Canada

Broadcast Interviews

2017/12/12 "Be gone Bacteria, you Baerly had a chance", GradCast, Radio Western, 30 minutes
2017/03/25 "All About the Lungs at Western Research Forum 2017", GradCast, Radio Western, 25 minutes.