

# A Theoretical Exploration of the Growth and Stability of Alveolospheres

by

Joseph Bonavia

Submitted to the  
Department of Mechanical Engineering  
in Partial Fulfillment of the Requirements for the Degree of

Bachelor of Science in Mechanical Engineering

at the

Massachusetts Institute of Technology

May 2022

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## **ABSTRACT**

An alveolosphere is a type of stem cell derived lung organoid. They have a distinct “balloon-like” structure which resembles the alveoli in human lungs. In recent years, they have become increasingly popular as model systems for disease research and treatment development, especially with the onset of COVID-19. Patients born with a rare mutation on both copies of their SFTPB genes face severe respiratory issues after birth which often lead to poor outcomes. Lung cells derived from the stem cells of patients with this double-mutation fail to form the complex structure indicative of successful alveolosphere development. The tension-dominated nature of this structure reveals that the biology of the formation of alveolospheres is heavily coupled with their mechanics. Therefore, in order to fully understand an alveolosphere’s biology it is essential to understand its mechanics. This thesis outlines a theoretical framework which, in conjunction with targeted experiments, could serve as the basis for a mathematical theory of the development and growth of alveolospheres. Such a theory would provide a better understanding of what needs to go right, and what can go wrong, during alveolosphere development. By extension, this framework offers a path forward towards the discovery of new treatments for genetic and pathological lung diseases that directly affect alveoli.

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## ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude to my thesis supervisor, academic advisor, and first UROP supervisor, Professor Ming Guo. During each of the past four years Professor Guo has offered encouragement, support, and mentorship. I do not think I would be pursuing academia now if I hadn't gotten that kind of encouragement from him and others. Next, I would also like to thank Professor Tal Cohen for welcoming me into her group this year, talking through this problem with me, and encouraging me to explore and grow. I also want to express thanks to a list of some of the many others who helped to guide me on my path through undergrad here at MIT. In no particular order, thank you to Professor Rohan Abeyaratne, Professor Asegun Henry, Professor Irmgard Bischofberger, Professor David Perreault, and (soon to be Doctor) Jessica Boles. A special thank you to the Pappalardo "Crew" who made sure I knew how to use my hands and keep a sense of humor.

I would like to thank my "safety net" of family and friends here and at home, who have always been there for me no matter how many thousands of miles away I was. Thank you to my girlfriend Tiana Monge for putting up with a lot of silence during the hours I spent working on PSets, classwork, and research and for always being there in the best and worst of times. Thank you to my parents, Maria and Edwin for being patient with infrequent calls, and for always offering me support in any way they could. Thank you to my sisters Karen and Cynthia for always being a phone call away and for all the help proofreading papers and emails. Thank you also to my brother-in-laws Ale and Angelo, my niece and three nephews, Chiara, Luca, and Alessio, and to my many aunts, uncles, and cousins.

One last final note of thanks to my grandmother Margaret "Gerita" Agius. She taught me the true meaning of grit and strength and always believed I could be someone special. Rest in peace, Nanna.



# TABLE OF CONTENTS

<b>Abstract</b>	3
<b>Acknowledgements</b>	5
<b>Table of Contents</b>	7
<b>List of Figures</b>	8
<b>List of Tables</b>	9
<b>1. Introduction and Background</b>	11
<b>1.1 Organoids and Organs-on-chips</b>	11
<b>1.2 Alveolospheres</b>	12
<b>1.2.1 Physical Overview</b>	12
<b>1.2.2 Applications</b>	14
<b>1.3 Developing a Framework</b>	15
<b>2. Structure Model of Alveolospheres</b>	16
<b>2.1 Force Balance</b>	17
<b>2.1.1 Radial Membrane Network</b>	17
<b>2.1.2 Outer and Inner Wall</b>	18
<b>2.2 Pressure as a Possible Limit to Growth</b>	19
<b>3. Thermodynamics of Alveolospheres</b>	20
<b>3.1 Transport Processes</b>	20
<b>3.1.1 Ion Conservation</b>	21
<b>3.1.2 Volume Conservation</b>	23
<b>3.2 Possible Energetic Limits</b>	25
<b>4. Large Alveolospheres in Static Equilibrium</b>	27
<b>4.1 Simplifications</b>	27
<b>4.2 Order of Magnitude Estimations</b>	28
<b>5. A Path Forward</b>	31
<b>5.1 Dynamics of Alveospheres</b>	31
<b>5.2 Suggested Experiments</b>	32
<b>#. Summary and Conclusion</b>	34
<b>#. Bibliography</b>	37

## LIST OF FIGURES

<b>Figure 1-1:</b>	Side-view and Cross-Sectional Images of 3D Alveolospheres	13
<b>Figure 1-2:</b>	Diagram of Bronchiole and Alveolar Sacs	13
<b>Figure 2-1:</b>	Alveolosphere Geometry and Pressure Diagram	16
<b>Figure 2-2:</b>	Alveolosphere Subsystem Free Body Diagrams	17
<b>Figure 3-1:</b>	Alveolosphere Transport Process Diagram	21



## LIST OF TABLES

**TABLE 4-1:** Estimated Values of Parameters

29



# 1. INTRODUCTION AND BACKGROUND

Historically, fundamental research into the effects of diseases and treatments on specific organs relied on the use of animal models, which can differ significantly from humans both structurally and chemically [1]. Until recently there were few alternatives to animal-based studies. Recent developments in our understanding of pluripotent stem cells (PSCs) have allowed for the development of organoids; small clusters of cells that resemble the tissue of a specific organ; for use as model systems. Organoids allow for investigation of the effects of diseases and treatments on human organs in living human cells. Organoids are often the much more biologically simple than similar *in vivo* tissue, but can still demonstrate complex structure. Understanding the structure and physics of organoids can also give great insight into the role mechanics plays in the development (or dysfunction) of human organs.

An alveolosphere is a fluid-filled, spherical lung organoid that gives insight into the development of human alveoli. Alveoli play an important role in gas exchange in the lungs, allowing for the absorption of oxygen into, and diffusion of carbon dioxide out of, the bloodstream [2]. The dysfunction of alveoli play a significant role in lung diseases including pulmonary fibrosis, COVID-19, and congenital lung disorders. Therefore, alveolospheres have become a valuable tool in research into lung disease and dysfunction [2-5].

Understanding how and when alveolospheres are able to form and maintain their complex spherical structure may prove an effective tool in lung-disease research. Their tension-dominated balloon like structure is mechanically complex. Because they are so tightly coupled, understanding the mechanics of alveolospheres is critical to understanding their biology. This thesis aims to create a mathematical framework which can be used to understand the growth and development of alveolospheres.

## 1.1 Organoids and Organs-on-chips

In recent years, human organoids have become increasingly important model systems in biology and biological engineering. In simplest terms, an organoid is a specialized cluster of cells, derived from pluripotent stem cells (PSCs) or adult stem cells (AdSCs), which resemble tissue of a specific organ chemically and functionally [1]. Stem cells can be thought of as the “most generalized” type of human cells. Through the addition of growth factors and other hormones,

PSC's and AdSCs can be coaxed into specializing into the cells that form organ-specific tissue. These specialized cells then form organoids which resemble the structure of their associated organ. As organoids develop, they often resemble the early stages of *in vivo* organ development [1].

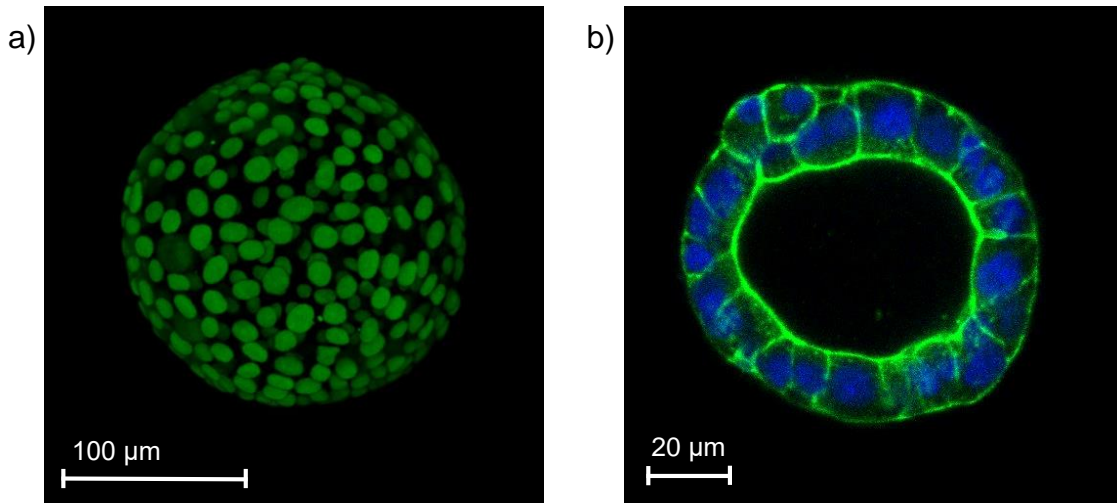
Organoids do not self-vascularize and therefore are often bounded in size by the limits of oxygen diffusion into their cores [6]. Non-vascularized PSC and AdSC derived organoids still present an exciting opportunity as model-systems for isolating and understanding the effect of specific diseases. They have already been utilized as model systems to better understand the diseases that affect at least eleven different organs ranging from the heart to the inner ear, and help to develop treatments for them [7]. In recent years, significant strides have been made in efforts to vascularize organoids to create systems more representative of whole organs. Many of these systems, often referred to as organs-on-chips (OOCs) are being designed with modularity in mind and aim to recreate as much organ functionality as possible [8]. The coming decade will prove instrumental in the development of successfully vascularized OOCs and the implementation of multi-organoid OOCs [9].

## **1.2 Alveolospheres**

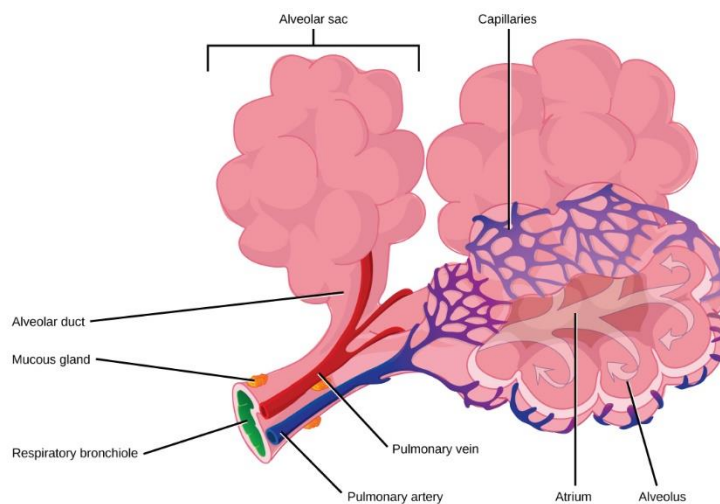
An alveolosphere is a lung organoid that can be derived from both AdSCs and PSCs. Alveolospheres are made up of the same cells and form a similar structure to that of alveoli, the millions of micrometer scale sacs that line the walls of the lungs and assist with the exchange of oxygen and carbon dioxide [2]. The importance of the of alveoli to lung function along with their unique balloon-like structure make alveolospheres an interesting and worthwhile candidate for study.

### **1.2.1 Physical Overview**

Alveolospheres are a particularly interesting in that they have a high degree of similarity to their *in vivo* counterparts, alveoli (singular alveolus). As their name suggests alveolospheres are spherical in shape (as shown in figure 1-1 a). However, unlike many other organoids, alveolospheres are not solid clusters of cells. Instead, the cells of an alveolosphere form a one-cell-thick spherical “bubble” filled with extracellular fluid (as shown in figure 1-1 b). During development alveoli are also relatively spherical in shape and remain so externally. *In vivo*, alveoli group together in structures called alveoli sacs and internally join together to form a much more complex structure (as shown in figure 1-2).



**Figure 1-1:** Side-view and cross-sectional view of 3D alveolospheres. a) Side-view of fully developed 3D alveolosphere roughly 180  $\mu\text{m}$  in diameter. Cell nuclei are marked in green. b) Cross-sectional view of a 3D alveolosphere earlier in development. Cell nuclei are marked in blue, and F-actin is marked in green. Cells in the wall of the alveolosphere remain a relatively constant 12 – 15  $\mu\text{m}$  throughout development of the alveolosphere. Captured using a Leica SP8 confocal microscope at a 25x/0.9 water objective. Images courtesy of Wenhui Tang, MIT GuoLab.



**Figure 1-2:** Diagram of a respiratory bronchiole and alveolar sacs. During development spherical alveoli (singular alveolus) join together to form alveolar sacs. Alveolar sacs facilitate gas exchange between the lungs and pulmonary blood vessels via their proportionally large surface area [2, 10]. This file is licensed under the [Creative Commons Attribution 4.0 International](https://creativecommons.org/licenses/by/4.0/).

Alveolospheres begin development as a single alveolar epithelial cell, derived from a PSC or AdSC through the addition of growth factors and other hormones. The epithelial cell is then placed into a growth medium such as matrigel. When the cell first splits, a fluid-filled, surfactant-

lined, almond shaped “bubble,” called a lumen, forms between its daughter cells [3-5, 11]. As the cells continue to divide, they form the aforementioned spherical balloon shape, which expands into the growth medium until growth halts at a relatively consistent size [3-5].

Because alveolospheres are not solid clusters of cells this means that they are not subject to the same oxygen diffusion limits as other organoids and are instead limited by many of the same physical phenomena which govern the development of *in vivo* alveoli. As a result, alveolospheres halt growth at a size remarkably similar to that of alveoli. Images provided in [2], point to a rough-estimate of the average diameter of a full grown alveolosphere of ~100 to 300  $\mu\text{m}$ . A 2004 study on the size and number of alveoli in human lungs found a “rather constant” mean diameter of  $200 \pm 10 \mu\text{m}$  “irrespective of lung size” [12]. This implies that the limits on alveolosphere growth may be controlled not only by biological regulation, but also fundamental mechanical principals.

As alveolospheres grow, one rather unusual trait is that the thickness of the cells that make up the wall of the alveolospheres remain relatively constant (12 to 15  $\mu\text{m}$ ) in thickness in the radial direction. As the cell grows the density of cells per unit surface area of the alveolosphere increases implying that the volume per cell decreases.

Surfactants are particularly important to the development, structure, and function of both alveoli and alveolospheres. Surfactants are chemicals secreted by the cell to reduce the surface tension at the air cell interface of an alveoli. They are present on the inner surfaces of both alveoli and alveolospheres. By decreasing the surface tension less internal pressure is required to maintain their spherical shape. Additionally, surfactants reduce fluid accumulation in the alveolar sacs and prevent alveoli from drying out. Dysfunction of alveoli affecting surfactant production is often deadly and therefore understanding the role surfactants play in the physics of alveoli may help save lives (as will be discussed in 1.2.2) [5].

### 1.2.2 Applications

Most lung diseases and disorders directly affect the alveoli. Dysfunction of the alveoli directly affects the body’s ability to absorb necessary oxygen from the air and expel carbon dioxide from the bloodstream. Therefore, understanding the structure, development, and dysfunction of alveoli is critical to developing treatments for said diseases [2].

Although they are a relatively new technology, lung organoids, and alveolospheres more specifically, have proven themselves valuable as model systems to assist in understanding lung

disease and dysfunction over most of the past decade [2]. For most of this time use of alveolospheres and other lung organoids has been focused on the investigation of pneumonia, fibroblasts and lung fibrosis, and lung cancer [7]. Since 2020, alveolospheres have gained some popularity as a model system for understanding the effect of COVID-19 on alveoli [4].

When an infant is born with a rare genetic mutation on both copies of *SFTPB*, a gene thought to control surfactant production in the alveoli, respiratory failure and death are a common outcome [12]. When epithelial alveolar stem cells are derived from the PSCs of patients who have had this rare double mutation, the epithelial cells do not form an alveolosphere, but instead cluster together in a much more disorganized structure [5]. The stability of bubbles and drops are canonical problems in mechanics because tension dominated systems are so clearly governed by fundamental physical laws. Alveolospheres, although also subject to the constraints of biological systems, are bound by these same fundamental physical laws. Therefore, understanding the mechanics of alveolospheres and what needs to go right to form them, may give great insight what exactly is going wrong in patients who suffer from rare lung diseases like this, and what possible directions to look for treatments or cures.

### **1.3 Developing a Framework**

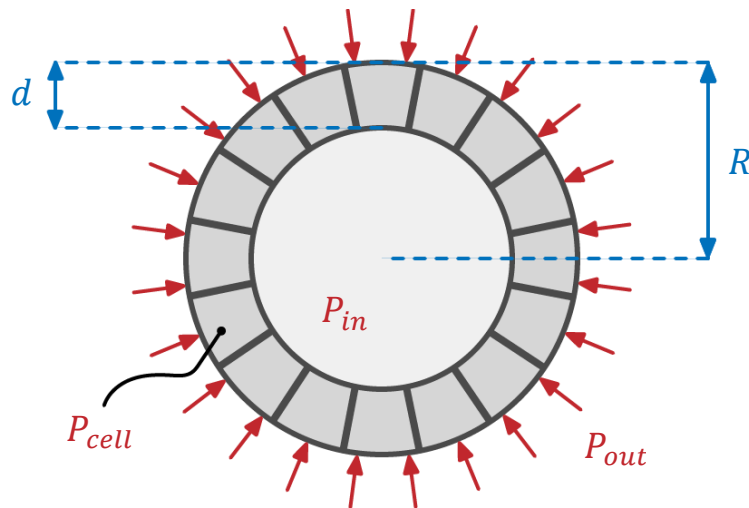
The goal of this thesis is to develop a basic mechanical and thermodynamic framework which can then be used to develop more complex models of the growth and development of alveolospheres. This framework is based on fundamental physical laws and bases many of its biological assumptions on those made in [11]. Chapters 2 and 3 of this thesis lay out this framework. Chapter 2 details a structural and mechanical model of an alveolosphere. Chapter 3 details a thermodynamic model of an alveolosphere and is broken up into two major sections. Section 1 outlines passive, active, and osmotic transport processes within an alveolosphere. Section 2 considers an energy limits that may halt alveolosphere growth.

Chapter 4 applies the framework of sections 1 and 2 in the limit of a large, thin-walled alveolosphere in static equilibrium. In this limit it is demonstrated that the framework can accurately attain an order of magnitude estimate for the size of alveolospheres. Chapter 5 outlines a set of experiments which may be used to verify the assumptions made in this framework, and what a dynamic model based on this framework may look like. By understanding the mechanics of alveolospheres and by extension the biology to which they are so strongly coupled.

## 2. Structural Model of Alveospheres

The non-linearity and complexity of the contents of a cell have led to the development of a large number of methods and models for describing the mechanics of cells [13]. In this thesis a cell will be modeled as a thin fluid filled membrane (as was the case in [11]).

We will begin by modeling an alveosphere as a spherically symmetric shell of radius  $R$  made of cells of radial thickness  $d$ , as shown in figure 2-1. We assume the shell is subdivided into  $N$  cells by a network of radially oriented cell walls. As discussed in section 1.2.1, it can be assumed that  $d$  remains a constant value throughout the alveosphere growth process. This means that as  $R$  increases  $d$  becomes proportionally smaller, which will aid in simplifying the model in the limiting case of a large alveosphere (in chapter 4). The cytoplasm in the cells is modeled as a fluid in constant pressure  $P_{cell}$  throughout the cell, and it is assumed that the cell is growing into some solid-continuous media with a known stretch-energy constitutive relationship (elastic, viscoelastic, poroelastic). Therefore, it is also assumed the pressure at the outer surface of the alveosphere  $P_{out}$  is a known function of  $R$ . It is assumed that the inside of the alveosphere is filled with extracellular fluid at a constant pressure  $P_{in}$ .

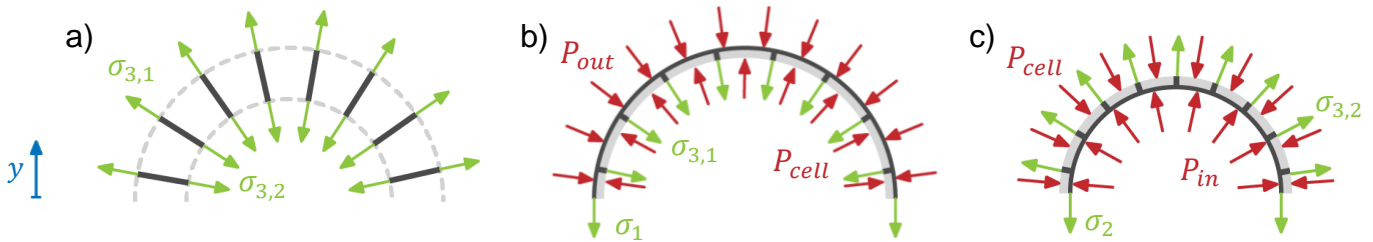


**Figure 2-1:** Diagram of an alveosphere of radius  $R$  and radial cell thickness  $d$ . It is assumed  $d$  remains constant throughout alveosphere growth (increasing  $R$ ). It is assumed that the alveosphere is subdivided into  $N$  cells via radially oriented cell walls. The cytoplasm in the cells is modeled as a fluid at pressure  $P_{cell}$ . The alveospheres outer is at a pressure  $P_{out}$  due to the deformation of the growth medium. Extracellular fluid in the alveosphere is at a pressure  $P_{in}$ .



## 2.1 Force Balance

By breaking down the alveolosphere into three subsystems and performing force balance on each of them, it is then possible to relate the pressures to the tensions in each section of the cells' membranes. These three subsystems are the radial network of cell walls (figure 2-2 a), the outer walls of the cells (figure 2-2 b), and the inner walls of the cells (figure 2-2 c).



**Figure 2-1:** Free body diagrams sub-systems of an alveolosphere. a) The radial membrane network which separates the cells can be assumed to be in a state of hydrostatic pressure  $P_{cell}$  circumferentially. At the boundary outer and inner boundary the membrane network has perimeter  $L_1$  and  $L_2$  in tension per length  $\sigma_{3,1}$  and  $\sigma_{3,2}$  respectively. b) The outer membrane can be assumed to be in circumferential tension per unit length  $\sigma_1$ . It also experiences radial tension per unit length  $\sigma_{3,1}$  on its inner surface from the radial membrane network and pressures  $P_{out}$  and  $P_{cell}$  on its outer and inner surfaces respectively. c) The inner membrane can be assumed to be in circumferential tension per unit length  $\sigma_2$ . It also experiences tension per unit length  $\sigma_{3,2}$  on its outer surface from the radial membrane network and pressures  $P_{out}$  and  $P_{cell}$  on its outer and inner surfaces respectively.

### 2.1.1 Radial Membrane Network

It can be assumed that due to the spherical symmetry, the network of membranes is in under hydrostatic pressure  $P_{cell}$  in the circumferential direction. This implies that we can consider only the radial forces due to tension in the membrane network. Because the perimeter of the network is not constant in the radial direction, but the force must remain constant in the radial direction to maintain equilibrium, the tension per length in the membrane changes in the radial direction.

The perimeter of the cell wall network at the outer wall is defined as a function of  $R$  and  $N$ ,  $L_1\{R, N\}$  and the tension per unit length in the membrane at the outer wall is defined as  $\sigma_{3,1}$ . Likewise, the perimeter and tension in the membrane network at the inner wall are defined as

$L_2\{(R - d), N\}$  and  $\sigma_{3,2}$  respectively <sup>1</sup>. In the following derivations it is assumed that all growth happens slowly enough to neglect inertial effects. By assuming the net of the membrane network is relatively spherically symmetric and evenly distributed, a force balance can be performed on the one half of the membrane network in the y direction (as shown in figure 2-2 a) yielding:

$$\Sigma F_y = \frac{\pi R^2}{0.5\{4\pi R^2\}} L_1 \sigma_{3,1} - \frac{\pi(R-d)^2}{0.5\{4\pi(R-d)^2\}} L_2 \sigma_{3,2} = 0. \quad (1)$$

The magnitude of the Cartesian force resulting from the tension in the membrane network on a hemispherical half of the alveolosphere at the inner and outer wall is thus given as:

$$F_3 = \frac{1}{2} L_1 \sigma_{3,1} = \frac{1}{2} L_2 \sigma_{3,2}. \quad (2)$$

### 2.1.2 Outer and Inner Wall

It is assumed that the cell walls that make up the outer wall of an alveolosphere are in a uniform state of circumferential tension per unit length  $\sigma_1$ . As shown in figure 2-2 b, the outer membrane also experiences pressure  $P_{out}$  on its outer surface due to compression of the growth medium, pressure  $P_{cell}$  on its inner surface due to pressure in the cytoplasm, and the tension per unit length  $\sigma_{3,1}$  on its inner surface from the outer perimeter of the radial membrane network. Using equation (2), and accounting for the 3D geometry of the alveolosphere, the following force balance in the y direction can be attained:

$$\Sigma F_y = \pi R^2 P_{cell} - \pi R^2 P_{out} - 2\pi R \sigma_1 - F_3 = 0. \quad (3)$$

The pressure difference across the outer membrane of the alveolosphere can be defined as:

$$\delta P_1 = P_{cell} - P_{out}. \quad (4)$$

Thus,

$$\delta P_1 = \frac{2\sigma_1}{R} + \frac{F_3}{\pi R^2}. \quad (5)$$

Likewise, it can be assumed that the inner wall is in a state of uniform circumferential tension per unit length  $\sigma_2$ . As shown in figure 2-2 c, the inner membrane also experiences pressure

---

<sup>1</sup>  $L_1$  and  $L_2$  can be approximated by assuming that the inner and outer surfaces of the cells in the wall of the alveolosphere form an organized and relatively symmetric shape such as a Goldberg polyhedron. When  $N$  is small ( $2 < N < 12$ ) it may be assumed that the cells take the form of a spherical triangles of equal size. For  $N = 2$  the force balance must change to reflect lumen formation [11].

$P_{cell}$  on its outer surface due to pressure in the cytoplasm, pressure  $P_{in}$  on its inner surface due to pressure in the extracellular fluid in the alveolosphere, and the tension per unit length  $\sigma_{3,2}$  on its outer surface from the inner perimeter of the radial membrane network. Again using equation (2), and accounting for the 3D geometry of the alveolosphere, the following force balance in the  $y$  direction can be attained:

$$\Sigma F_y = \pi(R - d)^2 P_{cell} - \pi(R - d)^2 P_{out} - 2\pi(R - d)\sigma_1 + F_3 = 0. \quad (6)$$

The pressure difference across the inner membrane of the alveolosphere can be defined as:

$$\delta P_2 = P_{in} - P_{cell}. \quad (7)$$

Thus,

$$\delta P_2 = \frac{2\sigma_2}{(R-d)} - \frac{F_3}{\pi(R-d)^2}. \quad (8)$$

## 2.2 Pressure as a Possible Limit to Growth

It is assumed that in order to maintain a “wrinkle-free” structure all sections of cell-membrane must remain under (at least some) tension. Therefore, we can further assume that because no wrinkles are observed in a healthy alveolosphere  $\sigma_1$ ,  $\sigma_2$ , and  $F_3$  are all positive. From equation (4) we find that in the case that both  $\sigma_1$  and  $F_3$  are both positive,  $\delta P_1$  must also be positive, enforcing that  $P_{cell} > P_{out}$ . This means that that the pressure inside the cell must always be greater than the pressure at the outer wall due to the deformation of the growth medium. In the case that  $P_{out}$  increases with  $R$  from its initial value, this means that pressure in the cell must also increase. If any of the processes within the cell are pressure sensitive this could lead to the halting of alveolosphere growth or even cell death.

### 3. Thermodynamics of Alveolospheres

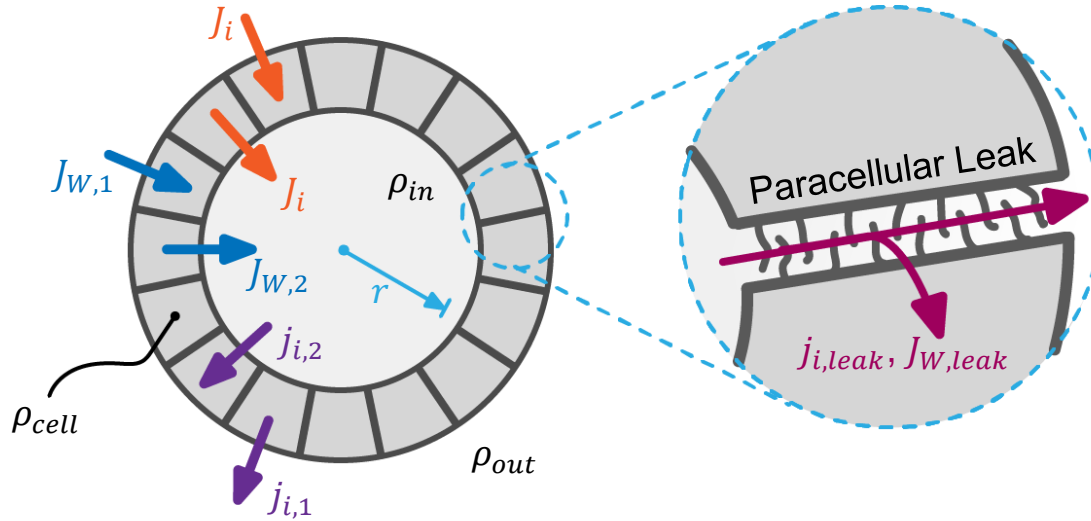
As in many other biological systems, thermodynamics, especially transport processes; are critical to the growth and development of alveolospheres. Much of the transport analysis in this chapter is based on Dasgupta's work on lumen growth [11], as the development of alveolospheres is governed by many of the same physical phenomena. Section 3.1 details a model for the transport processes in alveolospheres and using osmotic pressure, allows for coupling of the structural mechanics and thermodynamics. Section 3.2 considers possible energetic limits to the size to which alveolospheres can grow.

#### 3.1 Transport Processes

As shown in figure 3-1, there are three major transport processes that are considered significant across the cell membranes: active ion pumping, flow through passive ion channels, and osmotic flow of water. Additionally, leakage flow radially between cells from the central "bubble" of ions and water into the growth medium, known as a paracellular leakage, may be significant. It is assumed that diffusion across the membrane occurs at a much longer timescale than fluid mixing, and thus all concentration gradients except for those across a membrane or between cells can be neglected. It is assumed that the fluid which permeates the growth medium outside of the alveolosphere is at a uniform ion concentration  $\rho_{out}$ . Inside the cell, it is assumed that the cytoplasm is at a uniform ion concentration  $\rho_{cell}$ . Inside the alveolosphere, it is assumed that the extracellular fluid is at a uniform concentration  $\rho_{in}$ . A radial coordinate  $r$  is defined as the distance from the center of an alveolosphere to a given point.

Active ion pumping, represented by the molar flux  $J_i$ , is the active pumping of ions across a cell membrane. It is assumed that the pumps are always on. That is,  $J_i$  is a constant and positive molar flux into the cell from the outside and into the alveolosphere center from the cell. Passive ion channel flow is represented by the molar fluxes  $j_{i,1}$  and  $j_{i,2}$ , across the outer and inner membrane respectively, and is dependent on the concentration gradient across a membrane. Osmotic flow of water is represented by the water volume fluxes  $J_{W,1}$  and  $J_{W,2}$  across the outer and inner membranes respectively, and is dependent on the hydrostatic and osmotic pressure

gradients across a membrane. Paracellular leakage is dependent on each of these phenomena and can be determined by considering the chemical potential and pressure gradients between cells.



**Figure 3-1:** Alveolosphere ion concentrations and transport processes. The fluid outside the growth medium is at a uniform ion concentration  $\rho_{out}$ , the cytoplasm in the cells is at a uniform ion concentration  $\rho_{cell}$ , and the extracellular fluid inside the alveolosphere is at uniform ion concentration  $\rho_{in}$ . Active transport of ions across cell membranes is represented by the constant molar ion flux  $J_i$ . Flow through passive ion channels is represented by the concentration gradient dependent fluxes  $j_{i,1}$  and  $j_{i,2}$  across the outer and inner membranes respectively. Water flow due to osmotic and hydrostatic pressure gradients is represented by  $J_{W,1}$  and  $J_{W,2}$ , across the outer and inner membranes respectively.  $j_{i,leak}$  and  $J_{W,leak}$  represent the ion and water leakage from the alveolosphere to the growth medium via junctions between cells. A radial coordinate  $r$  is defined as the distance radial distance from the center of the alveolosphere.

### 3.1.1 Ion Conservation

As stated in the introduction to this chapter, the molar ion fluxes  $j_{i,1}$  and  $j_{i,2}$  through passive ion channels are related to the concentration gradients across the outer and inner membranes respectively. More specifically, a flux  $j_i$  through a passive ion channel from one fluid at concentration  $\rho_a$  to another fluid at concentration  $\rho_b$  is given by the relationship:

$$j_i = \Lambda_i k_B T \ln \left( \frac{\rho_a}{\rho_b} \right), \quad (9)$$

where  $\Lambda_i$  is the transport coefficient which is determined by the density of ion channels in the cell membrane [11],  $k_B$  is the Boltzmann constant, and  $T$  is the temperature of the system. It is assumed that  $\Lambda_i$ , and  $T$  are constant across the entire alveolosphere.

First, ion conservation is performed on the cytoplasm within the cells. For simplicity, it is assumed that all meaningful flow of ions into and out of the cell occurs across the inner and outer membranes. Thus, all leakage from the cellular junctions into the cells can be neglected. Applying ion conservation in the cell layer results in the following relationship between time rate of change in the number of moles of ions in the cell,  $n_{cell}$ , is related to the fluxes  $J_i$ ,  $j_{i,1}$ , and  $j_{i,2}$ :

$$\frac{dn_{cell}}{dt} = J_i(4\pi R^2) - J_i(4\pi(R-d)^2) - j_{i,1}(4\pi(R^2)) + j_{i,2}(4\pi(R-d)^2) . \quad (10)$$

Applying equation (9) to equation (10) results in the relationship:

$$\frac{dn_{cell}}{dt} = 4\pi \left\{ J_i(R^2 - (R-d)^2) + \Lambda_i k_B T \left[ -R^2 \ln\left(\frac{\rho_{cell}}{\rho_{out}}\right) + (R-d)^2 \ln\left(\frac{\rho_{in}}{\rho_{cell}}\right) \right] \right\} . \quad (11)$$

In order to make the solution analytically tractable it will be useful to linearize the logarithmic terms for small chemical potential gradients. In order to perform this linearization, it is useful to define the concentration difference across the outer and inner membrane respectively as.

$$\delta\rho_1 \equiv \rho_{cell} - \rho_{out} , \quad (12)$$

and

$$\delta\rho_2 \equiv \rho_{in} - \rho_{cell} . \quad (13)$$

Thus, the linearized relationship takes the form:

$$\frac{dn_{cell}}{dt} = 4\pi \left\{ J_i(R^2 - (R-d)^2) + \frac{\Lambda_i k_B T}{\rho_{cell}} \left[ -\delta\rho_1 R^2 + \delta\rho_2 (R-d)^2 \right] \right\} . \quad (14)$$

Next, ion conservation can be performed on the extracellular fluid inside the alveolosphere. In this case ion leakage is considered as the molar ion flux  $j_{i,leak}$ . Applying ion conservation in the cell layer results in the following relationship between time rate of change in the number of moles of ions in the alveolosphere,  $n_{in}$ , is related to the fluxes  $J_i$ ,  $j_{i,2}$ , and  $j_{i,leak}$ :

$$\frac{dn_{in}}{dt} = J_i(4\pi(R-d)^2) - j_{i,2}(4\pi(R-d)^2) - L_2 w j_{i,leak} , \quad (15)$$

where  $L_2$  is the inner perimeter of the cell membrane network (defined in chapter 1) and  $w$  is the width of the junctions between cells. Applying equation (9) to equation (15) and then linearizing for small  $\delta\rho_2$  results in the relationship:

$$\frac{dn_{in}}{dt} = 4\pi(R - d)^2 \left[ J_i - \Lambda_i k_B T \frac{\delta\rho_2}{\rho_{cell}} \right] - L_2 w j_{i,leak} . \quad (16)$$

Concentration between cells varies as a function of the radial coordinate  $r$ . In order to calculate  $j_{i,leak}$ , it is necessary to solve for the form of this concentration  $\rho(r)$ . It can be assumed that changes to  $\rho(r)$  in time are small [11]. The differential form of ion conservation can be applied to the junctions between the cells and then linearized for small concentration differences, yielding:

$$D_i w \frac{1}{r} \frac{d}{dr} r \frac{d\rho(r)}{dr} = 2 \Lambda_i k_B T \left( \frac{\rho(r)}{\rho_{cell}} - 1 \right) , \quad (17)$$

where  $D_i$  is the diffusion constant of ions through the junction. The factor of 2 accounts for the fact there are cell membranes on either side of the junction.  $J_i$  is neglected in this case, because it is not clear which direction active ion pumps would bias. This differential equation can be solved analytically resulting in a series of modified Bessel functions [11].

$j_{i,leak}$  is equal to the flux through the cell junction at the inner membrane ( $r = R - d$ ) of the alveolosphere, which via Fick's Law implies:

$$j_{i,leak} = -D_i \left. \frac{d\rho(r)}{dr} \right|_{r=(R-d)} , \quad (18)$$

which can then be substituted into equation (16) yielding:

$$\frac{dn_{in}}{dt} = 4\pi(R - d)^2 \left[ J_i - \Lambda_i k_B T \frac{\delta\rho_2}{\rho_{cell}} \right] + L_2 w D_i \left. \frac{d\rho(r)}{dr} \right|_{r=(R-d)} . \quad (19)$$

### 3.1.2 Volume Conservation

The volume in each layer of the alveolosphere is largely dictated by the amount of water in that layer. Because water can be considered incompressible, and mass must be conserved, volume must also be conserved. The flow of water between different layers of the alveolosphere is dictated by hydrostatic and osmotic pressure differences. The volume flux of water  $J_{W,a \rightarrow b}$ , which flows across a water permeable membrane and concentration difference and pressure difference  $\delta\rho_{a \rightarrow b}$  and  $\delta P_{a \rightarrow b}$  respectively is given by the osmotic pressure law:

$$J_{W,a \rightarrow b} = -\Lambda_V (\delta P_{a \rightarrow b} - \delta \Pi_{a \rightarrow b}) , \quad (20)$$

where  $\Lambda_V$  is the volumetric transport coefficient of water across the membrane (which is dependent upon the density of aquaporins in the membrane). It is assumed that  $\Lambda_V$  is constant across the entire alveolosphere.  $\delta \Pi_{a \rightarrow b}$  is the osmotic pressure difference between the two fluids defined as:

$$\delta \Pi_{a \rightarrow b} = 2 k_B T \delta \rho_{a \rightarrow b} , \quad (21)$$

Where the factor of two accounts for the equal treatment anions and cations.

Now, volume conservation can be applied to the cell layer of the alveolosphere. For simplicity, it is again assumed that all meaningful flow of water into and out of the cell occurs across the inner and outer membranes. Thus, all leakage from the cellular junctions into the cells can be neglected. Applying volume conservation in the cell layer results in the following relationship between time rate of change of volume in the cell,  $V_{cell}$ , is related to the fluxes  $J_{W,1}$ , and  $J_{W,2}$ :

$$\frac{dV_{cell}}{dt} = J_{W,1}(4\pi R^2) - J_{W,2}(4\pi(R-d)^2) . \quad (22)$$

Applying equations (20) and (21) to equation (22) results in the relationship <sup>2</sup>:

$$\frac{dV_{cell}}{dt} = 4\pi \Lambda_V \{ [ (R-d)^2 \delta P_2 - R^2 \delta P_1 ] - 2 k_B T [ (R-d)^2 \delta \rho_2 - R^2 \delta \rho_1 ] \} . \quad (23)$$

Next, volume conservation can be applied to the extracellular fluid within the alveolosphere. In this case ion leakage is considered as the molar ion flux  $J_{W,leak}$ . Applying volume conservation in the cell layer results in the following relationship between time rate of change in the volume of extracellular fluid in the alveolosphere,  $V_{in}$ , is related to the fluxes  $J_{W,2}$  and  $J_{W,leak}$ :

$$\frac{dV_{in}}{dt} = 4\pi (R-d)^2 J_{W,2} - L_2 w J_{W,leak} . \quad (24)$$

Equation (20) and (21) can then be applied to equation (24) resulting in the relationship:

$$\frac{dV_{in}}{dt} = 4\pi \Lambda_V (R-d)^2 (2 k_B T \delta \rho_2 - \delta P_2) - L_2 w J_{W,leak} . \quad (25)$$

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<sup>2</sup> In this thesis it is assumed that, across the outer membrane of the alveolosphere, the hydrostatic pressure term in the osmotic pressure law is equal to the mechanical pressure  $\delta P_1$ . However, in the pressure seen by the osmotic pressure law may be better modeled as a partial pressure found via a hydrogel mixture theory [14].



Pressure varies between cells varies as a function of the radial coordinate  $r$ . To calculate  $J_{W,leak}$ , it is necessary to solve for the form of the pressure  $P(r)$ . It can be assumed that changes to  $P(r)$  in time are small [11]. The differential form of volume conservation can be applied to the junctions between the cells yielding:

$$\kappa_V \frac{1}{r} \frac{d}{dr} r \frac{dP(r)}{dr} = -2\Lambda_V [(P(r) - P_{cell}) - 2 k_B T (\rho^3(r) - \rho_{cell})], \quad (26)$$

where  $\kappa_V$  is the Poiseuille coefficient that links pressure gradients to volumetric flow rate. The factor of 2 accounts for the fact there are cell membranes on either side of the junction. After inputting the solution for  $\rho(r)$ , derived from equation (17), this differential equation can be solved analytically resulting in another series of modified Bessel functions [11].

$J_{W,leak}$  is equal to the flux through the cell junction at the inner membrane ( $r = R - d$ ) of the alveolosphere, which via Poiseuille's Law implies:

$$J_{W,leak} = -\kappa_V \left. \frac{dP(r)}{dr} \right|_{r=(R-d)}, \quad (27)$$

which can then be substituted into equation (26) yielding:

$$\frac{dV_{in}}{dt} = 4\pi \Lambda_V (R - d)^2 [2 k_B T \delta\rho_2 - \delta P_2] + L_2 w \kappa_V \left. \frac{dP(r)}{dr} \right|_{r=(R-d)}. \quad (28)$$

### 3.2 Possible Energetic Limits

From the perspective of the growth medium, it can be assumed that the alveolosphere equates to a growing, pressurized spherical defect that deforms the medium around it. At any moment the alveolosphere can be described by its outer radius  $R$ , and the pressure which is required at its outer surface to deform the growth medium to that radius,  $P(R)$ . At a given  $R$ , the differential rate of work  $\dot{W}$  required to expand the by a volume rate  $\dot{V}$  is given by:

$$\dot{W} = P(R) \cdot \dot{V} = P(R) \cdot (4\pi R^2) \frac{dR}{dt}. \quad (29)$$

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<sup>3</sup> As in [11], it is assumed that flow is below the Poiseuille limit due to the short length scales and relatively high viscosity between cells.

It may be assumed that an alveolosphere has some finite amount of process available to it based on the maximum rate it is able to attain and process ATP into usable energy. If the process is limited by the cells' ability to attain and process ATP, it is likely that the available power scales with outer surface area. Therefore, we can write the maximum energy  $\dot{Q}_{max}$  as a function of the surface area  $A$  as:

$$\dot{Q}_{max} = q_{max} \cdot A = q_{max} \cdot (4\pi R^2), \quad (30)$$

where  $q_{max}$  is the maximum available energy available per unit surface area of the alveolosphere. When the alveolosphere is growing ( $\frac{dR}{dt} > 0$ ) it is necessary that the maximum available energy is greater than the work required to expand the alveolosphere to a larger radius ( $\dot{Q}_{max} > \dot{W}$ ). From equations (29) and (30) this implies:

$$q_{max} > P(R) \cdot \frac{dR}{dt}, \quad (31)$$

It can also be assumed that at a given  $R$ , without power input from the cells, the alveolosphere would decrease in size due to osmosis. Therefore, if the assumption is made that near some maximum size the velocity due to diffusion and growth interact linearly, there must be some minimum  $\frac{dR}{dt}$  to continue growth. As a result, the following must remain true during growth:

$$P(R) < \frac{q_{max}}{v_{shrink}}, \quad (32)$$

where  $v_{shrink}$  is the minimum velocity required to continue growth.

For materials where  $P(R)$  is increasing over any interval, this implies the possible existence of an energetic limit which may potentially be used to estimate the maximum size of the alveolosphere in especially stiff mediums.

## 4. Large Alveospheres in Static Equilibrium

As discussed in chapters 1 and 2, it has been observed that the thickness of the cell layer of the alveosphere remains relatively constant throughout development. This implies that in the limit of a large alveosphere, the thickness of this layer becomes negligible ( $R \gg d$ ). This allows for simplification of the equations derived in chapters 2 and 3 and, as a result, for a simple order of magnitude estimation for the equilibrium size of the alveospheres.

### 4.1 Simplifications:

In the limit of a large alveosphere ( $R \gg d$ ), it can be assumed that the radius of the inner membrane is approximately equal to the radius of the outer membrane ( $R - d \approx R$ ). Additionally, in the limit of a thin cell layer, it can be assumed that the concentration and pressure gradients in the cell junctions are approximately constant, yielding:

$$\left. \frac{d\rho(r)}{dr} \right|_{r=(R-d)} = \frac{d\rho(r)}{dr} = \frac{-(\delta\rho_1 + \delta\rho_2)}{d}, \quad (33)$$

and:

$$\left. \frac{dP(r)}{dr} \right|_{r=(R-d)} = \frac{dP(r)}{dr} = \frac{-(\delta P_1 + \delta P_2)}{d}, \quad (33)$$

These three simplifications can be applied to equations (5), (8), (14), (19), (23), and (28) resulting in the following set of equations:

$$\delta P_1 + \delta P_2 = \frac{2(\sigma_1 + \sigma_2)}{R}, \quad (34)$$

$$\frac{dn_{cell}}{dt} = 4\pi \frac{\Lambda_i k_B T}{\rho_{cell}} R^2 (\delta\rho_2 - \delta\rho_1), \quad (35)$$

$$\frac{dn_{in}}{dt} = 4\pi R^2 \left[ J_i - \Lambda_i k_B T \frac{\delta\rho_2}{\rho_{cell}} \right] - L_2 w D_i \frac{\delta\rho_1 + \delta\rho_2}{d}, \quad (36)$$

$$\frac{dV_{cell}}{dt} = 4\pi \Lambda_V R^2 [(\delta P_2 - \delta P_1) - 2 k_B T (\delta\rho_2 - \delta\rho_1)], \quad (37)$$

$$\frac{dV_{in}}{dt} = 4\pi \Lambda_V R^2 [2 k_B T \delta\rho_2 - \delta P_2] - L_2 w \kappa_V \frac{\delta P_1 + \delta P_2}{d}. \quad (38)$$

From experiments, it is clear that alveolospheres reach an equilibrium size at which growth halts. Using this framework to estimate this equilibrium size will help to evaluate its accuracy. Therefore, we assume that as the alveolosphere grows, it eventually reaches an steady state radius  $R_s$ . At this equilibrium, it is assumed that all derivatives with respect to time are necessarily equal to 0.

Applying this simplification to equations (35) and (37) reveals that in this limit:

$$\delta\rho_1 = \delta\rho_2 \equiv \delta\rho_s , \quad (39)$$

$$\delta P_1 = \delta P_2 \equiv \delta P_s , \quad (40)$$

and through substitution into equation (34),

$$\delta P_s = \frac{\sigma_1 + \sigma_2}{R_s} \equiv \frac{\sigma_s}{R_s} , \quad (41)$$

$\delta\rho_s$  and  $\frac{\sigma_s}{R_s}$  can then be substituted into equations (34), (36), and (38) resulting in the volume and ion conservation laws for a large alveolosphere in equilibrium:

$$0 = 4\pi R_s^2 \left[ J_i - \Lambda_i k_B T \frac{\delta\rho_s}{\rho_{cell}} \right] - \frac{L_2 w D_i}{d} \delta\rho_s , \quad (42)$$

and

$$0 = 4\pi R_s^2 \Lambda_V \left[ 2 k_B T \delta\rho_s - \frac{\sigma_s}{R_s} \right] - \frac{L_2 w \kappa_V}{R_s d} \sigma_s . \quad (43)$$

## 4.2 Order of Magnitude Estimations:

To test the general accuracy of this framework, it is useful to perform an order of magnitude estimation for the equilibrium size of alveolosphere (which is readily known from experiments). Table 4-1 contains estimations of the values of each of the parameters and sources for those estimations.

**Table 4-1:** Estimated values and sources for those values.

Parameter	Quantity	Value	Source
$R_s$	Alveolosphere Steady-State Radius	$\sim 100 \mu\text{m}$	MIT GuoLab Experiments
$d$	Cell Layer Thickness	$\sim 15 \mu\text{m}$	MIT GuoLab Experiments
$w$	Cell Junction Width	$\sim 1 \mu\text{m}$	MIT GuoLab Experiments
$L_2$	Inner Perimeter of Membrane Network	$\sim 10 \text{ mm}$	Estimated From Radius
$\sigma_s$	Alveolosphere Steady-State Tension	$\sim 10 \text{ mN/m}$	[15]
$D_i$	Cell Junction Ion Diffusion Coefficient	$\sim 10^{-10} \text{ m}^2/\text{s}$	[11]
$\Lambda_i$	Ion Permeation Constant	$\sim 10^{-9} \text{ mol}/(\text{J}\cdot\text{m}^3\cdot\text{s})$	[11]
$\Lambda_V$	Water Permeation Constant	$\sim 10^{-10} \text{ m}^3/\text{s}$	[11]
$\kappa_V$	Cell Junction Poiseuille Coefficient	$\sim 10^{-22} \text{ m}^3/\text{Pa}\cdot\text{s}$	[11], Poiseuille's Law
$k_B$	Boltzmann Constant	$8.314 \text{ J/mol}$	Boltzmann Constant
$T$	System Temperature	$310 \text{ K}$	Body Temperature
$\rho_{cell}$	Cell Ion Concentration	$\sim 1 \text{ mM}$	[11]
$\delta\rho_i$	Ion Balance Concentration	$\sim 1 \text{ mM}$	[11], given separately

Using these values, the coefficients  $\Lambda_i k_B T \frac{4\pi R_s^2}{\rho_{cell}}$  and  $\frac{L_2 w D_i}{d}$  can be compared. It becomes clear that the ion leakage term is roughly 4 orders of magnitude smaller and therefore can be neglected. This simplifies equation (42) further as:

$$\delta\rho_s = \frac{j_i \rho_{cell}}{\Lambda_i k_B T} \equiv \delta\rho_i, \quad (44)$$

Similarly, the coefficients  $4\pi R_s \Lambda_V$  and  $\frac{L_2 w \kappa_V}{R_s d}$  can be compared, after which it becomes evident that the water leakage term is roughly 9 orders of magnitude smaller and therefore can be neglected. This simplifies equation (43) further resulting in:

$$R_s = \frac{\sigma_s}{2 k_B T \delta\rho_i}. \quad (45)$$

It is important to note that at this stage, there are significant discrepancies with [11]. Firstly, one would expect an increase in size from increased pumping efficiency. This is seen in the order

of steady state approximations in [11]. It is also important to note that the ranges of values in [11] show some discrepancy with one another, which is understandable, as estimation of parameters is always a challenge in biological systems. These two discrepancies may be related as the values chosen to justify neglecting the leakage terms may have been flawed themselves.

That said, the order of magnitude estimation for the size of the alveolospheres is  $R_s \approx 1900$   $\mu\text{m}$ . This is about one order of magnitude of the observed value of 50 to 150  $\mu\text{m}$ . However, it is important to note that slightly changing parameters can give a large range of  $R_s$ , anywhere from 10  $\mu\text{m}$  to 1 cm. The order of magnitude estimation does not demonstrate that this framework is a polished or well-tuned theory for the development of alveolospheres. However, because the range is inclusive of the values of an actual alveolosphere, it demonstrates that with better estimations of parameters, and by extension, better application (or lack thereof) of simplifying assumptions, this framework may allow for a much more robust theory of alveolosphere development.

## 5. A Path Forward

The framework described in chapters 1-4 contains the bare bones equations and assumptions that may be reconfigured to develop a more robust theory of alveolosphere development, and to identify and model possible causes for disease and dysfunction in alveoli. To truly understand how alveolospheres grow and develop and what can go wrong during that process there are two main tasks that must be accomplished. First, a dynamic model which tracks alveolospheres from initial lumen formation to equilibrium size must be developed. Next, experiments must be performed to verify the relevance of parameters and assumptions chosen in this model. A final model should be able to predict, not only, the growth and equilibrium size of a healthy alveolosphere, but also changes in parameters that result in modes of failure seen in reality.

### 5.1 Dynamics of Alveolospheres:

In principle, there are two primary ways in which dynamics can be coupled into this framework. The first is through the volume and ion conservation laws, and the second is through the active membrane tension.

In a dynamic case,  $\frac{dV}{dt}$  and  $\frac{dn}{dt}$  are no longer set to zero, but instead enter into the conservation laws as  $4\pi R^2 \frac{dR}{dt}$  and  $\frac{4}{3}\pi \frac{d(\rho R^3)}{dt}$  respectively. These forms are highly non-linear and mean that finding a solution to the partial differential equations which result from the conservation laws will likely require numerical methods.

Although dynamics do not affect equilibrium force balance directly - because growth occurs on a timescale of days and the assumption can be made that the system is quasi-static from an inertial perspective - the form of  $\sigma$  in a membrane itself time dependent due to the presence of active actin fibers (as can be seen in figure 1-1). In [11], the tension in an active gel cortex is given as:

$$\sigma(t) = \sigma_0 \left[ 1 + \tau \left( \frac{1}{R(t)} \frac{dR(t)}{dt} \right) \right], \quad (46)$$

where  $\tau$  is the characteristic timescale of the interaction between the viscous and elastic effects in the membrane and  $\sigma_0$  is the steady state tension in the membrane. It may also be important to include time dependent effects from the growth medium. However, to try to extract the mechanics

of an alveolosphere at the most basic level it may be advantageous to also neglect viscoelastic and poroelastic effects in the growth medium, under the assumption that their timescales are relatively short.

During the beginning of growth, it may make the most sense to begin with the lumen model from [11] and then switch to this framework when the cell divides and  $N > 2$ . The cell division itself is something that will most definitely also be highly time dependent and should have an extremely significant effect on the trajectory of alveolosphere growth. For instance, equation (46) assumes a single piece of membrane that is being stretched, but more membrane will be added as the cells which make up an alveolospheres wall divide.

All in all, there is a lot of work which needs to be done, and a lot of thought that needs to be put into developing a robust dynamic alveolosphere model, but this framework along with the experiments outlined in this chapter should provide a strong starting point.

## 5.2 Proposed Experiments:

British statistician, George Box declared, in his ubiquitous quote, “All models are wrong: some are useful.” In order to verify the significance of the parameters and assumptions in this framework, and eventually the robustness and accuracy of a dynamic alveolosphere model, experiments must be performed which isolate said parameters and assumptions. It is often easy to detach oneself from reality when exploring a problem mathematically, but at the end of the day a model ceases to be “useful” when it is not grounded in experimentation. The following is a short list of possible experiments which may give insight into the relevance of certain parameters and assumptions:

1. *Disrupt active ion pumps.* Using inhibitors to disrupt the function of active pumps would decrease  $J_i$ , and give insight into its effect on alveolosphere development.
2. *Decrease adhesion between cells.* Decreasing adhesion between cells would widen the junctions gap between cells, increasing  $w$ , and by extension increasing  $J_{i,leak}$ . This would give insight into the importance of leakage.
3. *Increase hydrostatic pressure.* Attempting to grow alveolospheres under higher pressure conditions may give insight into whether pressure serves as a limit to growth.
4. *Increase external ion concentration.* By increasing  $\rho_{out}$  and observing the response of an alveolosphere, insight may be gained into the role of  $j_i$  during development.



5. *Decrease available ATP.* By progressively lowering the concentration of ATP outside the alveolosphere (and by extension the rate at which it can diffuse into the cells) it may be possible to determine if the energy limit is far above or below any other limits to the system.
6. *Measure relevant quantities.* To have any chance of accurately and precisely predicting alveolosphere behavior, the relevant material constants and cell dimensions must be well characterized.

## 6. Summary and Conclusion

Alveolospheres are PSC or AdC derived organoids which resemble alveoli in function. They have a unique, balloon-like structure, the mechanics of which is not thoroughly understood. Cells derived from diseased PSCs fail to create this complex structure. Therefore, understanding the development and growth of alveolospheres may give insight into developing treatments or cures for lung diseases. Developing a mathematical framework for understanding alveolosphere growth and development requires investigation of both their structure and thermodynamics.

In chapter 2, equilibrium force balance is applied to three sub-systems of alveolospheres: the cell junction membrane matrix, the outer membrane, and the inner membrane. These mechanical equilibrium equations lead to two equations ((5) and (8)) which relate the tensions in each of these sections ( $F_3$ ,  $\sigma_1$ , and  $\sigma_2$  respectively) to the pressure drops across the outer and inner membrane ( $\delta P_1$  and  $\delta P_2$  respectively). Pressure is also considered as a potential limit to alveolosphere growth.

In chapter 3, ion conservation and volume conservation are applied to both cytoplasm in the cell layer and extracellular fluid inside the alveolosphere. This results in a set of 4 equations ((14), (19), (23), and (28)) which relate the pressures across the outer and inner membrane, the concentration differences across the outer and inner membrane ( $\delta\rho_1$  and  $\delta\rho_2$  respectively), and the active pump ion flux ( $J_i$ ). Energetic arguments are also made to argue the existence of a power limited maximum alveolosphere size based on increasing pressure from the growth medium.

In chapter 4, additional simplifying assumptions are applied to this framework of equations for the limit of a large, thin-walled alveolosphere in steady-state equilibrium. Estimated values for relevant parameters are then inputted into the resulting set of simplified equations to attain an order of magnitude estimation for the maximum equilibrium size of an alveolosphere of 1900  $\mu\text{m}$ . This estimation is an order of magnitude large than the observed radius of alveolospheres of 50 to 150  $\mu\text{m}$ . However, by modulating parameters slightly away from what are assumed to be relatively middle of the road values, this equilibrium estimation can range from 10  $\mu\text{m}$  to 1 cm. The observed range is contained within these margins, which implies that this mathematical framework shows some promise as a basis for a more polished theory of alveolosphere development.

Finally, chapter 5 describes the next steps in creating such a robust theory. It first describes how the framework might be adapted to create a dynamic model of alveolosphere development. Time dependence arises from within the conservation laws, and due to the active actin filaments in the epithelial cells which make up an alveolosphere. Next, it describes a set of experiments which may help identify which parameters and assumptions from this framework are relevant to the reality of alveolosphere development.

The complex tension-dominated structure of alveolospheres showcases the mechanics which govern its development. The biology of alveolospheres cannot be separated from the set of fundamental physical laws which govern its mechanics. Therefore, a mechanical model is not just an exercise for nosey mechanics, but is *fundamental* to fully understanding the biology of the lungs. Like two pieces of a puzzle, they are so strongly coupled that one cannot be understood without the other.

Derived from basic physical principals, this thesis offers a loose, yet thorough framework which can be used as a tool to develop a more accurate and robust theory for the growth and development of alveolospheres. Crude, order of magnitude estimations for the equilibrium radius of an alveolosphere made using this framework already demonstrate potential by successfully bounding the experimentally observed radius. This framework may serve as a guide for careful experimentation and measurement of relevant parameters, and as a basis for a more polished model of alveolosphere growth and development.



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