7-16-2015

Polyelectrolyte Coatings for Sequential Delivery of Growth Factors: Automation and Characterization

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by

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B.S., University of Connecticut, 2013

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

at the

University of Connecticut

2015

APPROVAL PAGE

Masters of Science Thesis

Polyelectrolyte Coatings for Sequential Delivery of Growth Factors: Automation and Characterization

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2015

Acknowledgements

I would like to acknowledge my advisor Dr. Liisa Kuhn for all of her guidance and help during my graduate education. I would also like to thank Emily Jacobs and Li Zhu for teaching me new lab techniques and sharing their knowledge with me. In addition to Dr. Kuhn's lab I would like to thank other members of the UConn Health Center: Dr. Gronowicz and Dr. Hurley. I would furthermore like to thank Dr. Donald Peterson for the last 3 years of advice that has led me to finishing my masters' degree this year. Lastly I would like to thank my friends and family for all the encouragement.

I would also like to dedicate this thesis to my family: Bill, Sheree, Kade, Matt, Billy, and Danny. They have been a constant support throughout my entire education and especially during my last year here at UConn.

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Abstract

Mimicking the body's natural healing process, which involves a variety of growth factors and cytokines produced by cells in a staged and sequential manner, has been shown to be a favorable technique in the field of tissue engineering. This project is focused on the fabrication and characterization of a polyelectrolyte multilayer (PEM) coating with and without a calcium phosphate (CaP) barrier layer capable of multiple growth factor delivery. The PEM coatings are created using a layer-by-layer (LBL) process in a dip coating machine in order to create uniform layers of oppositely charged material that can be used to deliver embedded growth factors. A custom sample holder was designed and fabricated using 3-D printing in order to minimize use of the expensive reagents. Diffusion and mixture of multiple growth factors through PEM-only coatings motivated the introduction of a CaP barrier layer. The CaP layer is precipitated using the simulated body fluid technique over the first growth factor followed by the PEM coating and additional growth factors. Confocal imaging of fluorescent model proteins was investigated as a means to assess the effectiveness of the CaP barrier layer in blocking diffusion of the model protein through poly L lysine/poly L Glutamic acid PEM coatings. Unfortunately, confocal imaging doesn’t have the resolution to discriminate the fluorescent model protein location within these nanometer thick PEM coatings, and the rough surface of the CaP further complicated the analysis. Cell-based bioassays are recommended to study ability of CaP to block diffusion of embedded factors within PEM coatings.

1. Chapter One: Introduction to Bone Tissue Engineering

With over 6.8 million patients in the United States with bone fractures per year, bone regeneration is a growing field. [1] Bone regeneration is a complex process that involves multiple cell types, proteins, and growth factors. The overall research goal of the Kuhn laboratory is to follow a developmental tissue engineering paradigm [2] where an implanted biomaterial will mimic and enhance the body's regenerative processes to a tipping point which will then trigger the body to take over the rest of the healing process. The idea behind developmental tissue engineering is to give the body a boost in the right direction without fully taking over the healing process.

Bone healing occurs as a cascade of events as seen in Figure 1. [3] The bone healing and regeneration process occurs in three main steps: inflammatory phase, reparative phase, and finally remodeling phase. [4] The inflammatory phase occurs within minutes of the fracture or damage and can last several hours. During this stage blood from the broken vessels will fill the wound site and a clot will form. This clot will cut off nutrition to the cells at each end of the break and cause cell death. Acute edema of the area will also occur at this time and signal for inflammatory cells as well as leukocytes, macrophages and mesenchymal cells. [5] Mesenchymal cells are a type of adult stem cells that can be found in bone marrow as well as peripheral blood, skeletal muscle, the heart, and the liver. [6] These cells are multipotent, meaning they are able to differentiate into various cells, such as bone or cartilage cells. [7] During the reparative phase the cells infiltrate the hematoma and a callus is formed. This callus contains cartilage, fibrous tissue, and the early stages of bone fibers. The mesenchymal cells will differentiate into osteoprogenitor cells and further into osteoblast cells. [7] Osteoblast cells are responsible for the building of bone within the body and during this reparative stage the

osteoblast will begin to form bone at the wound site. The last stage that occurs is the remodeling phase, this phase can last weeks up to years in the body. During this phase osteoblasts and osteoclasts build and resorb bone based on the mechanical stimulation. Since bone is a dynamic tissue, it responds to mechanical forces, with the lack of force leading to the resorption of bone. While bone may heal after fracture, it will never fully return to its original state. [8]

There are multiple types of bone grafts that are used in the body to help induce or speed up the healing process of bones. Bone grafts materials are broken down into 2 major categories: natural or synthetic. The natural bone graft materials include autografts, allografts, and sometimes xenografts. The synthetic substitutes include ceramic and/or polymer based bone grafts. The idea behind using a bone graph in a clinical setting is that as the surrounding natural bone grows the bone graft material will be replaced. These grafts can also contain growth factors that will stimulate bone production cells in the body or help differentiate cells into osteoblasts that build bone. [9] The healing process is also slower is older patients and so creating a bone graft that is supplemented with multiple growth factors that can specifically rejuvenate the healing process in the elderly is needed. [10] [11]

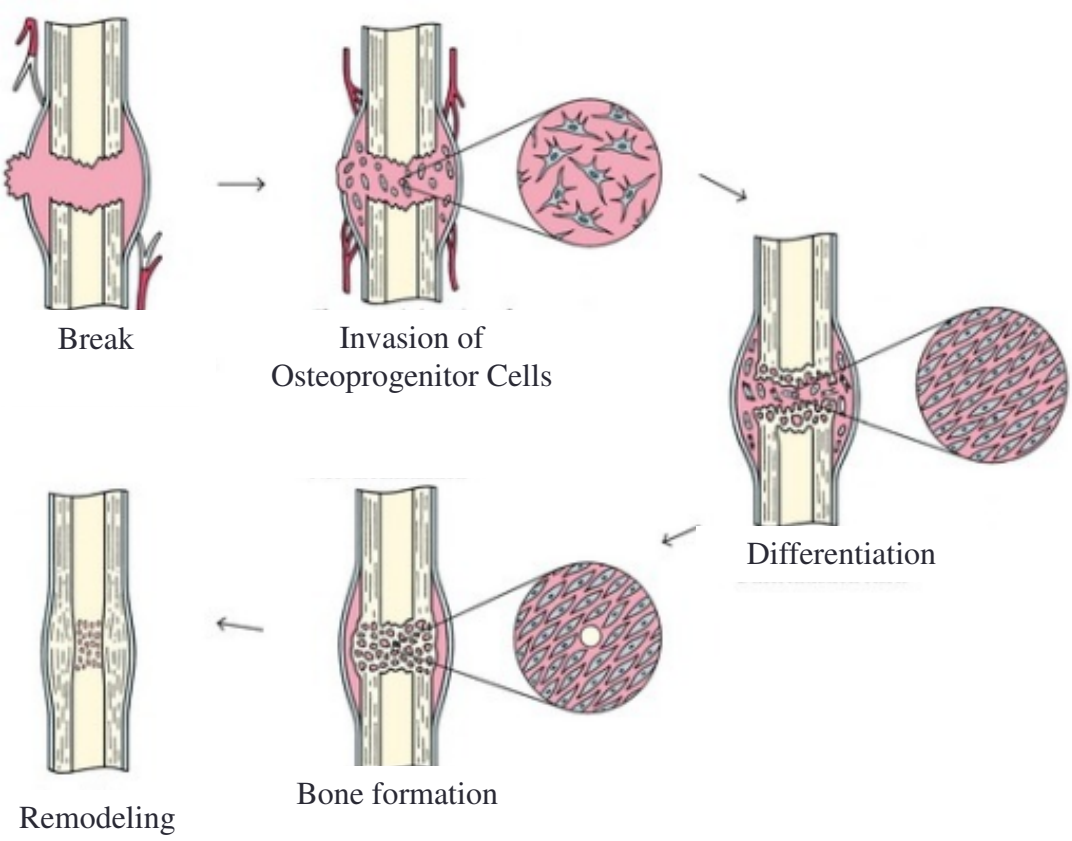


Figure 1: The Healing Process of Bone

1.1. Clinical need: A Biomaterial Coating Process that is Capable of Dual Delivery of two Growth Factors

In the proposed coating, two growth factors would be released to help with bone regeneration. Fibroblast Growth Factor-2 (FGF-2) would be used to proliferate the osteoprogenitor cells that are called to the wound site. The addition of FGF-2 also increases the sensitivity of the cells to Bone Morphogenetic Protein-2 (BMP-2), a protein that stimulates the production of bone in the body. The increased sensitivity to BMP-2 reduces the amount of BMP-2 needed in the second phase, which lowers the risk of adverse reactions. [12] Ideally after 5-7 days the second growth factor BMP-2 would be released to induce osteoblast differentiation. [13]

The increased number of osteoblasts in the wound site should decrease the healing time for bone fractures or damage as seen in Figure 2. [3]

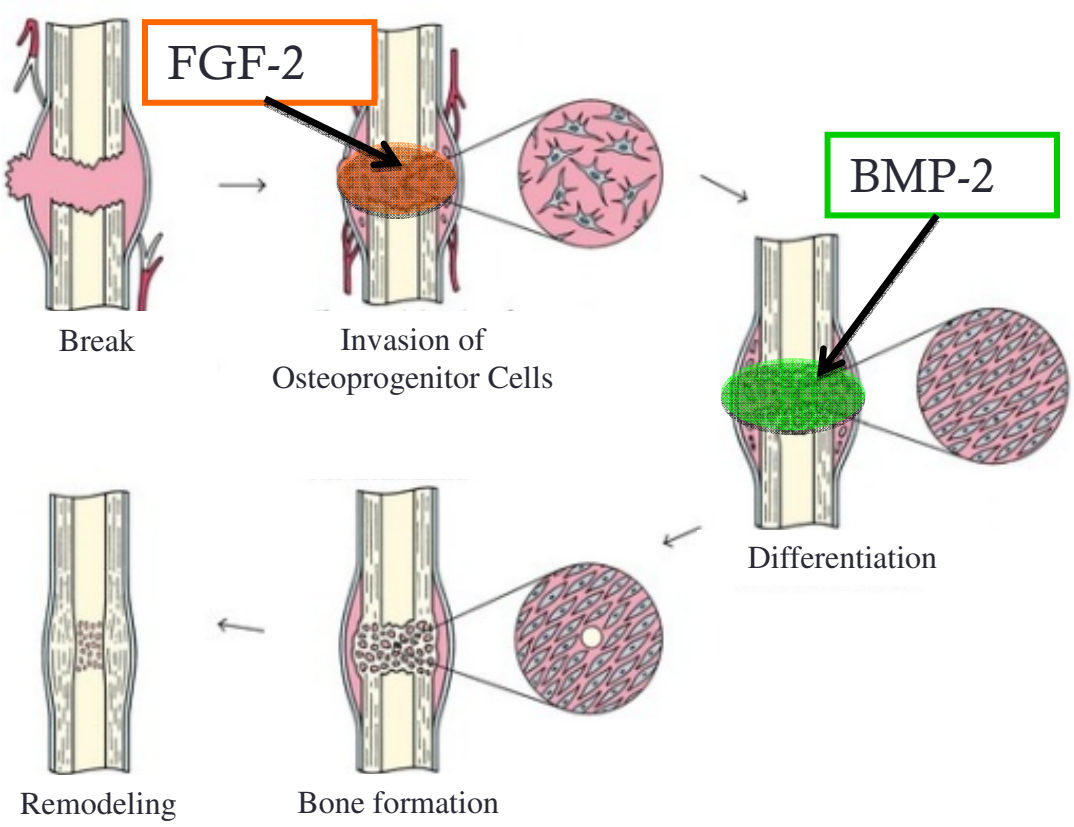


Figure 2: The Healing Process of Bone with Growth Factors Release Time Points

Our lab has previously shown an increased amount of mineralization and differentiation of mouse calvarial cells with the sequential delivery of the growth factors FGF-2 and BMP-2 when compared to a co-delivery of the same growth factors. [13] Those studies were completed without using a biomaterial scaffold. The importance of staged delivery of FGF-2 and BMP-2 was proven through growth factor delivery achieved by spiking in solutions of growth factor. Therefore, this project was focused on the development of a biomaterial coating technique that

can be applied to either flat or 3-D biomaterials to achieve sequential delivery of the growth factors. The unique design aspect of the coating is a calcium phosphate (CaP) barrier layer in combination with a polyelectrolyte multi-layer (PEM) made by sequential deposition of two polyelectrolytes, poly-L glutamic acid (PG-) and poly-L lysine (PL+) as seen in Figure 3. [14,15]

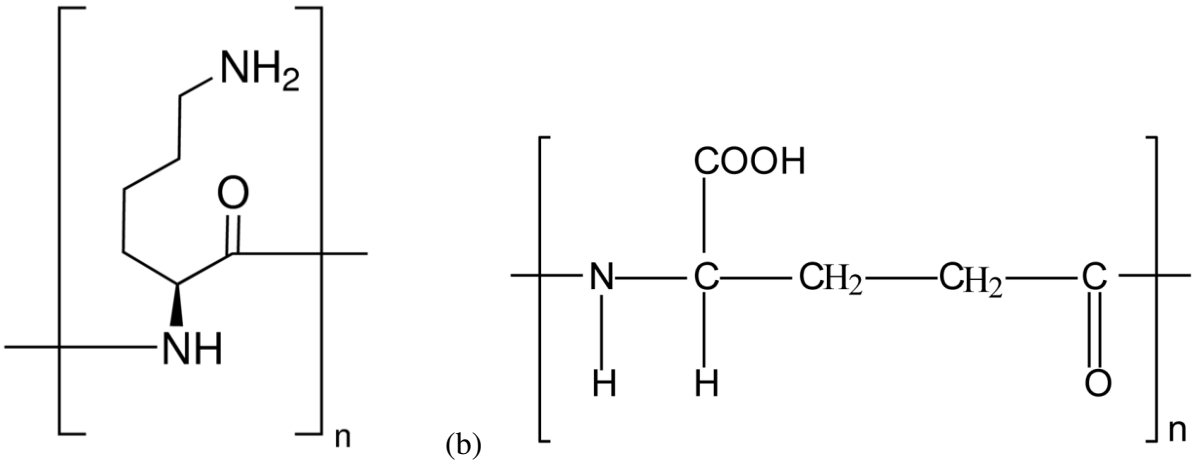


Figure 3: (a) Poly-L-Lysine and (b) Poly Glutamic Acid Chemical Structure

It is also relevant that this is a biomaterial coating, not an entirely new biomaterial. This allows for products that are currently on the market, and already FDA approved, to be coated and approved at a faster pace. Bone graft substitutes that are commercially available, such as Healos© (Depuy, West Chester, PA) can be coated. Healos© is a porous, spongy material made of Type I collagen that is coated with calcium phosphate in the form of hydroxyapatite. The layer-by-layer (LBL) process of the coatings also allows for multiple shapes and sizes to be easily coated rather than only 2D flat surfaces.

1.2. Polyelectrolyte Multilayers

In order to achieve sequential delivery of growth factors polyelectrolyte multilayers (PEM) are proposed for coatings on biomaterials. Polyelectrolytes are polymer solutions that

have either negatively charged units (polyanions) or positively charges units (polycations). [16] The electrostatic interaction between the positive polymer segments and the negative polymer segments allow for PEM to be fabricated most often by a layer-by-layer technique (LBL). A layer of a polycation (positive layer) is layered on a polyanion (negative layer) or vice versa depending on the substrate material charge. A single layer of a polycation and single layer of polyanion layer make a bi-layer. [17] It has been reported that the buildup of each layer has exponential growth due to diffusion of the positive and negative layers [18]. Because the electrostatic interactions are weak in the system during the layering technique the positive and negative polymers will move up and down the system. This provides the mechanics for the exponential growth. For example if a positive polycation was being layered all the negative polyanions would move to the top of the coating since opposite charges will attract, because of the stronger negative presence more deposition of the positive polycation will occur. [18] Figure 4 below depicts the exponential growth of a PEM coating. The initial pink line represents the positive polycation polymer and the teal line represents the negative polyanion polymer.

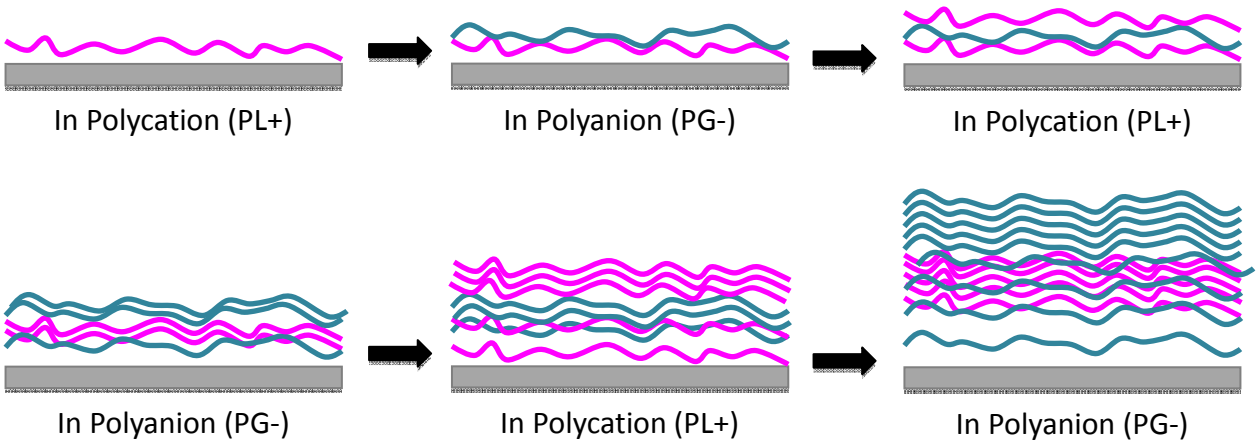


Figure 4: Exponential Growth of PEM Coatings

1.3. Calcium Phosphate Layer

PEM coatings will act as reservoirs for embedded growth factors, but because of the weak electrostatic binding, the growth factors can easily diffuse throughout the coating which prevents the ability of the PEM coating alone to provide sequential release. The diffusion of the growth factors can be seen in Figure 5(a). The addition of a calcium phosphate (CaP) layer is being investigated by the Kuhn lab as a barrier layer between growth factors within the biomaterial coating. The CaP layer is hypothesized to act as a barrier between the first and second growth factor. The PEM coating will contain the first growth factor that is easily accessible to the cells. The CaP layer is a poorly crystalline material that is easily degraded via cell mediated degradation [19] so in order for the cells to be exposed to the second growth factor they would have to degrade the PEM coating and also degrade the CaP layer. This system will allow for a sequential delivery of two growth factors from the same biomaterial as seen in Figure 5(b). [20]

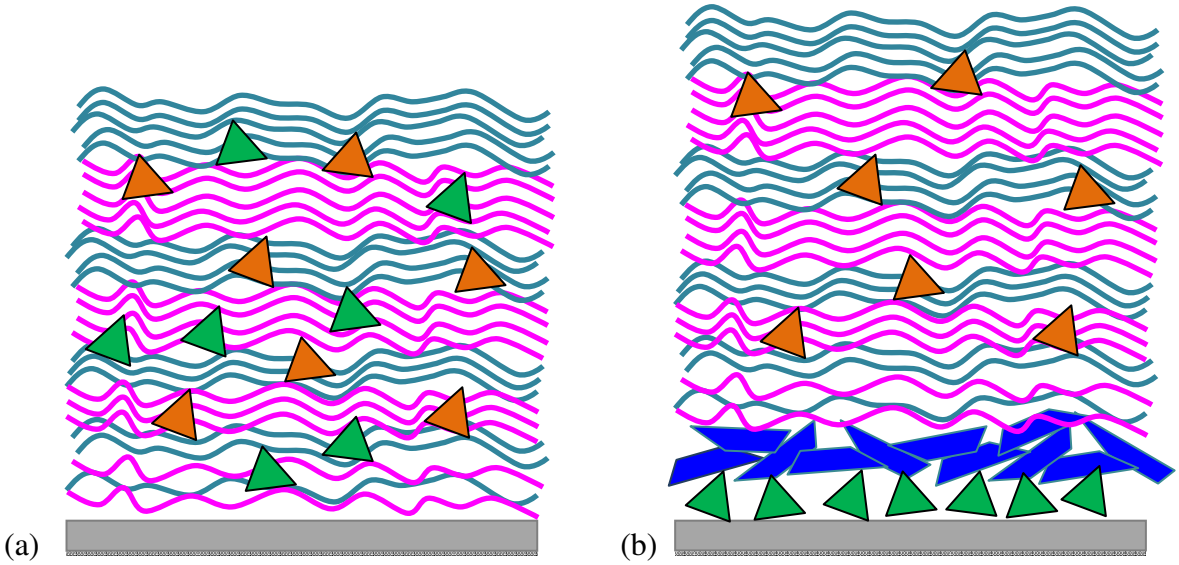


Figure 5: (a) Diffusion of Growth Factor and (b) Addition of CaP Blocking Diffusion

Figure 5 above represents a PEM coating that has embedded growth factors (green and orange triangles). The second image represents a CaP layer (blue polygons) that has a blocking effect and prevents the embedded growth factors from diffusing into the PEM coating.

Prior to this Master's thesis, the Kuhn lab made PEM coatings by hand pipetting each polyelectrolyte into a 12 well tissue culture plastic dish. While great care was taken to slowly add the solutions and rotate the angle at which solutions were added, it was not possible to pipette on coatings without disrupting the fragile bilayers of the PEM coating. The layers that were also hand pipetted were also not uniform due to human input, and thus automating the procedure would allow for an identical coating process between each insert. The second rational for automation was user input time. The previous method needed an individual to hand pipette each layer and wait the appropriate 10 minutes during each binding step. This caused a single bilayer to take just under an hour to create by hand. This time requirement limited the amount of layers that could be added to an experiment. The final motivation for automating the process was monetary constraints. In order to gain the most information out of *in vitro* studies it is important to have cost effective procedures. Previous to automating the process the cost of performing a 12-sample experiment was $434.16. After automation the cost of a 12-sample experiment was reduced to $201.00. By automating and reusing the coating material the lab is able to cut the costs by more than half per experiment. The breakdown of the costs can be seen below.

PG-: $\frac{\$123.50}{100mg} = \$1.23\ per\ mg * \frac{1ml}{1mg} = \$1.23\ per\ ml$

PL: $\frac{\$391.00}{500mg} = \$0.78\ per\ mg * \frac{1ml}{1mg} = \$0.78\ per\ ml$

0.6ml of solution is used per layer when hand pipetting

50ml of solution is used to coat 6 disks when using automated procedure

12 inserts per experiment

30 bilayers of PEM

<u>One Time Use:</u>

$0.6mL * 30\ layers * 12\ wells = 216ml$ of each solution

$216ml * \$1.23 = \$265.68$

$216ml * \$0.78 = \$168.48$

Total=$434.16

<u>Reused:</u>

$50ml * 2\ buckets = 100ml$ of each solution

$100ml * \$1.23 = \$123.00$

$100ml * \$0.78 = \$78.00$

Total=$201

1.4. <u>Project Statement</u>

This project is focused on automating the fabrication techniques and characterizing PEM coatings with and without a CaP barrier layer deposited on tissue culture plastic inserts capable of sequential delivery of multiple growth factors. These studies will all be performed *in vitro* in order to act as a base study for future *in vivo* studies performed with the PEM and CaP coating on a Healos© scaffold that will be placed in a mouse calvarial defect.

2. Chapter Two: Quartz Crystal Microbalance

2.1. Introduction

In order to evaluate if the polyelectrolyte solutions could be reused multiple times during an automated dip coating process without a substantial change in their concentration and without a change in the rate of deposition of the coating a Quartz Crystal Microbalance (QCM) was used, as seen in Figure 6. [21]

The machine used was a qCell T by a company called 3T Analytik. The goal of this study was to compare if polyelectrolyte solutions used multiple times (e.g. 30) during the dipping procedure resulted in the same rate of deposition of PEM coatings as compared to the use of new solutions for each step. A flowchart of the experiment can be seen in Figure 7.

Figure 6: 3T QCM Machine and Gold Sensor

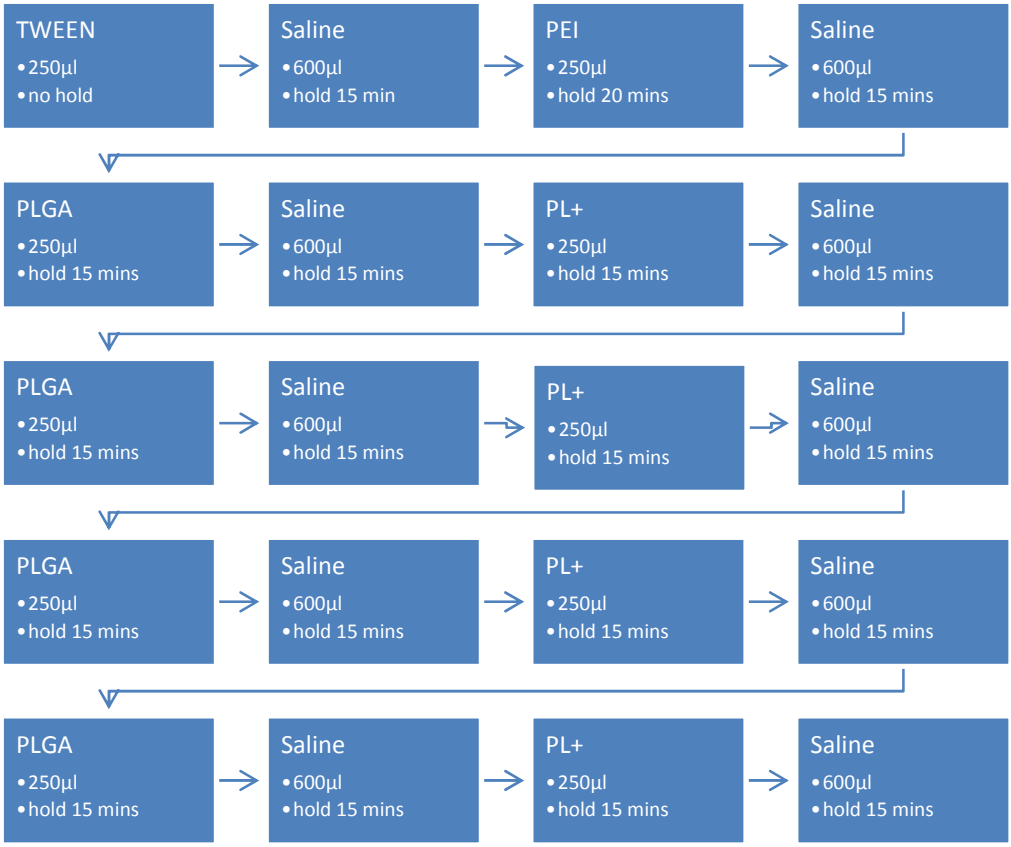


Figure 7: Flow Chart for QCM Experiment

2.2. Principle of QCM

The general principle behind how QCM works is based on the frequency of the quartz crystal in the system. “The measuring principle of quartz sensor technique is based on the precise oscillation of the quartz sensors at their resonant frequency when an alternating voltage is applied.” [22] The changes in both the observed frequency and the damping correlate to changes in the properties of the deposited layer. The polyelectrolyte solutions are added one after another starting with the negative polyanion (PG-) because the gold sensor, after being cleaned and prepared with polyethylenimine (PEI), has a positive surface charge. Polyethyleneimines are used in the cell culture of weakly anchoring cells to increase attachment. PEI is a cationic

polymer; the negatively charged outer surfaces of cells are attracted to dishes coated in PEI, facilitating stronger attachments between the cells and the plate. However, polyethylenimine has a very strong toxicity. Because it has toxicity it is never used when making PEM coatings for cell culture.

The change in the measured resonance frequency is caused by the polyelectrolyte absorption onto the gold sensor. The greater the frequency shift correlates to greater amount of polyelectrolyte deposition and greater coating thickness. When layers of polymers are added to the surface of the gold sensor, because they are viscoelastic and not purely Newtonian liquids, the frequency and damping will shift in opposite directions. [22]

2.3. Study Design

The goal of this study was to compare if polyelectrolyte solutions used multiple times (e.g. 30) during the dipping procedure resulted in the same rate of deposition of PEM coatings as compared to the use of new solutions for each step.

2.4. Materials and Methods

Two sets of data were collected in order to test the repeated use of polyelectrolyte solutions during the PEM coating process. A control of fresh solution (0x) was used as a baseline reading to check the changes of frequency that occur with each addition of polyelectrolytes for a total of four bilayers. The second set of data was collected using solution that was reused in the automated dip coating process. The solutions were reused to make a total of 30 bilayers (30x). The changes in frequency were normalized to start at zero during the initial saline coating.

The 3T Analytic Quartz Crystal Microbalance was turned on an allowed to warm up for 2 hours before any experimental data was collected as instructed by the user manual. The PEI,

saline, and polyelectrolyte solutions were degassed on a stir plate and under vacuum for 10 minutes.

Formula for Piranha:

70% sulfuric acid

30% hydrogen peroxide

Example: 5ml Piranha solution= 3.5ml sulfuric acid, 1.5ml hydrogen peroxide

Dangerous Material

MUST use glass for container for Piranha

*** WASTE DOES NOT go down the drain***

****ALWAYS ADD THE PEROXIDE TO THE ACID VERY SLOWLY*****

WARNING SOLUTION IS HOT

Proper personal protective equipment was used: heavy duty rubber gloves (with nitrile gloves as base glove layer, full face mask, and rubber apron. Under a fume hood, Piranha solution was created using the formula below and by adding peroxide component into acid component slowly and beaker was swirled in order to mix. Once Piranha solution was created, drops were added to cover the gold sensor, not the surrounding plastic holder. The Piranha was allowed to remain on the gold surface for 60 seconds until rinsed 4-5 times with Ultrapure water. Sensor was dried compressed air. Equipment was properly disposed in biohazard waste bin after drying and Piranha and waste solutions were collected in waste jar. The QCM machine set up can be seen in Figure 8.

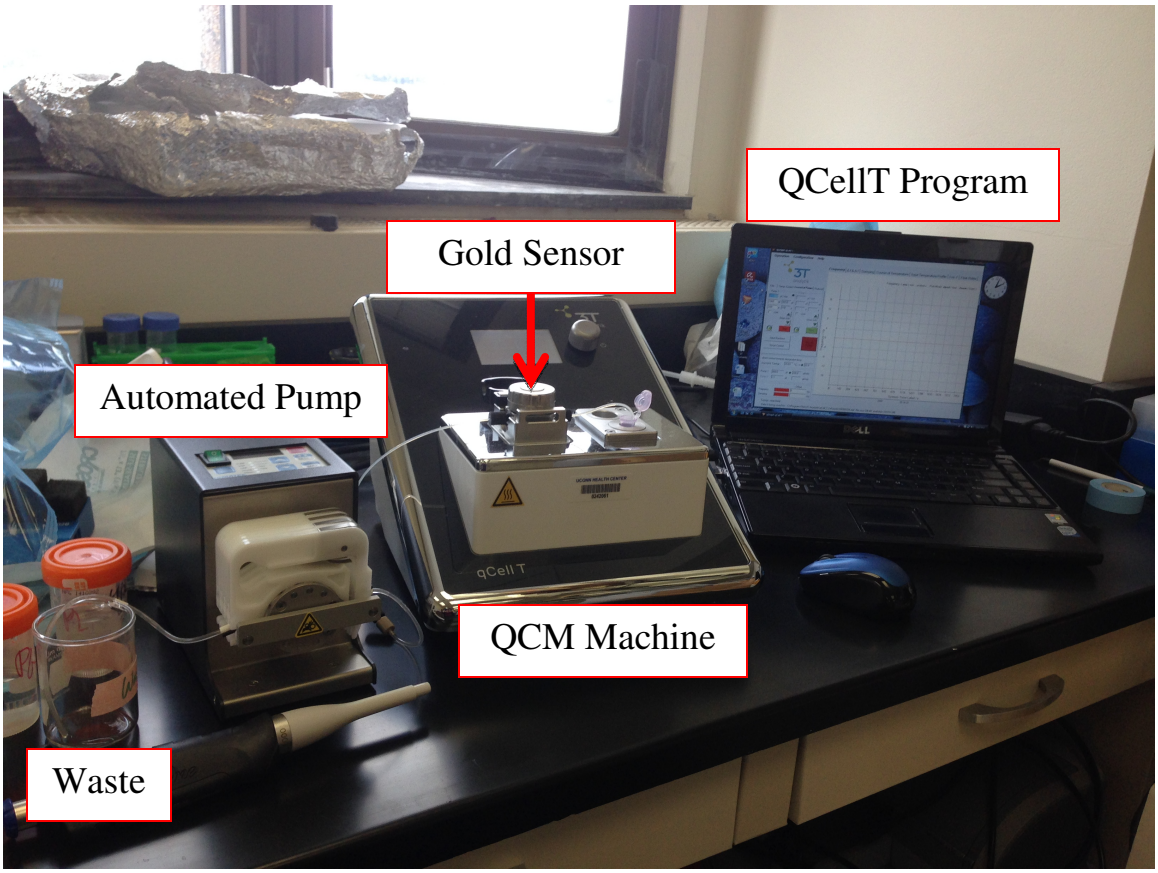


Figure 8: QCM Machine Experimental Setup

A solution of 0.5% SDS was run through QCM at a flow rate of 200µl/min for 3 minutes and held for 10 minutes. SDS should not be run through the machine with gold sensor in place because it clogs the sensor surface

Ultrapure water was run through the system at a flow rate of 200µl/min for 5 minutes. Air was run through system at a flow rate of 200µl/min for a minimum of 540µl to allow tubes to fully empty.

Newly Piranha cleaned sensor was placed into QCM and metal cover was replaced and locked into proper flow direction. The "QCellT" program was reset and the frequency was tarred. Program temperature was set to room temperature of 25ºC. Protocol feature in "QCellT" program was used to make note of changes in solutions. A solution of 1wt% TWEEN at a flow

rate of 200µl/min was run through the machine for 250µl to ensure no air bubbles formed on sensor. Saline at a flow rate of 200µl/min was run through the machine for 3 minutes and held for 15 minutes to allow frequency to plateau. A solution of 5mg/ml PEI at a flow rate of 200µl/min was run through the machine for 250µl and held for 20 minutes to allow frequency to plateau. The machine was rinsed with saline at a flow rate of 200µl/min was run through the machine for 250µl and held for 20 minutes to allow frequency to plateau. PG- solution at a flow rate of 200µl/min was run through the machine for 250µl and held for 15 minutes to allow frequency to plateau. Saline solution at a flow rate of 200µl/min was run through the machine for 250µl and held for 15 minutes to allow frequency to plateau. PL+ solution at a flow rate of 200µl/min was run through the machine for 250µl and held for 15 minutes to allow frequency to plateau. PG-, saline, and PL+ solutions were repeated for a total of 4 bilayers. After final step of experiment Ultrapure water at a flow rate of 500µl/min for 2ml was run through the system followed by air only until tubing is dry throughout the system. Experiment was repeated using solutions that had been used for 30 layers of PEM coating.

2.5. Results and Discussion:

RAW QCM data from the software is seen in Figure 10; the different color lines represent the addition of the polymer solutions starting with PG-. The bilayers were repeated for a total of 4 bilayers. There are slight increases in frequency when the saline solution is added as the rinse step removes any particles floating above the sensor that may cause incorrect layer thickness readings.

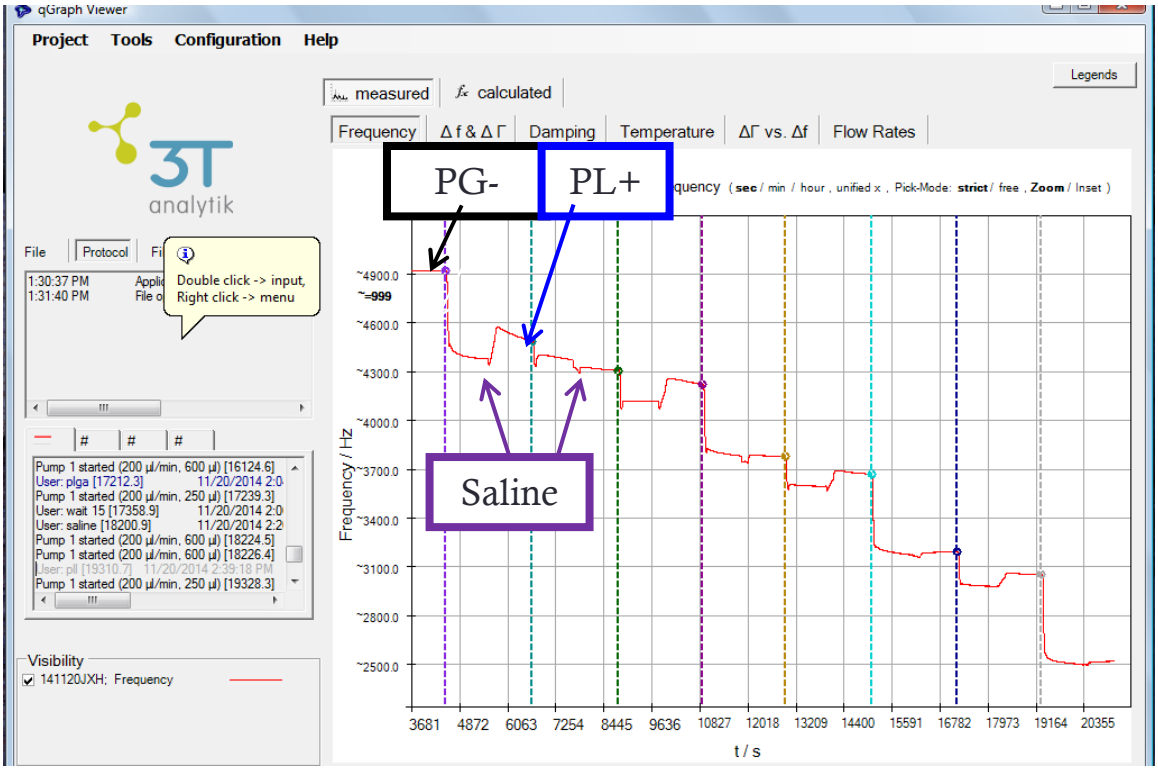


Figure 9: Raw QCM Data

From the graph below in Figure 10 it can be seen that the changes in frequency have similar trends between the 0x and the 30x used solutions. While there is less of a decrease in frequency with the 30x solution, indicating less of a layer buildup, it is only a minor decrease and should not have a major impact on the layer buildup of 30 bilayers. It is also know that there is some carry-over from the automated machine between the dip steps so it might also affect the 30x solutions in the QCM. Lastly, numerous studies using PEM coatings use automated dip-coating machines for multiple bi-layers with little or no mention of changing solutions. [23-26]

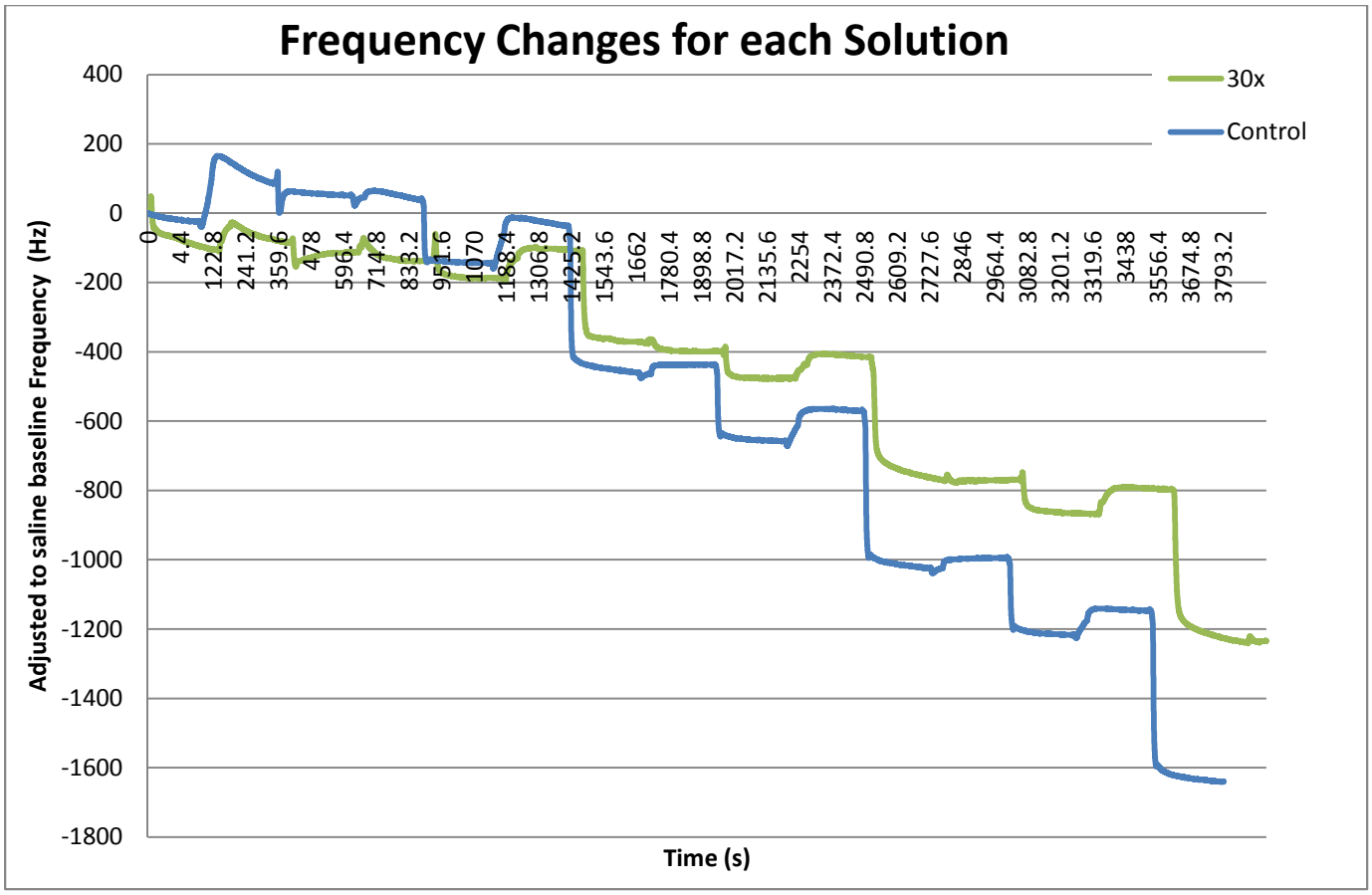


Figure 10: QCM Frequency Shifts for 0x and 30x PEM Solutions

The results indicate that reusing the polyelectrolyte solutions can be used with minor impact on film build-up. From this data, it can be concluded that it is safe to reuse solutions for 30 bilayers without compromising the PEM coatings on biomaterials.

3. Chapter 3: Automation of PEM Coating Procedure

3.1. Introduction:

The layer-by-layer (LbL) procedure for PEM coatings allow for an easily automated procedure. There are numerous studies that have used programmable dipping machines to build PEM coatings. [23-26] Unfortunately the majority of the machines that were used had dipped single rectangular glass slides and used larger volumes. [23,25] For the purpose of cell studies in our lab, 22mm circular tissue culture plastic disk inserts are used and need to be coated and the goal was to coat 12 or more at a time. The tissue culture plastic disk inserts are sandblasted in order to have a rougher surface for the PEM and the calcium phosphate to bind to. A Varistain 24-4 histology machine was able to perform the dipping process needed to create the PEM coatings but only had attachments for rectangular slides. The other problem with the machine was that the buckets used to hold histology stains were too large (holding up to 750ml). This amount of material would be too costly to use for the PEM coatings.

Two devices were designed in SolidWorks: a disk holder and a bucket insert. SolidWorks is a computer assisted design (CAD) program that allows users to take 2D engineering sketches and create 3D models of designs. The disk holder was designed to hold up to 6 disks, allowing for studies to have an experimental replicates of n=6. The bucket inserts were designed to hold 3 specimen cups in a set position to allow the disks holders to easily dip into each specimen cup holding the polyelectrolyte or saline. Up to 6 different experimental groups each with n=6 can be run in a single trial using the Varistain machine and the custom design parts. The basis procedure for the PEM coating process is a dip in PL+ followed by rinses in saline then dip in PG- followed by rinses of saline and repeated until the desired number of bilayer is reached as seen in Figure 11.

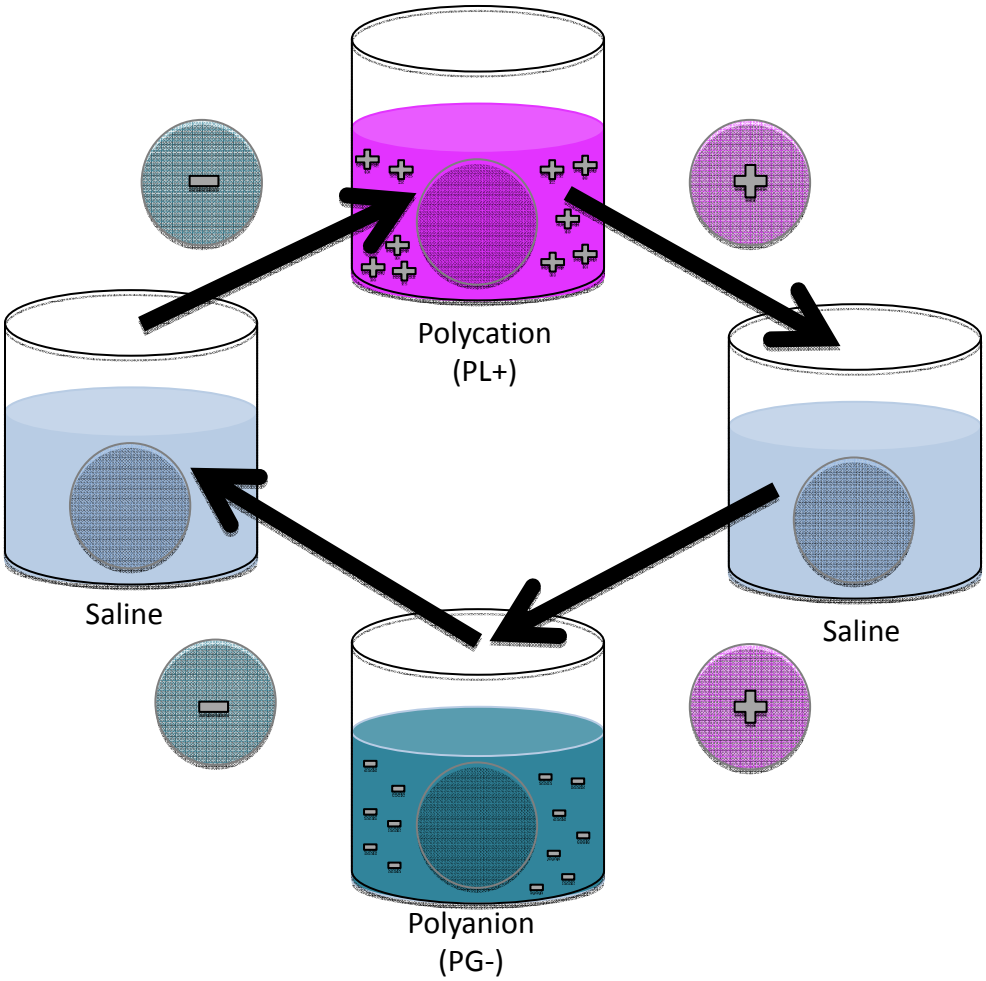


Figure 11: Layer-by-Layer Process of PEM Coatings

The SolidWorks files were used to 3D print custom disk holders and bucket inserts. A MakerBot Replicator 2 was used for 3D printing as seen in Figure 12. [27] The basic goal of 3D printing is to create 3D physical objects from a computer file. The process is performed similar to an ink printer where material is extruded and laid on a flat surface in a specific pattern. For an ink printer the ink will be extruded onto a sheet of paper while for a 3D printer the material is extruded onto a build plate. The 3D printer will continuously build onto of the first layer creating a 3D object layer-by-layer. The MakerBot 3D printer used for the project used a technique called fused deposition modeling (FDM).

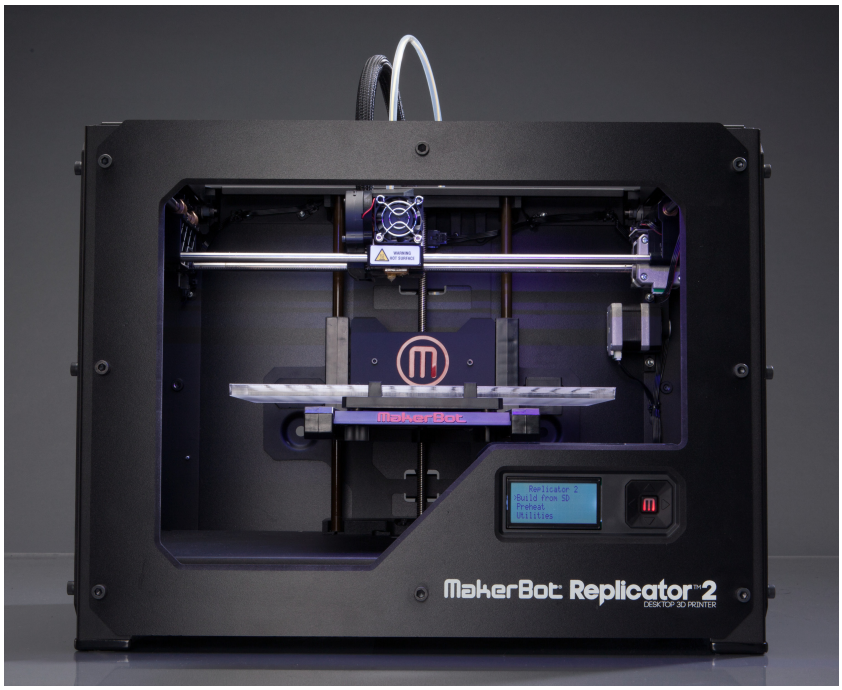


Figure 12: MakerBot Replicator 2 3D Printer

During FDM the nozzle of the 3D printer will heat up and melt the material, most often a type of plastic, and extrude the material onto the build plate. The material almost hardens immediately after it is extruded. [28] The MakerBot Replicator 2 used polylactic acid (PLA) plastic as the material for building the 3D model.

The Varistain 24-4 (Thermo Scientific, Waltham, MA) machine is primarily used for histology slide staining as seen in Figure 14. The manual is available at www.thermoscientific.com. The machine consists of 24 buckets that have a solution capacity of 750ml each. The machine operates based on programed steps. Each step the canopy of the machine will lower into the buckets, wait the allotted time, raise and revolve clockwise to the next step. The machine has the capability to store up to 3 programs each containing up to 24 steps. The programs are able to have an “end” step where the holders will remain in the last step or a continuous program where the machine will continue to perform the same 24 steps until the user stops the program.

Figure 13: Varistain 24-4 Histology Machine

3.2. Materials and Methods:

SolidWorks Software was used to design 3D models of a custom 22mm tissue culture plastic disk insert holder and a custom Varistain 24-4 bucket insert. The dipping heights as well as the step movement of the machine was taken into account when designing the disk holder. The bucket size of the Varistain 24-4 as well as the circumference of the specimen cup used to hold the experimental solutions was taken into account when designing the custom bucket insert. After the two devices were designed ".STL" files were created of the devices. The ".STL" files were loaded into the MakerBot Desktop Software program. The devices were rotated and moved to align properly on the build plate. The "Make" function was used to create a build design for the MakerBot Replicator 2 3D printer. During this step a raft was added to secure the thin sections of the devices to the build plate. The resolution was also adjusted to have 0.2mm thick layers. The MakerBot file was saved onto a USB and imputed into the MakerBot Replicator 2 and the devices were printed. Polylactic Acid (PLA) was used the building material for the devices. The extruder was set to heat to 215 °C in order to melt the PLA and print layers. On

average a single disk holder would take just over an hour to print and a bucket insert would take just over a half hour. The MakerBot user manual is available at www.MakerBot.com.

In order to ensure there is no contamination between various experimental groups, the custom disk holders were cleaned before each use. The holders were rinsed in 0.5% SDS and hand washed ensuring disk grooves were thoroughly cleaned. A plastic test tube brushed was if needed to remove calcium phosphate or calcium phosphate buildup. The disk holders were then rinsed 3 times with DI water. Following the DI rinses the disk holders were rinsed 3 times with Ultrapure water. Once rinsed the holders were places in a sonicator machine and sonicated for 3 times, or a total of 30 minutes. The holders were allowed to air dry and stored under vacuum when not in use.

The protocol for general programming of the Varistain 24-4, seen in the appendix, was followed to create a program for PEM coating with 8 saline rinse steps. The flow chart for the steps needed to be included in a program for 30 layers of PEM and 8 saline rinse steps can be seen in Figure 14.

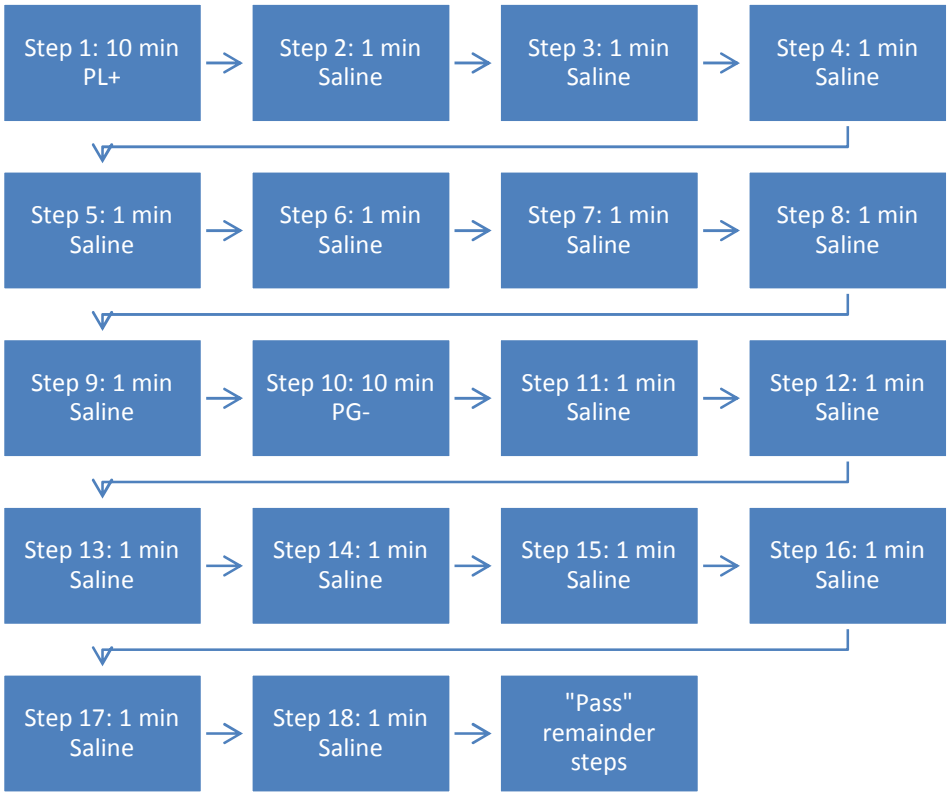


Figure 14: Flow Chart of Varistain Programming

In order to sterilize the tissue culture plastic disk inserts they are placed under UV light for 10 minutes on each side.

Labels were added to 21 specimen cups broken into appropriate groups. Cups were filled with 50ml of saline. Labels were added to 6 specimen cups broken into the same groups, each with a PL+ and PG- label. Specimen cups were filled with 50ml of the appropriate polyelectrolyte solution as seen in Figure 15.

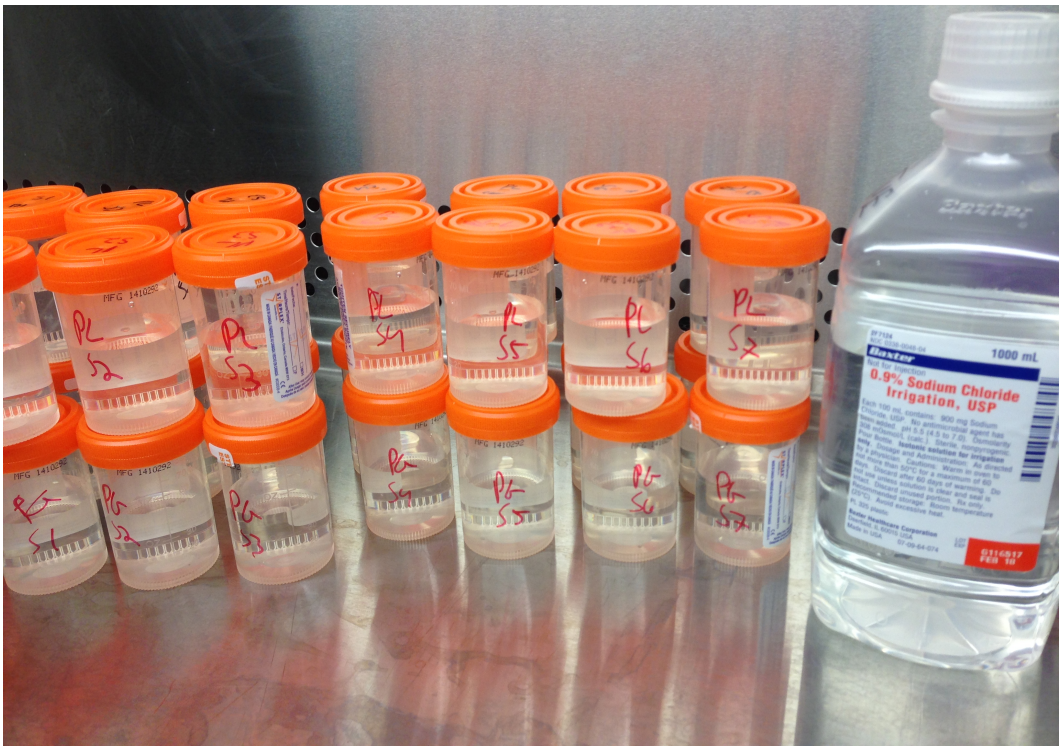


Figure 15: Labeled and filled Saline Rinses in Specimen Cups

Program for 30 bilayers was loaded into Varistain machine, custom 3D printed histology bucket guides were added, and specimen cups were loaded into appropriate locations. Custom 3D printed holders were attached to the machine starting above the PL+ solutions. Machine was allowed to run for 20 hours and 42 minutes to complete 30 bilayers of PEM coating on disks.

3.3. Results and Discussion:

The original proposal for the 3D custom printing was to have one holder that was capable of holding 6 different experimental groups that would be dipped into a custom bucket. This design would allow 6 different groups to be preformed at the same time with 6 samples per group. This design would also only use 30ml of polyelectrolyte solutions. The design can be seen in Figure 16.

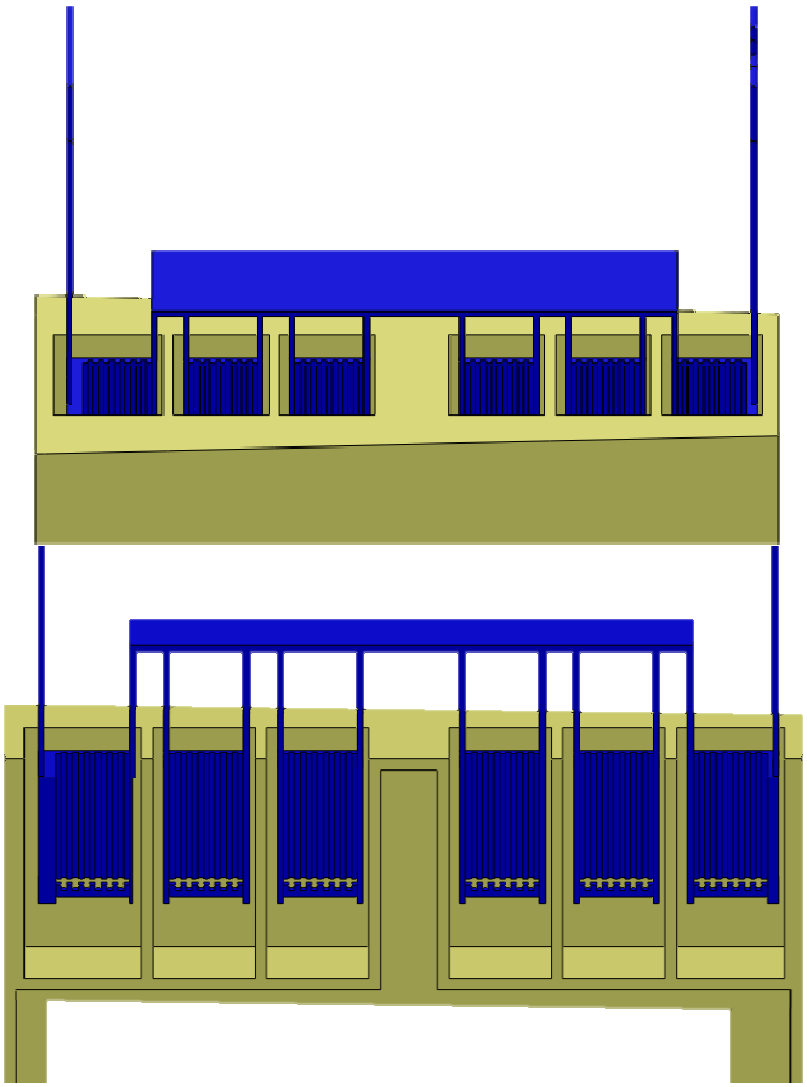


Figure 16: Two views of original SolidWorks design for automation of PEM coating

After the initial designs were printed it was discovered that the Varistain machine did not have enough horizontal movement control to accurately dip the holders into the custom buckets. Often the holders would not properly align when moving between steps and the edges of the holders would stop on the edge of the bucket.

New SolidWorks models for the disk holder were designed as individual holders for 6 disks. Upon further design specimen cups were found to be optimal solution containers as well. The specimen cups would allow for only 50ml of solution to be needed as well as the sterility

needed for future cell cultures. In each histology bucket 3 specimen cups can fit in a linear pattern. In order to ensure the same placement of specimen cups in the histology buckets a custom bucket insert was designed. The design consisted of 3 holes to act as a guide to ensure the specimen cups were properly aligned in each bucket. The design can be seen below in Figure 17.

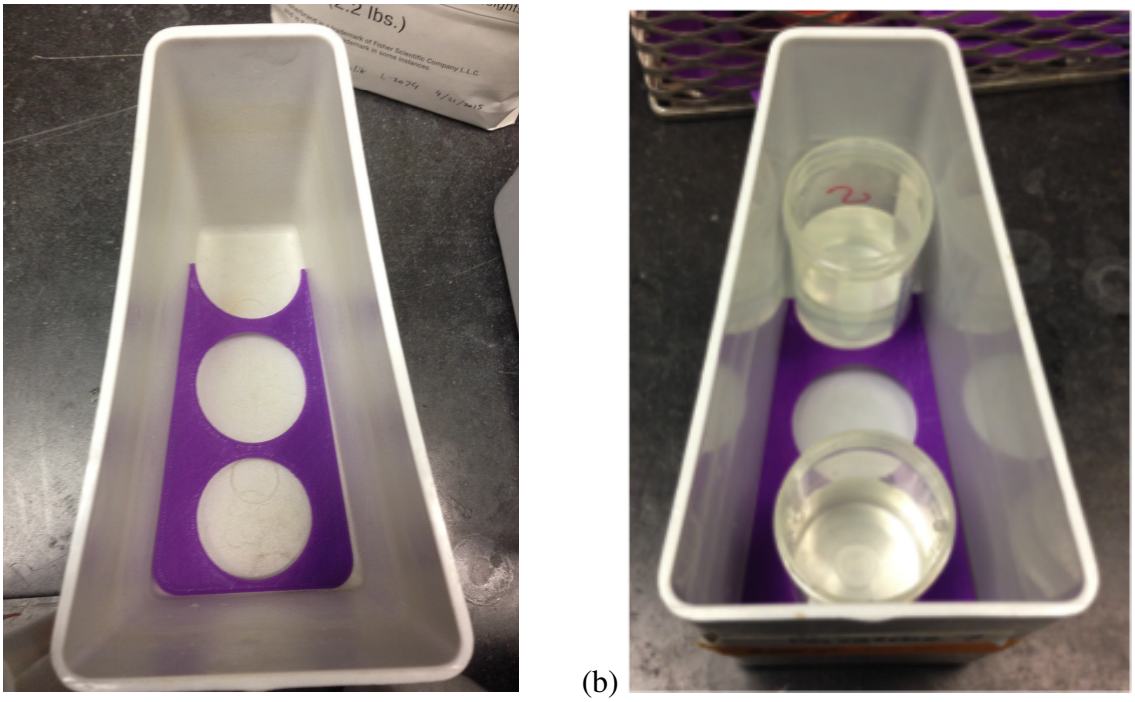


Figure 17: Custom 3D Printed Bucket Guide inside Varistain Machine Bucket without (a) and with (b) Specimen Cups

Multiple prototypes of the disk holder was created in order to achieve the lowest amount of surface area as well as preventing the disk inserts from floating out of the holder when submerged. The lowest amount of surface area was due to the solution carryover that occurred during the dip coating process. During the early runs of the disk holders, the polyelectrolyte solutions because progressively cloudy due to the formation of small particulates due to a mixture of PL and PG. A simple experiment was performed with green food coloring added to

saline as a model of the polyelectrolyte solution to examine the amount of carryover of solution that occurred during the dip coating process.

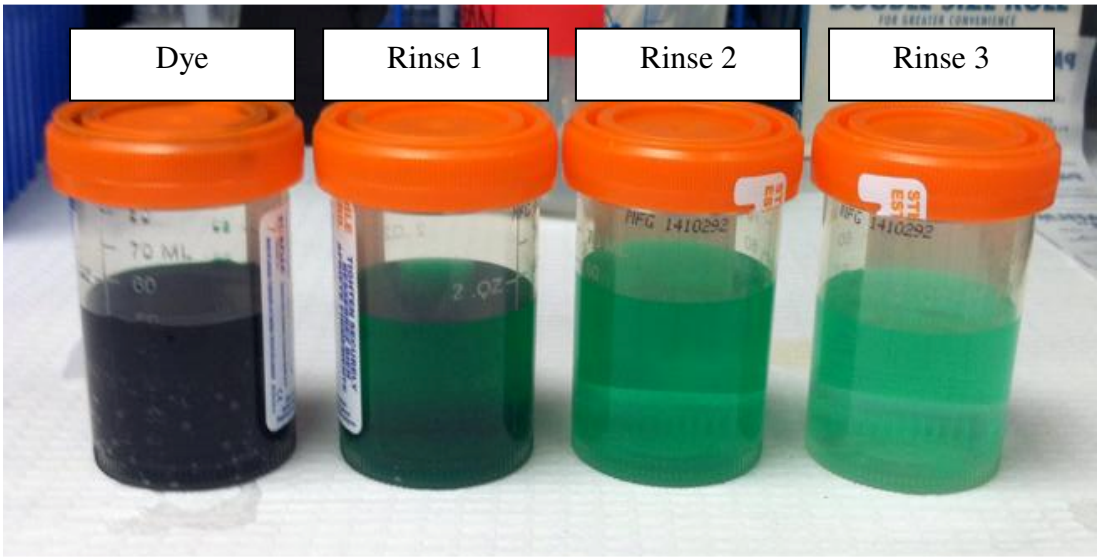


Figure 18: Dye Carry Over Between Saline Rinses

Because of the carryover that was observed to occur by the food coloring, Figure 18, additional saline rinse steps were added to the dip coating program raising the number from 3 to 8 rinses after each polyelectrolyte solution step. The carryover can be reduced in the future by printing the disk holders at a higher resolution and also using acetone. The acetone can smooth the side of the holders which are grooved due to the layering printing technique.

The final SolidWorks design and 3D printed version of the tissue culture plastic disk insert holder can be seen in Figure 19.

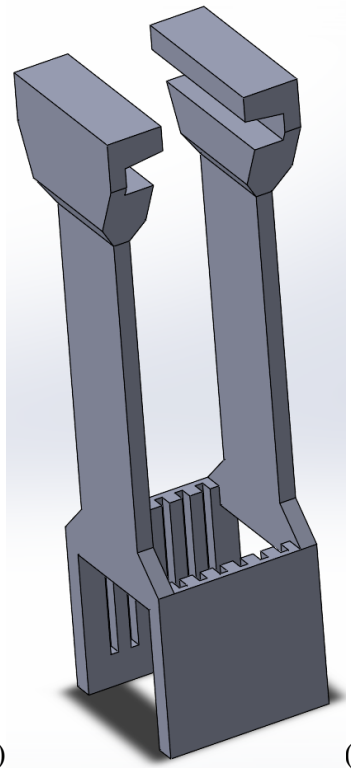
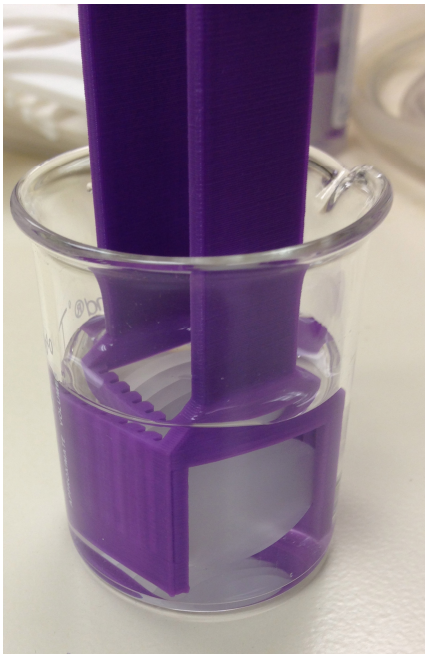
(a)  (b)  (c) 

Figure 19: (a) SolidWorks Design, (b) 3D print, and (c) device holding tissue culture plastic inserts

The final automated design can be seen below in Figure 20. The bucket guides hold the specimen cups in proper alignment and the slide holder is aligned over the specimen cups. This automation allows for polyelectrolyte solutions to be reused 30 times. This process also the user to set the machine and leave for 20 hours and 43 minutes to create PEM coatings of 30 bilayers. The automation of the process has not only saved the lab over 50% of the costs per experiment, but it has also saved time. The lab is now able to create PEM coating of 30 layers overnight which used to be performed over 3 days with each day consisting of 10 hours of hand pipetting coatings. This custom automation also allows more freedom in the number of PEM coating bilayers. While solutions must be changed every 30 layers, the number of PEM bilayers can far exceed 30.

Figure 20: Image of final PEM coating Automation Set-up

After repeated use of the 3D printed PLA plastic sample holders there was slight degradation of the material. It was found in a study that the PLA material with a thickness of 0.2mm fully degraded in 168 hours of *in vitro* incubation. [29] For a PEM coating of 30 bilayers the process takes just under 21 hours in solutions to complete. The degradation of the PLA material into the polyelectrolyte solutions and the saline rinses could cause errors in the PEM coatings. While the MakerBot Replicator 2 is only able to print PLA, the CAD model can be sent out to another company and additional plastics can be used with less degradation.

Polyphenylsulfone is a possible alternative because of the high chemical resistance of the material and it has the ability to easily sterilized using common methods such as an autoclave. The company Stratasys has the ability to 3D print multiple types of thermoplastics.

4. Chapter 4 Confocal Microscopy

4.1. Introduction

Confocal imaging has been used to create Z-axis images of PEM bi-layers that contain different colored fluorescently labeled proteins [24]. In order to investigate the ability of the CaP/PEM to block the diffusion of a protein within PEM layers, model proteins (BSA Alexa Flour) fluorescently tagged at 448 (green) and 594 (red) were used as representations of the growth factors BMP-2 and FGF-2 respectively. The main difference between confocal microscopy and normal microscopy is the ability of a confocal microscope to only detect in-focus light. The process works by a laser exciting the sample, the excited fluorescence being emitted from the sample, and the microscope detecting the emitted fluorescence though a pinhole which will remove any out of focus light emissions. This light pathway can be seen in Figure 21. [33] This process allows the user to view different depths of their sample and from the different depths create z-axis images of their sample.

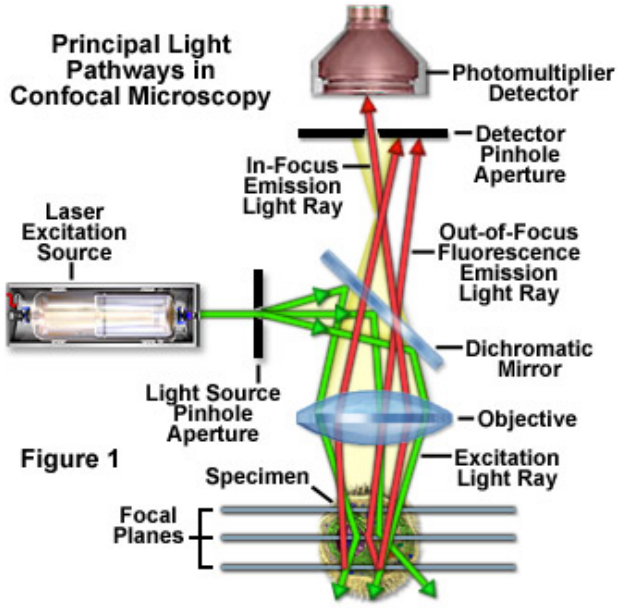


Figure 21: Confocal Microscopy Light Pathway

4.2. Materials and Method

In order to test the diffusion of BSA 488 into the PEM coating with and without a calcium phosphate layer 3 groups were tested. The first group consisted of BSA 488 embedded under 30 bilayers of PEM. The second group consisted of BSA 488 embedded under a layer of CaP and then 30 bilayers of PEM. The final group consisted of a layer of CaP, 30 bilayers of PEM and BSA 488 bound to the top in order to test if BSA-488 had an affinity to the CaP layer.

Group 1: TCP_{SB}-BSA_{488}-PEM_{30} (n=3)

Group 2: TCP_{SB}-BSA_{488}-CaP-PEM_{30} (n=3)

Group 3: TCP_{SB}-CaP-PEM_{30}- BSA_{488} (n=3)

Clean tissue culture plastic inserts (TCP_{SB}) were obtained for an n=3 for each group. The 9 inserts were divided into 3 petri dishes (1 per group). Groups 1 & 2 had 250µl of BSA 488 binding solution added to the center of each insert. Group 3 had 250µl of saline added to the center of each disk. These groups can be seen in Figure 22.

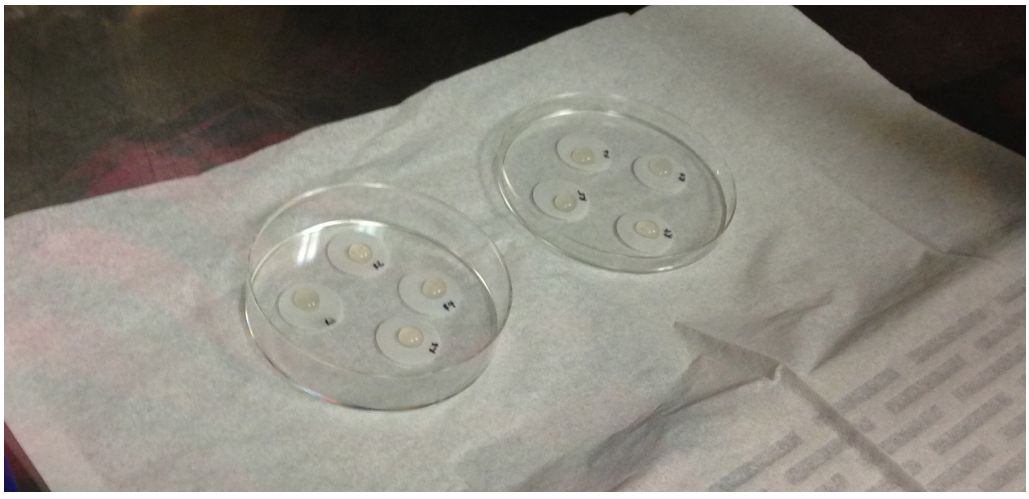


Figure 22: BSA 488 Binding to Tissue Culture Plastic Disk Inserts

Tin foil was used to cover inserts when not in use since BSA 488 is a light sensitive material. Solutions were allowed to bind for 1 hour and 30 minutes. Supernatant was removed

from each disk and disks were rinsed 3 times with saline. Disks were placed in cleaned custom 3d printed disk holders, one group per holder.

A layer of calcium phosphate was deposited onto Group 2 & 3 inserts via simulated body fluid with 5 times ionic concentrations (SBFx5). Group 1 was treated in saline under same conditions. Groups 2 & 3 inserts were rinsed with Ultrapure water 3 times. Groups 2 & 3 inserts were placed in clean custom 3d printed disk holders. Groups 2 & 3 were hand dipped into PG- solution for 10 minutes and rinsed in saline for 1 minute for a total of 8 rinses.

Labels were added to 21 specimen cups broken into Groups 1, 2 & 3. Cups were filled with 50ml of saline. Labels were added to 6 specimen cups broken into the same groups, each with a PL+ and PG- label. Specimen cups were filled with 50ml of the appropriate polyelectrolyte solution. Program for 30 bilayers was loaded into Varistain machine, custom 3D printed histology bucket guides were added, and specimen cups were loaded into appropriate locations. Custom 3D printed holders were attached to the machine starting above the PL+ solutions. Machine was allowed to run for 20 hours and 42 minutes to complete 30 bilayers of PEM coating on disks. Samples were removed from machine and inserts were placed in saline to prevent coating from drying out.

A Zeiss LSM 780 confocal microscope (Zeiss, Oberkochen, Germany) was used for the imaging as seen in Figure 23. Blank inserts and PEM only inserts were used to set gain settings on confocal microscope to remove any background fluorescence. All settings were held constant for each sample imaging. Z-stack images were taken from each sample in order to see layering of PEM coating and diffusion of BSA 488. Each sample was made into a “sandwich” using coverslips on the bottom and top as well as BSA 594 to act as a red line to mark the bottom of the insert and a drop of BSA 488 to mark the top of the insert as seen in Figure 24.

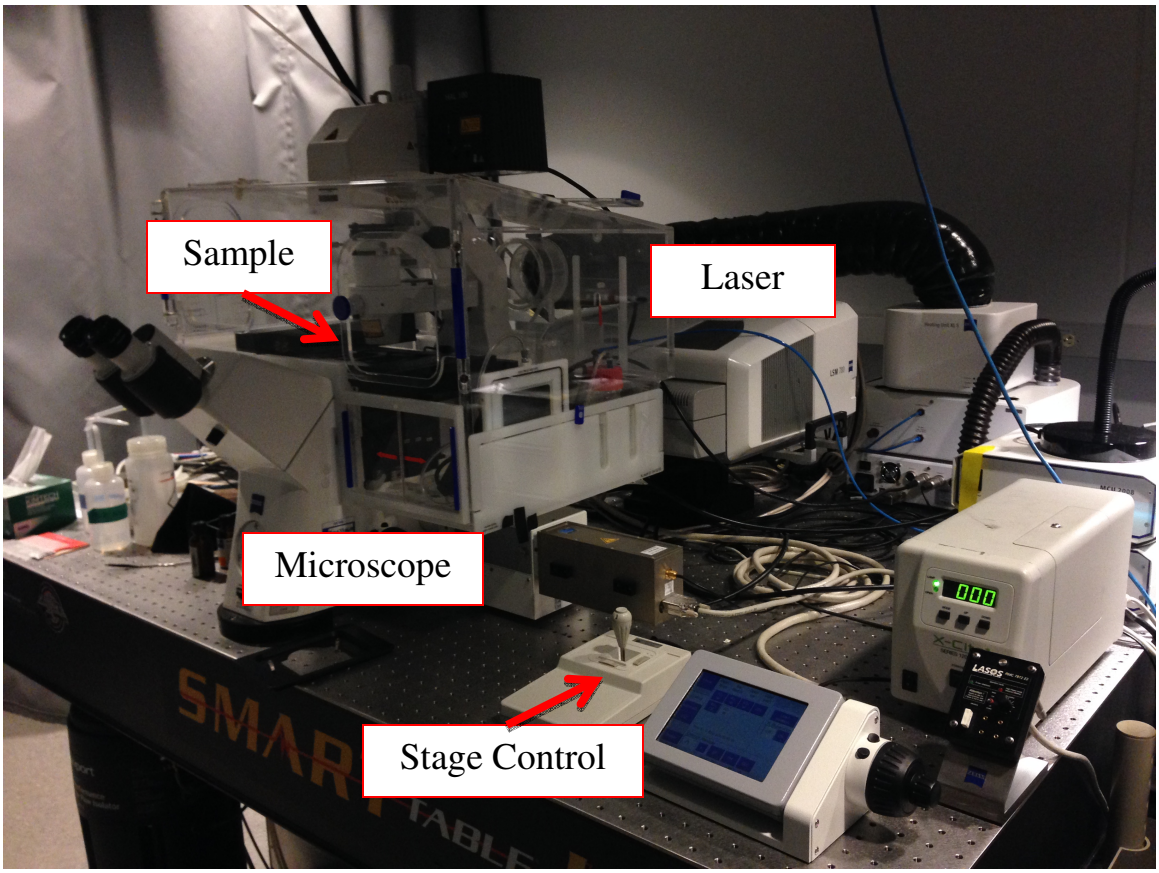


Figure 23: Confocal Microscopy Machine Set Up

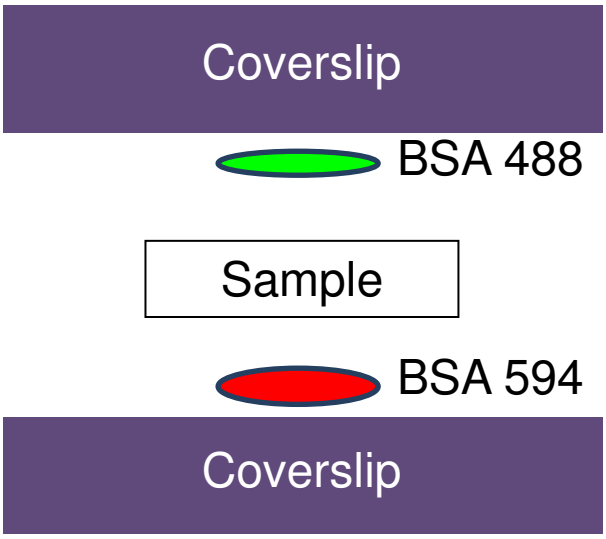


Figure 24: Confocal Sample "Sandwich"

Collected images were analyzed using ZEN Software (Zeiss, Oberkochen, Germany). Diffusion distances were measured and labeled.

4.3. Results and Discussion

Four controls were used to set the gain settings for the confocal microscope. The blank insert, insert with 30 bilayers of PEM, insert with Cap layer, and an insert with both 30 bilayers of PEM and a CaP layer. Again a drop of BSA 594 was used as a marker in order to determine the location of the bottom of the disk and BSA 488 to mark the top of the disk. The layering procedure for the controls can be seen below in Figure 25.

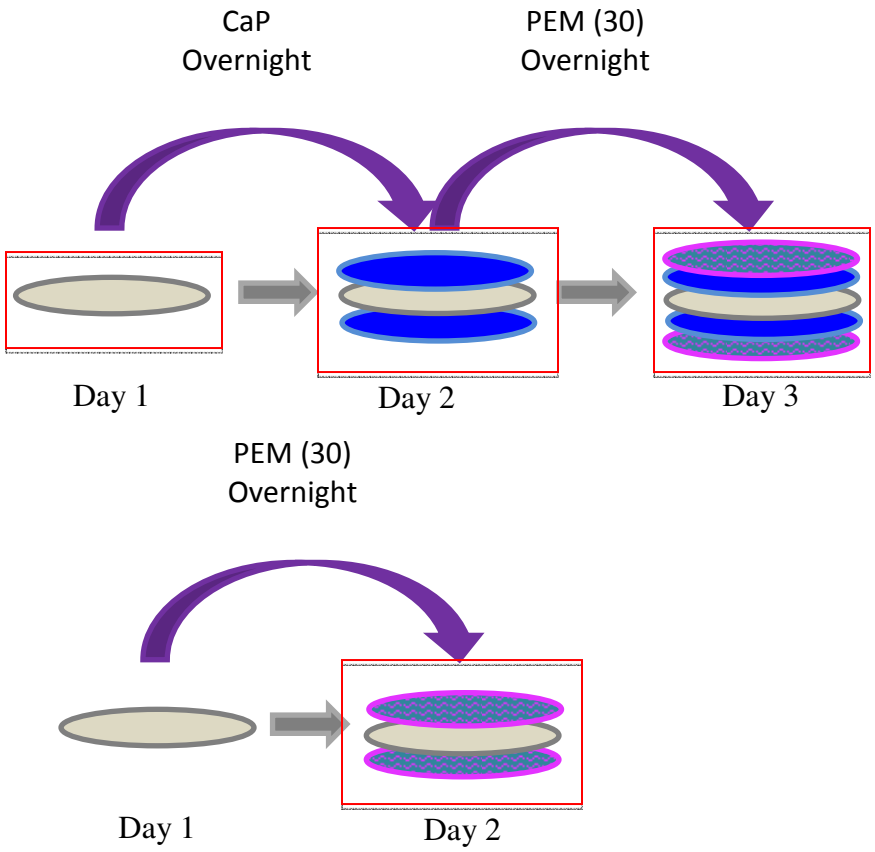


Figure 25: Layering Procedure for Confocal Controls (red boxes indicate control insert)

The gains were set to remove any background fluorescence from the inserts, PEM, and CaP coatings. These controls were set to ensure that any fluorescence detected by the confocal microscope was from the BSA proteins added to the samples. The below images in Figure 26 represent the architecture of each sample with the BSA 594 (red) drop on the bottom and the BSA 488 drop on the top of the sample.

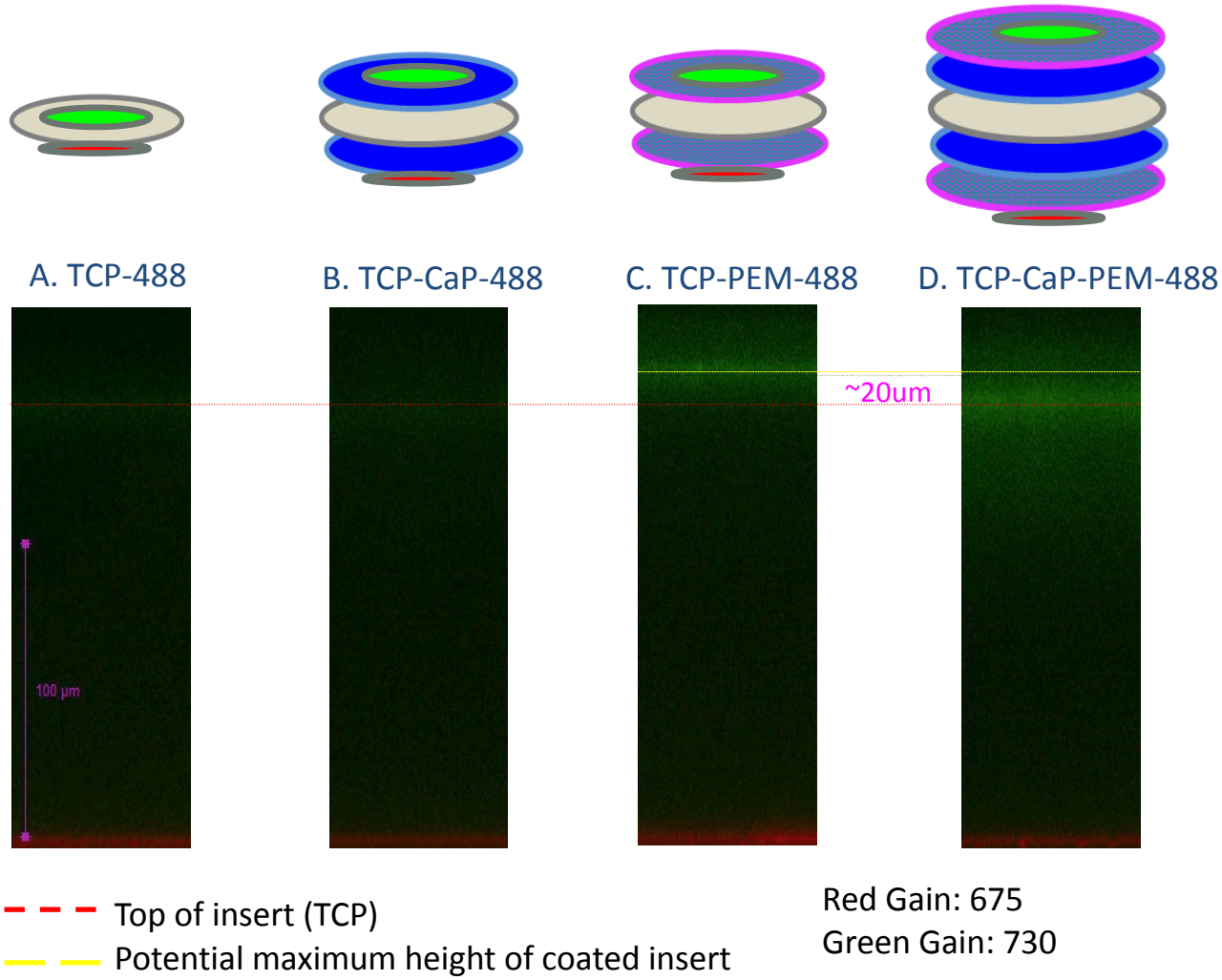


Figure 26: Architecture and Confocal Images for the Control Data

The control data confirms PEM acts as reservoir for BSA as indicated by more green visible in PEM coated inserts when comparing Figure 26 C+D to A+B. The images also showed

that BSA diffuses down through PEM in Figure 26 C, and has an affinity for CaP as indicated by concentrated green along CaP layer on top of insert in Figure 26 D. The data also suggested that the PEM coatings were around 20 µm in thickness and the CaP layer thickness was negligible as indicated by similar coating thicknesses with and without the CaP layer. Based on the information and gain settings found with the controls, two experimental groups were fabricated to test the blocking effect a CaP layer has on embedded BSA. The layering procedure for the experimental groups can be seen below in Figure 27.

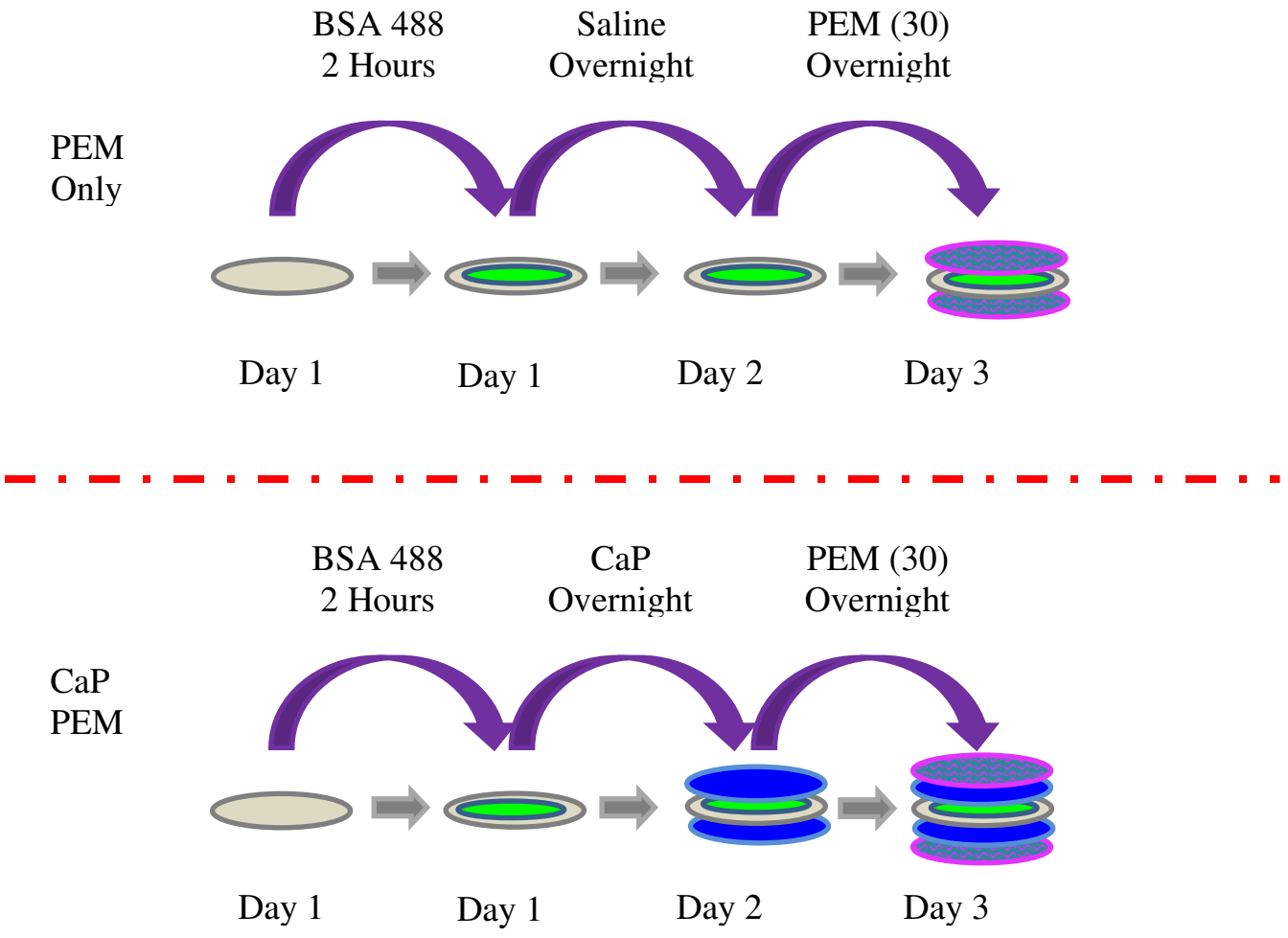


Figure 27: Layering Procedure for PEM Only and CaP PEM Experimental Groups

Because the coating procedure is performed in a vertical setting, the coatings are applied to both sides of the tissue culture plastic insert. Only the topside of the insert was coated with the BSA-488 in order to easily visualize any diffusion or lack of diffusion in the experimental samples. The architecture of each experimental group can be seen below in Figure 28.

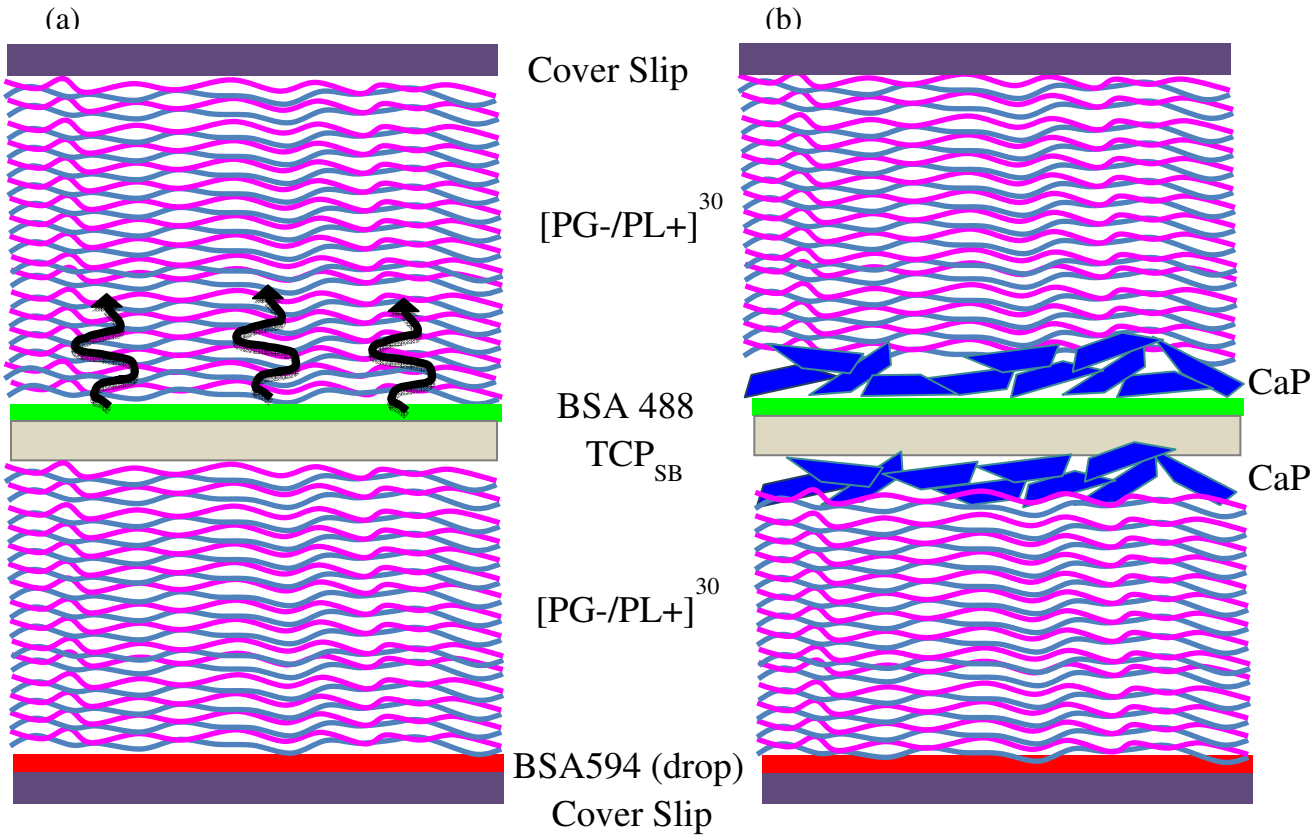


Figure 28: Architecture of (a) PEM Only and (b) CaP PEM Groups

Based on our preliminary data, it was expected that the BSA 488 embedded under the CaP layer will have little to no diffusion while BSA 594 absorbed to PEM layers will diffuse down through the PEM coating to the CaP layer and it could be seen using confocal imaging. The expected confocal images can be seen below in Figure 29.

(a)

(b)

Figure 29: Expected Confocal Results for (a) PEM Only and (b) CaP PEM Groups

The red line represents the drop of BSA 594 that marks the underside of the tissue culture plastic insert. Without the addition of the CaP layer the BSA 488 should diffuse throughout the PEM coating, as indicated by a wider band of green on the confocal image. With the addition of the CaP layer the BSA 488 should remain under the CaP layer and be indicated by a thin green band on confocal images.

The confocal results for the PG-/PL+ PEM coatings were not as expected. In order to image the bound BSA 488 the gain had to be increased which caused the detector to pick up fluorescence from the tissue culture plastic insert. It was determined that the BSA 488 concentration used, 25µg/ml, was not detectable at the control gain settings. The images below in Figure 30 are a PEM coated insert with no bound BSA 488 compared to the two experimental groups with bound BSA 488 with and without a CaP layer.

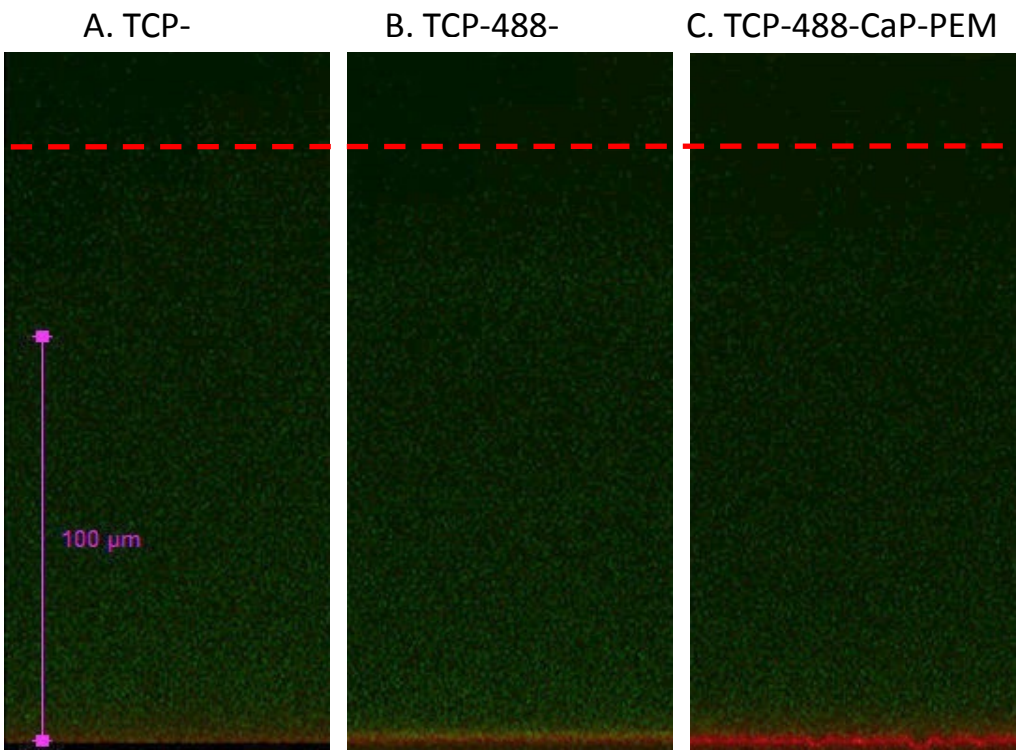


Figure 30: Confocal Images of (A) Fluorescing Disk with PEM Coating (B) PEM Only Experimental Group and (C) CaP PEM Experimental Group

As seen by the figure above in Figure 30, the diffusion or lack of diffusion of the BSA 488 could not be determined. As seen by the architecture in Figure 28, the confocal image was always taken through the disk insert rather than directly at the PEM coatings. This may have caused some of the z-stack image slices to be out of focus. The fluorescence detected that was originally thought to be diffusion could in reality be out of focus scatter. This scatter caused the coatings to appear thicker than they actually were. When concentrations of the BSA 488 or BSA 594 were increased, the images were distorted by fluorescence "bleeding." When the concentrations were too high the microscope would detect BSA at incorrect locations. An example of image "bleeding" can be seen below in Figure 31.

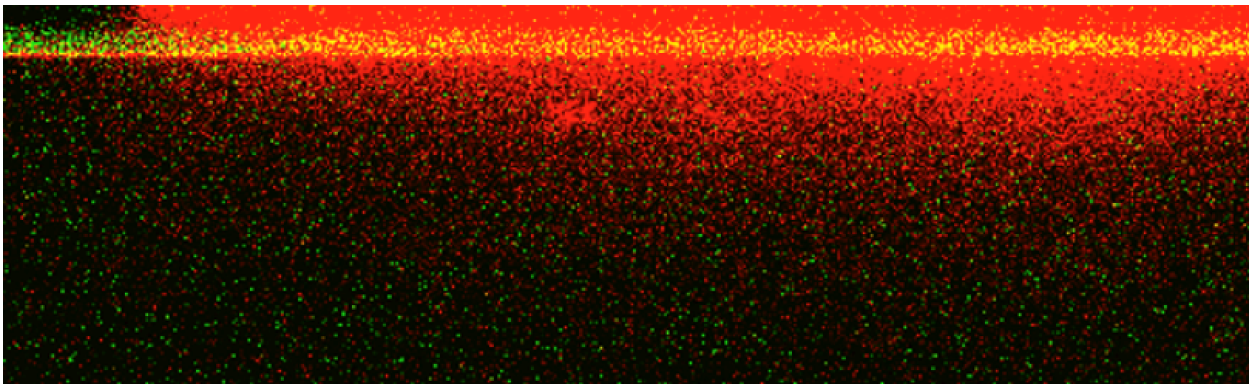


Figure 31: Confocal Image “Bleeding”

The red seen in the image, higher concentration BSA 594, was added above the green line, lower concentration BSA 488. The red color can be seen below the green line due to the confocal microscope picking up fluorescence before reaching the actual location of the BSA 594. This image “bleeding” prevented us from using a higher concentration of BSA 488 bound to the tissue culture plastic insert because with the “bleeding” the true location of the BSA 488 and therefore the diffusion or lack of diffusion could not be determined. While investigating the literature for possible reasons for the bleed through, it was discovered that PEM coatings made with PG- and PL+ are much thinner than those previously analyzed by confocal imaging [32]. This explains why the confocal images were not able to accurately determine the thickness of the coating and could not confirm the blocking effect of the CaP layer from the diffusion of the bound BSA 488.

5. Chapter 5: Conclusions and Future Work

Studies with the QCM machine proved that repeated use of the polyelectrolyte solutions would not hinder the buildup of the PEM coatings. Based on these results, an automated process for dip coating 2-D disk inserts was designed using a Varistain machine and custom 3D printed sample holders. The protocol allowed for 30 bilayers of PEM coating to be produced automatically in less time and also at a reduced cost. The PLA plastic material used for the sample holders will need to be changed for long-term use in the dip-coating machine. The sample holders should be printed from the polyphenylsulfone material as it has a high chemical resistance and can be easily sterilized using common sterilization techniques. [33] The automated techniques made possible by this thesis research are now being used in the Kuhn lab to create multi-layered structures to further investigate sequential delivery of BMP-2 and FGF-2 for bone regeneration.

The automation techniques designed from this research can be beneficial outside of the Kuhn lab as well. This automation technique allows for tissue culture plastic inserts to be dipped into any type of liquid or even semi-solid materials such as powders. The inserts can than be placed into culture disks for cell growth. The benefit of using a Varistain Machine is that it can be easily housed in a clean box that allows for the dipping to be performed in a sterile environment, which is crucial for experiments with cells. Research and Development labs in industry could use this technique for small-scale experiments given that the tissue culture plastic inserts are smaller than the rectangular slides the Varistain machine was originally designed to use and the 3 separate specimen cups prevent contamination between experimental groups unlike the original large buckets in the machine.

Due to the limitation of confocal imaging resolution to evaluate the effects of the CaP layer on limiting diffusion of a model protein within nanometer thick PEM coatings prepared in these studies, the recommended next step is the use of more sensitive bioassays involving effects of the coatings on living cells, such as osteoprogenitors. For example, our lab has shown that FGF-2 at high doses will reduce MC3T3 E1 cell proliferation (unpublished data). Therefore if it is embedded under a PEM layer that allows for diffusion there should be a reduction of proliferation if the FGF-2 has diffused throughout the PEM coating. The CaP should show no inhibition of proliferation if it is effective. Another approach is to use Antimycin-A which is cytotoxic so there should be cell death if diffusion occurs through the PEM coatings and a reduction or delay of cell death of the CaP/PEM group. These experiments are underway.

In order to perform animal studies to evaluate PEM coating delivery of growth factors the automated procedure developed for 2-D coatings will need to be revised to coat the porous 3-D Healos© scaffold. The current automated dip coating procedure will not be sufficient to enable coating of the interior pores of the scaffold due to the capillary forces within the small pores that retain liquid during transfer from one solution to the other. A greater fluid flow is needed to push the solution into the pores and a centrifuge step is currently being used to accomplish this for manual layer deposition. In order to ensure even coatings, the Healos© scaffold could be cut and stacked into a cylindrical container with an entry and exit for the solution and a pump could force polyelectrolyte and saline solutions through the stack of scaffolds to ensure coating through the entire scaffold. A multichannel peristaltic pump, Figure 32, could be used to pump the different solutions through the container at different amounts and durations.

Figure 32: Multichannel Pump for Healos Coating

6. References

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7. Appendix

7.1. General Programing of Varistain 24-4

The protocol for general programming of the Varistain 24-4 is adapted from the manual and is as follows.

1. Turn on Machine
2. Press “Save”
3. Select the program number using numeric keypad on right (1-3)
4. Press “Reset” to being programming at Step 1
5. Enter desired time using numeric keypad
6. Press “Enter” to confirm time, Press “Cancel” to cancel time, Press “Pass” to skip step
7. Press “Step” to move to next programming step
8. Repeat 5-7 until 24 step program is entered
9. Press “End” to end program
    a. Programs with no “End” command will run continuously
10. Press “Save” to store program in machine
11. To run a program select “Recall” and selected number using keypad on right
12. Select “auto” to run program
    a. When starting a program with no designated “end” step the machine will flash “end” until “enter” is pressed to begin the program

To raise and lower canopy press “load”

7.2. Protocol for Programming 30 Bilayers with 8 Rinse Steps

The protocol for programming the Varistain 24-4 for 30 layers of PEM and 8 saline rinses is as follows.

1. Turn on Machine
2. Press “Save”
3. Select program 3
4. Press “Reset” to being programming at Step 1
5. Enter 10 minutes (PL+)
6. Press “Enter” to confirm time
7. Press “Step” to move to next programming step
8. Enter 1 minute
9. Press “Enter” to confirm time
10. Repeat 7-9 for a total of 8 rinse steps that each last 1 minute
11. Enter 10 minutes (PG-)
12. Press “Enter” to confirm time
13. Repeat 7-9 for a total of 8 rinse steps that each last 1 minute
14. Press “Pass”
15. Press “Enter” to confirm pass
16. Repeat 14-15 until 42 step program is entered
    a. Note machine will only program up to 24 steps but steps 25-42 need to be programed as “pass”
17. Press “Save” to store program in machine

7.3. Confocal Imaging Protocol

1. Turn ON Zeiss 780
    a. Turn ON "Main Power" switch (gray box)
    b. Turn ON 488 laser
        i. Black laser box behind computer system
        ii. Toggle to ON main power
        iii. Turn key ¼ to the right
    c. Turn ON the "Systems PC" switch (gray box)
    d. Turn ON "X-Cite" lamp
    e. Turn ON PC and login to account
        i. Login: Kuhn
        ii. Password: Spring2015!
    f. Turn on "Components" switch (gray box)
        i. Wait till filters stop moving before proceeding
    g. Start Gray Zen software
    h. Turn on argon laser (black box)
        i. Push toggle to run position
        ii. If green light is not on slowly increase power till green light then back down until it goes off
2. Press "show all" under "acquisition" tab in Zen
3. Press "show all" under "view" tab
4. Load 32x water objective
5. Add water drop to objective
6. Set channels to 1Au starting with red channel
    a. Check that green and red channels have similar Au values
7. Prep Sample
    a. Place 4µl of BSA 594 on coverslip in center
    b. Place disk sample on BSA drop in center right side up
8. Turn transmitted on and load sample
9. Focus with oculars
10. Load configuration "488 and 594" under "acquisition " tab
11. Press "continuous"
12. Adjust gain on samples for both red and green channels
13. Press "continuous" to stop imaging
14. Adjust Z-stack to bottom ("set first") and top ("set last") of sample
15. Press "run experiment"
16. Save image
17. Turn OFF Zeiss 780
    a. Decrease argon laser power (black box) and toggle to "standby"
    b. Turn key on 488 laser back ¼ to noon position
        i. Wait until system has cooled and fans have stopped running before turning off laser
        ii. Can proceed to next step while laser cools
    c. Exit Zen software, log off, and shut down PC
    d. Turn OFF "X-Cite"
    e. If laser is cooled proceed, if not wait

f. Turn OFF power supply to laser

Turn OFF “Systems PC, Components and Main” power switches