**THREE-DIMENSIONAL SCAFFOLDS FOR MAMMARY EPITHELIAL CELL
GROWTH**

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of the Requirements for the Degree
Master of Science in Engineering**

By

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ABSTRACT

THREE-DIMENSIONAL SCAFFOLDS FOR MAMMARY EPITHELIAL CELL GROWTH

By

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Mammary epithelial cells are highly efficient secreting cells. This natural phenomenon has triggered research efforts to focus on using these cells to assist in the production of viable liquids. The application of this technology is nearly endless. If developed successfully, mammary epithelial cells could be used by industries from farming to pharmacology.

The feasibility of culturing epithelial mammary cells which secrete within a three-dimensional scaffold was investigated and is documented within this paper. A biocompatible film with suitable mechanical characteristics and processing properties for use in a bioreactor was developed through multiple iterations. The film that was developed also withstands extended periods of time in an aqueous solution at 37 degrees Celsius (the incubation temperature for epithelial cell lines) and can be optimized for cell survival (adjustable permeability). A prototype of a self-contained system that facilitates the growth of epithelial mammary cells and promotes their secretion of lipid proteins was also developed through this exploratory research.

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Chapter 1. Purpose

Gene “pharming” is an emerging scientific field with the objective of producing genetically altered animals which produce pharmaceuticals. “Pharming” is a portmanteau of “farming” and “pharmaceutical” and refers to a practice which represents a projected US market value of \$3 billion dollars annually. Using genetic engineering to inject genes, which code for useful pharmaceuticals, into host animals is a novel idea in theory, but a difficult practice to perfect. Mammary tissue is one of the most prolific of all secretory tissue, and has been argued to be the ideal site for producing complex bioactive proteins. However, creating transgenic cattle requires 1600 egg injections per animal, yielding a 50% success rate. The inefficiencies associated with “pharming” in mammals including low rates of gene integration, poor embryo survival, and unpredictable transgene behavior, make the practice very expensive [41].

The goal of this project focuses on isolated mammary cells, thereby eliminating complications of genetic engineering due to other gene or system interactions. These cells are easily cultivated in vitro; however they lose their secretory functions when grown under standard cultivating conditions. Therefore the scope of this project is to develop a three-dimensional scaffold which simulates the physical morphology and chemical gradient of the natural environment for mammary cells, with the anticipation that this simulated environment will stimulate secretory function. This paper focuses on the development of the material systems and bioreactor design for growing mammary epithelial cells in synthetic, polarizing alveolar structures that support increased secretion.

Chapter 2. Background

Section 2.01 Function of mammary cells

Mammary cells are a specific type of epithelial cell, primarily responsible for the production of milk proteins. Secretory mammary tissue is organized into lobes, with each lobe made up of many lobules. Lobules contain the milk synthesizing units known as aveoli. The lumen of each aveoli is lined with a single layer of secretory epithelial cells, which in turn is surrounded by contractile myoepithelial cells. The rough endoplasmic reticulum, within the secretory cells, synthesizes the milk proteins, which are then processed in the Golgi apparatus. Within the Golgi apparatus, casein micelles and lactose are produced. These products are stored in secretory vesicles until they are secreted.

The lumen is connected to a duct system and upon milking stimuli and release of oxytocin (a hormone released from the pituitary which begins the milk-let-down process), the myoepithelial cells compress the aveoli, putting pressure on the lumen and releasing the milk products into the duct system.

The epithelial cells lining the aveoli are polar in nature, which means that the basal side of the cells has distinctly different functions compared with the apical (lumen) side. The contents of the cells are polarized such that the nucleus is located on the basal half of the cell and the Golgi apparatus along with the secretory vesicles and fat droplets are usually located on the apical half. This polarization is key to the secretion of milk by mammary epithelial cells.

In order for mammary cells to function similarly *in vitro*, it is important that the same polarization and establishment of the basal and lumen sides (see Figure 1) as well as simulation of geometry are achieved. By providing a polarized setting with a nutrient supply (basal) side and a secretory (lumen) side, and providing the necessary nutrients for

milk production, it is hypothesized that the mammary epithelial cells will be able to secrete products *in vitro* comparable to how they secrete *in vivo* [14].

Figure 1 Schematic of a mammary alveolus
(<http://www.foodsci.uoguelph.ca/deicon/mammary.gif>)

(a) Tissue Engineering

Tissue engineering can be thought of as the combining of clinical medicine, engineering and science. It involves the ‘application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal pathological mammalian tissues and the development of biological substitutes that restore, maintain or improve tissue function’ [29]. The *in vitro* seeding and adhering of cells to a synthetic or organic scaffold is the basis of tissue engineering. Therefore, choosing the correct scaffold material is very important in tissue engineering [28].

Scaffold materials can be made of synthetic or organic materials. Examples of synthetic materials include polyglycolic acid (PGA), polylactic acid (PLLA) or

polycaprolactone (PCL). These three polymers are the most common synthetic materials used for tissues engineering scaffolds. The most popular natural polymer used for tissue engineering is collagen [28].

(b) Collagen

Collagens are the primary structural protein in most connective tissues, including skin, bone and tendons, where they provide structural support to the tissues [6]. Collagen is composed of three polypeptide α -chains, each consisting of over 1000 amino acids [13]. Each of the three polypeptide chains are coiled into a left-handed helix and then the three are joined together into a right-handed superhelix which is stabilized by interchain hydrogen bonds and covalent crosslinks [6]. See Figure 2.

Figure 2 Chemical structure of collagen type I. (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix structure and (c) staggered quaternary structure. [13]

Currently, 13 different types of collagen have been identified, which differ in length of helix and nature and size of the non-helical portions [13]. Type I collagen is most prominent in higher order animals, and can be isolated from skin, tendon, bone,

cornea, dentin, fibrocartilage, large vessels, intestine, and uterus. This type of collagen is characterized by two identical polypeptide chains, referred to as $\alpha 1$, and one $\alpha 2$ chain with a different amino acid composition. The amino acid compositions for the two chains of Type I collagen are listed in Figure 3. Collagen is an attractive material for tissue engineering because it is a natural material with low immunogenicity and because its structural complexity provides properties not present in synthetic materials. These properties can be modified to meet the needs of the material application. For example, thermal, chemical or physical denaturation of collagen breaks down the triple helix into single coils, resulting in gelatin [6].

Amino acid	$\alpha 1(\text{I})$ -chain	$\alpha 2(\text{I})$ -chain
Alanine	124 (2)	111 (3)
Arginine	53 (2)	56 (1)
Asparagine	13	23
Aspartic acid	33 (3)	24 (2)
Glutamic acid	52 (2)	46 (2)
Glutamine	27(3)	24 (1)
Glycine	345 (6)	346 (6)
Histidine	3 (1)	8
Hydroxylysine	4	9
Hydroxyproline	114	99
Isoleucine	9 (1)	18
Leucine	22 (3)	33
Lysine	34 (2)	21 (1)
Methionine	7	4
Phenylalanine	13 (1)	15 (3)
Proline	127 (4)	108 (1)
Serine	37 (5)	35 (1)
Threonine	17 (1)	20
Tyrosine	5 (5)	4 (3)
Valine	17 (1)	34
Total	1056 (42)	1038 (24)

Figure 3 Amino acid composition of type I collagen from calf-skin (the values in parentheses are residues contributed by non-helical telopeptide regions) [23]

(c) Gelatin

Gelatin is the product of denaturation and structural degradation of collagen.

Because of this, gelatin has many of the same properties of collagen, but is less

expensive. To extract gelatin from collagen, the collagen is treated with either alkali or acid, which each result in different types of gelatin (see Figure 4). Type A gelatin results from acid treatment, which is most commonly used with pigskin. Type B gelatin is formed when collagen (usually from bovine bone) is treated with alkali. The main difference between the two gelatin types is the pH at which they are neutral, also known as the isoelectric point (IEP). Type A gelatin is neutral between pH 8.0 and 9.0 and type B gelatin between pH 4.8 and 5.4.

Figure 4 Processes for breakdown of collagen into gelatin [35]

There are many glycine, proline and 4-hydroxyproline residues in gelatin. A common structure is $-\text{Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro}-$. Mainly due to the nature of the side chains of gelatin, it has the ability to form reversible gels. Modifications of these side chains result in higher or lower solid to liquid transition temperatures [18]. The average melting temperature of gelatin is about 37°C , although it

can vary slightly based on the gel strength, or Bloom value. Bloom values are used to categorize gelatin based on strength, where the higher Bloom values indicate higher gel strength and therefore higher melting/solidification temperatures [33]. To increase the melting temperature of gelatin above what high-bloom gelatin can offer, several modification methods are used to modify the chemical side chains, including chemical treatment and gamma irradiation.

Albert Yang conducted preliminary research in 2005 towards the identification and selection of a material suitable to the needs of the project goals. His work focused on varying concentrations of food grade gelatin poured into thin films. While Albert narrowed the process for pouring and creating films of consistent thickness, the food grade gelatin was not able to withstand the incubation temperature of 37 degrees Celsius. Therefore, it is necessary to identify an effective modification technique to increase the solid-liquid transition temperature of gelatin.

Section 2.02 Treatment Methods for Gelatin

(a) Glutaraldehyde

Gelatin in its natural state is soluble in water if the temperature is above 20-25 C, where the “physical crosslink” is destroyed. After treatment with glutaraldehyde, the gelatin becomes “chemically crosslinked” and insoluble in any solvent. The free aldehyde groups of glutaraldehyde form a Schiff base with the amino groups of lysine in gelatin. This crosslinking action between glutaraldehyde and gelatin occurs in various states: 1. both aldehyde groups of glutaraldehyde form the Schiff base with amino groups of lysine, 2. one of the two aldehyde groups reacts with an amino group of gelatin, but the

other remains unreacted, 3. some glutaraldehyde is included in complex graft polymers on the protein [23]. The unreacted aldehyde groups of glutaraldehyde are toxic to cells. In order for the glutaraldehyde treated gelatin sheets to be used for cell cultures, it is necessary that the sheets be rinsed in glycine, which converts the aldehyde groups to carboxyl groups [23]. One protocol for treating gelatin films with glutaraldehyde suggests treating the films with 0.3% glutaraldehyde for 1 hour, washing with double distilled water for 3 hours under agitation, followed by Milli Q water for 1 hour under agitation, rinsing with 100 mM glycine for 2 hours and finally washed in Milli Q water for 1 hour under agitation three times and dried in a sterile hood [1].

Figure 5 Chemical reactions between gelatin and glutaraldehyde [23]

(b) Transglutaminase

Enzymatic treatment of gelatin with transglutaminase catalyzes the formation of isopeptide bonds between glutamine and lysine. Transglutaminase provides an alternative to treatment with glutaraldehyde, which has some drawbacks due to its toxicity [2]. Microbial transglutaminase has been approved for use in food and is

protected by and distributed exclusively by Ajinomoto [25]. It is important to note that Type A gelatin and not Type B gelatin should be used with transglutaminase treatment because base treatment hydrolyzes the amide groups and suppresses enzymatic crosslinking.

(c) UV Irradiation

Physical crosslinking may be induced by exposure to ultraviolet (UV) light. One source documented a dose of $500 \mu\text{W}/\text{cm}^2$ for 40 minutes. Depending on the thickness of the sheets or films, this technique could allow for one side to be crosslinked, while the other side remains in its native state. The benefit to that would be increased cell viability on the side not treated [15].

Section 2.03 Geometry of Scaffold

(a) Conventional Scaffold Fabrication Techniques

Solvent-casting particulate-leaching – This technique is performed using PLLA in chloroform and adding salt particles, which suspend uniformly. Once the solvent evaporates, the polymer is submerged in water and the salt dissolves leaving a porous matrix [28].

Gas foaming – PGLA is saturated with carbon-dioxide at high pressures and then rapidly brought back to atmospheric pressure, resulting in the formation of gas bubbles in the polymer [28].

Fiber meshes/fiber bonding – PLLA is dissolved in methylene chloride and casted over PGA mesh. The solvent is dissolved and then the matrix is heated above the melting temperature of PGA. The PLLA is then removed and a matrix of PGA fibers, joined at the cross-point, remains [28].

Phase separation – Biodegradable synthetic polymer is dissolved in molten phenol, while biologically active molecules are added. The mixture is brought down in temperature to form a liquid-liquid phase, and even further to form a two-phase solid. The solvent is removed and a porous structure is left behind [28].

Melt molding – A Teflon mold is filled with PLGA powder and gelatin microspheres and the mold is heated above the glass transition temperature of PLGA. The PLGA particles bond together and the gelatin is then dissolved in water leaving a PLGA matrix. This technique allows matrices to be formed in specific geometries [28].

Freeze drying – Synthetic polymers or natural polymers, such as collagen, can be frozen and freeze-dried to produce porous matrices. Freezing rate and pH can control pore size [28].

Solid Freeform Fabrication (SFF) – Involves building 3-D objects using layered manufacturing technology. Starting from the bottom and building layers up allows each layer to form a cross-sectional division [28].

(b) Cell Sheet Engineering

Cell sheet engineering provides an alternative to the conventional 3-D biodegradable scaffolds described above. This relatively new concept is based on creating 3-D scaffolds by layering 2-D cell sheets. Cells are cultured to confluence and then instead of using enzymes that disrupt the cell-to-cell junction proteins, the cells are harvested as a continuous sheet by decreasing temperature. The resulting 3-D matrix is composed of viable cell sheets without any scaffolds [32].

The geometry of the scaffold is very important for cell viability. The cells are accustomed to living in a certain geometry *in vivo*, so it is important that their environment is mimicked as closely as possible *in vitro*. The options for geometric configurations include flat films, tubes which provide one dimension of curvature, or hemispheres providing 2 dimensions of curvature. The hemispheres would most closely replicate the geometric configuration of an alveoli, the natural environment for mammary epithelial cell, however this geometry is very difficult to consistently produce. Collagen and gelatin films are the most common geometry for culturing fibroblasts and endothelial cells. Mammary epithelial cells grow to confluence on flat sheets, however, they are not able to secrete efficiently in this geometry. Tubes allow one dimension of curvature,

which is closer to replicating the cells' natural environment than flat sheets, and are reasonably efficient to manufacture.

Chapter 3. Objective

Create a biocompatible film with suitable mechanical characteristics and processing properties for use in a bioreactor that also withstands extended periods of time in an aqueous solution at 37 degrees Celsius (the incubation temperature for epithelial cell lines) and can be optimized for cell survival (adjustable permeability). Develop a prototype of a self-contained system that facilitates the growth of epithelial mammary cells and promotes their secretion of lipid proteins.

Chapter 4. Outline

The preliminary research for the project was more exploratory and qualitative than quantitative. Without a background in cell biology or much knowledge of gelatin or collagen, the two suggested materials for use, a lot of trial and error observational experiments were conducted. The various phases of the project are outlined below and are discussed in further detail throughout the next several pages.

Material Development

Phase I: Optimization of Gelatin Films

Phase II: Optimization of Collagen Films

Phase III: Modification of Gelatin Films

Phase IV: Growing Viable Mammary Epithelial Cell Cultures

Phase V: Developing Repeatable, Sterile Process for Cell Cultures on Treated Gelatin Films

Geometry Development

Phase I: Scaffold Geometry

Phase II: Bioreactor Geometry

Phase III: Modified System Proof of Concept

Chapter 5. Material Development

As stated in the purpose of this research, the first goal was to develop a substrate that would facilitate the growth of epithelial mammary cells. Previous efforts to accomplish this revealed that the main constraints in selecting appropriate materials are melting temperature, biocompatibility, and ease of use. The melting temperature of the material must be above 37 degrees Celsius so that the material doesn't lose its integrity when it is subjected to incubation temperature. The biocompatibility of the material is important so that the cells are able to grow abundantly on the chosen substrate. Some materials promote cell growth better than others and materials should be analyzed comparatively so that the best substrate for cell growth is chosen. Ultimately, whatever material is chosen, it is important that it is easy to work with and will give repeatable results. A material that encourages cell growth but cannot be easily manipulated into working sheets, tubes or other configurations is not beneficial to the ultimate goal of this project.

Section 5.01 Phase I: Optimization of Gelatin Films

(a) Materials and Methods

Since the previous research was completed using gelatin, the experiment began by exploring the properties of gelatin. Most food grade gelatin has a melting temperature of 35-37 degrees Celsius so that it will "melt in your mouth". For this reason, it was obvious that food grade gelatin alone would not be an adequate material for the purposes of this project. However, based on previous observations and experiences, gelatin is known to be a very versatile substance and one with which it is very easy to work.

Because of this, it was decided to continue to use gelatin as a base material and look into treatment options for increasing the melting temperature of the gelatin.

Gelatin was to be the base for scaffold, therefore it was important that the properties of the gelatin sheets created were optimized. A Design of Experiments (DOE), shown in Table 1, was generated and performed to find the thickness, drying rate and concentration of gelatin that combined to create the optimal gelatin sheet for this application. For our purposes, the thickness of the gelatin sheets was measured and controlled by varying layers of masking tape that constrained the gelatin during the drying process. The drying rates were based on high, 38 degrees Celsius, and low, 4 degrees Celsius, temperature settings. Powdered gelatin was dissolved into water at 7 g per 50 mL and 2 g per 50 mL.

DOE	Concentration (g/50 mL)	Drying Temperature (°C)	Thickness (Layers of tape)
HIGH	7	38	3
LOW	2	4	1

Table 1 Design of experiments with varying concentrations of gelatin, drying temperature and thickness for optimization of gelatin films.

The experiment was conducted using stainless steel plates with masking tape forming square constraints for the gelatin to be poured into. Because of the fluid properties of liquid gelatin, it will pool together unless it can adhere to something. The edges of the masking tape absorb minimal amounts of the liquid gelatin mixture and allow it to dry conforming to the shape that the masking tape outlines. This helps control

the thickness of the resulting sheets as well since the gelatin is forced to occupy a specified area instead of pooling together in the center. Initially, a thermal controller was used to control the temperature cycles. The thermal controller was set to subject the samples to the extreme temperatures for either one hour at 38 degrees Celsius or two hours at 4 degrees Celsius and then the samples were to sit for a least two hours before being removed from the plates. Unfortunately, the thermal cycler broke in the midst of these experiments, so instead the experiments were repeated using a hot plate for the high temperature setting and a refrigerator for the low temperature setting. This set up did not allow for as accurate temperature control, but for observational purposes it gave adequate data.

The sheets from the DOE were tested in 37 degree Celsius water to determine if they would remain sheets at incubation temperature and visual observations were also made.

(b) Discussion

The following observations were made regarding the gelatin films that resulted from altering the three variable of the DOE. Higher drying temperatures and higher concentrations of gelatin led to very brittle gelatin films. Thin sheets were also very brittle and flaky to the point that they were often difficult to remove from the plates. In addition to the single layer of tape, low concentration of gelatin also made very thin, brittle sheets of gelatin. The low temperature resulted in thick sheets of gel which didn't dry until they were left at room temperature for a least an hour, and even then they resulted in non-uniform sheets.

In addition to visual observations, the sheets from the DOE were also tested in 37 degree Celsius water. The thicker sheets made with three layers of tape and higher concentrations of gelatin lasted longer in the water, but eventually all of the sheets dissolved. It was apparent that something had to be added to the gelatin sheet to increase their melting temperature. However, the DOE resulted in a consistent process for producing manageable gelatin sheets.

Section 5.02 Phase II: Optimization of Collagen Films

(a) Materials and Methods (First Iteration)

Type I bovine collagen isolated from achilles tendon was ordered from Sigma Aldrich. The collagen arrived in powdered form. The challenge for the applications of this project was to transform the collagen from its natural state to a form that would meet our objectives of pliable sheets with high melting temperatures. Having never worked with collagen before, this turned out to be a much bigger task than anticipated. An attempt was made to dissolve the collagen using 4 mg collagen per mL 0.017 M acetic acid.

(b) Discussion (First Iteration)

The collagen failed to dissolve into the acetic acid solution. Several other concentrations of acetic acid, up to .2 M, also failed to dissolve the collagen, even over the course of several weeks and at varying temperatures. Titration with 0.1 M NaOH to reach a pH of 5.5, which was described in one referenced paper, also failed [8]. After several weeks and multiple attempts without success, reference back to the Sigma description of the product ordered showed that the collagen type we were working with was classified as "insoluble".

(c) Materials and Methods (Second Iteration)

Soluble type I bovine collagen isolated from calf skin was ordered from Sigma to replace the insoluble collagen. The soluble collagen was dissolved successfully into 0.017 M acetic acid. The collagen solution was poured directly onto the metal plate and onto a dried gelatin film.

(d) Discussion (Second Iteration)

The layer of collagen applied to the plate did not form enough of a film to remove from the plate. The layer of collagen solution applied to the surface of a gelatin sheet and dried was tested in 37 degree Celsius water. The gelatin dissolved leaving behind a very thin collagen film floating in the water. The film was so thin that it collapsed on itself when it was removed from the water and did not regain its integrity. Although this wasn't fully successful, it was promising and led to further experiments down a similar path.

Section 5.03 Phase III: Modification of Gelatin

(a) Materials and Methods (First Iteration)

Further experiments with gelatin were conducted in parallel with the collagen experiments. In order to increase the mechanical properties and melting temperatures of gelatin, chemical crosslinking was applied. Two treatments were considered; transglutaminase and glutaraldehyde. Transglutaminase creates an irreversible covalent bond between glutamine and lysine, whereas glutaraldehyde forms an irreversible lysine-lysine bond [25]. These irreversible bonds allow the gelatin to survive higher temperatures. Transglutaminase is a proprietary formulation and is therefore difficult to obtain. Glutaraldehyde is easily obtainable through Sigma-Aldrich, and so was chosen as the chemical of choice for the following experiments. The 25% glutaraldehyde in water solution from Sigma was diluted with Milli-Q water to obtain a 2.5% solution for the first

experiment. Below are the procedures that were followed for the first experiment with glutaraldehyde.

Process:

1. Mix gelatin and glutaraldehyde and cast films

- a. Mix 20% gelatin solution (made with 300 bloom gelatin and water)
- b. Add glutaraldehyde in three different ratios of gelatin to glutaraldehyde : 64:1, 128:1, 265:1
- c. Cast three sheets (30mm x 30mm) of each solution with two layers of tape on a metal plate
- d. Let dry at room temperature

2. Treat gelatin sheets with glutaraldehyde solution

- a. Cast 20% gelatin solution sheets (30mm x 30mm) with two layers of tape on metal plates
- b. Submerge gelatin sheets into 2.5% glutaraldehyde solution for 20 minutes
- c. Rinse with de-ionized water
- d. Submerge sheets into 1% glycine solution for 30 minutes
- e. Rinse with de-ionized water

3. Treat only part of gelatin sheet

- a. Cast 20% gelatin solution sheets (30mm x 30mm) with two layers of tape on metal plates
- b. Use pipet to treat part of gelatin sheet with 2.5% glutaraldehyde solution
- c. Rinse with de-ionized water
- d. Submerge sheets into 1% glycine solution for 30 minutes

e. Rinse with de-ionized water

(b) Discussion (First Iteration)

When the gelatin and glutaraldehyde were mixed together it almost immediately transformed from a liquid to a semi-solid. The liquid turned from clear to an orange dense gel. The glutaraldehyde caused cross-linking in the gelatin that made it impossible to pipet the liquid. Further experiments were performed with differing concentrations of gelatin and glutaraldehyde, none of which resulted in a solution that was pipetable. It was concluded that the reaction between the lysine bonds happens too quickly for this method to work. As a result, Method 1 of the above processes was eliminated.

The gelatin sheets prepared and then treated with glutaraldehyde performed very well. The glutaraldehyde again caused cross-linking of the gelatin which was noted visually with the change of color to orange. The same was observed for the gelatin sheet treated with Method 3, which consisted of a square of glutaraldehyde in the center of the gelatin sheet. The sheets processed with Method 2 and 3 above, were tested in 37°C water and did not dissolve. The treated sheets were tested along side untreated sheets which dissolved within 2-3 minutes. In the case of the Method 3 sheet, which was partially treated, the untreated portions dissolved leaving behind the square which had been treated with glutaraldehyde. The treated sheets were left in the water and kept their integrity for over an hour at temperatures up to 60 degrees. These results were very promising towards the goal of the project. However, while the design constraint of a high melting temperature seemed to have been met, the 2.5% glutaraldehyde solution created sheets of gelatin which were very rigid.

(c) Materials and Method (Second Iteration)

Experiments were performed with lower dilutions of glutaraldehyde and different gelatin concentrations, utilizing the DOE shown in Table 2, until an optimal combination was obtained.

		Glutaraldehyde Concentrations			
Glutaraldehyde Concentrations		3.0 %	5.0 %	7.0 %	10.0 %
	0.1 %	X	X	X	X
	0.3 %	X	X	X	X

Table 2 Design of experiments for varying glutaraldehyde and gelatin concentrations

(d) Discussion (Second Iteration)

The DOE with lower glutaraldehyde concentrations proved that 10% gelatin treated with 0.1% glutaraldehyde showed to be the best combination at this point. This combination created a gelatin film that was strong and durable, yet very flexible and pliable.

Section 5.04 Phase IV: Developing Viable Mammary Epithelial Cell Cultures

(a) Material and Methods

Once a reliable and repeatable process was developed for creating sheets of gelatin with the desired properties, the cells could finally be introduced to the experiments. Bovine mammary cells were thawed from cryovials to create our control plates. M199 growth medium with bovine serum, penicillin and insulin was used to incubate the cells. The

cells grew abundantly in the control plates which were empty petri dishes with cells and growth medium only. The cells became confluent on the bottom of the dishes overnight. Once confluent, the cells were split into petri dishes containing various combinations of gelatin sheets treated with glutaraldehyde following the combinations listed in Table 2. The cells were left to incubate overnight.

(b) Discussion

After 24 hours the cells were observed under a microscope and unfortunately the Petri dishes with gelatin film contained mostly dead cells floating in the medium. The control plate contained confluent, healthy cells. It was thought that perhaps the glutaraldehyde was toxic to the cells so lower concentrations of glutaraldehyde were tested. Petri dishes were prepared following the process described above with lower concentrations of glutaraldehyde. The cells were left to incubate overnight and after 24 hours most of the cells in the experimental plates were dead again.

Section 5.05 Phase V: Creating Repeatable, Sterile Procedure for Culturing Cells on Treated Gelatin Films

(a) Materials and Methods (First Iteration)

More literature was consulted and it was discovered that rinse times for water and glycine needed to be increased dramatically. The rinse time for the first MQ water rinse was increased to 3 hours, the 30 minute glycine rinse went up to 2 hours, and the final MQ water rinse went to 3 hours with fresh water every hour. Along with the increased times, the rinses are also to be done under agitation to increase that amount of glutaraldehyde that is rinsed off. The DOE shown in Table 2 was repeated using these new rinse times. The process is shown below in Figure 6 Process for glutaraldehyde treatment of gelatin films in sterile hood.

Figure 6 Process for glutaraldehyde treatment of gelatin films in sterile hood

(b) Discussion (First Iteration)

After 24 hours the cells looked better in the experimental plates than they had before, but still weren't confluent. An additional observation was that the cells were more confluent on the bottom of the Petri dish than they were on the gelatin. There was a small barrier of dead cells and bare Petri dish around the square sample of gelatin. There was definitely something about the gelatin sheets that was not conducive to cell growth.

Part of the problem of having a very limited background in cell biology, is that there is a large learning curve that translated into a lot of trial and error for this project. The problem with the current process turned out to be very simple. While the glycine serves to neutralize the free glutaraldehydes and sterilize any microbial contamination, it does not eliminate material contamination. The sheets that we were using were prepared with

gelatin and tap water which contains many trace metals that contaminate the sheets and compromise cell growth.

(c) Materials and Methods (Second Iteration)

Several more steps were added to the preparation of the films to ensure sterility. The gelatin and MQ water mixture was autoclaved before the sheets were poured onto plates that were also autoclaved. Dense polycarbonate plates were used instead of metal plates and the tape used was kept in the sterile hood to avoid introducing additional contamination. The sheets were prepared in the hood and gloves and sterile forceps were used at all times. In addition, the process shown above in Figure 2 was modified to minimize handling of the films. Instead of transferring the sheet from Petri dishes for each treatment, the liquid was aspirated from the Petri dish after each treatment using a vacuum pump. Cells were plated once again according to the DOE in Table 2.

(d) Discussion (Second Iteration)

This time, after 24 hours there were healthy cells growing on the gelatin sheets. A summary table is shown below with several combinations of the DOEs that were performed during the experimental phases of this project. The low concentrations of gelatin with low glutaraldehyde concentrations create films that are structurally too weak to survive the incubation temperature for mammary epithelial cells of 37 °C. Films with concentrations of glutaraldehyde greater than 3.0%, were structurally too rigid for three dimensional matrices desired. It was also observed that at concentrations of glutaraldehyde greater than 3.0%, cell growth was prohibited. Films with less than 3% gelatin, were very flimsy and difficult to manipulate. These films were too fragile for the application of this project. Films that were made with more than 10% gelatin were too

thick for use in a bioreactor. These high concentrations of gelatin yield sheets with low permeability and often developed brittle mechanical properties after treatment with glutaraldehyde. The unshaded regions represent the optimal ranges for both structural use and for cell proliferation.

Gelatin Concentrations		< 3.0%	3.0 %	5.0 %	7.0 %	10.0 %	> 10.0 %
Glutaraldehyde Concentration	< 0.1 %						
	0.1 %						
	0.3 %						
	> 0.3 %						

Table 3 Un-shaded region shows optimal gelatin and glutaraldehyde concentrations for mammary epithelial cell growth and structural use.

Figure 7 Mammary epithelial cells on glutaraldehyde treated gelatin under 10x magnification

Figure 7 shows the cells under 10x magnification. This was a huge breakthrough for the project and gave proof of concept to move onto the next level, which was creating a three-dimensional area for cell growth.

Chapter 6. Geometry Development

Section 6.01 Phase I: Scaffold Geometry

(a) Preliminary Exploratory Research

The first configuration that was attempted was a very simple three-dimensional shape, a tube. This was most logical since the current process produced square sheets that in theory could easily be rolled into tubes. As simple as this concept sounds, there were still many obstacles that had to be overcome before a repeatable process was developed. The first process that was attempted involved rolling the untreated gelatin tubes around a sterile rod. When hydrated with water, the gelatin becomes sticky and will easily stick to itself, so the method involved re-hydrating the edges and sticking them together. While this worked, re-hydrating only part of the sheet created uneven and unsymmetrical tubes. Another method that was attempted without success was casting films on the rods and letting them dry. These proved impossible to remove from the rod, leaving them unusable. Yet another method was to treat the sheets with glutaraldehyde prior to forming tubes. This method was also unsuccessful, as the glutaraldehyde makes the sheets very slippery and hydrophobic, eliminating the possibility of adhering the sheet to itself. A combination of two of the previous methods proved to be successful.

(b) Materials and Methods

The gelatin sheets were prepared and dried completely as sheets. Then the sheets were fully re-hydrated in water and carefully wrapped around the rod. Glass and Teflon rods were both used and for this last method, the Teflon rods gave the most consistent results. First, an attempt was made to remove the tubes from the rod and treat the gelatin tubes with glutaraldehyde. However, the tubes didn't maintain their structure very well

during treatment without the rod for support. So a second attempt was to treat the gelatin tubes prior to removing them from the rods.

(c) Discussion

The rods provided support during treatment and also made it very simple for handling purposes. Being able to transport the tube using the rod eliminated possible contamination. Removing the tubes from the rod before they were completely dry seemed logical to prevent them from adhering to the rod; however they were too fragile at that stage and began to tear. The tubes were left to dry overnight and were easily removed from the rods once they were dry. A second proof of concept was achieved. By developing a method that could consistently produce gelatin tubes, the next step of integrating the materials into the system could begin.

Section 6.02 Phase II: Bioreactor Geometry

(a) Material and Methods

The goal for developing a system was to create a three-dimensional environment that would induce mammary cells to grow and secrete as they do in their natural environment. Previous research had shown that mammary cells plated on the bottom of Petri dishes will produce secretions, but cannot secrete their products as they do naturally. The goal of this project was to create a three-dimensional system that could be polarized giving the cells direction in which to secrete their products. In order to integrate the materials and process for creating and treating the gelatin tubes, the design for the system had to be modular. Another design constraint was that all components of the system had to be autoclavable to ensure sterility. The ultimate goal for the project

was to collect the secretions from the cells and therefore the system had to have an outlet.

Based on these design constraints the design concepts below were the first brainstormed.

Figure 8 Bioreactor Design - Option 1

Figure 9 Bioreactor Design - Option 2

Figure 10 Bioreactor Design - Option 3

Development continued with the prototype shown in Figure 6. Autoclavable parts were purchased from Sigma Aldrich. The system went together easily; the challenging part was integrating the gelatin tubes and cells into the system. Since the process for creating the tubes involved using the Teflon rod for support, that concept was incorporated into the system design. The gelatin sheets were secured to two quick release valves that easily snap in and out of the system. Holes were drilled into the valves that allowed the Teflon rods to pass through and provide support for the gelatin during the glutaraldehyde treatment. After the treatment, the rod was removed, leaving the gelatin tube secured to the valves on either end (Figure 11).

Figure 11 Modular section of bioreactor with unseeded gelatin tube.

(b) Discussion

The modular design that was chosen made the system easy to create and made integrating the previously developed material relatively easy. With the geometry created and the materials chosen, the only thing left to do was to prove that everything would work together as a system.

Section 6.03 Phase III: Modified system for proof of concept

(a) Materials and Methods

To simplify the experiment, an attempt to grow cells inside the tubes was isolated to the modular section shown in Figure 12.

Figure 12 Modular section of bioreactor with seeded gelatin tube

The falcon tube was filled so that the gelatin tube was surrounded with growth medium. Cells suspended in growth medium were injected into the top valve using a pipette. The weight of the top valve caused the gelatin tube to collapse slightly, but there was still enough shape to carry out the experiment.

(b) Discussion

After two days, the section was dissected and examined under a microscope (Figures 8-10). There were cells alive and adhering to the gelatin tube. This proved the concept that mammary cells would grow in a three-dimensional environment.

Figures 8-10 10x Images of dissected gelatin tube showing healthy, adhered mammary cells

Chapter 7. Proposed Continuation Efforts

The information presented above demonstrates a proof of concept for culturing epithelial mammary cells in a three-dimensional environment. The extensive experiments performed to optimize the materials used, as well as processing steps, proved to be effective when combined with the modular bioreactor system. The cell viability shown within the bioreactor provides enough confidence in the materials and system to be able to continue research in this area. As mentioned previously, the ultimate goal of this ongoing bioreactor project is for the cells to secrete as they do *in vivo*. As a follow up to this project, it is recommended that the secretory functions of the epithelial mammary cells within the bioreactor system are tested. Now that it has been proven that the mammary cells are capable of growing to confluence within the tubular section of the bioreactor, the next step would be to polarize the system to induce cell secretion. Recommendations for polarization include replacing the media on the basal side (outside of tube) with lactation media to induce cell differentiation and milk synthesis. The media on the lumen side (inside the tube) can then be replaced with phosphate buffered saline (PBS). The PBS should aid in the polarization process, and will also make it easy to purify the cell secretion products due to their suspension in PBS. A quantitative comparative analysis of the lactogenic response of mammary cells grown on flat gelatin substrates versus those grown in a tubular polarized environment would be the next recommended step for this project.

Chapter 8. Conclusion

This preliminary research has provided a very solid basis for further development of a three dimensional polarized environment conducive to mammary cell viability and increased secretion. This system will complement the extensive genomic and proteomic research in mammary gland biology. This area of research is currently limited by the expense and low success rate associated with using transgenic animals as bioreactors. This system will provide an alternate solution and will provide complementary data to the genomic or proteomic analysis of the mammary gland. The possible applications for this system, once fully developed, are nearly endless. The fields of health and secretion biology for commercial applications could benefit from a new model for biopharmaceutical production, which may contribute to the improvement of current bioreactor systems. Once fully developed and scaled up, the system could be applied to tissue engineering projects. Since mammary cells are a specific type of epithelial cells, this research should be compatible with other epithelial cell types and therefore applicable to tissue, such as skin. Preliminary research suggests that this system could possibly be a solution to the limitation of growing multiple cell types functioning within a larger-scale system. Components of this research could also be applied as a seed to the development of manufacturing and packaging techniques for “living mechanisms” such as biosensors, bio filters, and bioremediation efforts. The primary goal of this research however, was to develop a system that would be used to mimic a living system. The resulting system would serve as an in vitro factory for the production of biopharmaceuticals. Thus, the understanding and controlling of mammary epithelial

cells, partially accomplished through this research, is of high value to the pharmaceutical industry as a potential means for the production of complex drugs.

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