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ABSTRACT

The pharmaceutical industry not only includes infinite areas of specialization, but also consists of distinct areas that do not typically overlap. Biotechnology is the branch of medicinal research that bridges the gap between the fields within the pharmaceutical industry by being able to take on the challenges that require knowledge of a vast range of information. This practicum was organized to put the scientific knowledge and the interdisciplinary practices of biotechnology to use in a modern day, pharmaceutical company specializing in wound therapy and skincare: Smith & Nephew Biotherapeutics. Wound therapy has the widest range of application due to being one of the few fields that affects everyone, regardless of medical disposition. The specific goals of this practicum were: to develop novel purification and biochemical characterization protocols for *C. collagenase* from *Clostridium histolyticum* to replace current production methods of Santyl®, to develop a working prototype of a venom-based, hemostatic film, and perform reformulation, quality control, troubleshooting, and verification testing on samples of Regranex®, Iodosorb "Max", and EU-Collagenase. Every goal presented was approached with the end results of saving Smith & Nephew costs, reducing bioburden of production, and creating more efficient protocols to bring Smith & Nephew into the modern age.

Creating novel purification and biochemical characterization protocols for *C. collagenase* from *Clostridium histolyticum*, developing a new emergency medicine product, and formulating several novel therapies for chronic and acute wound treatment.

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CREATING NOVEL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION PROTOCOLS FOR C. COLLAGENASE FROM *Clostridium histolyticum*, DEVELOPING A NEW EMERGENCY MEDICINE PRODUCT, AND FORMULATING SEVERAL NOVEL THERAPIES FOR CHRONIC AND ACUTE WOUND TREATMENT.

INTERNSHIP PRACTICUM REPORT

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LIST OF ABBREVIATIONS

CF	Clotting Factor
FDA	Food & Drug Administration
GMP	Good Manufacturing Practice
HBSS	Hank's Buffered Salt Solution
kD	Kilodalton (molecular weight)
MC1	Modified cellulose 1
MC2	Modified cellulose 2
MES	2-(N-Morpholino)-ethane-sulfonic Acid
MW	Molecular Weight
NUPAGE®	Brand of precast electrophoresis gels
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PDGF	Platelet-Derived Growth Factor
PEG	Polyethylene Glycol
PPI	Pound per Sq. Inch
QA	Quality Assurance
QC	Quality Control
R&D	Research and Development
S&N	Smith and Nephew
SDS	Sodium Dodecyl Sulfate
TFF	Tangential Flow Filtration
U	Units
V	Volts
µL	MicroLiter

CHAPTER 1

PRACTICUM INTRODUCTION

Protocol Development for Collagenase Purification by TFF for Improved Santyl® Production

The following practicum completed at Smith and Nephew Biotherapeutics (S&N) will lead to, among others, the completion of creating a new protocol to replace an existing production step in the development of one of S&N's largest income products, Santyl®. This product has been produced by S&N since the 1970's and utilizes the technology and methods from that era. This practicum will include the development and methods needed to transform a portion of the development process to use new technologies to make the production more efficient, cost-effective, and greener production. This experimentation will focus on concentrating and purifying collagenase proteins in a single solution using a new method of filtration, Tangential Flow Filtration (TFF), and isolating the pure proteins of interest, testing for the activity, and verifying protein specificity and characterization. In the distant future these accomplishments could lead to process/formulation changes that would become a new standard for production of this multi-million dollar product and replace a decades-old procedure to introduce modern-day technology to make the process more efficient, cost-effective, and environmentally conscious.

Development of Military-Contracted, Hemostatic Bandage

For the Hemostatic bandage prototype, trials will be performed to make synthetic films that will be used to create bandage-like wraps for treating extremity wounds. We will be experimenting with Chitosan and snake venom to create a hemostatic process when in contact with blood using an industrial clotting apparatus. Scientists at S&N believe that the Chitosan will create a lattice network within the film, acting as an immobilization matrix, not only trapping the snake venom proteins within, but also aiding in the clotting process. Upon contact with the film, according to abundant literature, data shows

that the venom will cause an almost instantaneous clot, stopping blood flow out of the wound where the film is placed. We tested many formulations to determine how to make this process not only work, but to discover a formulation that can lead to mass production of this product for the Armed Forces to be used for in-theater battle wounds with the potential of integration into civilian emergency medical treatment.

QC Testing for Regranex® Reformulations

The remaining portions of this practicum will cover the experimentations implemented during the reformulation processes of several S&N products. The first reformulation included is Regranex®, a debriding gel used in the treatment in diabetic lesions, pressure ulcers, and various chronic skin wounds. This product, although effective has a poor shelf life and requires low temperature storage, making it undesirable compared to various similar products. This practicum includes several texting procedures acting as Quality Control and verification of reformulated samples compared to the commercial product.

Iodosorb "Max" comparison testing for in-vivo contradiction studies

The next portion of this practicum will focus on Iodosorb®; Iodosorb® is a product made by S&N that contains Iodine encapsulated in Cadexomer (carbohydrate) beads formulated in Polyethylene Glycol (PEG) mixture. The mode of action for Iodosorb is dispersing iodine into chronic wounds to manage the bioburden, preventing infection. The R&D team at S&N Fort Worth site reformulated the product so that it is more efficacious toward Gram (-) bacteria, has a better iodine release profile and it is easier to apply. Problems arose from the first *in vivo* study. All *in vitro* studies confirmed aforementioned claims, but the *in vivo* studies resulted in somewhat unexpected data. The primary objective is to be instructed on how to make a synthetic mimic of an *in vivo* model and to test the Iodosorb "Max" vs. the Commercial Iodosorb in every scenario that could happen in a live organism. The intent of these studies was to discover why the results from the *in vivo* study contradicted *in vitro* data and to find out what went wrong in the testing stages. This will lead to clarification on why the contradictory results were obtained from *in vivo* and *in*

vitro studies. When the results of this study are attributed to the reformulations, it should lead to a more successful version of Iodosorb®, propelling it closer to implementing commercial manufacturing changes.

Troubleshooting the cause of failing batches of EU-Collagenase from S&N's German Facility

Lastly, this practicum will attempt to tackle the problems associated with batches of EU-Collagenase, the European counterpart of Santyl®. Although the two products are similar in their mode of action, the production methods and formulations are slightly different. The R&D Team in Germany believes that a compound in their formulation, chloramphenicol, is causing the problems but they need second opinions and expert protein chemists to determine the root cause of the product's failure. The report will include the step-by-step processes in this determination. The quicker the problem is pinpointed, the quicker Germany can start to make the recommended changes produce successful batches of EU-Collagenase.

CHAPTER 2

DEVELOPMENT OF CHARACTERIZATION PROTOCOLS USING TANGENTIAL FLOW FILTRATION AND VARIOUS ASSAYS FOR SANTYL® PRODUCTION

Background & Literature

Wound debridement has to occur in both chronic and acute wounds in order for wound healing to be successful [5]. By definition, debridement is a process of removing dying, damaged, and/or infected tissue from a wound and is crucial for successful wound management [18]. There are many ways to perform debridement including surgically, autolytically, biologically, mechanically, and enzymatically [26]. Although surgically is the most direct form of debridement, enzymatic debridement is the most effective and preferred treatment among medical professionals for use on chronic and unstable wounds as well as for patients who cannot afford surgery [35]. Much advancement has been made using the protein collagenase to assist in the wound healing process by aiding in the debridement of wounds. Many studies have found the most effective type of collagenase used for pharmaceutical work is the collagenase from the N-Group of *Clostridium histolyticum* [23]. Collagenase breaks down collagen in the skin and promotes breakdown of dead/decaying skin in the right conditions; it unwinds and destabilizes the alpha helixes of collagen starting at the alpha-2 chain [8, 21]. Collagen is the most plentiful protein in the skin making up 75-80% of the composition, while also making up 30% of the proteins in the human body [3, 19]. In a vast majority of cases, collagenase has been proven the best agent to use for wound debridement [20]. Collagenase has been proven to be one of the most effective debridement enzymes in every level of mammalian organism from mice to humans [35]. Many debridement products on the market include some form of collagenase, usually in a topical cream that is applied repeatedly over the course of a chronic wound [24]. Devastating wounds, whether military or civilian, can have prolonged healing times and have the potential to become chronic due to infections, dying/decaying skin, and extreme trauma to effected

tissues [10]. Not only do chronic wounds take psychological tolls on victims, but cost billions of dollars annually to treat and consist of over 50% of skin disease costs [17]. Collagenase-based ointments give patients who are not financially able to afford surgical debridement a cheaper and efficient alternative [33]. Although collagenase is broadly used in ointments of many sorts, this practicum will focus on the extensive purification and concentration of collagenase from *Clostridium histolyticum* fermentation using a process called Tangential Flow filtration. This process, in contrast to gravity filtration, uses a multi-surface filtration in a vertical, upward flow. Due to a pressure-inducing valve on the outward flow channel, pressure is increased on the system and forces compounds under a certain molecular weight out of the system through a permeate valve. This concept and mechanics behind this apparatus are shown in Figure 1 and Figure 2 below.

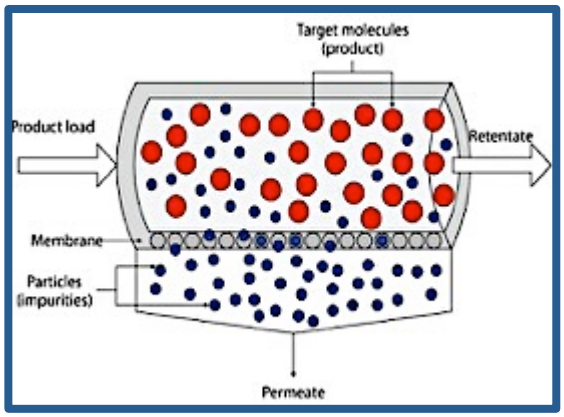


Figure 1: TFF Concept Art

The pressure gauges for most TFF apparatuses are recommended to stay below 25 ppi; anything above 25 ppi can cause backflow in the pump and alter purification time. The screw clamp on the retentate controls the amount of filtrate excreted. The higher the pressure on the screw clamp, the more filtrate is forced out of solution. When the remaining fluid in the sample is depleted, most protocols call for adding either water or buffer to assist in the washing and cleaning of the sample

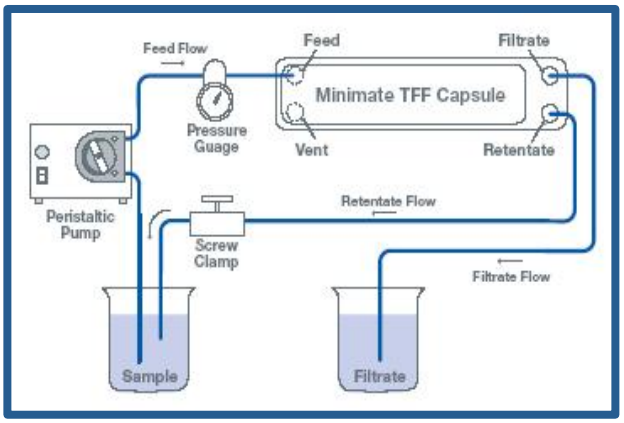


Figure 2: TFF Apparatus

A pump sends the sample of interest through a filter with a specific cut-off limit. Any compound with a molecular weight smaller than the specified cut-off weight is able to pass through the filter and be excreted through the "permeate", also called the "filtrate." The retentate is the portion of the sample that has the compound of interest above the weight limit and doesn't cross the filter. These compounds are then sent back to the original sample container.

Purpose & Significance

Collagenase is an active metabolite in a number of pharmaceutical products, and in particular, one of S&N's highest earning products, Santyl®. Santyl® is a Food & Drug Administration (FDA)-approved prescription ointment that helps in the debridement stage of wound healing. Santyl® removes dead and decaying tissue from wounds to kick-start the healing process. It has been prescribed nationally for over 25 years for wounds such as but not limited to: pressure ulcers (bed sores), diabetic foot ulcers, venous ulcers, severe burns, etc. Santyl® was originally patented in the 1970's [34]. According to FDA regulations, the method of production has to be replicated exactly unless changes are approved and filed for verification. Due to the advancement of manufacturing capabilities, S&N is faced with the decision to take advantage of modern technological advancements. Introducing the TFF process to the production of Santyl® would save S&N hundreds of thousands of dollars by replacing multiple expensive steps in the

production [41]. While the specifics of the current production and manufacturing of this product are proprietary, this new method of purification could be utilized to replace the outdated method that has been in place for decades. TFF will save costs not only in reagents needed for production but will also decrease the environmental impact of the process while also reducing the time necessary to perform the improved purification. Reducing bioburden of industrial level manufacturing would also improve the already glowing reputation and public relationships S&N has with the public. Below in Figure 3, the process of making Santyl® is listed by the main production phases.

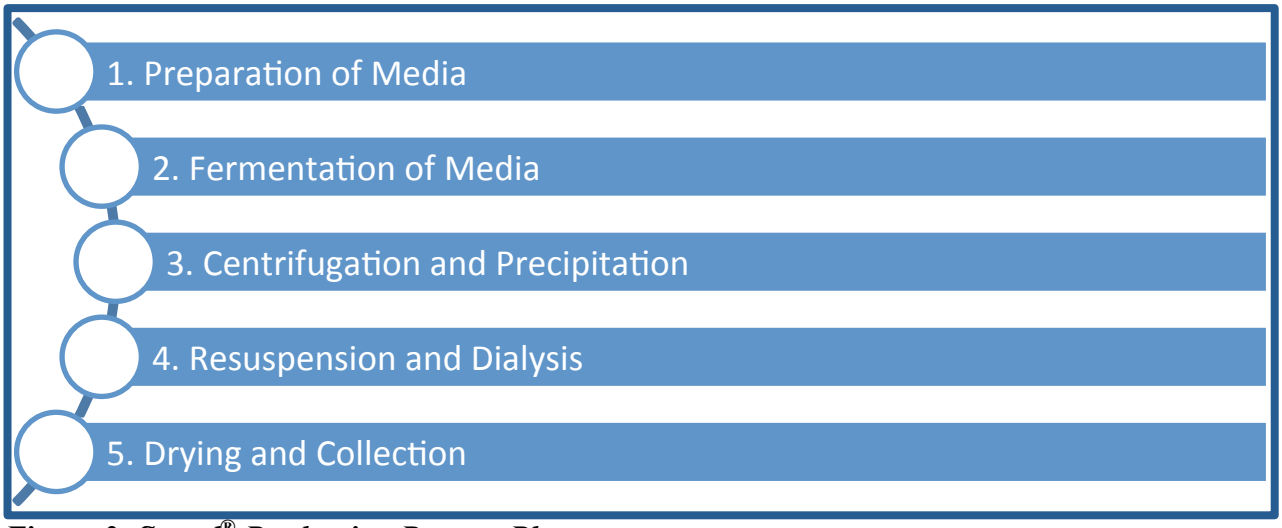


Figure 3: Santyl® Production Process Phases

The current method of collagenase isolation involves hundreds of pounds of salts during the Precipitation and Suspension phases to help force the collagenase into solution while also aiding in the removal of the solution's organic components; these salts are then simply disposed of, increasing the environmental effects of this production. TFF would negate the need for these salts as well as provide a more efficient method of disposing of the majority of the smaller components that aren't removed by the salts. Calculating the costs of the salts, the depositing costs of the used reagents, and the time/effort from the dialysis procedures, utilizing TFF in place of the current process could save roughly $12 million per year on an already $300 million annual product [32].

Materials and Methods

TFF Purification

The Collagenase was purified using Tangential Flow Filtration (TFF), also known as Cross-Flow Filtration. Each batch began by dissolving a chosen amount of the Collagenase/Clostridium Biomass in a 10 liter flask of Nano-pure water (100 grams per 10 liters of water). We filtered using a peristaltic pump and Millipore 10 kilo-Dalton, 30 kilo-Dalton and 50 kilo-Dalton filtering cartridges. The cartridge yielding more promising results upon analysis will be taken forward for the rest of the project. The filtration will run until one liter of solution is left in the flask. This should multiply the concentration of collagenase by a factor of 10 considering one removes 90% of the unwanted material that is less than the molecular weight cut-off. It will then be diluted back to 10 liters with more Nano-pure water. This process will be repeated until one liter of solution is left, again adding another factor of 10 to the collagenase concentration. It will then be diluted a third time using a Tris Buffer fixed to a pH between 7.4-7.6. It will run until there is less than one liter of retentate. It will then be transferred into either a stainless steel, lyophilizer tray or into a 1200 mL beaker and be frozen overnight in a -80°C freezer. This process will be repeated until there are two or three samples of each container type worth of purified collagenase solution. Once frozen, the trays will be placed in the lyophilizer which will be run for multiple days until the samples are fully freeze-dried so that all that is left is purified collagenase powder.

The first seven cycles of TFF performed were using just one 10 kD filter cartridge. On cycle 7, we started stacking cartridges; the apparatus can hold up to three Millipore® cartridges at once, expanding the surface area for filtration to occur. Cycles 8-30 used the three-cartridge system. At cycle 31, we transitioned to a cylindrical, rod-style 50,000 kD filter and a 500,000 kD filter. The 500,000 kD filter was used to filter out collagenase directly from the bacterial fermentation to get rid of any and all bacterial components. Once the majority of the collagenase was removed and placed into a different holding container, it was then filtered through the 50,000 kD filter to remove any impurities, small proteins, and broth media from the collagenase-containing solution. For these double-filtration cycles, the apparatus

was constantly monitored due to the rapid speed at which the filters operated. The same protocols were used when approaching the concepts and modes of actions for these experimentations.

Protein Banding Gel Electrophoresis

Once the collagenase is purified, the first step in verification of the product is by calculating the percent yield of protein. This will be a basic comparison of the starting weight before TFF and the final weight of the same material after TFF and Lyophilization. This will later play a role in determining the efficiency of the purification process. The first protein characterization step is the analysis of the powder's composition by running a protein weight gel to verify that the material contains the exact molecular weights of collagenase. If other distinct bands appear below this molecular weight, this protocol would have put to large of a strain on the proteins and caused them to become degraded. This process involves preparing various solutions for dissolving and preparing the collagenase samples as well as a step-by-step protocol on how to run the test. Collagenase from *Clostridium histolyticum* has two main subunits: collagenase G and collagenase H with molecular weights of 112,000 kD and 116,000 kD respectively; this should be reflected in the gels. The protocol for this process of verification is listed in Table 1 below.

Table 1: Gel Electrophoresis Protocol

Solutions:
• 1x MES: Total volume of 1 liter. • 1x Working Solution (1xWS) • 2x Sample Buffer: Per 10 mL final volume. • Coomassie Blue stain: Gently mixed prior to use. • Molecular Weight Markers: PagePlus MW markers, thaw and set aside.
Methods:
1. Turn on heat block. 2. Weigh out your samples and make the desired concentration in solution of 1x WS. 3. Mix an aliquot of your samples 50:50 with the 2x Sample Buffer 4. Heat samples at 95°C in the heat block for allotted time 5. Assemble Gel box and prepare the pre-decided gel. 6. Run gel at a constant voltage for about desired amount of time 7. Remove and rinse gel in ultrapure water for multiple washes 8. Coomassie Stain (cover gel with stain). 9. De-stain in ultrapure water until bandings are clear (add banded kim-wipes to help bind up the Coomassie)

FALGPA Assay

After one full batch of collagenase powder is fully dehydrated, a FALGPA Assay will be performed in the sample to test activity compared to the original NP-powder. FALGPA is an N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Alanine peptide. Collagenase chops off the glycine, proline and alanine amino-acids from the substrate leaving the "FAL" portion; the assay analyzer reads the concentration of

the original FALGPA protein by reading the absorbency at the wavelength matching the original substrate, 345 nm. This assay tracks the concentration of the beginning substrate to calculate the activity of the collagenase as compared to the standards made using the original biomass powder. The results of this test will give a rate of digestion per unit of time and will appear as a negative slope. Theoretically, there should be a higher activity in the purified sample since those samples should have higher concentrations of collagenase per milligram. For the activity tests, a similar Tris buffer will be used to dissolve the collagenase. This data is provided in Figure 4 below.

Large Scale Manipulations

Once the final parameters were set (i.e. sample collection, buffer selection, and wash cycle determination), the team set out to use the TFF system on a large-scale batch made in a new-age Bioreactor, monitored by a Biostat Device. This fresh fermentation would be 50 liters and would be filtered first through a 500 kD TFF cartridge to filter out the bacteria and organic components, controlling bioburden. Tests were run at this stage to guarantee no bacteria or spores got through the filter. Once those tests were confirmed negative for bioburden, the solutions were then sent through the 50 kD filter to filter out anything with a molecular weight smaller than 50 kD. The solution was sent in a cyclical manner and as it ran, the volume decreased due to filtration of smaller compounds out of the solution. Once the overall volume had decreased to 5 liters, the buffer washes began. At the end of the buffer washes; there was an almost clear solution that was divided up equally between four 1.2 liter beakers and frozen. After a night in the -80 degree freezer, the samples were placed on the lyophilizer for a few days until the sample was a dry powder. These samples were tested in the same method as the small scale manipulations completed previously, (i.e. FALGPA Assays, Gel electrophoresis, BSA Screens, etc). Images of the large-scale procedure are located below in the results section.

Optimal Assay for Bacterial Collagenase by TFF Protocol
Smith & Nephew Biotherapeutics – Jason Mars

Solutions:

1. Assay Buffer: Tris Buffer (50 mM N-[Tris(hydroxyethyl)methyl]glycine, 400 mM NaCl, 10 nM CaCl2, pH=7.5
   a. Per 1 liter of solution, combine
      i. 9.0 g Tris
      ii. 23.4 g NaCl
      iii. 1.50 g $CaCl_2$
   b. Dissolve into 1 liter of Ultra-pure H_2O and adjust pH=7.5.
   c. Keep in fridge or in ice bath during use; when done, keep in fridge; it doesn't go bad.

2. Collagenase Standard Solutions
   a. Weigh out 60 mg of the raw collagenase NP Powder (Pre-TFF) and dilute into 50 mL of the Assay Buffer (1.2mg/mL). Mix until fully dissolved or let sit on mixing rotor.
   b. From this solution, create a separate 1:2 dilution using the Assay Buffer (0.4 mg/mL).
   c. From these two standard solutions, you'll make two-fold dilutions in the plate, giving 10 standards for each assay.
      i. 1.2 mg/ml → 1.2, 0.6, 0.3, 0.15, 0.75
      ii. 0.4 mg/mL → 0.4, 0.2, 0.1, 0.05, 0.025
   d. Keep in fridge or in ice bath during
   e. Make new standards solutions each time you test.

3. Product Sample Solutions
   a. Weigh out 40 mg of lyophilized collagenase powder into 14 mL Assay Buffer and mix thoroughly until fully dissolved (2.87 mg/mL).
   b. From this solution, pipet out 0.5 mL and add to 9.5 mL of Assay Buffer (0.14 mg/mL).
      i. This will be the sample you use in the assay.
      ii. Keep in fridge or in ice bath during use.
      iii. Samples stay good for 2-3 weeks

4. FALGPA Solution
   a. Weigh out enough N-(3[2-Fury] Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) from **Sigma (F-5135)**.
   b. Make a 1mg/ml solution in Assay Buffer.
   c. Keep on ice during use.
   d. Freeze in -40C Freezer when not in use

Procedure:

1. Set up the BioReader settings before plating samples.
   a. Set kinetics to 345 nm
   b. Read @ 30 sec. intervals for 20 min.
   c. Shake before first read
   d. Plot readings against time, use linear plot
   e. Read from -4 to 4
   f. Create lag of 100 seconds
2. Prepare the above solutions needed using Assay buffer as stock, dilute to the concentrations above.
3. Pipet 100 µL of the Assay Buffer into each of the blank wells.
4. Pipet 100 µL of the Assay Buffer into the wells for standards 2-4 for both standard sets.
5. Pipet 200 µL of the standard solutions into the first well of each standard set.
6. Pipet 100 µL from well A1 put in Well A2 and mix, repeat process until you've diluted the standard 2-fold 4 times.
7. Pipet 100 µL of each sample into its designated well & let incubate for 2 minutes.
8. Pipet 150 µL of the FALGPA Solution into every well, lightly shake, & let sit for 2 minutes.
9. Start Read

Figure 4: FALGPA Assay developed for calculating Collagenase Activity

Results

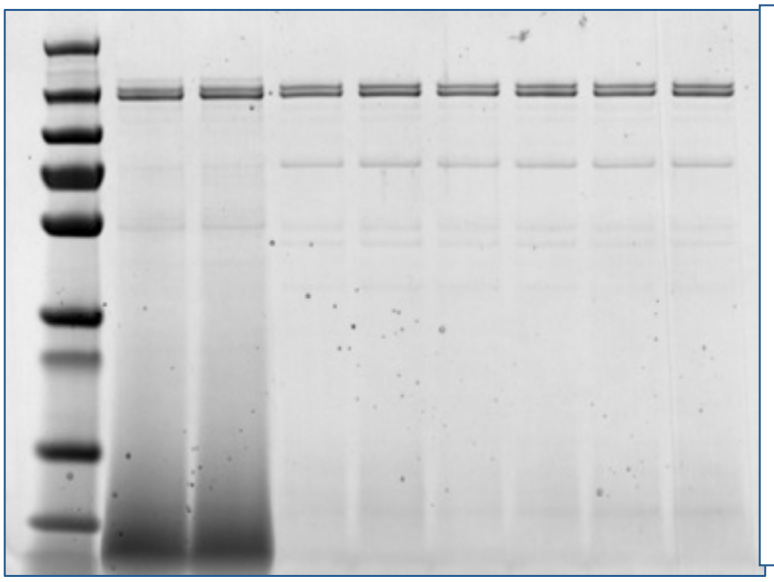
After each cycle of TFF performed, the samples were frozen at -80°C, lyophilized, and weighted out to calculate percent yield. The samples were then prepared for gel electrophoresis and the FALGPA Assay and sent through these respective protocols. The data and qualifications of each cycle of TFF are listed and organized below in Table 2. These values will be used to quantify and compare each NP-powder lot and delineate the future steps for changes in these processes. Gels were run to verify that the product of the purification is in fact collagenase. Two gels for example purposes are found below in Figures 5 & 6. Once the right variables were agreed upon from the test results, a large scale was performed to compare for efficiency as well to test for future use. By the time the large-scale was pursued, the TFF wore out and wasn't filtering as well as when it was new. The first batch of large-scale filtration resulted in no activity and no collagenase. The TFF filter had not held up to the large amount of use. The process was repeated with a brand new TFF filter cartridge and the experimentation was a success. Below are a host of graphs, pictures, and data tables illustrating the findings throughout this experimentation stage.

Table 2: Collagenase from TFF Collection

Cycle #	Lot #	Status	Tray/ Beaker	NP Conc.	Initial Weight (w/o H2O)	Purified Weight	Weight Percent Yield	Collagenase Activity	Purity Banding
Cycle #1	Lot 13-115 Set 3	Completed	Tray	~10 mg/mL	100.01 g (98.3 g)	35.73 g	36.35% Yield	0.410 µmol/min	~102/110 kD
Cycle #2	Lot 13-115 Set 3	Completed	Tray	~10 mg/mL	100.12 g (98.42 g)	36.85 g	36.91% Yield	0.387 µmol/min	~101/109 kD
Cycle #3	Lot 13-115 Set 3	Completed	Tray	~10 mg/mL	101.07 g (99.35 g)	28.7 g (Tween)	28.89% Yield	0.209 µmol/min	~110/112 kD
Cycle #4	Lot 13-115 Set 3	Destroyed	Beaker	~10 mg/mL	100.06 g (99.36 g)	N/A	N/A	N/A	N/A
Cycle #5	Lot 13-115 Set 3	Completed	Beaker	~10 mg/mL	88.5 g (86.99 g)	23.1 g (Tween)	26.56% Yield	0.552 µmol/min	~110/112 kD
Cycle #6	Lot 13-151 Set 1	Completed	Tray	~10 mg/mL	101.19 g (99.47 g)	26.6 g	26.74% Yield	0.560 µmol/min	~101/108 kD

Cycle #7	Lot 13-151 Set 1	Completed	Beaker	~10 mg/mL	100.54 g (99.13 g)	26.9 g	27.15% Yield	0.498 µmol/min	~102/110 kD
Cycle #8	Lot 13-151 Set 1	Completed	Beaker	~10 mg/mL	101.2 g (99.48 g)	22.9 g	23.05% Yield	0.558 µmol/min	~104/110 kD
Cycle #9	Lot 13-151 Set 1	Completed	Tray	~10 mg/mL	102.01 g (100.27 g)	31.1 g	31.02% Yield	0.545 µmol/min	~98/104 kD
Cycle #10	Lot 13-151 Set 1	Completed	Tray	~10 mg/mL	103.5 g (101.74 g)	21.7 g	21.33% Yield	0.507 µmol/min	~99/104 kD
Cycle #11	Lot 13-151 Set 1	Completed	Beaker	~10 mg/mL	100.4 g (98.69 g)	19.9 g	20.19% Yield	0.537 µmol/min	~100/106 kD
Cycle #12	Lot 13-131 Set 3	Completed	Beaker	~10 mg/mL	100.2 g (98.5 g)	20.2 g	20.09% Yield	0.44 µmol/min	Approved
Cycle #13	Lot 13-131 Set 3	Completed	Tray	~10 mg/mL	100.16 g (98.46 g)	26.4 g	23.97% Yield	0.515 µmol/min	Approved
Cycle #14	Lot 13-131 Set 3	Completed	Beaker	~10 mg/mL	100.1 g (98.41 g)	21.2 g	21.51% Yield	0.555 µmol/min	Approved
Cycle #15	Lot 13-131 Set 3	Completed	Beaker	~10 mg/mL	100.56 g (99.14 g)	21.2 g	21.34% Yield	0.555 µmol/min	Approved
Cycle #16	Lot 12-158 Set 3	Completed	Beaker	~10 mg/mL	100.05 g (99.35 g)	13.9 g	14.00% Yield	0.749 µmol/min	Approved
Cycle #17	Lot 12-158 Set 3	Completed	Beaker	~6 mg/mL	60.3 g (59.27 g)	9.8 g	16.25% Yield	0.756 µmol/min	Approved
Cycle #18	Lot 12-158 Set 3	Completed	Tray	~6 mg/mL	62.6 g (61.54 g)	11.9 g	19.34% Yield	0.602 µmol/min	Approved
Cycle #19	Lot 12-158 Set 3	Completed	Tray	~6 mg/mL	61.4 g (60.36 g)	12.6 g	20.87% Yield	0.717 µmol/min	Approved
Cycle #20	Lot 12-158 Set 3	Completed	Tray	~6 mg/mL	61.8 g (60.71 g)	13.2 g	21.74% Yield	0.714 µmol/min	Approved
Cycle #21	Lot 12-158 Set 3	Completed	Tray	~6 mg/mL	60.21 g (59.25 g)	14.1 g	23.8% Yield	0.612 µmol/min	Approved
Cycle #22	Lot 13-111 Set 4	Completed	Tray	~15 mg/mL	150.27 g (147.7 g)	32.1 g	21.74% Yield	0.768 µmol/min	Approved
Cycle #23	Lot 13-111 Set 4	Completed	Tray	~15 mg/mL	150.08 g (147.53 g)	28.6 g	19.40% Yield	0.765 µmol/min	Approved
Cycle #24	Lot 13-111 Set 4	Completed	Tray	~15 mg/mL	150.59 g (147.91 g)	25.3 g	17.10% Yield	0.752 µmol/min	Approved

Cycle #25	Fresh Ferm	Completed	Tray	~10 mg/mL	~55 g	8.7 g	~15.82% Yield	0.785 µmol/min	Approved
Cycle #26	Lot 13-131 Set 4	Chilling	Tray	10 mg/mL	100.45 g (94.99 g)	20.1 g	21.16% Yield	0.755 µmol/min	Approved
Cycle #27	Lot 13-151 Set 3	Deep Freeze	Tray	10 mg/mL	102.44 g (96.87 g)	N/A	N/A	N/A	N/A
Cycle #28	Lot 13-151 Set 3	Deep Freeze	Beaker	10 mg/mL	110.54 g (104.57 g)	N/A	N/A	N/A	N/A
Cycle #29	Lot 13-151 Set 3	Lyo	Beaker	10 mg/mL	102.48 g (96.91 g)	18.1 g	18.68% Yield	0.875 µmol/min	Exceptional
Cycle #30	Lot 13-151 Set 3	Lyo	Beaker	10 mg/mL	101.56 g (98.83 g)	20.4 g	20.70% Yield	0.789 µmol/min	Exceptional
Cycle #31	Lot 13-151 Set 3	Completed	Beaker	10 mg/mL	104.2 g (97.01 g)	15.3 g	15.78% Yield	0.764 µmol/min	Exceptional
Cycle #32	Lot 13-131 Set 4	Completed	Beaker	10 mg/mL	102.4 g (100.65 g)	14.5 g	14.41% Yield	0.867 µmol/min	Exceptional
Cycle #33	Fresh Ferm	Completed	Beaker	~10 mg/ml	~60 g	14.7 g	24.50% Yield	0.066 µmol/min	Failed
Cycle #34	Lot 13-131 Set 4	Completed	Beaker	10 mg/mL	107.3 g (105.47g)	13.4 g	12.7% Yield	0.575 µmol/min	Exceptional
Cycle #35	Lot 13-131 Set 4	Lyo	Beaker	10 mg/mL	115.2 g (113.24 g)	17.2 g	15.2 % Yield	0.765 µmol/min	Exceptional
Cycle #36	Lot 13-131 Set 4	Lyo	Beaker	11 mg/mL	102.5 g (100.76 g)	16.1 g	16 % Yield	0.682 µmol/min	Exceptional



Lane Key:

Lane 1: Ladder

Lanes 2& 3: Unfiltered sample

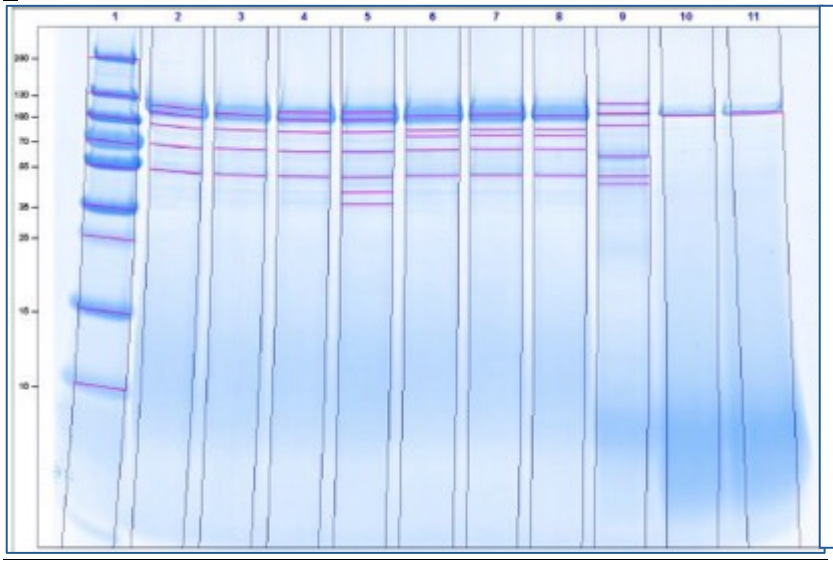
Lanes 4 & 5: 50 kD filter

Lanes 6 & 7: 30 kD filter

Lanes 8 & 9: 10 kD filter

Figure 5: Filter Differences - 50 vs 30 vs 10 (kD)

c



Lane Key:

Lane 1: Ladder

Lanes 2-7: Samples 5,7,8,9,10,11,13

Lanes 8: Fresh Fermentation Supertenant Pull-off

Lanes 9 & 10: TFF Precipitate

Figure 6: TFF Batches 1-7 w/ Mystery Precipitate

The distinct bands at the top are also the subunits of collagenase. Each lane was a separate lot accompanied by the last two lanes containing the mystery precipitate. Although the mystery precipitate had bands at the collagenase molecular weight marker, the samples had negligibly no activity.

Once the gels were run and the molecular weights were verified as containing pure collagenase, the samples were then dissolved into specified concentrations listed in Figure 3: FALGPA Assay Protocol. When completed, the analysis of the samples is presented as rates of reagent loss and rates of

increased degraded products. The analysis of the samples and the data recorded from this assay is located below in Table 3.

Table 3: FALGPA Activity Data

FALGPA Assay Results Master Sheet								
Sample	OD Value (mU)	Vmax (mU/min)	Result	Rate	CV	Time (min)	Date of Test	Conc. Of Unknown
Batch #1	-8.995	-0.67459127	0.403	0.41	2.1	13.334	10/3/2015	.285 mg/mL
	-9.109	-0.68314084	0.408					
	-9.341	-0.70053997	0.42					
Batch #2	-8.575	-0.64309285	0.382	0.387	1.7	13.334	10/3/2015	.285 mg/mL
	-8.824	-0.66176691	0.394					
	-8.604	-0.64526774	0.383					
Batch #3	-5.292	-0.39688016	0.219	0.209	7.8	13.334	10/3/2015	.285 mg/mL
	-4.705	-0.35285736	0.19					
	-5.259	-0.39440528	0.218					
Batch #5	-10.579	-0.70526667	0.552	0.552	2.6	15	10/3/2015	.285 mg/mL
	-10.289	-0.68593333	0.537					
	-10.844	-0.72293333	0.566					
Batch #6	-11.531	-0.86478176	0.528	0.56	6.1	13.334	10/3/2015	.285 mg/mL
	-12.889	-0.96662667	0.596					
	-12.06	-0.90445478	0.555					
Batch #7	-9.333	-0.6222	0.487	0.498	4.8	15	10/3/2015	.285 mg/mL
	-9.219	-0.6146	0.481					
	-10.061	-0.67073333	0.525					
Batch #8	-11.314	-0.75426667	0.591	0.558	5.1	15	10/3/2015	.285 mg/mL
	-10.48	-0.69866667	0.547					
	-10.294	-0.68626667	0.537					
Batch #9	-11.65	-0.87370631	0.534	0.545	1.9	13.334	10/3/2015	.285 mg/mL
	-12.057	-0.90422979	0.554					
	-11.894	-0.8920054	0.546					
Batch #10	-11.136	-0.83515824	0.509	0.507	1.5	13.334	10/3/2015	.285 mg/mL
	-11.217	-0.84123294	0.513					
	-10.917	-0.81873406	0.498					
Batch #11	-10.293	-0.6862	0.537	0.537	1.7	15	10/3/2015	.285 mg/mL
	-10.107	-0.6738	0.527					
	-10.448	-0.69653333	0.545					
Batch #12	-6.732	-0.57701209	0.339	0.44	31.7	11.667	10/12/2016	.285 mg/mL
	-7.448	-0.63838176	0.382					

	-11.072	-0.94900146	0.599					
Batch #13	-10.078	-0.67186667	0.526	0.515	2.1	15	10/3/2015	.285 mg/mL
	-9.67	-0.64466667	0.505					
	-9.869	-0.65793333	0.515					
Batch #14	-12.035	-0.90257987	0.553	0.555	1.2	13.334	10/3/2015	.285 mg/mL
	-12.218	-0.91630418	0.562					
	-11.945	-0.89583021	0.549					
Batch #15	-11.87	-0.89020549	0.545	0.555	2.6	13.334	10/3/2015	.285 mg/mL
	-11.931	-0.89478026	0.548					
	-12.41	-0.93070346	0.572					
Batch #16	-13.517	-1.1585669	0.745	0.749	0.6	11.667	10/12/2016	.285 mg/mL
	-13.676	-1.17219508	0.755					
	-13.571	-1.16319534	0.748					
Batch #17	-13.737	-1.1774235	0.758	0.756	0.5	11.667	10/12/2016	.285 mg/mL
	-13.645	-1.16953801	0.758					
	-	-	-					
Batch #18	-10.295	-0.88240336	0.5522	0.602	7.2	11.667	10/12/2016	.285 mg/mL
	-11.478	-0.98380046	0.623					
	-11.605	-0.99468587	0.631					
Batch #19	-13.202	-1.13156767	0.726	0.717	1.8	11.667	10/12/2016	.285 mg/mL
	-13.15	-1.12711065	0.723					
	-12.802	-1.09728293	0.702					
Batch #20	-12.957	-1.11056827	0.712	0.714	404	11.667	10/12/2016	.285 mg/mL
	-13.547	-1.16113825	0.747					
	-12.508	-1.07208365	0.685					
Batch #21	-11.2	-0.95997257	0.606	0.612	1	11.667	10/12/2016	.285 mg/mL
	-11.408	-0.97780063	0.619					
	-11.295	-0.9681152	0.612					
Batch #22	-13.888	-1.19036599	0.767	0.768	0.6	11.667	10/12/2016	.285 mg/mL
	-13.822	-1.18470901	0.764					
	-13.972	-1.19756578	0.772					
Batch #23	-10.386	-0.5193	0.763	0.765	1	20	11/7/2016	.142 mg/mL
	-10.323	-0.51615	0.758					
	-10.515	-0.52575	0.774					
Batch #24	-10.656	-0.5328	0.785	0.752	4.2	20	11/7/2016	.142 mg/mL
	-9.865	-0.49325	0.721					
	-10.23	-0.5115	0.751					
Batch #25	-10.852	-0.5426	0.801	0.785	1.8	20	11/7/2016	.142 mg/mL
	-10.626	-0.5313	0.783					
	-10.499	-0.52495	0.772					
Batch #26	-13.196	-0.87973333	0.754	0.755	1.9	15	2/1/2017	0.142

	-13.615	-0.90766667	0.779					mg/mL
	-13.181	-0.87873333	0.753					
Batch #27	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A					
	N/A	N/A	N/A					
Batch #28	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N/A	N/A	N/A					
	N/A	N/A	N/A					
Batch #29	-15.628	-1.04186667	0.9	0.875	2.3	15	2/1/2017	0.142 mg/mL
	-15.315	-1.021	0.882					
	-15.115	-1.00766667	0.878					
Batch #30	-14.075	-0.93833333	0.807	0.789	3	15	2/1/2017	0.142 mg/mL
	-14.438	-0.96253333	0.829					
	-13.649	-0.90993333	0.781					
Batch #31	-14.415	-0.961	0.827	0.764	64.6	15	2/1/2017	0.142 mg/mL
	-13.151	-0.87673333	0.751					
	-13.128	-0.8752	0.75					
Batch #32	-16.249	-1.08326667	0.938	0.867	4.9	15	2/1/2017	0.142 mg/mL
	-15.244	-1.01626667	0.877					
	-14.697	-0.9798	0.844					
Batch #33	-2.583	-0.1476	0.058	0.066	0.007	17.5	1/11/2017	0.125 mg/mL
	-2.849	-0.1628	0.071					
	-2.815	-0.16085714	0.069					
Batch #34	-14.502	-0.82868571	0.603	0.575	0.029	17.5	1/11/2017	0.125 mg/mL
	-13.241	-0.75662857	0.545					
	-13.908	-0.79474286	0.576					

Although the 500 kD filtration step was successful, the 50 kD filtration put too much strain on the proteins in the solution and the final sample had no activity and contained no protein. To test this, we put the same 50 kD column through one more round of NP-Powder purification. If the sample were inactive with no protein, our hypothesis would be correct. If the sample tested active under the FALGPA Assay, our R&D team would have to investigate further to determine the cause of the double-TFF failure. Below is the assay image showing the activity of the hypothesis test of the “broken” filter purification. The activity of the positive control was 1.171 U/mg whereas the activity of the hypothesis sample had an

activity of 0.979 U/mg. Statistically these samples are similar within calculated deviations. This would be substantial information

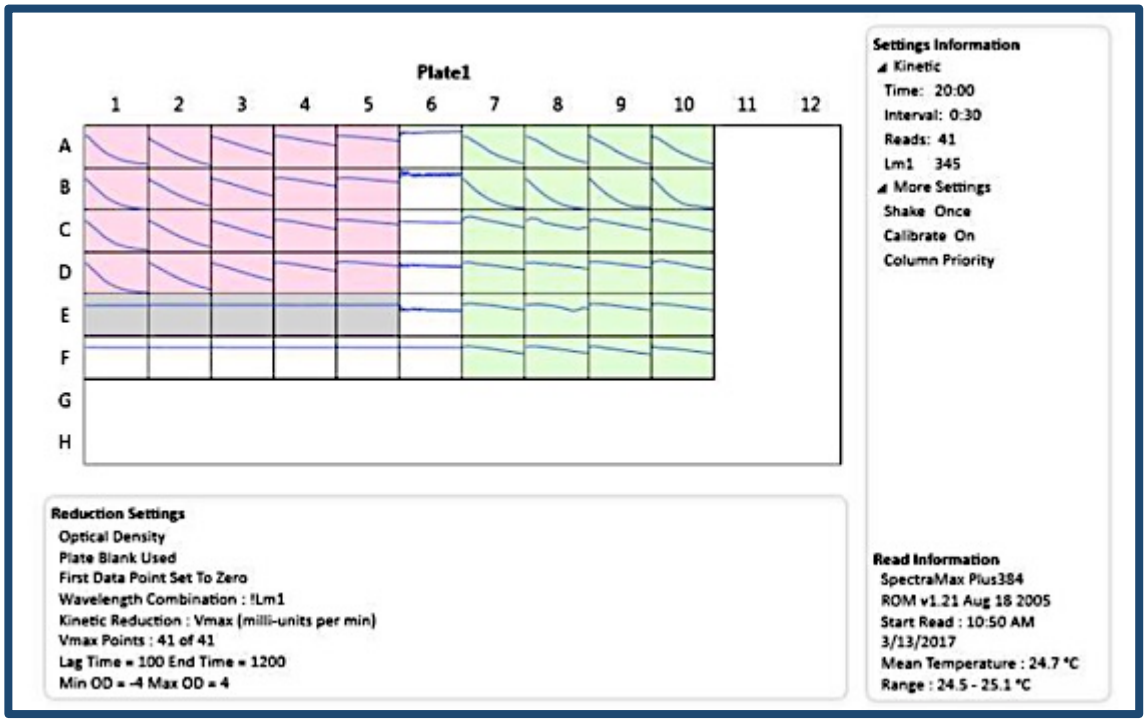


Figure 7: Degradation Hypothesis TFF FALGPA Test.

Wells A1 through D5 contain the standard reference samples used to create the standard curve.

Wells A7 through A10 contain the sample from the degradation hypothesis purification cycle.

Wells B7 through B10 contain the positive TFF control sample for comparison.

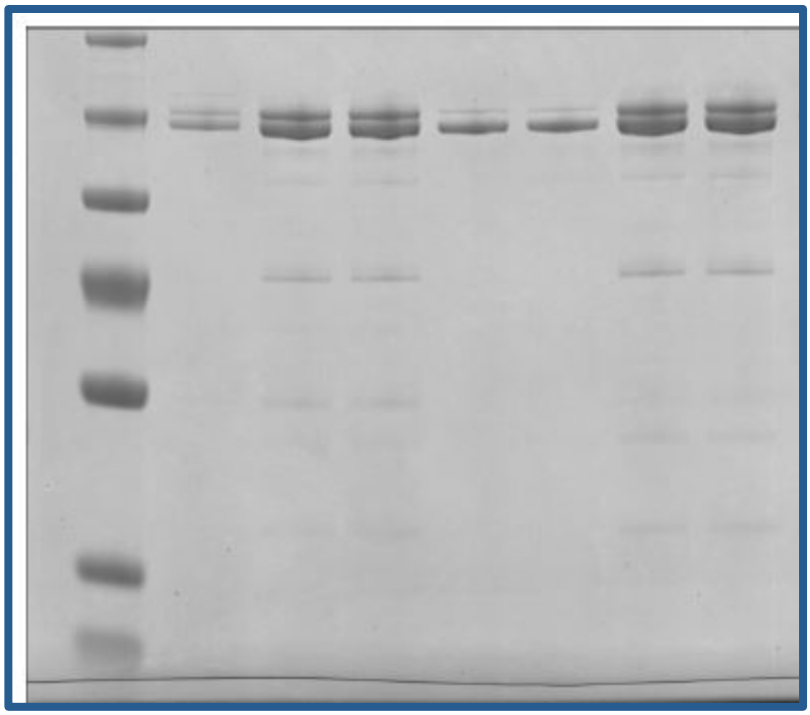


Figure 8: Purification vs Enrichment

As mentioned previously, the goal of this TFF process is to create a protein solution that is more pure and potent than the current protocols in use today. The figure above shows the difference in the compositions of different preparation mechanisms. Lanes 2-4 shows the different in a pure TFF system vs. the ammonium sulfate process. Lane 2 was prepared using the 500 kD filter and the 50 kD filter, where as lane 3 and 4 were prepared using the old method of protein isolation and the 50 kD filter. Lanes 5-8 were prepared using the 30 kD filter in place of the 50 kD filter but keeping the remaining test conditions. The lanes where the samples included the 500 kD preparation had a substantially purer product of collagenase than the older method of production. This last gel cast shows the difference the new protocol can make for the quality and purity for the final collagenase product.

Summary & Conclusions

After the large scale was completed, we were confident in moving forward with this new TFF protocol. After we performed the 500 kD TFF filtration, we compared the activities and they were not statistically different, meaning that this technique can easily be used to replace the centrifugation steps in

the manufacturing process. From the information presented, we believe that the double TFF procedure put too much pressure on the collagenase proteins by forcing it into too small of a solution; we believe this pressure forced the collagenase out of solution or strained them enough to degrade them into inactivity. More tests will be completed in the coming months to further investigate these processes and how to perfect these techniques.

CHAPTER 3

DEVELOPMENT OF HEMOSTATIC BANDAGE FOR MILITARY USE

Background & Literature

From Ancient Egypt and biblical times to current Chinese pharmacopoeia and oceanic medicinal uses, venom has been historically honed to fight off various illness and diseases [6]. The past few decades have shown the first in depth studies of the pharmaceutical use of snake venom isolates. For example, a peptide from the Brazilian viper, *Bothrops jararaca* has been isolated and used to produce the angiotensin-I converting enzyme (ACE)-inhibiting drug, Captopril® [30]. Many more examples can be found to support the evidence that venom can have substantial medicinal benefits if used carefully and correctly. Snake venoms can widely differ in the way they affect the human body. Some snake venoms are rich with neurotoxins that attack the nervous system, shutting down nerve function and stopping all muscle movements (mambas, cobras, rattlers, etc.), while some venoms have cytotoxic capabilities which destroy cells and entire tissues, dissolving flesh and causing horrific pain (vipers, sea snakes, etc.). On contact with blood, some venoms cause extreme coagulation and solidification within seconds while others can cause the inactivation of clotting [25]. Hemostasis involves not only clot clearing, but also clot formation, keeping the hemodynamic homeostasis of blood constant. Venoms from the Viperidae family have been observed to cause instant coagulation of blood by acting as serine proteases, mimicking the action of thrombin, and cleaving fibrinogen, thus creating clots [4]. Many people have seen the video posted by BBC world news showing an exposé on snake venom and its effects on healthy human blood [16]. Very low concentrations of snake venom can cause instant coagulation of a cup of blood within seconds. This coagulation process is why snake venom has been an interest among researchers for years for its potential use in medical treatment for coagulative disorders, like Lupus. This practicum plans on showcasing a novel therapy using snake venom to assist in the formulation of a Navy-contracted project.

Chitosan is a derivative of chitin, the second most important/abundant polymer, following cellulose, in the world according to many researchers in the biomedical field [29]. Both of these polysaccharides form exoskeletons in marine crustaceans, shrimp, crabs, etc. and have been utilized in cosmetics, food, and for biomedical and pharmaceutical applications. Naturally, chitin and chitosan exist as ordered crystalline fibrils in arthropod shells and the cell walls of fungi and yeast usually functioning as reinforcement. What distinguishes chitosan from chitin is that chitosan is soluble in aqueous solutions. This gives it a unique characteristic that makes it, literally, one of a kind; it is the only pseudo-natural cationic polymer [29]. This solubility factor plays a huge role in the history of its use in medical research, specifically in solidifying structures like gels and films. When chitosan is dissolved in solution and introduce into a gelatin matrix, it formed cross-linking lattices within the gel, giving it unique anti-permeation properties [2]. This process of immobilization in the gel has two prominent properties: first, it allows the gel to become more stable and hold up to the elements better, and secondly, it creates an immobilization matrix within the gel. This opens up the possibility of trapping compounds within the gel for various purposes. This process of immobilization has been tested by trapping fluorescently labeled proteins in the matrix, allowing the film matrix to sit in a dissolving solution where they would track the escape of the labeled proteins over time, showing a delayed release of the matrix contents [38]. This specific example opens up countless doors in chronic injury treatment by allowing the possibility of any drug or treatment compound to be trapped in these matrices, applied to a specific area of interest and allowed to degrade over time, slowly releasing the treatment for hours if not days. There are also films that will be attributed to this project that will be starch based and classified as rapidly dissolving films. For instances where you need an expedited process, these films would be preferred. If needed, these films can be immediately removed upon negative reaction and the treatment would instantaneously stop, whereas topical ointments have to be thoroughly cleaned and naturally flushed out of the tissues where they have been absorbed. This film apparatus is more efficient and physician friendly when trouble shooting diagnosing an ailment. This technology has piqued interest from S&N considering their expertise in wound therapy and other external injury treatment. Chitosan immobilization films are

currently being used with bee venom in the treatment of arthritis, back pain, cancerous tumors, and various skin diseases [1]. Bee venom causes acute inflammation around the applied area, acting upon cyclooxygenases and phospholipases to slow degradation of the joints as well as help stabilize the joints with their inflammatory properties [15]. Due to the immobilization matrix created by the chitosan film, the bee venom was administered slowly overtime. This same concept will be making appearances in chronic wound treatments in upcoming years.

Purpose & Significance

Smith and Nephew Biotherapeutics has been in the wound care industry for decades, long before they acquired the Smith and Nephew name. They have on many occasions worked with the military to produce new battle wound therapy products. The newest development in this relationship is the need for a coagulative bandage. This bandage needed to create a hemostatic environment on a wound to stop blood flow in less than one minute, it needed to have debridement capabilities, it needed to have the capacity to deliver antibiotic therapy, and lastly, it needed to provide the wound with nourishment and moisturizing components. This hemostatic bandage/film could revolutionize the way we treat in-theater battle wounds as well as extreme and traumatic civilian injuries. During this practicum, I led the lab-based effort in creating this new product, its first prototypes, *in-vitro* lab testing apparatuses, and the plans for the first stages of clinical trials.

Materials & Methods

Film Preparation

The Navy-contracted, hemostatic bandage project consisted of creating many synthetic films and experimenting with the viper venom. We used a chitosan-containing film to attempt to immobilize the snake venom protein, batroxobin. Chitosan has the property of creating a network-like matrix within gels and films that could be used to encapsulate the snake venom, trapping them inside the gel [38]. Theoretically, on contact with a blood-flow, if covered, the blood cells and platelets would come into

contact with the gel, partially flow through, and react with the batroxobin [27]. The theoretical reaction would result in an almost instantaneous clot on the wound site. This could be a huge step forward in combat wound treatment. In collaboration with Dr. Jovanovic, we plan to formulate how to begin making the film. We will be starting with a combination of Modified cellulose 1 (MC1) and/or Modified cellulose 2 (MC2) to make a basic gel and then make a second one made from MC1, MC2, and chitosan. The MC1 and MC2 will be individually added to separate amounts of water to make two separate gels [7]. The gels, after fully dissolved and cooled, will be added together to make one gel solution, spread across a large petri dish and allowed to air-dry to dehydrate for 48 hours. The same process will be done with the chitosan. We will make a gel out of chitosan, water, and enough acetic acid to fully dissolve. This gel will be added with calculated amounts of MC1 and MC2 gels to create a framework for the venom gel. Our team created 58 film combinations throughout these trials, each containing various components that can be viewed in the results section below. The components were added to a beaker and mixed using a basic, IKA® Eurostar 60 Mixer. Once fully incorporated and homogenized, the final gel mixture was split across four small petri dishes and dried in two separate ways. Each petri dish contained 30 grams of hydrogel and approximately 1 gram of solids. Half of the petri dishes will air-dry to dehydrate for 48 hours whereas the other half of the gels will be frozen in an -80°C freezer and once frozen, placed in a lyophilizer to freeze dry. For the Lyophilization process, the films were placed in a multi-adapter, Virtis Vacuum Pump Lyophilizer. The lyophilizer pulled a vacuum 0-50 torr and maintained a temperature of approximately -90°C. We will be performing various tests to make sure the matrix within the film has been formed. Once we are certain that the films are as we want, we will be experimenting and formulating to create a film that will respond well under the desired circumstances. Once the best films are chosen, the team will focus on determining most efficient way to infuse the viper venom.

Film Dissolving & Coagulation Tests

Once the films were guaranteed to have the venom fully incorporated successfully, these films were tested by dissolving them in a basic Tris buffer and introducing them to a solution of 5 mg/mL

fibrinogen to observe the coagulative effects. To simulate a natural human wound environment, a chosen amount of thrombin was introduced to each test to mimic a normal level in the average human. Using basic standard curve techniques we discovered that the smallest amount of venom to achieve desired coagulative effects in the sample in the presence of human thrombin was 0.2 mg/mL. Once the coagulative properties were validated, we started incorporating starches, PEG-600, glycerol, cellulose powders, and various substances to create quick-dissolving films and also manipulated the environments of the forming gels to create specific textures as well as flexibilities. After each film was created, it was tested for dissolving capabilities with the best candidates taken to further testing. Through this process two distinct goals were decided. The first goal would be to create a highly absorptive film that had high water uptake ability and to create a quick dissolving film that would dissociate fully in liquid within 2 seconds. The methodology behind this decision is to have a bilayer bandage. The bottom/first layer will be the fast dissolving film that will dissociate in one second or less and the highly absorptive film above it to create a suction immediately following contact with blood. When the first film dissolves, its components will be immediately absorbed with the blood into the second film. With this design, the clotting would begin inside the highly absorptive film and start the clotting cascade through the blood, initiated by the venom on blood fibrinogen. This cascade of clotting would then fast track the actions of Clotting Factor (CF) 4 and CF7. Expediting the body's natural clotting processes would give the patient exponentially more time to seek medical attention for his wound.

Mechanical and Tensile Strength Testing

Tensile strength and mechanical capabilities are a major force behind the strength of bandages and films. An Instron is a machine used in many medical device labs to test the strength of various materials. The Instron 2000 that was used in the qualification testing of these films specifically measured tensile strength. This was done by adhering a piece of film between two locked clamps and slowly pulling it in opposite directions while measuring how much force is being applied to the films. The machine measured the moment the films had a solid break in their tension and it measured the peak force

withstood during the stretch. The data was given in table-form and compiled into a master data sheet listed below in the results section.

Venom Infusion and In-vitro Models

According to calculations, to approach the targeted concentration in the film, one milliliter of a 1-mg/mL solution of the snake venom will be added to the film mixture. This amount gives approximately 0.2 milligrams of venom per 30 mg film solution. Our team chose Films 26 and 28 as the best candidates to use as the quick dissolving, first layer film. Films 9, 10, and 12 were chosen to be used as the highly absorptive, second layer films. These combinations of venom-containing films were labeled specifically so the testers could easily tell which films to be cautious with. These films were 26V, 28V, 26V12, and 28V12. As a control, films were also created to be a 1:1 combination of films 26/28 and 12 to compare film differences: this would be done to see which would be more efficient, layering two separate films side-by-side or combining the two actions in one film. Once the films were created, they had to be used sparingly due to the price and availability of the venom at the time.

To test these films, a device had to be reconstructed/repurposed to create an *in-vitro* lab model. For this, we repurposed a glass Franz Cell, normally used in a Hanson Research Vertical Diffusion Device. Usually, both chambers have fluid flowing through them, but for this experiment, only the center section was used. To test the apparatus to verify its use for this concept, we clamped a small circular cut of the film between two white washer-like adapter pieces and locked them on top of the Franz cell with a fastener and a clamp device. On the bottom outlet port, we attached a tube that ran to a syringe full of the fibrinogen solution and used this to fill the inner chamber. Attached on the upper port, we attached an empty syringe with the plunger fully depressed. The bottom syringe filled with the fibrinogen solution was given small pulses to slowly fill the inner chamber until it reached the films surface. Theoretically, if the venom in the film responds as expected, it will create a solid clot and prevent the solution from flowing through the top, open-air adapter [31]. If the venom-film held, it would hold back the pressure

created by the pulse-action of the syringe and force fluid out into the empty syringe. If the film did not clot, the solution would flow through and escape out of the top of the Franz cell. The design of this *in-vitro* model can be seen below, showing the images of the device and its setup.

A final test our team included was a simple, solution-based coagulation study. To test our venom films against the three commercial films provided by the Navy, we set up five 20 mL wide vials with each containing 20 mL of the fibrinogen solution used in previous protocols. Equal weight cutouts were made of each film/bandage and placed on top of the fibrinogen solution to be observed over the course of two hours. Pictures were taken every 30 minutes to help create a time-lapse of this test. This was the final test performed during this practicum; fortunately, the R&D team at S&N will continue these efforts to create a new product based on the following data sets.

Figure 9: In-Vitro Model Apparatus

Left: Front View of the Franz cell with the large ports going to the inner chamber and the shorter ports going into the outer chamber. **Middle:** The side view showing the clamp device securing the white holster pieces. **Right:** The full apparatus with tube and syringes attached, also showing view of holsters. Bottom syringe is full, top syringe is empty, and the top piece on Franz cell is open-air.

Results

All gels made during this testing were designed to contain 5 g solid per 150 mL of solution. The MC1 solution was prepared by delicately adding 15 g MC1 to 500 mL H_2O and similarly, the MC2

solution was made by delicately adding 12.5 g to MC2 to 500 mL of H_2O. Table 4 shows not only the reagents used to make these film but also details the observations of the films once dried and suggestions on what changes should be made to further the creation of the goal film. Once we figured out that the venom did work in the MC1/MC2 films, testing procedures were planned.

Table 4: Venom-Slow Film Preparations and Results

Film Trial	Gel Components/	Observations/ Suggestions for further trials
Film #1	42% MC1 (2.1 g) 35% MC2 (1.75 g) 23% PEG-600 8.5 g H_2O	Successful solidification Long dissolving time Clear film, transparent *Introduce Chitosan
Film #2	34% MC1 (1.68 g) 28% MC2 (1.4 g) 18% PEG-600 (0.92 g) 20% Chitosan (1 g)	Successful solidification Long dissolving time opaque, slightly transparent film *Ready to add venom
Film #3	34% MC1 (1.68 g) 28% MC2 (1.4 g) 18% PEG-600 (0.92 g) 20% Chitosan (1 g) 1 mL Batroxobin (1 mg)	Successful solidification Long dissolving Time Very opaque film *Used for the Coagulation tests in Tables 5-7 *Move to a quick dissolving gel

Once we figured out that the venom did work in the MC1/MC2 films, we moved to the development of quick dissolving films. Since the first trials of films took substantial time to fully dissolve in every solution attempted, we moved to a starch-based films removing MC2 from the reagents. These films, their preparation, and observations of their finished stage can be found below in Table 5. A circle cutout was taken and placed in an empty petri dish and 2 mL of saline was added to the film. Observations were made on how the gel responded when placed in saline (swell, dissolve, break, etc.)

Table 5: Quick-Absorption Film Preparations and results

Film #	Gel Components	Observations/Suggestions for further trials Within 5 minutes
Film #1	50% MC1 (2.5 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 10% Glycerin 90.5 g)	Very thick with high plasticity. After placed in 40°C incubator it became somewhat slimy and dry. *Swelled up to twice its starting size when added to 2 mL Saline
Film #2	40% MC1 (2 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 20% Glycerin (1 g)	Very thick with high plasticity. After placed in 40°C incubator it became much drier than the 50% film. *Swelled up slightly when added to 2 mL Saline
Film #3	30% MC1 (1.5 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 30% Glycerin (1.5 g)	Very thick, but not completely solidified. Very little plasticity and tore easily. After being placed in the 40°C incubator, it simply melted and didn't stand up to the heat *Melted and became gel-like when added to 2 mL Saline
Film #4	55 % MC1 (2.75 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 5% Glycerin (0.25 g)	Slight swelling, not quickly enough. Reached about the same size as gel 1
Film #5	Miscalculated ratios and ended up a solid white mass, wrong consistency. **Redid as Film#6	N/A
Film #6	35 % MC1 (1.75 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 10% Cellulose (0.5 g) 10% Starch (0.5 g) 5% Glycerin (0.25 g)	Dried: Expanded beautifully, within the entire circumference of saline within 12 minutes Lyo'd: Full flow through in 2 seconds, full absorption within 1 minute Water Uptake: 904.4 g (1861% increase)
Film #7	50% MC1	No change… no dissolving, no expanding,

	12.5%Starch 12.5% Cellulose 20% PEG-600 5% Glycerin	Lyo'd: Little to no inflation Water Uptake: 0.558 g (1276% increase)
Film #8	35 % MC1 (1.75 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 10% Cellulose (0.5 g) in Saline 10% Starch (0.5 g) in Saline 5% Glycerin (0.25 g)	Slight inflation, but became sticky and very unstable and very weak Water Uptake: 0.427 g (1001% increase)
Film #9	25 % MC1 (1.25 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 15% Cellulose (0.75 g) in Saline 15% Starch (0.75 g) in Saline 5% Glycerin (0.25 g)	Dried: Great inflation, expanded fully within 20 minutes when added to the saline. Slightly smaller than #6 Lyo'd: Full uptake in 1 minute Water uptake: 0.957.6 g (1829% increase)
Film #10	25 % MC1 (1.25 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 20% Cellulose – Explotab (1 g) 10% Starch (0.5 g) 5% Glycerin (0.25 g)	Dried: Not much change, slow inflation over 2 minutes Lyo'd: Stable, Partial inflation within 30 sec. Water uptake: 1.079 g (2340% increase)
Film #11	35 % MC1 (1.75 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 20% Cellulose – Explotab (1 g) 5% Glycerin (0.25 g)	Dried: Stable, Full inflation within 2 minutes. Lyo'd: Stable, Full inflation within 35 seconds Water uptake: 1.916 g (4059% increase) Lost tensile strength within 10 minutes
Film #12	35 % MC1 (1.75 g) 15% Chitosan (0.75 g) 15% PEG-600 (0.75 g) 20% Cellulose – Explotab (1 g) 10% Carpamoxal MC (0.5 g) 5% Glycerin (0.25 g)	Dried: Stable, full inflation within 2 minutes Lyo'd: Stable, Full inflation within 35 seconds Water Uptake: 1.911 g (4065% increase) Kept tensile strength after 10 minutes

Film #13	30 % MC1 (1.5 g) 10% Chitosan (0.5 g) 15% PEG-600 (0.75 g) 25% Cell - Explotab (1.25 g) 15% Carpamoxal MC (0.75 g) 5% Glycerin (0.25 g)	Dried: Stable, inflated, retained 10.7x weight Water Uptake: 398.1 g (1074% increase) Lyo'd: After 5 minutes it still had a dry middle Water uptake: 1.0467 g (1880% increase) Lost stability after 6 minutes, started flaking
Film #14	35 % MC1 (1.75 g) 15% Chitosan (0.75 g) 10% PEG-600 (0.5 g) 25% Cell - Explotab (1.25 g) 10% Carpamoxal MC (0.5 g) 5% Glycerin (0.25 g)	Dried: Slight inflation, but not significant within 5 minutes Water Uptake: 0.2919 g (858% increase) Lyo'd: Same as Film 13, but with quicker flaking of edges. Dry areas in middle. Water Uptake:0.9109 g (1980% increase)
Film #15	35% Cellulose (1.75 g) 20% Chitosan (1 g) 20% PEG-600 (1 g) 10% Cellulose-Explotab (0.5g) 10% MC1 (0.5 g) 5% Glycerin (0.25 g)	Dried: Self-coiling t first, but still stiff with slight swelling after 2 min. After 5, expanded 1.5 x diameter, still stiff Lyo'd: Immediate penetration into film. Swelled in thickness. Became Goo-like. Did not dissolve, but decreased in structural integrity
Film #16	35% Cellulose-Explotab (1.75 g) 20% PEG-600 (1 g) 15% MC1 (0.75 g) 10% Chitosan (0.5 g) 10% CMC (0.5 g) 10% Glycerin (0.25 g)	Dried: 2 min: Slight inflation, somewhat soft. 5 min: 1.5 diameter expansion, pliable Lyo'd: Immediate absorption, dry layer in center, became gel-like within 2 minutes
Film #17	30% Cellulose-Explotab (1.5 g) 20% PEG-600 (1 g) 15% MC1 (0.75 g) 10% Chitosan (0.5 g) 15% CMC (0.75 g) 10% Glycerin (0.25 g)	Dried: 2 min: Little if any inflation. 5 min: less expansion than 16, but similar in action Lyo'd: Immediate Absorption, dry center layer larger than Film 16, slightly more gel-like.
Film #18	25% Cellulose-Explotab (1.25 g) 20% PEG-600 (1 g) 15% MC1 (0.75 g)	Dried: 2 min: Very stiff, self-coiling, no expansion. 5 min: Slight expansion, slightly pliable,

	10% Chitosan (0.5 g) 20% CMC (1 g) 10% Glycerin (0.25 g)	Lyo'd: Instant absorption, small dry center, noticeable vertical expansion, complete loss of structural integrity by lifting
Film #19	20% Cellulose-Explotab (1 g) 20% PEG-600 (1 g) 15% MC1 (0.75 g) 10% Chitosan (0.5 g) 25% CMC (1.25 g) 10% Glycerin (0.25 g)	Dried: 2 min: Stiff, no expansion 5 min: Slight expansion, slightly pliable, similar to 18, but smaller. Lyo'd: Instant FULL absorption, no dry spots, because like a thick gel with barely any integrity, didn't tear on lifting
Film #20	20% Explotab (1 g) 15% MC1 (0.75 g) 43.3% PEG-600 (2.165 g) 21.6% Glycerin (1.08 g)	Dried: 2 min: Slightly inflated, but became fragile upon touch. 5 min: Because translucent, but barely dissolved. Lyo'd: Instantly dissolved. Small fragments floating in saline
Film #21	30% Explotab (1.5 g) 22.5% MC1 (1.125 g) 31.67% PEG-600 (1.5835 g) 15.8% Glycerin (0.79 g)	Dried: Same as film 20 but with more structural integrity, Lyo'd: Instant absorption, small dry areas in middle. Kept structural integrity.
Film #22	40% Explotab (2 g) 30% MC1 (1.5 g) 20% PEG-600 (1 g) 10% Glycerin (0.5 g)	Dried: Same as film 21 but with greater structural integrity (20 < 21 < 22) Lyo'd: Instant absorption with very few dry spots. After 2 min, it became translucent but maintained structural integrity.
Film #23	30% Explotab (1.5 g) 10% Maltodextrin (0.5 g) 35% MC1 (1.75 g) 15% PEG-600 (0.75 g) 10% Glycerin (0.5 g)	Dried: Translucent in a matter of seconds, became very soft, but kept structural integrity Lyo'd: Partial absorption, kept large internal dry areas, no change after 30 seconds. Maintained condition.
Film #24	20% Explotab (1 g) 20% Maltodextrin (1 g) 35% MC1 (1.75 g) 15% PEG-600 (0.75 g) 10% Glycerin (0.5 g)	Dried: Same as 23, but slightly more fragile with slightly more inflation Lyo'd: Partial absorption, more internal dry areas than 23, no change after 30 seconds. Maintained condition.

Film #25	10% Explotab (0.5 g) 30% Maltodextrin (1.5 g) 35% MC1 (1.75 g) 15% PEG-600 (0.75 g) 10% Glycerin (0.5 g)	Dried: Same as 24, but slightly softer and much more delicate. But kept enough structural integrity to remove and measure. Lyo'd: Partial absorption, more internal dry areas than 24, no change after 30 seconds. Maintained condition.
Film #26	40% Explotab (2 g) 20% MC1 (1 g) 20% PEG-600 (1 g) 10% Sorbitol (0.5 g) 10% Glycerin (0.5 g)	Dried: Slight inflation, quick absorption, remained somewhat stiff. Not as quick as previous films. Lyo'd: Instant, full absorption in less than 2 seconds, maintained structural integrity.
Film #27	40% Explotab (2 g) 10% MC1 (0.5 g) 20% PEG-600 (1 g) 20% Sorbitol (1 g) 10% Glycerin (0.5 g)	Lyo'd: Immediately absorbed fully, but lost all physical differentiation, basically disintegrated into tiny pieces
Film #28	40% Explotab (2 g) 15% MC1 (0.75 g) 20% PEG-600 (1 g) 15% Sorbitol (0.75 g) 10% Glycerin (0.5 g)	Lyo'd: Immediate absorption but while expanding, it broke off into pieces and became very unstable

Once the films have been synthesized they will go through testing to determine their tensile strength and durability. An important aspect of this product will be how well it could stand up against friction and natural movements while on the wound. These films will be cut in identically equal pieces and placed in a pneumonic clamp device and run through the Instron, a device that stretches and extends products while measuring how far they can stretch before breaking and how much force can be applied to the product before the film splits. Table 6 below outlines the raw data obtained from testing each film created. Films with a 'D' in the name were air-dried whereas films with an 'L' in the name were prepared using the Lyophilizer.

Table 6: Raw Instron Data - Tension Tests

Film Sample	Maximum Load (N)	Extension @ Maximum Load (mm)	Extension @ Break (mm)	Load @ Break (N)
6-L-1	3.5	1.0	7.0	0.6
6-L-2	4.5	0.9	9.6	0.4
6-L-3	9.2	0.8	8.2	0.5
6-L-4	6.5	0.6	10.0	0.2
9-L-1	4.2	2.3	10.4	0.3
9-L-2	5.6	2.0	8.0	0.4
9-L-3	3.9	2.2	6.8	0.5
9-L-4	4.0	2.3	7.8	0.3
10-L-1	16.2	2.6	9.8	0.2
10-L-2	8.1	3.1	11.9	0.0
10-D-1	51.6	3.3	3.3	51.6
10-D-2	36.0	2.0	2.0	36.0
10-D-3	84.7	1.4	1.4	84.7
10-D-4	77.1	1.7	1.7	77.1
11-L-1	6.4	1.5	6.6	0.5
11-L-2	6.3	2.2	12.1	0.4
11-D-1	35.3	0.9	0.9	35.3
11-D-2	75.5	1.5	1.5	75.5
11-D-3	83.98	1.3	1.3	83.98
11-D-4	60.6	1.0	1.0	60.6
12-L-1	2.2	1.3	5.3	0.1
12-L-2	2.9	2.0	8.8	0.1
12-D-2	43.8	1.1	1.1	43.8
12-D-2	37.0	0.9	0.9	37.0
12-D-3	73.5	1.0	1.0	73.5
12-D-4	83.6	1.0	1.0	83.6
13-L-1	1.6	5.3	18.7	N/A
13-L-2	1.9	3.4	27.3	N/A
13-L-2	3.1	1.0	9.5	N/A
13-L-4	2.3	1.3	7.3	N/A
13-D-1	55.7	2.0	2.0	55.7

13-D-2	54.9	2.1	2.2	53.3
14-L-1	2.9	1.2	11.7	N/A
14-L-2	3.0	1.0	13.8	N/A
14-L-3	2.8	2.2	10.5	N/A
14-L-4	2.7	1.4	9.9	N/A
14-D-1	137.7	1.9	1.9	137.7
14-D-2	77.1	1.0	1.0	77.1
14-D-3	125.6	2.3	2.3	125.6
14-D-4	115.2	1.6	1.6	115.2
15-D-1	67.9	1	67.9	1
15-D-2	89.3	0.9	89.3	0.9
15-D-3	89.3	0.7	89.3	0.7
15-D-4	106	0.8	106	0.8
15-L-1	13.6	0.8	11.2	1.3
15-L-2	6.5	0.6	1.3	7.6
15-L-3	8.7	0.4	8.7	5.5
15-L-4	14.3	0.7	6.7	1.2
16-D-1	36.6	0.6	0	32.8
16-D-2	77.2	1	0.3	13.1
16-D-3	41.2	0.7	0.3	5
16-D-4	60.1	0.8	0.4	4.7
16-L-1	8.8	0.6	8.3	0.7
16-L-2	5.4	0.9	4.1	1
16-L-3	12.1	0.9	10.1	1.1
16-L-4	2.2	1	0.3	5.5
17-D-1	62.6	1	62.6	1
17-D-2	82.1	1.1	82.1	1.1
17-D-3	79.6	1	79.6	1
17-D-4	68.3	1.4	68.3	1.4
17-L-1	2.1	1	0.3	5.4
17-L-2	6.1	0.9	0.4	6.9
17-L-3	1.9	4.3	0.4	9.7
17-L-4	3.8	0.8	0.5	5
18-D-1	59.3	1.6	59.3	1.6

18-D-2	60.5	1.1	60.5	1.1
18-D-3	46.7	0.9	45.3	1
18-D-4	40.9	1.3	30.1	1.7
18-L-1	7.7	1.3	0.1	4.8
18-L-2	6.7	1.1	0.5	5.2
18-L-3	6.5	1.1	1.1	5
18-L-4	6.2	0.8	1.1	4.5
19-D-1	42.1	4	42.1	4
19-D-2	44.2	1	34	1.2
19-D-3	35	1.6	21.1	2.3
19-D-4	42.1	1.3	21	1.8
19-L-1	2.4	1.5	0.2	6.9
19-L-2	2.1	2.3	0.1	7.6
19-L-3	2.6	1.2	0.4	6
19-L-4	2.6	2.3	0.3	7.3
23-D-1	27.8	4.7	27.8	4.7
23-D-2	24.7	2.3	24.7	2.3
23-D-3	25	4	25	4
23-D-4	24	1	23.9	2.5
24-D-1	42.4	0.9	41.4	1.8
24-D-2	47.5	1.2	46.8	1.5
24-D-3	30.6	1.5	30.6	1.5
24-D-4	36.9	0.9	36.7	1
25-D-1	21.9	3.9	21.9	3.9
25-D-2	34.3	5	32.2	5.1
25-D-3	156.3	9.6	133.3	12.8
25-D-4	157.5	10.7	133.4	12.4
26-D-1	13.5	0.9	0.3	5.9
26-D-2	15.6	0.4	12.9	6.8
26-D-3	12.1	0.7	0.1	7.6
26-D-4	13.7	0.9	11.2	4.9

*Note: Films 20-22 and the Lyo'd films of numbers 23-26 were far too delicate and didn't hold up to the Instron for proper readings and would not be able to be read. These numbers were all "0" and were excluded from the table.

The Instron Data was the next major step in identifying the best direction for further film preparation. Analysis of the data in the above table and the table detailing the response to water uptake had to be done to decide on which percentages of components would be the most efficient for our final prototype. Below are two figures showing the relationship between the percentages of MC1 and the average amount of water uptake as well as the relative tension in the films. Below those are two more figures showing the relationship between the levels of Cellulose-Explotab and CMC to the tensile strength as well as the average water uptake per equal size film.

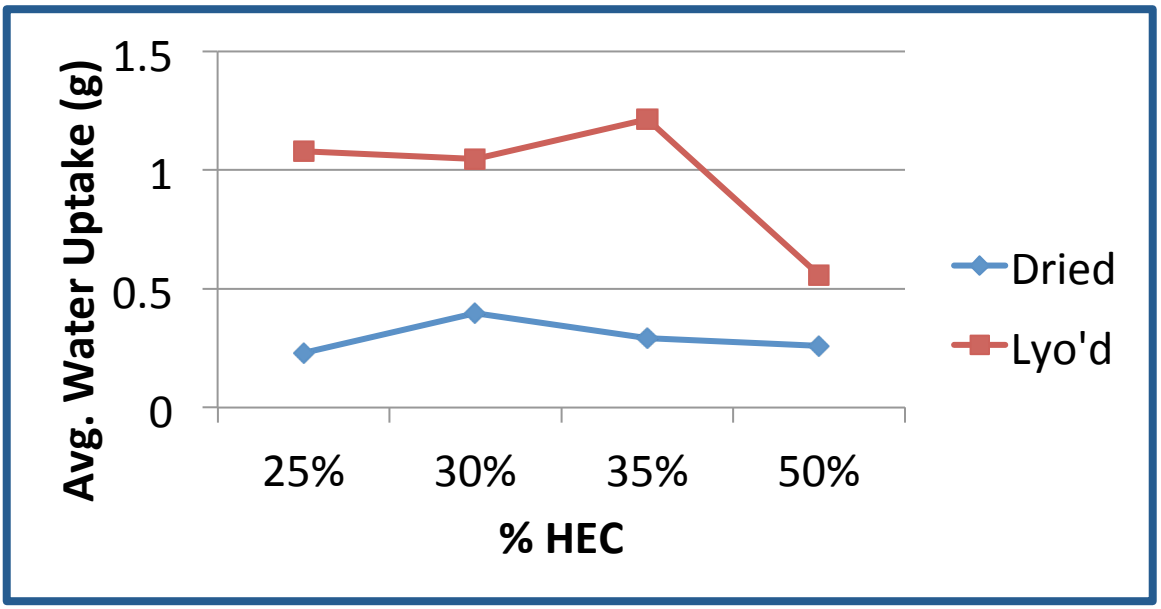


Figure 10: MC1 Percentage vs Avg. Water Uptake

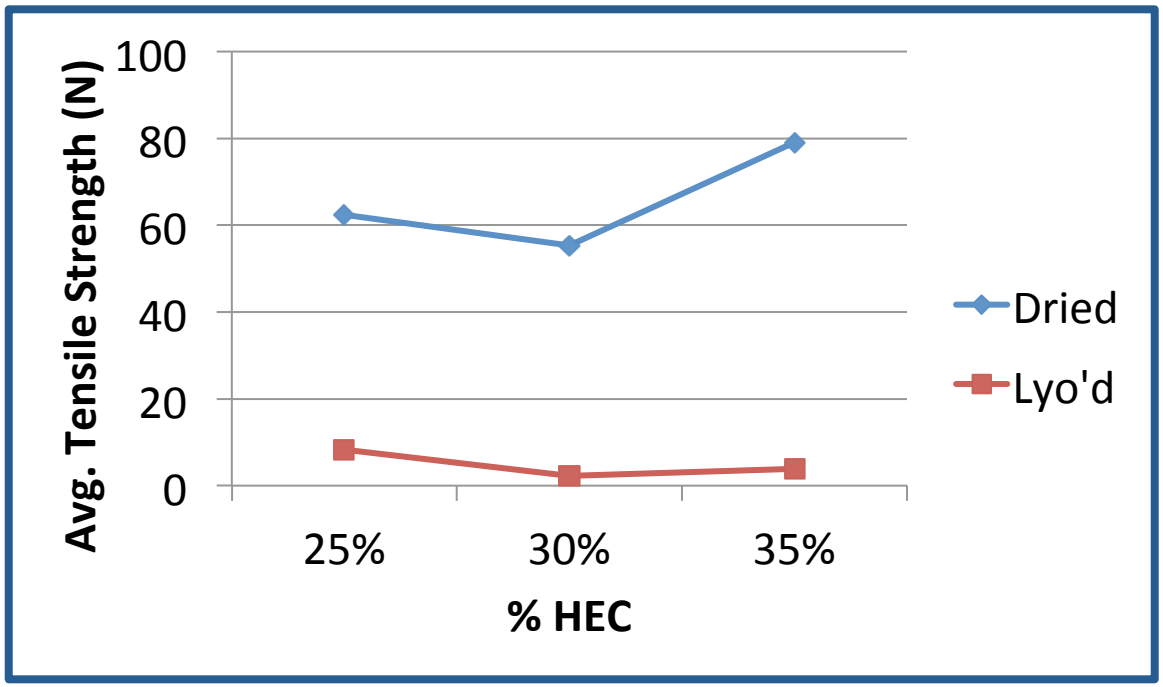


Figure 11: MC1 Percentage vs Tensile Strength

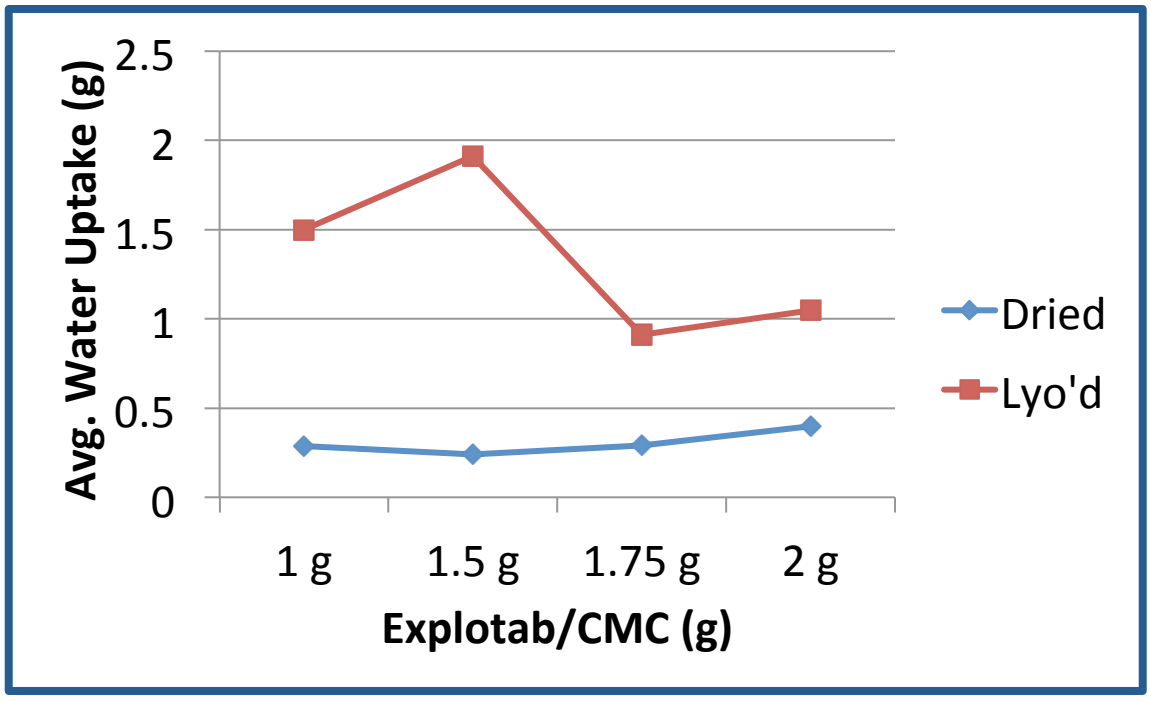


Figure 12: Explotab/CMC vs. Water Uptake

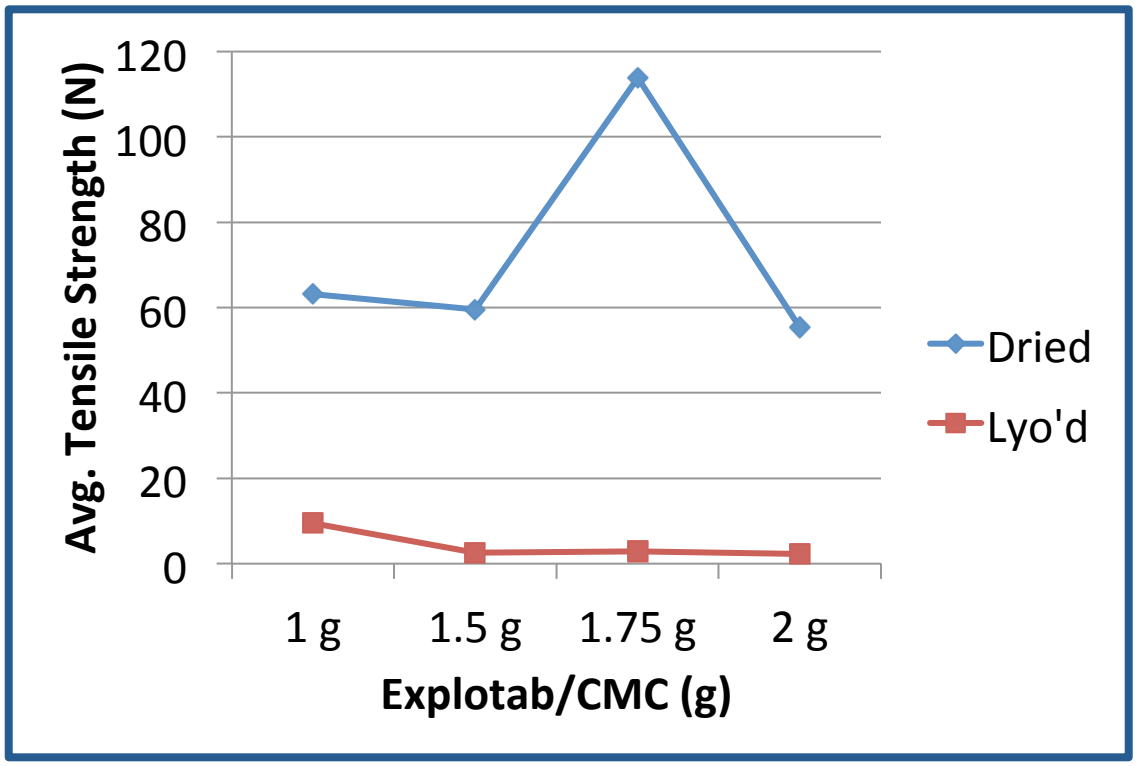


Figure 13: Explotab/CMC vs. Tensile Strength

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The final step once the best film combinations were chosen was to create a film with those specifications and to add the venom in the film preparation. But before the final venom-containing prototype could be created, the percentage and concentration of the venom protein needed to be calculated and decided. To do this we created a selected number of films containing various concentrations of the venom protein to test on separate coagulation experiments. The next stage in development was to dissolve it in an in vitro system and observe the effects it had on a sample containing fibrinogen. For testing regulation, the fibrinogen sample was prepared to mimic the expected levels of fibrinogen in the average person. To validate the results, we used a sample of CPT2, a human cell-based thrombin product as a positive control to view how the normal reaction should be in the circulatory system naturally. To fulfill this experimentation, we added 40 µL of the venom solution to 200 µL of a 5.5 mg/mL fibrinogen solution. The results of this validation test are located below in Table 7.

Table 7: Venom Coagulation Standard Curve

Venom Concentration	Observations @ 5 minutes
CPT2 Thrombin	Positive Control: Full coagulation, large opaque clot-cell
0.5 mg/mL	Full coagulation, opaque clot-cell; Worked slightly better than Thrombin
0.1 mg/mL	Slight coagulation in center; Noticeable solidified mass
0.05 mg/mL	Increase in viscosity, strand-like structures through droplet
0.01 mg/mL	Slight "sticky" response to agitation with needle
0.001 mg/mL	No noticeable reaction
0.0 mg/mL	Negative control –ultrapure H_2O

Now that the results prove the positive reaction of venom and the fibrinogen, the next step in experimentation is verifying the lowest concentration of thrombin that would result in full coagulation of the fibrinogen samples. This result would give us another data point to consider when calculating the level of venom needed to cooperatively coagulate the fibrinogen samples in later tests. The results of the thrombin response standard curve are listed in Table 8 below. These results were accompanied by observing the effects of the thrombin with the fibrinogen by cMC1king to sample after the 5, 12, and 20 minutes. The experimentation was done by adding 40 µL of the Thrombin solution to 200 µL of the 5.5 mg/mL fibrinogen solution.

Table 8: Thrombin Coagulation Standard Curve

Thrombin Concentration	Observations
10 U/mL	Solid mass, 100% coagulation
5 U/mL	Solid, stable central mass
4 U/mL	Increased viscosity and strand-like, internal structures within 5 minutes Full solid mass within 12 minutes
3 U/mL	Increased viscosity and strand-like, internal structures within 5 minutes Full solid mass within 12 minutes
2 U/mL	Increased viscosity and strand-like, internal structures within 5 minutes Full solid mass within 12 minutes
1 U/mL	Slightly strand-like and sticky yet still liquid at five minutes, increased surface tension at 15 minutes
0.5 U/mL	No significant response within 5 minutes. Slight surface rigidity at 15 minutes. Failed Concentration
0.25 U/mL	No significant response within 5 minutes. Slight surface rigidity at 15 minutes. Failed Concentration
0.125 U/mL	No significant response within 5 minutes. Slight surface rigidity at 15 minutes. Failed Concentration
0.0625 U/mL	No significant response within 5 minutes. Slight surface rigidity at 15 minutes. Failed Concentration
0.0 U/mL	Negative control –ultrapure H_2O

Once the Thrombin coagulation tests were completed, we observed that the lowest concentration of thrombin that created a full coagulate was the 2 U/mL sample. This sample would be taken on to the next testing system. We needed to figure out the response of the batroxobin proteins would have with the fibrinogen in the presence of human thrombin, mimic the molecules that would be present in an active, bleeding wound. The results from the Thrombin/venom tests are below in Table 9 with observations made

at a 5-minute and a 15-minute time-lapse. Experimentation was done by adding 40 µL of venom solution to 40 µL of 2 U/mL Thrombin solutions and 200 µL of the 5.5 mg/mL fibrinogen solution.

Table 9: Venom & 2 U/mL Thrombin Coagulation Standard Curve

Venom Concentration	Observations @ 5 minutes	Observations @ 15 minutes
0.5 mg/mL	Substantial solid, center of mass Very opaque in color	Full coagulation
0.25 mg/mL	Medium-large center of mass Opaque in color	Large/Full coagulation
0.1 mg/mL	Small/medium center of mass Slightly opaque	Medium-large coagulation, Opaque
0.05 mg/mL	Increased viscosity Stringy/but still clear liquid	Small center of mass, Opaque
0.01 mg/mL	Water-like, tiny strand-like structures	Increased viscosity, small clumps, very sticky, strand-like structures
0.001 mg/mL	No change	Increased viscosity, small clumps, very sticky, strand-like structures
0.0 mg/mL	Negative Control: No change	Increased viscosity, small clumps, very sticky, strand-like structures

Based on the above results, the best venom concentration to use for this product should be 0.25 mg/mL of batroxobin in the final film. Assuming our qualifications for fibrinogen and thrombin concentrations in these tests matches the information backed by the literature, this will be the ratios taken forward in the next formulation processes. According to our coagulative tests, the two venoms taken to the infusion test were decided to be films 26 and 28.

Venom Infusion Tests

After the films were tested on the Franz cell apparatus, the thresholds of each film was observed and recorded for comparisons. The venom-containing films created a solid barrier within the films and

held back the increasing pressure from the solution in the Franz cell and forced solution out of the output syringe. The flow rate thresholds varied from 3-25 mL/min using different absorbent, second-layer films. The average thresholds of these tests are diagramed below in Figure 12. These data shows that the best choice of film to pair with the venom-containing films would be Film 10.

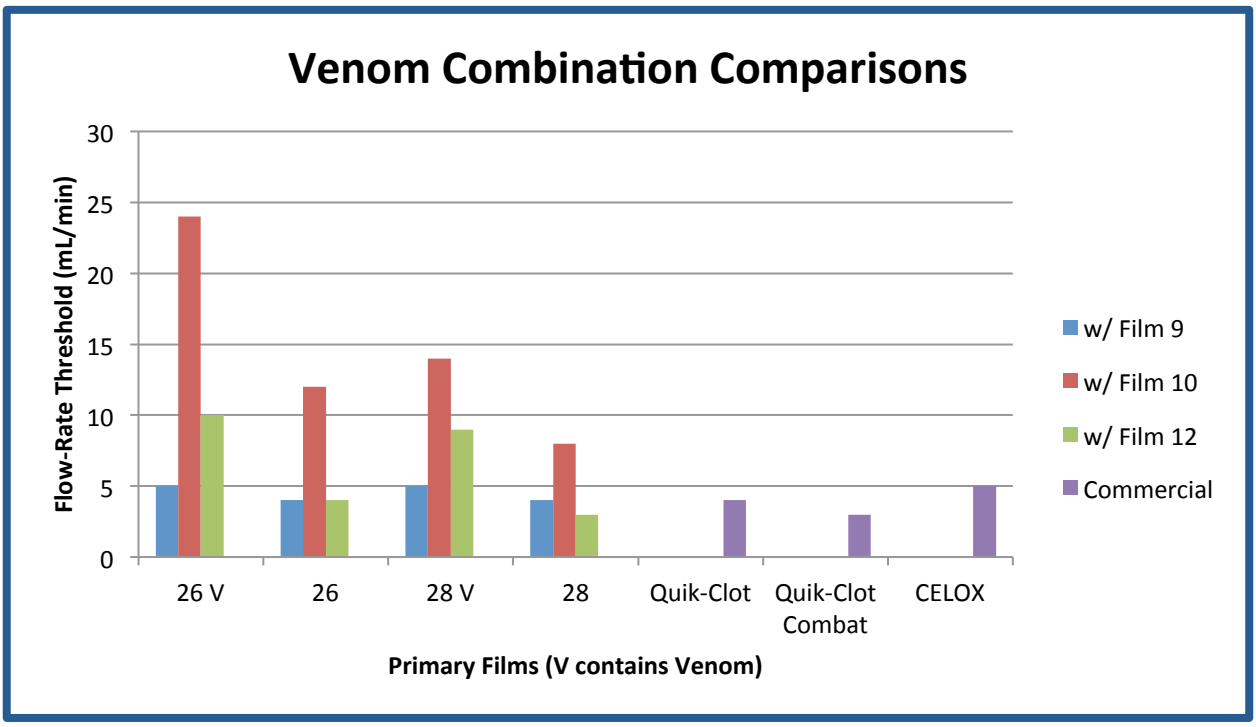

Figure 14: Film Comparisons via Franz Cell Resistance Tests

The third and final test was performed over a 2-hour period to observe the coagulative effects of the films on 10 mL of a fibrinogen solution. We compared the two venom-infused films, Quikclot, Quikclot-Combat, and CELOX. Below are the before and after images of this simple test. In order, the vials are: (1) Film 26V (2) Film 28V (3) Quikclot (4) Quikclot-Basic (5) CELOX.

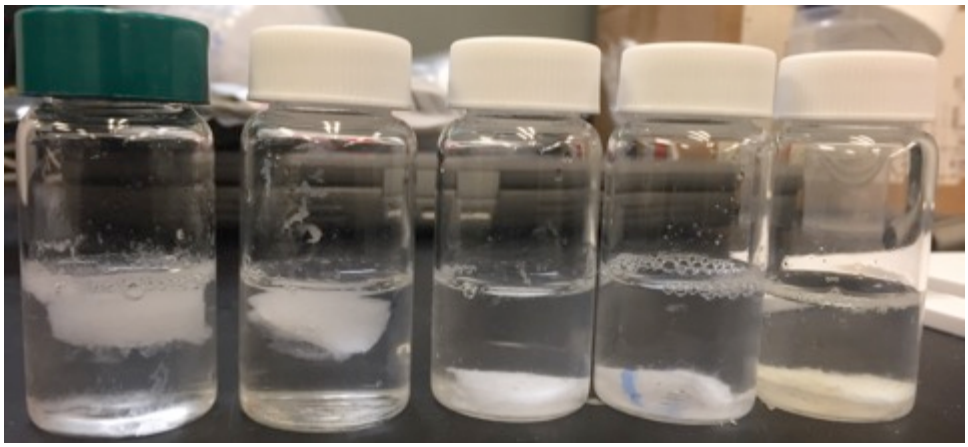

Figure 15: Firbinogen Solution Comparison: Before

The immediate response of the venom films was a rapid absorption of the solution. Due to their porous nature, they remained floating on the surface while the current industrial models simply floated to the bottom of the vial. An opaque substance leeched out of the CELOX film and filled the bottom of the vial. Upon further observation, this substance remained aqueous and never solidified.

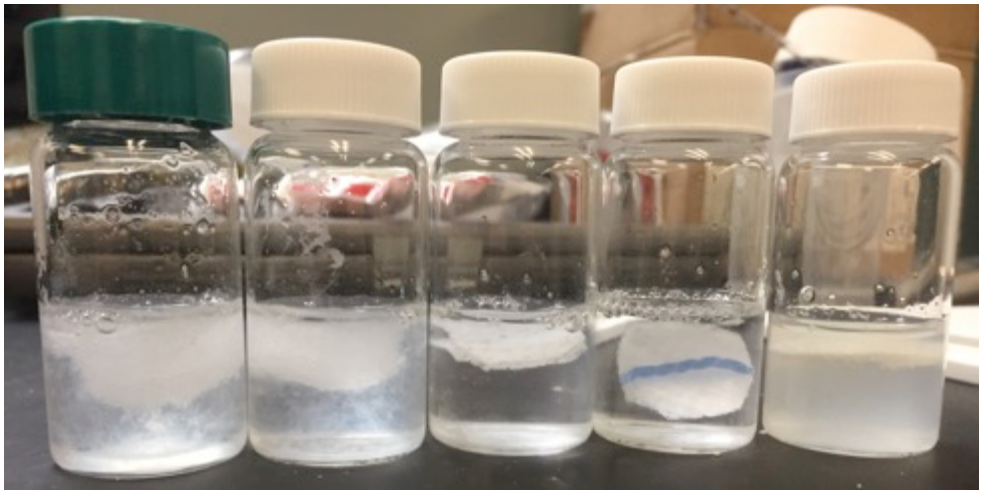

Figure 16: Fibrinogen Solution Comparison: After 2 hours

After 2 hours, distinct fibrin formed throughout the venom containing vials. On movement, these two vials had substantial solid clots formed throughout the vial whereas the commercial products were completely liquid. Although the CELOX vial looks as if some action took place, the aforementioned substance that seeped out of the film caused this discoloration. No solidification took place in vials 3-5.

Summary & Conclusions

During this experimentation, we have established a viable framework for this hemostatic film. While this practicum covers the first stages in the development of this film, we believe that the work done during this time has propelled this product into a stage of development where S&N can continue its production. This practicum covers the production of the films creation and the venom infusion. The next stages of development are to infuse the antibiotic, iodine substrates, the nutritional reagents, and a debridement protein similar to collagenase. The *in-vitro* apparatus proves the concept of the films and provides substantial evidence of the need for this product. When my time at S&N comes to an end, the R&D team will start attributing these actions to the films. When all of the preferred actions are added to the films, they will be sent to a San Antonio Pre-Clinical Trial lab and tested using porcine models due to their similar circulatory systems.

CHAPTER 4

REGRANEX® REFORMULATION QUALITY TESTING USING SILVER STAINING AND VARIOUS TESTS

Background & Literature

Regranex® gel acts as a treatment for lower extremity diabetic neuropathic ulcers; its effects extend into subcutaneous tissue and various tissues with adequate blood supply. The gel is composed of 0.1 mg/mL of Becaplermin and when combined with good wound-keeping, accelerates wound healing by approximately six weeks compared to placebo gels. Regranex® is an S&N product used across the country mainly in geriatric treatment centers and nursing homes. Becaplermin is a cicatrizant, promoting skin healing through the formation of scar tissue [36]. Regranex® is a human platelet-derived growth factor (PDGF) denoted along with good wound care for various treatments of lower limb diabetic ulcers [28]. PDGF is a protein that helps maintain equilibrium of cell growth and division and plays a significant role in angiogenesis [11]. PDGF, acting as a tyrosine kinase receptor, promotes cell migration; Regranex® promotes this migration to the wound site to create new scar tissue to help the healing process [36]. Analysis and experimentation involving healing wounds revealed that PDGF causes proliferation and differentiation of fibroblasts and was observed to increase the healing capabilities of people with pre-existing decreased healing capacities [14]. Many tests have shown that the actions of Becaplermin also promote collagen production and the rate of re-epithelialization and revascularization [13].

For this experimentation, the testing method of silver staining will be introduced to replace classical analytical methods. Silver Staining is a process that is used to specifically identify proteins ad micromolar and nanomolar level in solution [22]. Silver staining increases the sensitivity of detection by 50 times due to the accuracy of the binding of silver to the proteins [42].

Purpose & Significance

Becaplermin, the active ingredient of Regranex® requires storage at 4°C and is good for 12 months. Each sample takes approximately four to six months to reach consumer shelves and weeks to actually get into the hands of people who use it. This gives approximately six months of use per batch of Regranex® made. Due to the rise of new, competitive products with longer shelf lives, S&N have begun reformulation of Regranex® to develop a new version of this product with a significantly longer shelf life. The first stage is trying to create new versions of the ointment by using a variety of different solutes and stable matrices. The problem lies with the absence of a viable testing procedure to quantifiably compare the different formulations. The first stage will be repurposing a silver-staining method to fit our products and then compare the different formulations of Regranex® to see which would be the optimal formulation. If the proposed protocols are improved and perfected within a reasonable time-frame, this practicum will include the QC & QA testing of these new formulations to quantifiably answer the question of which of these formulations retain the active PDGF substrates for the longest duration as well as which formulations respond correctly to the production of the product.

Materials & Methods

Regranex® has a 0.1 mg/ml concentration of Becaplermin, making it very difficult to do normal SDS PAGE or MES PAGE electrophoresis & molecular weight analysis due to the very dilute amount of protein. Due to this incredibly small concentration of active protein the method of silver staining was introduced into this analysis. Silver staining is used on samples with proteins in the micromolar and nanomolar concentrations. This gave results that were substantially clearer and more precise. Once a gel is run with the designated samples, the gels are then put through 4 hours of intricate and specific washes. This process followed the Sigma-Aldrich protocol and used the reagents included in their ProteoSilver™ Silver Stain Kit. Five solutions were made to conduct the multiple washing of the gel: Fixing solution, 30% Ethanol solution, Sensitizer solution, Silver solution, and Developer solution. Two versions of each gel were made, one native (unchanged) and one reduced (partially degraded). The gels were run through a

4-12% Bis-Tris SDS-PAGE gel for 25-40 minutes at 225 volts (V). The preparation of each staining solution and the Silver Stain Process are listed and described below in Table 10. Anyone performing this protocol needs to read through entire protocol before attempting to begin due to the volatile and time-sensitive nature of these solutions.

Table 10: Silver Staining Solutions and Process

Solutions:

- Fixing Solution: Ethanol/water/Acetate Solution
- 30% Ethanol Solution: Prepare.
- Sensitizer Solution: Allow the precipitate particles in the bottle to settle to the bottom before removing.

  *Use within 2 hours of preparation.
- Silver Solution:.

  *Use within 2 hours of preparation.
- Developer Solution: Add ProteoSilver Developer 1 and ProteoSilver Developer 2 to ultrapure water.

  *Use within 20 minutes.
- Stop Solution: Have on hand for the end of the procedure.

Methods:

1. Upon completion of gel electrophoresis, Record Room Temperature and get a clean gel box for the staining procedure.
2. Fixing: Remove gel from precast box and carefully place it in a gel box, add the Fixing Solution to gel and incubate on rocking platform.

   *Gel may shrink and become discolored during this step; this is expected and will be reversed.
3. Ethanol Wash: Decant Fixing Solution, add ethanol solution and place on rocking platform

4. Water Wash: Decant the ethanol solution and add ultrapure water and incubate on rocking platform.
5. Sensitization: Decant the water and add Sensitizer solution and incubate on rocking platform.
6. Water Wash: Decant the Sensitizer solution and add ultrapure water and incubate on rocking platform.
7. Repeat Water Wash: Decant the water and add ultrapure water and incubate on rocking platform.
8. Silver Equilibration: Decant the water and add Silver Solution and incubate on rocking platform.
9. Water Wash: Decant the Silver solution and add ultrapure water. Use gentle agitation by-hand for **1 minute** and do not exceed **1.5 minutes** before decanting.
10. Development: Add Developer solution and incubate on rocking platform for 4-5 minutes prior to adding stopping solution reagent. DO NOT exceed 5 minutes.

    *Note: bands will start to develop within 2 minutes. The gel background will also darken with increased development time.

Once these gels have been through the staining process, they were then imaged on a gel analyzer. The data was collected from the sample on the gel and quantifiably compared.

Results

During these staining tests, we were testing the possibility of using Silver staining in future QC/QA testing. These procedures were performed on various formulations of Regranex® using an array of solvents. The protocol shown above was put together by one of our analytical chemists using a kit, but changing the protocol to try and fit our purposes. The first two gels taken through the staining process did not have any response to the staining. As a quality control cMC1kpoint, the process was repeated with the same results. It was determined that the Silver-Staining Kit may have been mislabeled and expired without our knowledge. With this information, we changed kits and performed a new round of testing, using the same gel format and the same samples. On this new gel, the ladder used in lane one responded

to the staining whereas the individual samples had no response. This was repeated a second time with no change in results. Our team concluded that the only explanation was the possibility of a problem with the sample preparation. The project was put on hold for 3-4 weeks while our analytical specialist made new samples of the Regranex® formulations using a different technique. Since this practicum only focuses on the staining process and its data collection, the sample preparation will not be provided. After the temporary hiatus on the testing, new samples were provided and were tested as previously described. The result of the silver staining experiment only worked for the reduced samples and never worked for the native samples despite two rounds of repeated efforts to rule out user-error. The only silver-stained gel that we were able to verify is available below. The ladder is clear, the positive controls serially diluted in lanes two, three, and four are clear, and the PDGF standard reference in lanes eight and nine are clearly visible; unfortunately, all three of the formulations in lanes five, six, and seven didn't respond to the staining process. This gel and staining procedure was repeated a second time with no change in results. The reduced sample gel that gave any data is shown below with a legend describing each well's contents.

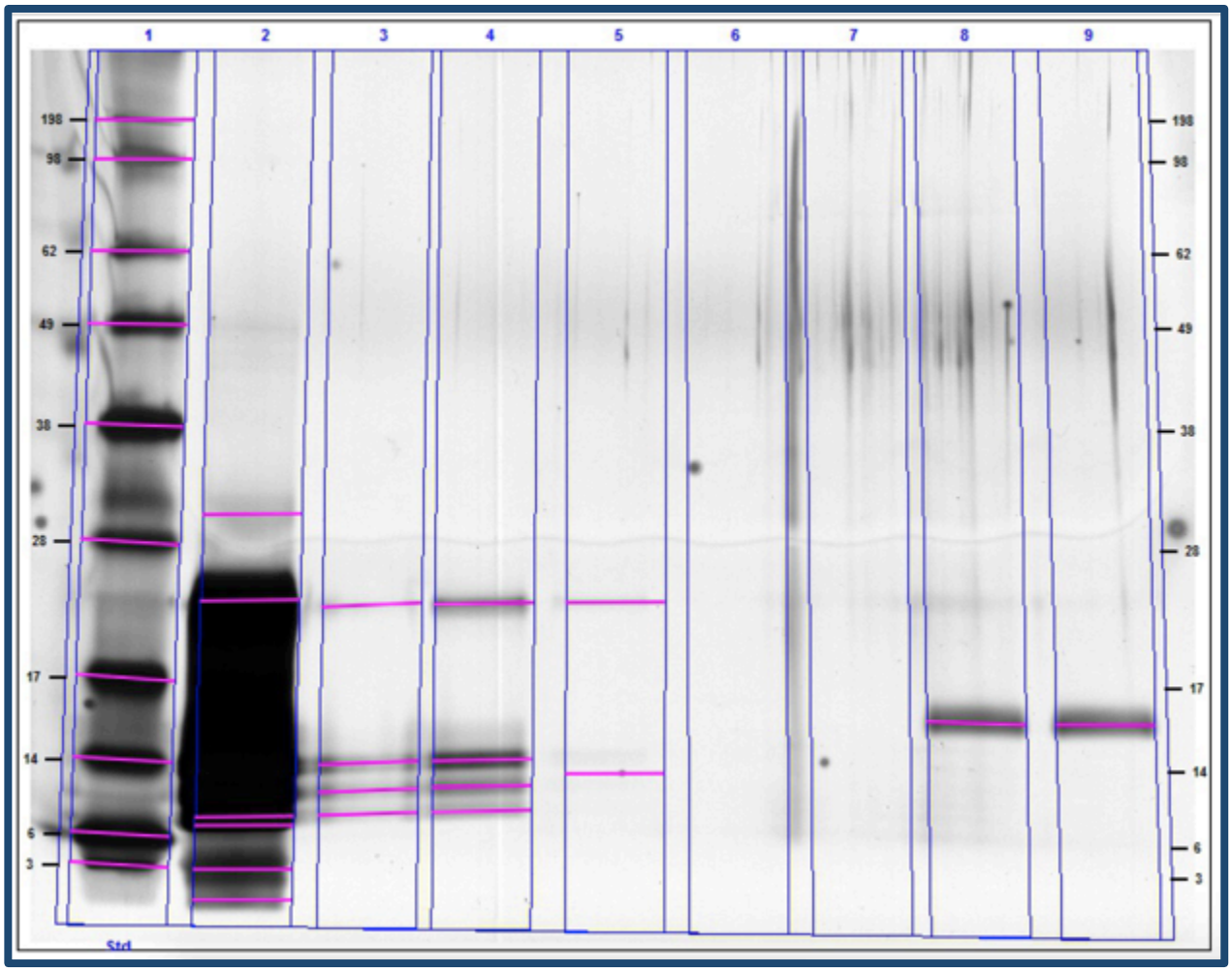

Figure 17: Silver Staining – 8th Attempt

Lane 1: Ladder.

Lane 2-4: Positive Control Regranex® Ointment Samples: 1 µg/mL, 10 ng/mL, 100 ng/mL respectively

Lane 5-7: Formulations 31-1, 34-2, and 37-1 respectively

Lane 8-9: PDGF Standard Reference

Summary & Conclusions

Due to the unforeseen results of this process, the possibility of using Silver-Staining as the new qualification test for QC/QA was abandoned. S&N's R&D team is now exploring other options to find a suitable test for the Regranex® formulation testing. Dr. Sumith Kottegoda is leading this effort and was

able to use the information from these gels to make decisions on how to change these protocols as well as assisting him to find a better replacement to this process. Until further instruction is given on the directions that this project will take, this practicum will conclude its coverage of the Regranex® reformulation.

CHAPTER 5

IODOSORB "MAX" EFFICACY TESTING AND DEVELOPMENT OF AN IN HOUSE *IN-VITRO* MODEL TO MIMIC LIVE WOUND ENVIRONMENT.

Background & Literature

Iodosorb® is a cadexomer-bead containing dressing that is used to remove barriers to healing by using its dual action antimicrobial and sloughing properties. A cadexomer is a 3D cross-linked polysaccharide starch matrix. These cadexomer are highly absorbent micro beads that contain 0.9% iodine that is bound to the inner matrix. Iodine is a proven combatant to a broad spectrum of bacterial and microbial growth in healing wounds, both chronic and acute. Iodosorb® helps to create a moist wound environment, prevents infection, and possesses a high absorbency to perpetuate the antimicrobial activity. The iodine overtime seeps out of the cadexomer beads and creates a prolonged protection for up to 24-36 hours per application. Upon the release of the encapsulated iodine, the brown color of the gel will slowly fade to an opaque, off-white, marking the end of its use. The specific mode of action of Iodosorb® can be found in Table 11 below.

Table 11: Iodosorb® Mode of Action*

Cadexomer with Iodine: The Mode of Action*
1. Wound fluid and exudate are absorbed into the cadexomer beads of the product, allowing the iodine to be released slowly. Please refer to the animation below.*
2. When Cadexomer with iodine is applied to the wound surface, exudate, pus and debris are absorbed into the cadexomer beads. The beads will swell resulting in the formation of a demonstrable gel.*
3. The presence of exudate and the consequent swelling of the beads results in the cross-linked bonds of the cadexomer matrix breaking and the iodine being released into the surrounding wound environment. When the iodine is released, the amount of iodine released will be to a level such that the concentration of iodine in the dressing and the wound environment reach

equilibrium. The equilibrium will remain and no further iodine will be released until the balance is disturbed.*

4. Once the iodine in the surrounding wound environment has been depleted, more will be released from the product until the equilibrium is reached again and will remain until disturbed. This process will continue until all the 0.9% iodine within the product has been exhausted. The conversion of iodine is also associated with a color change from orangey brown to white. Therefore once all the iodine has been converted and utilized, the Iodosorb® will appear white in color. At this point it is time to make a dressing change.*

*Taken directly from S&N's product site

Purpose & Significance

Iodosorb®, although effective, is being drowned out in the pharmaceutical market by products that have a more long-lasting mode of action. If this product is to be successful, it needs to be reformulated to have an even slower escape of iodine, making its effectiveness much longer than 24-48 hours. Comparing it to other on the market products gives us a picture of what changes need to be made. During this reformulation process, our *in-vivo* results didn't match the *in-vitro* results and these tests were performed to create a synthetic test system that would mimic the environment of a mammalian model.

Materials & Methods

The Iodosorb® gel studies were conducted by making basic gels for control testing. Each gel represented another added stage of testing making these gels more and more complex. The goal of these studies is to produce an efficient model that will mimic the conditions Iodosorb® when introduced into on a live, animal model. Table 12 below lists the gels, as well as the order in which they will be made. At each stage, we made a control as well as a gel containing sodium thiosulfate; a compound that we know reduces the iodine to iodide; therefore, we can follow the rate of the diffusion of iodine from the formulation into the environment (e.g., gel loaded with sodium thiosulfate). For these gels, Hank's Buffered Salt Solution (HBSS) will be used as the main buffer considering it has a comparably high level of success when solubilizing both organic and nonorganic substances. Any gels containing stubborn or

highly insoluble compounds will be prepared using a Silverson L4RT-A Mixer, an industrial homogenizer to force all gel components into their respective solutions.

Results

The gels made were created with a new component at each advancing stage. Below in Table 12, you will see the advancing process of creation of these gels and the significance to each stage along with the gel contents. At each stage the corresponding gel contains Sodium thiosulfate to act as a control to force iodine out of the Iodosorb®. Comparing the two shows how each component affected the release of iodine from the cadexomer beads. Each stage was specifically chosen to introduce a new aspect to help get to a model that would represent and mimic the environment of epithelial skin.

Table 12: Iodosorb Gel Study data

Gel Trial	Gel contents	Significance
Gel #1	10% Gelatin in Water	Negative control
Gel #2	10% Gelatin, 20% Na_2SO_3 in Water	To prove that Na_2SO_3 causes rapid discoloration of both Iodosorbs to compare to Gel#1 control
Gel #3	9% Gelatin, 0.3% H_2O_2 in HBSS	Possible increase of peroxides activity in damaged cells. Added salts to introduce one more living component.
Gel #4	10% Gelatin, 0.3% H_2O_2, 20% Na_2SO_3 in HBSS	To compare to Gel #3
Gel #5	10% Gel, 1% Lipid in HBSS	To see how Iodosorb interacted with mimic of skin interaction.
Gel #6	10% Gelatin, 1% Lipid, 20% Na_2SO_3 in HBSS	To use in comparison with Gel #5
Gel #7	10% Gelatin, 1% Lipid in 50% HBSS +50% Fetal Bovine Serum	To introduce compounds, nutrients, vitamins, and various organic components that would also be present in animal models.
Gel #8	10% Gelatin, 20% Na_2SO_3, 1% Lipid in 35% HBSS +35% Fetal Bovine Serum	To compare to Gel #7
Gel #9	10% Gelatin, 1% Lipid, in 50% HBSS and	To act as the penultimate *in vivo* mimic to

	50% FBS. Gel then soaked in glutaraldehyde solution and kept at 40°C	simulate body heat to the gel. Glutaraldehyde used to cross-link the gel and keep it solid while in higher temps.
Gel #10	10% Gelatin, 20% Na_2SO_3, 1% Lipid in 35% HBSS +35% Fetal Bovine Serum. . Gel then soaked in glutaraldehyde solution and kept at 40°C	To compare with Gel #9

Each gel was continuously monitored and photographed on an hourly and daily basis to create a visual time lapse of the progression of color loss of Iodosorb. Over time the iodine in these cadexomer beads leaches out and the brown color disappears from the gel. Below is a set of figures showing the progression of this discoloration over time. The time lapses show the progression of the release of iodine from the gels. On all of the gels, the commercial Iodosorb® is on the left and the new formulation, Iodosorb "Max", is on the right. On the vast majority of the gel tests the Iodosorb "Max" lasted longer than the older, commercial Iodosorb®. The order of the time lapses below include the progression of adding, step-by-step, components that at each stage, get the model to approach the environment that would be expected in a model, mammalian organism.

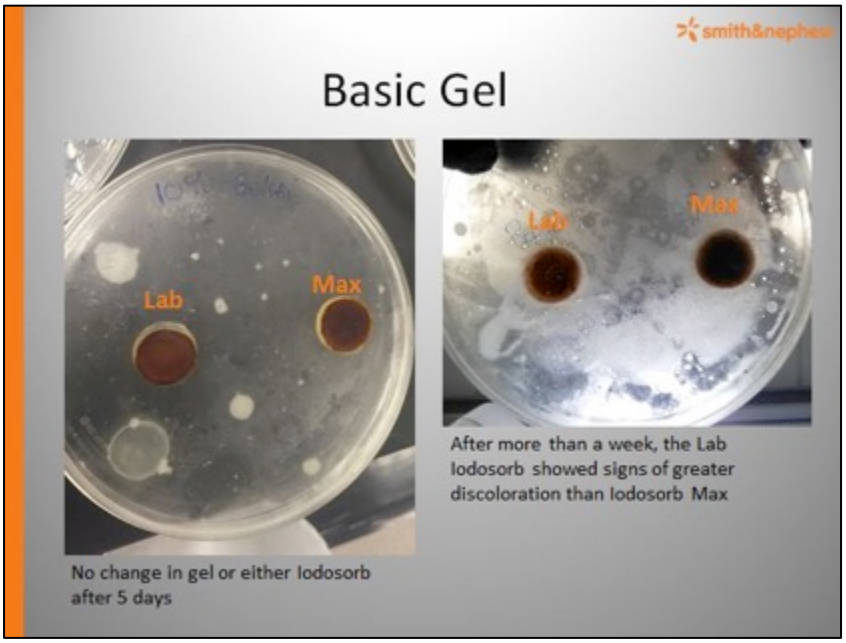

Figure 18: 10% Agarose Gel

After more than a week, The Iodosorb "Max" as well as the commercial, lab Iodosorb®, both had little change, yet Iodosorb "Max" kept a deeper color

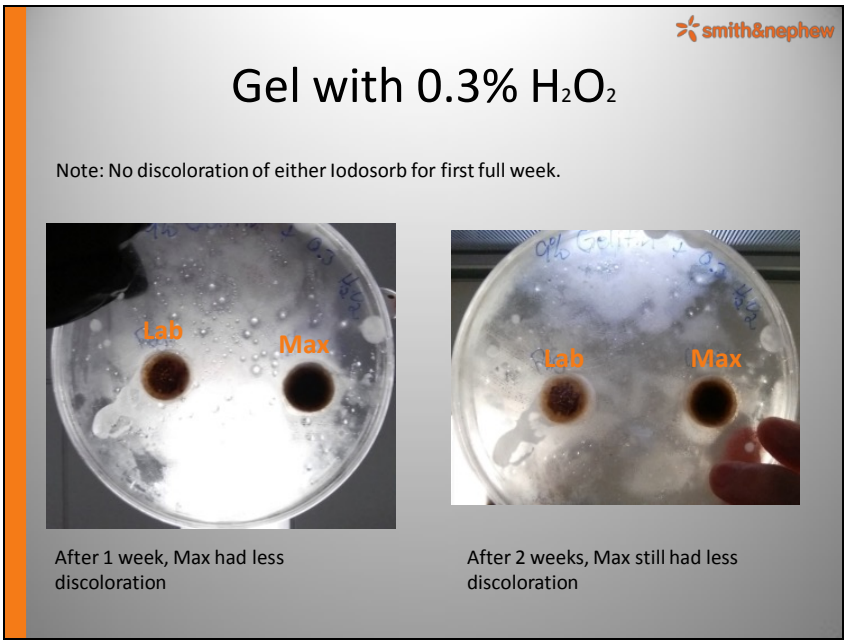

Figure 19: Gel w/ H2O2

After 2 weeks, Iodosorb "Max" showed greater retention of Iodine and maintained a deeper color.

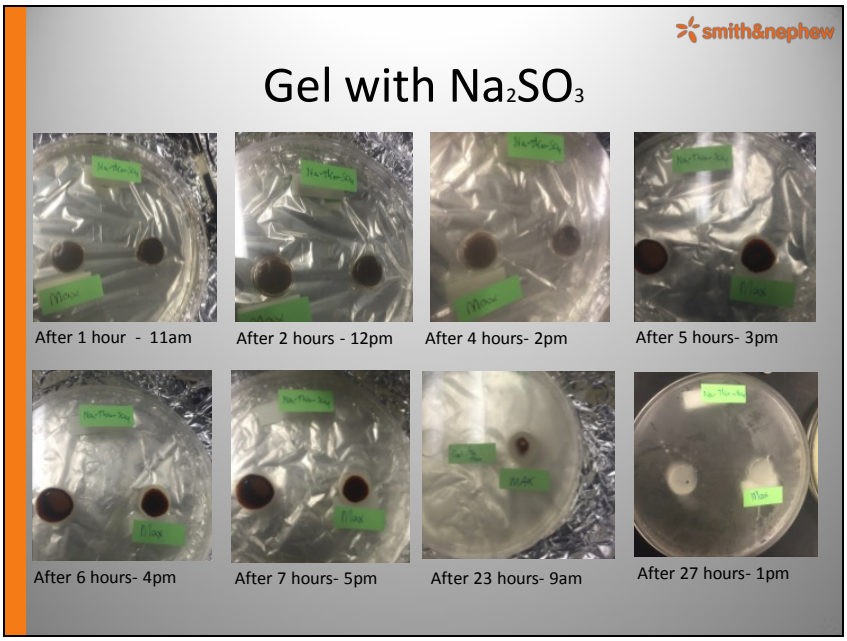

Figure 20: Gel w/ Sodium Thiousulfate
After 23 hours, the Iodosorb "Max" proved to last longer than the Commercial Iodosorb®.

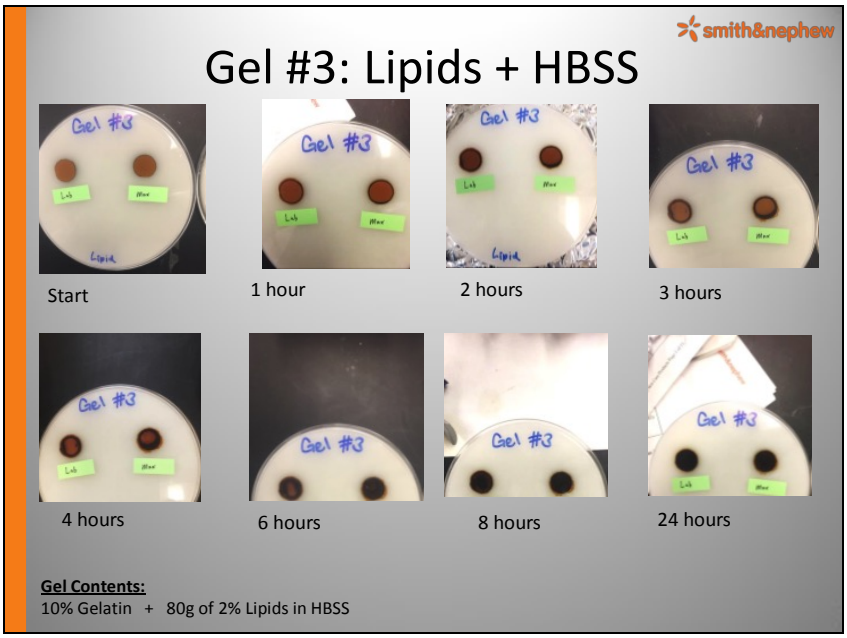

Figure 21: Gel w/ Lipids in HBSS
The discoloration of both Iodosorb® Lab and "Max" can be observed over the first 24 hours here. The initial discoloration is slower in Iodosorb® lab.

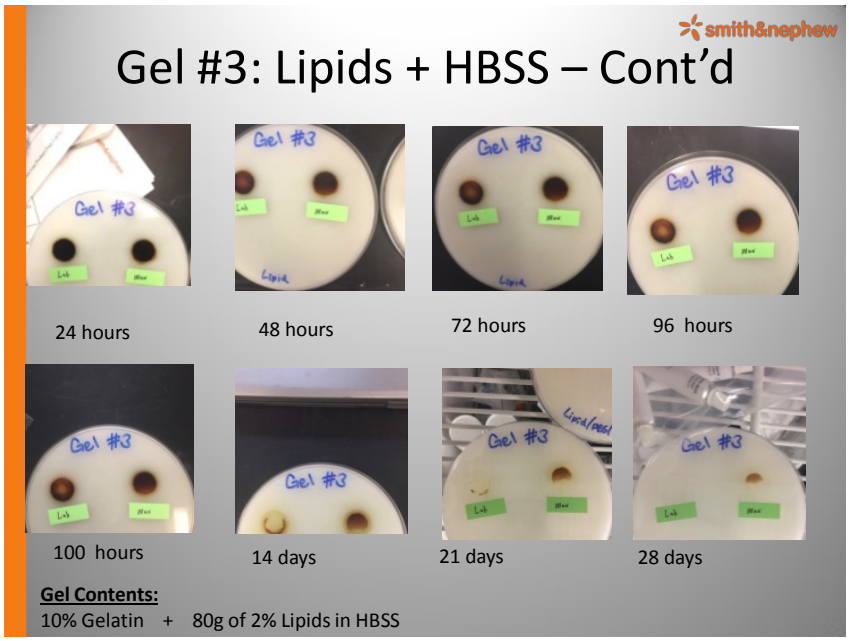

Figure 22: Gel w/ Lipids in HBSS-Cont'd

Although Iodosorb® Lab has a slower initial discoloration, Iodosorb "Max" outlasts commercial Iodosorb long term and lasts significantly longer.

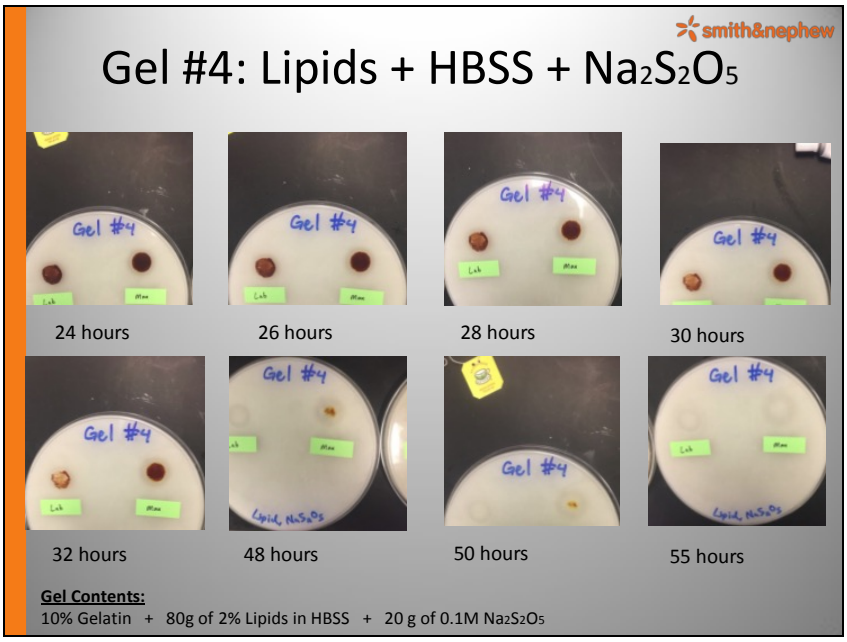

Figure 23: Gel w/ Lipids in HBSS and Sodium Thiosulftae

In the presence of Sodium Thiosulfate, Iodosorb "Max" lasts significantly longer than then commercial Iodosorb®, proving a higher retention of iodine over time.

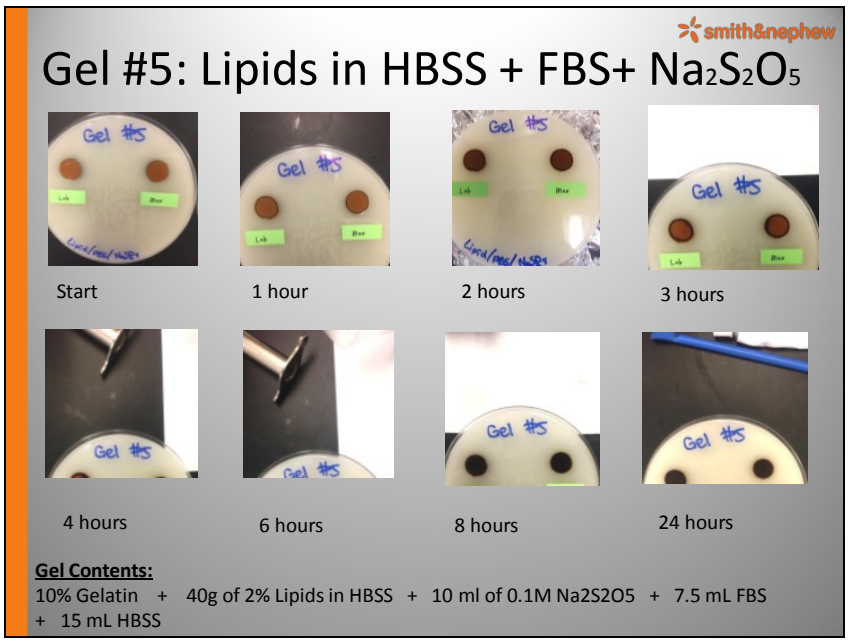

Figure 24: Gel w/ Lipids in HBSS, FBS, and Sodium Thiosulfate

The discoloration of both Iodosorb® Lab and "Max" can be observed over the first 24 hours here. The initial discoloration is similar for each sample. No significant difference.

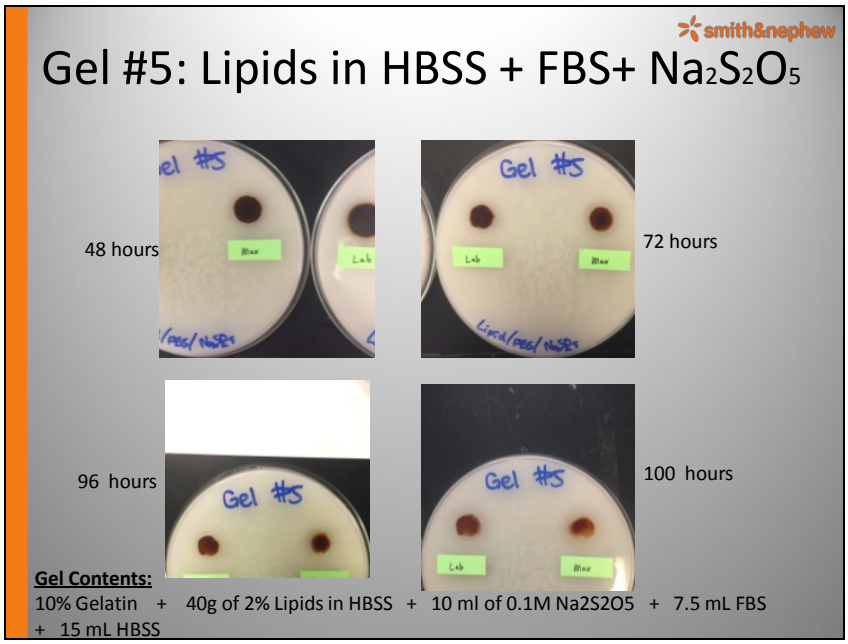

Figure 25: Gel w/ Lipids in HBSS, FBS, and Sodium Thiosulfate Cont'd

Final pictures were not available due to time constraints. Iodosorb "Max" is lighter than commercial Iodosorb® but compared to the other gels, this light stage still outlasts commercial Iodosorb® samples.

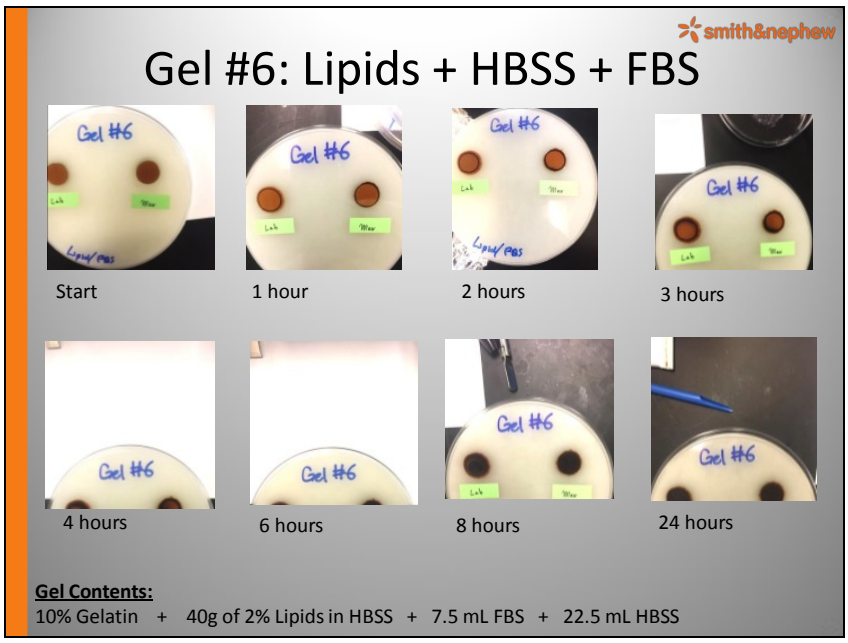

Figure 26: Gel w/ Lipids in HBSS and FBS

The discoloration of both Iodosorb® Lab and "Max" can be observed over the first 24 hours here. The initial discoloration is slower in the commercial Iodosorb®.

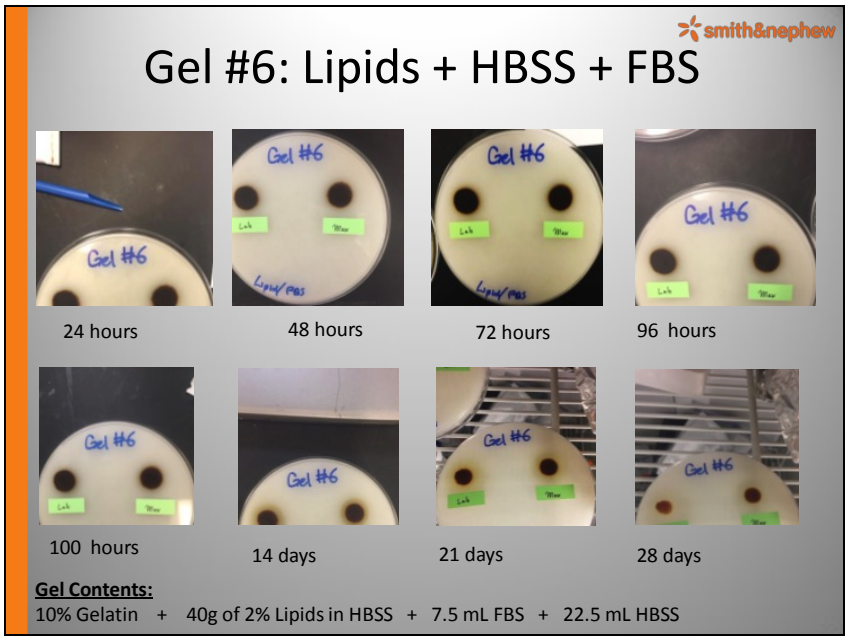

Figure 27: Gel w/ Lipids in HBSS and FBS - Cont'd

Although Iodosorb® Lab has a slower initial discoloration, Iodosorb "Max" maintains a higher retention over time. Current results show Iodosorb "Max" retaining more iodine after 4 weeks.

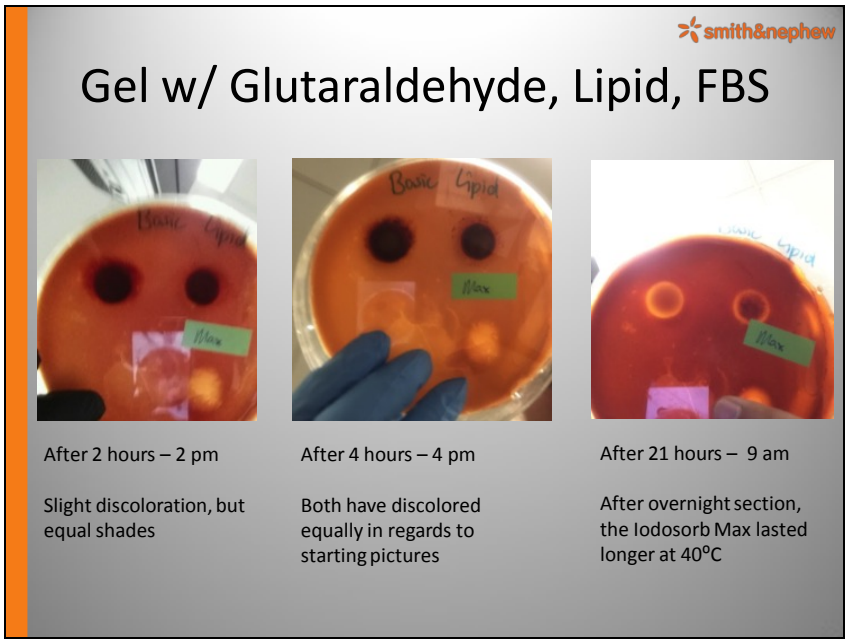

Figure 28: Gel w/ Lipids in HBSS, FBS, Glutaraldehyde, and Heat.
Iodosorb "Max", without the presence of Sodium Thiosulfate, lasted longer than the Commercial Iodosorb®. In the final, heated model.

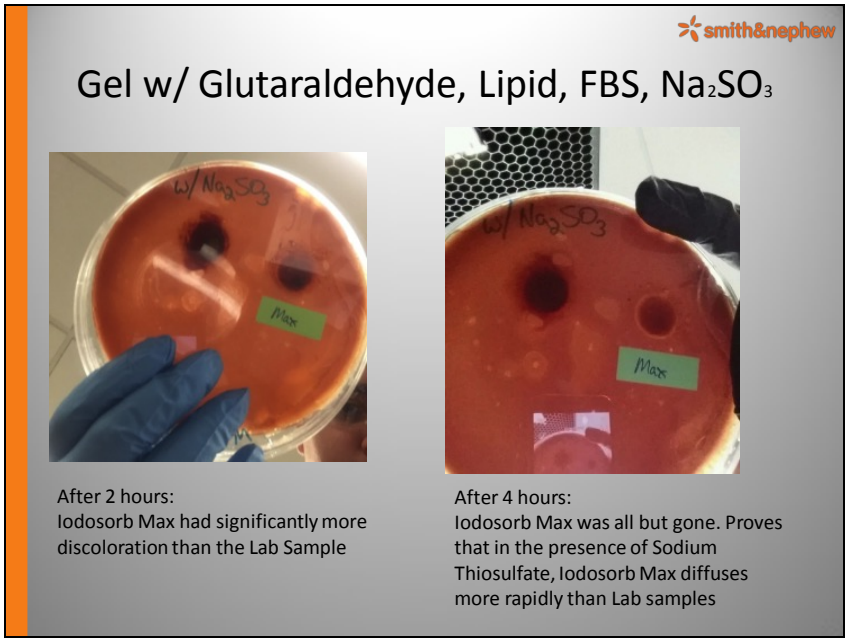

Figure 29: Gel w/ Lipids in HBSS, FBS, Glutaraldehye, Sodium Thiosulfate, and Heat.
In the presence of sodium thiosulfate, the commercial Iodosorb® outlasted Iodosorb "Max".

Summary & Conclusions

This testing apparatus gave S&N a conclusive view on the differences in Iodosorb® and Iodosorb "Max". The efforts from this practicum section prove that Iodosorb "Max", in the vast majority of models outlasted the commercial grade Iodosorb®. Sodium Thiosulfate should have acted equally on the iodine in each sample, causing identical iodine release but gave different results depending on the model's contents. In every model where sodium thiosulfate was absent, the Iodosorb "Max" outlasted the commercial sample. This shows that the newest formulation of Iodosorb "Max" retains iodine longer and would create a longer-lasting antibiotic property on a wound bed. Although the majority of this stage of testing was qualitative, it gave us the information needed to send to S&N's headquarters in London for final decisions for the future of Iodosorb®.

CHAPTER 6

TROUBLESHOOTING AND PROBLEM SOLVING TECHNIQUES TO STUDY FAILED BATCHES OF EU-COLLAGENASE

Background & Literature

As discussed earlier in the Collagenase and Santyl® chapter, EU-Collagenase is the European counterpart to Santyl and has the same mode of action, but what sets it apart is a different strain of Clostridium histolyticum. EU-Collagenase uses a strain of Clostridium histolyticum that produces an extra protease, Clostripain. Clostripain, also referred to as Endoprotease Arg-C, is a cysteine-activated protease that hydrolyzes arginyl bonds and lysyl bonds [40]. Clostripain can be controlled and negated by adding the compound Phenylmethylsulfonyl Fluoride (PMSF). PMSF is a serine protease inhibitor and is known for its inhibition of clostripain; and will be used to control clostripain's activity in relation to the sample solution. The R&D team at S&N believes that the Clostripain is interacting with the Chloramphenicol in the ointment, somehow lowering the activity of the collagenase in the final product. Chloramphenicol is an antibiotic that helps fight many bacterial infections [9]. Although it is effective, it is only recommended when safer antibiotics cannot be used or are ineffective. Chloramphenicol stops protein synthesis in targeted bacteria by blocking the binding of amino acids [9]. This action led our team to believe that the chloramphenicol could be interacting with the clostripain and collagenase at a particular amino acid on the collagenase active chains. This hypothesis was thoroughly tested and the results are provided below.

Along with the hypothesis of the causes of the failed batches of EU-Collagenase, our team decided to tackle the method of extraction that has been used at our European counterpart in Germany. This practicum will also be analyzing the protocols used by our German facility to extract the active

metabolites out of the ointment to see if there are more efficient options available. We will be testing the efficacy of these protocol changes by using a Ninhydrin Assay. Ninhydrin is a chemical used to detect ammonia and amines (both primary and secondary); on contact with free amines, it produces a deep purple color known as "Ruhemann's Purple" [12]. This color intensity correlates with the amount of substrate reacting with the protein of interest. The test creates a simple analysis: the greater the intensity, the greater the absorbency reading, and the greater the activity of the target protein.

Purpose & Significance

EU-Collagenase is the European counterpart of the American product, Santyl®. EU-Collagenase is a $54 Million/year product and is one of the top wound care products globally. EU-Collagenase is made by Smith & Nephew's German facility and the last few batches of EU-Collagenase have been failing due to what they believe is their antibiotic, Chloramphenicol (CLMF). The specific strain of C. histolyticum creates an extra protease, Clostripain, that is not included in the American cell bank. Our R&D team at the Fort Worth S&N site was asked to troubleshoot the problems associated with the failed batches of EU-Collagenase and determines the best course of action to eliminate the cause(s) of the issues.

Materials & Methods

The samples of EU-Collagenase, both failed and passing, were shipped from Germany accompanied by multiple samples of chloramphenicol, controls, and reference standards. After translating the protocols from German into English, our team started a series of tests to establish the root causes behind why the recent lots of EU-Collagenase have been failing. For all tests this practicum will cover, our team used four specific product samples from the German, sister-site. The four samples include: 1: The commercial lot (Passed), 2: The Failed lot (Failed). 3: The Passing sample with no chloramphenicol (No CLMF). And 4: The Collagenase Standard Reference (Col. Std. Ref.). All of these samples have minute amounts of collagenase and clostripain while all but one have chloramphenicol. Every round of tests comparing the failed ointments began with the extraction of the active metabolites in EU-

Collagenase. During this extraction protocol a compound called "Brij 35" is typically added to help aid the separation of the active metabolites from the ointment in question. Brij 35 is a surfactant similar to Tween and has a similar mode of action. Later in the stages of testing, our team starts adding Brij 35 to different steps in the extraction process to observe the impact of adding extra surfactant. The protocol for the EU-Collagenase extraction is as follows:

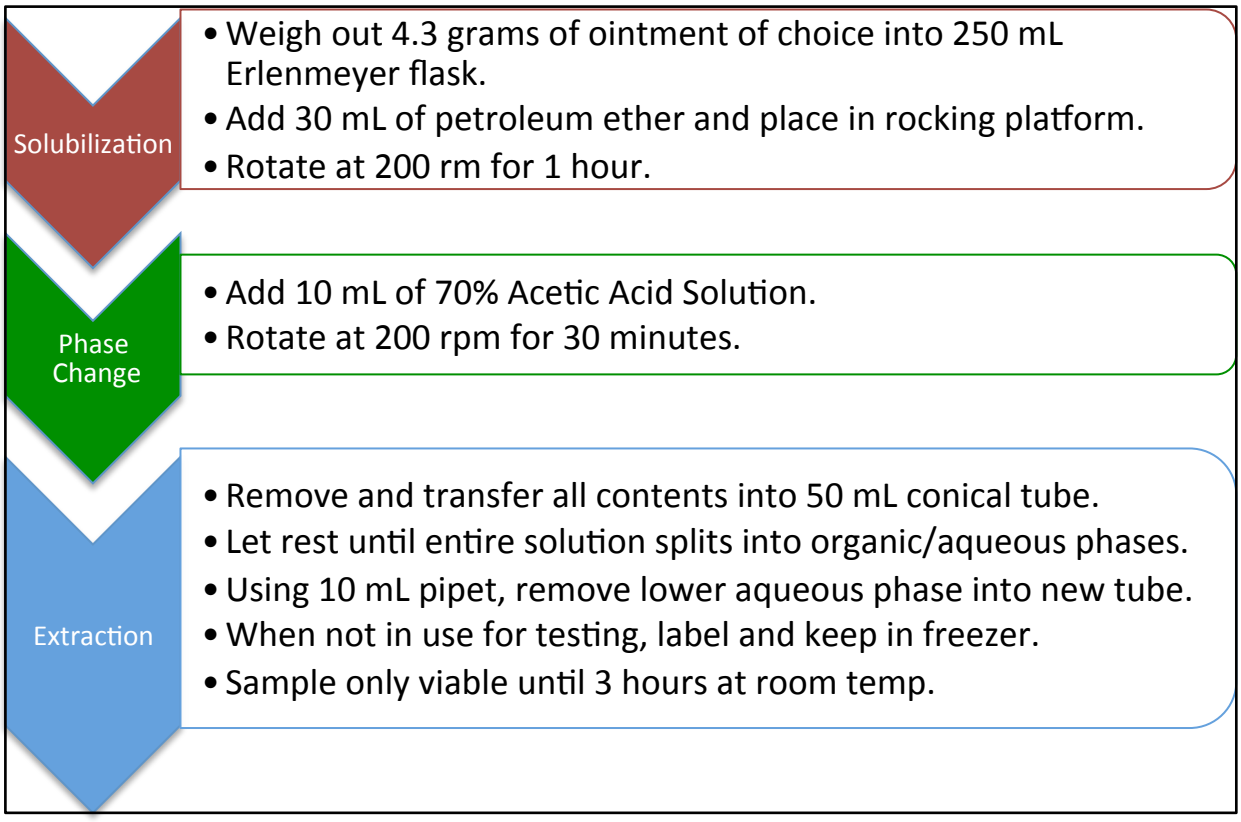

Figure 30: EU-Collagenase Extraction Protocol

The first stage of analysis included activity tests of clostripain using a Casein-FITC Assay. Collagenase is the main active metabolite that will break the bond between the Casein and its FITC label. Once it acts upon the bond, the label is released and will fluoresce; its intensity will be measured by the plate-reader. These samples were measured using a TECAN Safire^2, which measured the intensity of fluorescence. The greater the fluorescence is, the larger the activity of collagenase. As negative controls, Protein Inhibitor Cocktails (PICs) were added to identical samples to block the actions of both

collagenase and clostripain. This step was to guarantee that all enzymes in the solution would be deactivated to create a negative control for each sample. For the Standard curve, a lab-made solution of collagenase was used and serially diluted the solution by 2-fold dilutions starting with 1 mg/mL. The protocol used for this process is below:

Table 13: FITC-Casein Test Preparaton for Clostrpain Effects

Solution	Preparation
TBS (Assay Buffer)	Dissolve contents of the BupH TBS Pack in 500 mL ultrapure water. *Contents: 25 mM Tris, 0.15 M NaCl, pH 7.2
FITC-Casein Stock Solution	Dissolve 2.5 mg of FITC Casein with 500 µL of ultrapure water to make 5mg/mL stock solution. Make 30 µL aliquot's and store frozen. Thaw new aliquot each time an assay is performed.
FITC-Casein Working Reagent	Thaw one aliquot and dilute 1:500 in TBS. Prepare 10 mL of WR for use with a 96 well plate. Do not refreeze a thawed aliquot
Sample Stock Solutions	Use directly from the extraction solution for each sample.
Collagenase Standard	Make a 1 mg/mL solution in TBS and perform 2-fold serial dilutions to yield 6-8 standards to construct a standard curve.

Table 14: FITC-Casein Assay for Clostripain Protocol

Assay Step	Procedure
Step 1	Add 100 µL of each prepared sample to 4 wells for each sample. Use TBS as a blank for comparison.
Step 2	Add 100 µL of the Working Reagent (Casein) to all wells
Step 3	Incubate for 5-60 minutes at room temp, covered.
Step 4	Measure fluorescence on plate reader using fluorescein excitation filter set
Step 5	Subtract the average blank value from each sample and standard measurement and then prepare standard curve.

The second stage of testing was to determine the effects of chloramphenicol on the clostripain in EU-Collagenase. The German facility sent us three batches of chloramphenicol that had been used in various lots of EU-Collagenase. The Casein-FITC test was repeated and was reformatted to include

samples of clostripain alone, clostripain with chloramphenicol, and Chloramphenicol alone. This would give us a direct visual representation of the effect of the interaction between the two enzymes. Along with this comparison, the rest of the plate was reformatted to include samples of the different EU-Collagenase samples. For this round of testing, PMSF was added to each sample to compare to the original sample. Since PMSF blocks the actions of clostripain, PMSF was added to observe the interactions between clostripain and collagenase. If the samples containing PMSF had a value lower than the original samples, it would prove that Clostripain enhances the effect or activates collagenase. If the samples containing PMSF had greater values of fluorescence, it would prove that clostripain has an inhibitory effect on collagenase. This test used the same protocol provided above and its results are located in the following section.

The third stage of testing was a quality control test to test the relative actions of the three different lots of chloramphenicol sent from Germany. Once all three have been introduced to an industrial sample of collagenase, the interactions can then be analyzed with all the information received previously. A collagen-FITC Assay was performed to test the relative activities of the collagenase standard in comparison to collagenase with chloramphenicol. The protocol for the chloramphenicol comparison test is provided below:

Table 15: Collagen-FITC Assay for Chloramphenicol Comparison

Solutions	Preparation
Tris-HCl Buffer	Make a 1 M Tris Solution. Add 0.1 M HCl to adjust pH to 7.2
Substrate Suspension	Weigh 20 mg collagen-FITC into as many 1 mL tubes as needed samples. To each substrate sample, add 1 mL of Tris-HCl buffer. Stir gently until all particles are wetted.
Collagenase Standard Curve Solution	Make a 5 mg/mL solution of collagenase in Tris Buffer. Make multiple dilutions ranging from 0.1 – 2.5 mg/mL
Sample Preparation	Weigh out 10 milligrams of each chloramphenicol and mage a 2 mg/mL solution in TBS. Combine each sample 1:1 with the 5 mg/mL collagenase solution. **This gives each unknown sample a 2.5

	mg/mL collagenase conc. and a 1 mg/mL chloramphenicol conc.

Table 16: Collagen-FITC Assay Protocol for Chloramphenicol Comparison

Assay Step	Procedure
Step 1	To each tube of substrate, add 100 µL of sample and incubate at 37°C for 30-60 minutes.
Step 2	Place tubes in ice bath for 5 minutes before centrifugation.
Step 3	Set temperature control on centrifuge at 0°C and centrifuge all samples at 10,000 rpm for 5 minutes
Step 4	On a non-binging plate, pipet 100 µL of supernatant from each tube to the assigned wells.
Step 5	Read the plate at 485 nm
	From the absorbencies, find out the amount of solubilized collagen (mg) from the standard curve. Calculate the rates (mg/min)/ Plot the rates against enzyme concentration for the enzymes.

The fourth stage of testing included a series of Ninhydrin Assays to calculate specific activities of the active metabolites in all of the EU-Collagenase samples. This also provided the R&D team with ample data and analysis to quantifiably compare the different EU-Collagenase lots. The Ninhydrin reaction protocol is listed below.

Table 17: Ninhydrin Reaction/Assay Protocol.

Protocol Step	Directions
1. Substrate Preparation	1. Create a 1.5 mg/mL solution of the substrate (GPG-GPA) in Buffer 2. Add one drop of 30% Brij Solution
2. Ninhydrin Preparation	Completely dissolve 1.5 grams of Ninhydrin in 50 mL 2-methoxyethanol in a volumetric flask.
3. Enzyme Stock Preparation	Create a 0.18 U/mL solution of the Collagenase Std. Reference in Buffer
4. Digestion	1. Add 0.5 mL of each Sample into a labeled 10 mL, glass,-capped, test tube. 2. Add 1 mL of the Brij 35-containing Substrate Solution to each tube.

	3. Close tubes and incubate in 37° C water bath for 6 minutes.
5. Ninhydrin Reaction (In Fume-Hood)	1. Add 0.5 mL of Rosen Buffer to each tube (contains cyanide). 2. Add 0.5 mL of Ninhydrin Solution to each tube. 3. Close tubes and incubate in 100° C water bath for 15 minutes.
6. Reaction Stop (In Fume-Hood)	Add 5 mL of 70% Isopropanol to each tube.
7. Sample Dilution	Make a 2 fold dilution of each sample using 70% Isopropanol.
8. Absorbency Analysis	Measure and record absorbency at 520 nm.

Results

The Casein-FITC test shows the relative levels of clostripain per sample of EU-Collagenase ointment. Below are the data sets from the Casein-FITC test of clostripain accompanied by their respective graphs to give a visual representation of the data. From this data, you can see that the clostripain and collagenase do respond to negative inhibition by the PIC; this should help prove that the PIC also effects clostripain response to the PMSF for the comparison testing in the future analysis.

Table 17: Collagenase Standard Curve Data

Standard Curve							
Sample	Conc	BackCalcConc	Wells	Value	Mean Value	SD	CV
0.0625 mg/mL	0.063	0.068	E1	0.045	0.034333333	0.016773	49.9
		0.03	E2	0.015			
		0.065	E3	0.043			
		Range?	E4	Masked			
0.125 mg/mL	0.125	0.151	D1	0.107	0.093333333	0.015822	17.1
		Range?	D2	Masked			
		0.137	D3	0.097			
		0.108	D4	0.076			
0.25 mg/mL	0.25	0.263	C1	0.181	0.179333333	0.005686	3.1
		0.268	C2	0.184			
		Range?	C3	Masked			
		0.25	C4	0.173			
0.5 mg/mL	0.5	0.502	B1	0.297	0.291	0.02358	8.2
		0.542	B2	0.311			
		Range?	B3	Masked			

		0.423	B4	0.265			
1 mg/mL	1	0.675	A1	0.347	0.363	0.019	5.2
		Range?	A2	Masked			
		Range?	A3	0.384			
		0.739	A4	0.358			

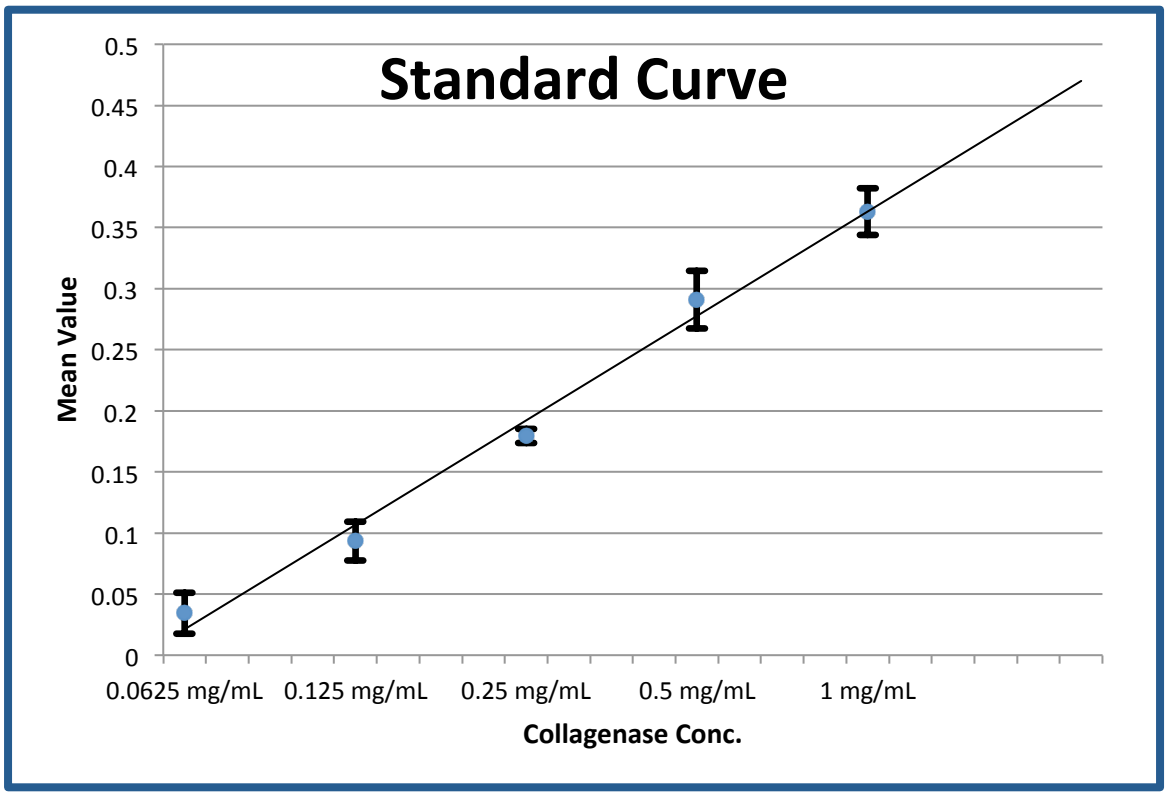

Figure 31: Collagenase Standard Curve Plot

This Standard Curve will give the machine the data needed to compare the approximate collagenase levels in each solution as well as its effect on each sample. The activity of each sample can be seen below in both a data table for each well and in a graph showing the comparison of each sample.

Table 18: Collagenase Activity w/ PIC Data Using Casein-FITC Assay: Data

Ointment Samples							
Sample	Wells	Value	R	Result	Mean Value	SD	CV
No CLFM	A5	Masked	R	Range?	0.698	0.020075	N/A
	A6	0.712	R	Range?			
	A7	0.707	R	Range?			
	A8	0.675	R	Range?			
No	A9	-0.325	R	-0.312	-0.321	0.003606	1

CLFM+PIC	A10	-0.318	R	-0.306			
	A11	-0.32	R	-0.308			
	A12	Masked	R	Range?			
Failed	B5	0.572	R	Range?	0.581333333	0.008083	N/A
	B6	0.586	R	Range?			
	B7	0.586	R	Range?			
	B8	Masked	R	Range?			
Failed+PIC	B9	-0.357	R	-0.34	-0.33166667	0.032517	8.9
	B10	-0.343	R	-0.328			
	B11	-0.295	R	-0.286			
	B12	Masked	R	Range?			
Passed	C5	0.581	R	Range?	0.592333333	0.009866	N/A
	C6	Masked	R	Range?			
	C7	0.599	R	Range?			
	C8	0.597	R	Range?			
Passed+PIC	C9	Masked	R	Range?	-0.388	0.027221	6.2
	C10	-0.413	R	-0.386			
	C11	-0.359	R	-0.341			
	C12	-0.392	R	-0.369			
Std. Ref	D5	Masked	R	Range?	0.586	0.005292	N/A
	D6	0.59	R	Range?			
	D7	0.588	R	Range?			
	D8	0.58	R	Range?			
Std. Ref+PIC	D9	-0.323	R	-0.311	-0.32	0.017692	5
	D10	-0.336	R	-0.322			
	D11	-0.301	R	-0.291			
	D12	Masked	R	Range?			
PIC only	E9	-0.97	R	-0.778	-0.931	0.033867	2.8
	E10	-0.909	R	-0.74			
	E11	-0.914	R	-0.744			
	E12	Masked	R	Range?			

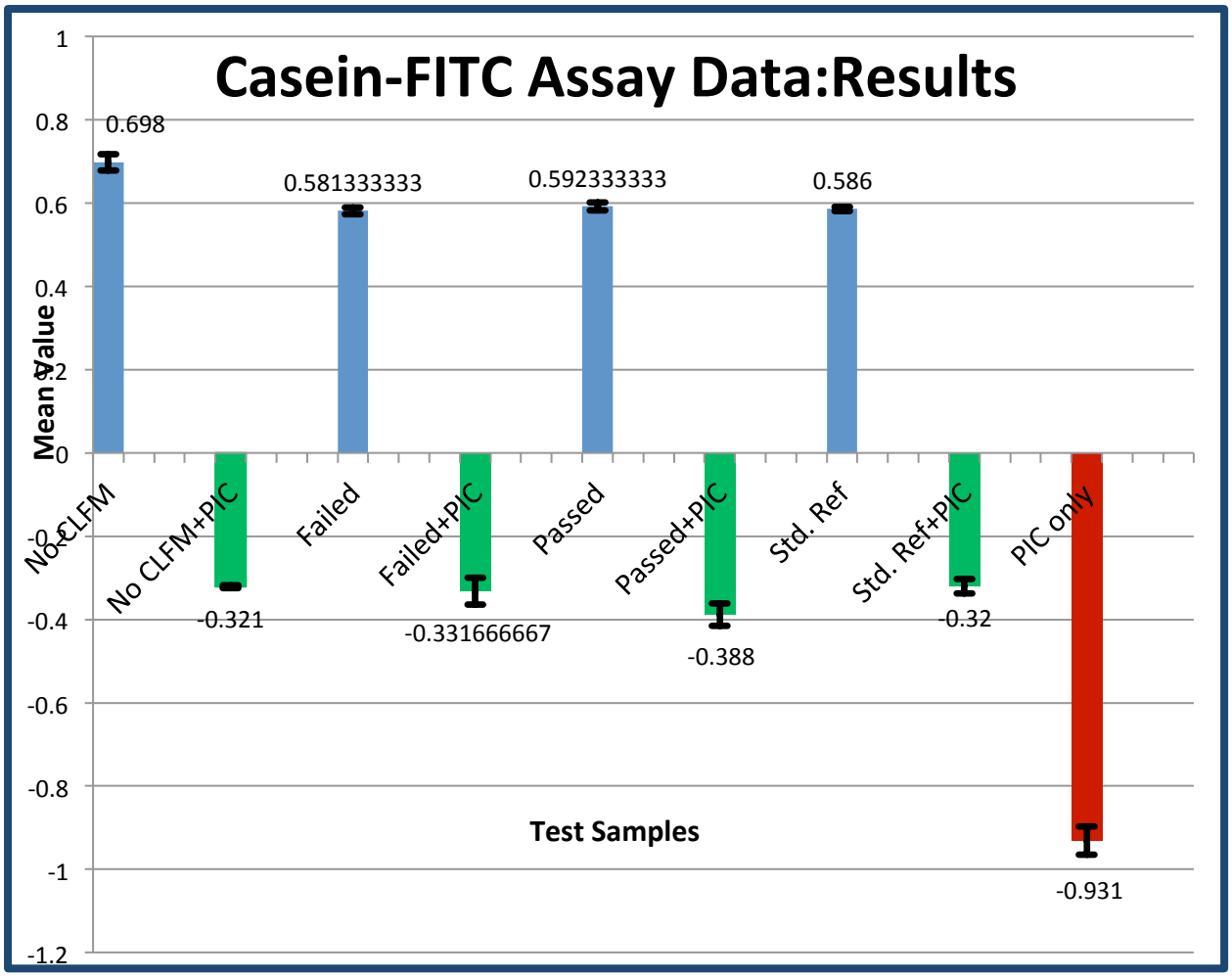

Figure 32: Collagenase Activity w/ PIC Data Using Casein-FITC Assay: Plot

From the graph above, we can see how the PIC affects the activity of the collagenase. Since the PIC negates all protein activity in the solutions, we can assume clostripain responds the same way t negative inhibitors as collagenase. The next step was performing the Casein-FITC test while adding PMSF to each sample and comparing the direct action of clostripain on collagenase. Below are the data and analysis of the PMSF infused Casein-FITC assay. For this series of tests, we used solutions of industrial clostripain as the standard and positive control. The standard curve data and the sample data were compared using the sample protocol as the PIC infused assay. The standard curve uses a 1 mg/mL concentration and performs 2-fold dilutions.

Table 19: Clostripain Standard Curve: Data

Standard Curve							
Sample	Conc	BackCalcConc	Wells	Value	MeanValue	SD	CV
0.0625 mg/mL	0.063	Range?	B5	Masked	Masked	Masked	Masked
		Range?	B6	Masked			
		Range?	B7	Masked			
		Range?	B8	Masked			
0.125 mg/mL	0.125	0.41	A5	0.073	0.071	0.00469	6.7
		0.486	A6	0.074			
		0.38	A7	0.073			
		-0.0625	A8	0.064			
0.25 mg/mL	0.25	Range?	C1	Masked	Masked	Masked	Masked
		Range?	C2	Masked			
		Range?	C3	Masked			
		Range?	C4	Masked			
0.5 mg/mL	0.5	0.333	B1	0.072	0.07325	0.002217	2.5
		0.698	B2	0.076			
		0.475	B3	0.074			
		0.228	B4	0.071			
1 mg/mL	1	1.115	A1	0.079	0.0765	0.004509	1.8
		1.204	A2	0.08			
		0.894	A3	0.077			
		0.901	A4	0.07			

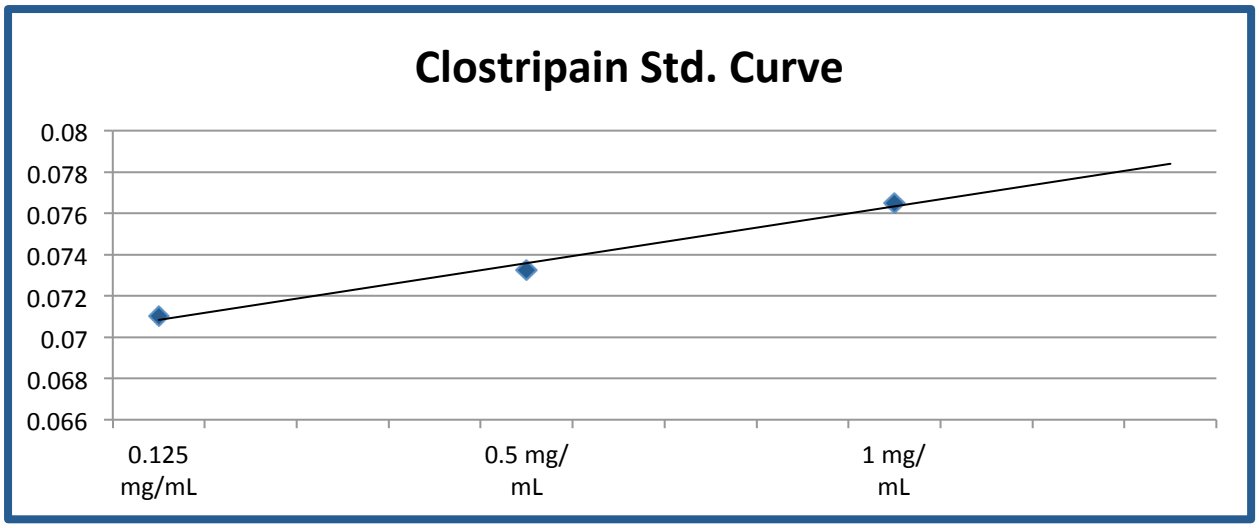

Figure 33: Clostripain Standard Curve: Plot

During this test, a secondary comparison was also performed to analyze the interactions between chloramphenicol and clostripain. If significant interactions occur between these two compounds in the solution, a new test will have to be created to separate these compounds. If there is no significant interaction, the analysis will go on as planned. The data from this comparison is below.

Table 20: Chloramphenicol vs. Clostripain Assay: Data

Conceptual CLFM vs Clostripain Sample Data							
Sample	Wells	Value	R	Result	Mean Value	SD	CV
CLFM	D9	0.009	R	-6.674	0.006666667	0.002082	5.2
	D10	0.006	R	-6.958			
	D11	0.005	R	-7.391			
	D12	Masked	R	Range?			
CLFM+CLOST	E9	0.081	R	1.246	0.082666667	0.002082	17
	E10	0.082	R	1.417			
	E11	0.085	R	1.737			
	E12	Masked	R	Range?			
CLOST	F9	0.082	R	1.422	0.078	0.005292	70.9
	F10	0.08	R	1.191			
	F11	0.072		0.243			
	F12	Masked	R	2.592			

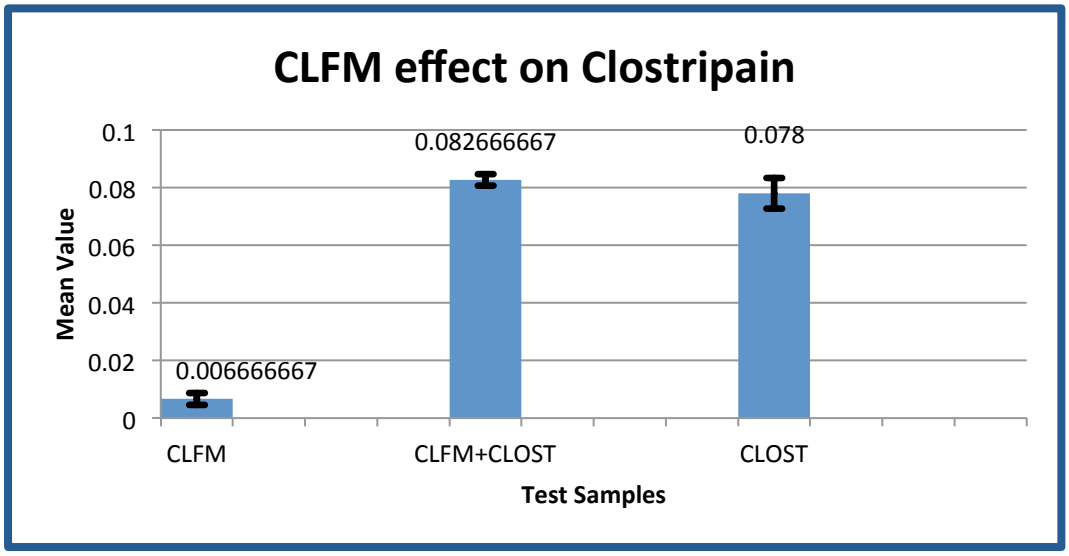

Figure 34: Chloramphenicol vs. Clostripain Assay: Graph

From the data above, we can se that chloramphenicol has no significant effect on the Casein-FITC substrate and has no interaction with clostripain. From this information, we ruled out the possibility of chloramphenicol having any significant relationship with clostripain or its mode of action. Using the data points in the standard curve above, the assay calculated the activity of the ointment samples with and without PMSF. The data and graph showing these comparisons are below.

Table 21: PMSF-Infused Assay: Data

Ointment Samples							
Sample	Wells	Value	R	Result	Mean Value	SD	CV
No CLFM	E1	0.089	R	2.207	0.089	0.00216	3.5
	E2	0.086	R	1.891			
	E3	0.09	R	2.292			
	E4	0.091	R	2.421			
No CLFM+PMSF	E5	0.101	R	3.494	0.102	0.003162	3.7
	E6	0.104	R	3.803			
	E7	0.105	R	3.967			
	E8	0.098	R	3.223			
Failed	D1	0.072		0.297	0.071	0.001414	29.3
	D2	Masked	R	Range?			
	D3	0.07	R	0.062			
	D4	Masked	R	Range?			

Failed+PMSF	D5	0.102	R	3.656	0.0965	0.00526	3.9
	D6	0.1	R	3.377			
	D7	0.092	R	2.531			
	D8	0.092	R	2.529			
Passed	F1	0.104	R	3.856	0.101333333	0.002517	13.1
	F2	Masked	R	Range?			
	F3	0.099	R	3.296			
	F4	0.101	R	3.496			
Passed+PMSF	F5	Masked	R	Range?	0.112	0.005292	5.5
	F6	0.114	R	4.946			
	F7	0.116	R	5.146			
	F8	0.106	R	4.043			
Std. Ref	G1	0.082	R	1.353	0.08725	0.005737	7.2
	G2	0.083	R	1.459			
	G3	0.094	R	2.768			
	G4	0.09	R	2.294			
Std. Ref+PMSF	G5	0.093	R	2.609	0.09	0.00383	3.8
	G6	0.093	R	2.649			
	G7	0.089	R	2.218			

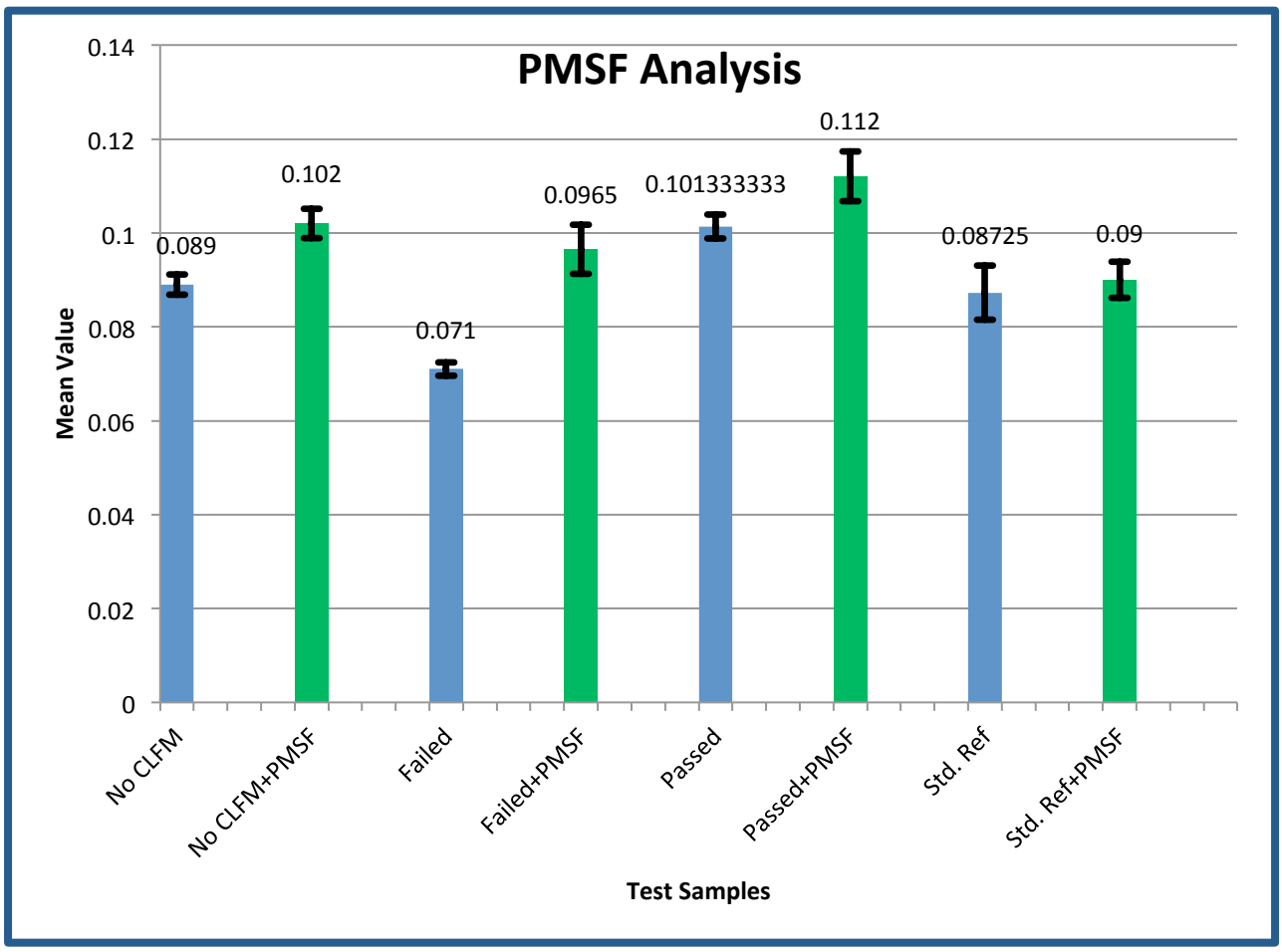

Figure 35: PMSF-Infused Assay: Graph

From the PMSF data above, clostripain has a significant inhibitory effect on collagenase. In every sample where PMSF blocks the action of clostripain, the sample increased in fluorescence and, in relation, increased activity of collagenase. Now that we know clostripain has a significant inhibitory effect on collagenase, the three chloramphenicol samples from Germany were tested to compare their relative effects on standard industrial collagenase. The results of that test are below. The samples used for the comparison were three lots used in the German manufacturing process of EU-Collagenase: Lot 26811, Lot 33505, and Lot 35382. This test was done in a blind manner not knowing which samples were passing or failing.

Table 22: Chloramphenicol Effects on Collagenase: Data

Chloramphenicol Effects on Collagenase											
A	43766	43199	44167	45304	46247	47883	41570	44010	...	...	...
B	35452	35105	35620	34250	34854	35800	36047	34892	...	...	...
C	34296	34760	34131	34428	33497	34512	34918	34172	...	...	...
D	33514	33878	33863	34194	34771	34913	34544	34045	...	...	...
E	11669	11418	11985	11615	11432	11563	11406	11197	...	...	...
							Subtraction using N-Control				%
Avg. Fluorescence w/ Collagenase					44518	Rel. Units		32983	Rel. Units		100%
Avg. Fluorescence w/ Coll & CLMF 26811					35253	Rel. Units		23717	Rel. Units		72%
Avg. Fluorescence w/ Coll & CLMF 33505					34339	Rel. Units		22804	Rel. Units		69%
Avg. Fluorescence w/ Coll & CLMF 35382					34215	Rel. Units		22680	Rel. Units		69%
Avg. Fluorescence w/ CLMF (N-Control)					11536	Rel. Units		Negative Control			0%

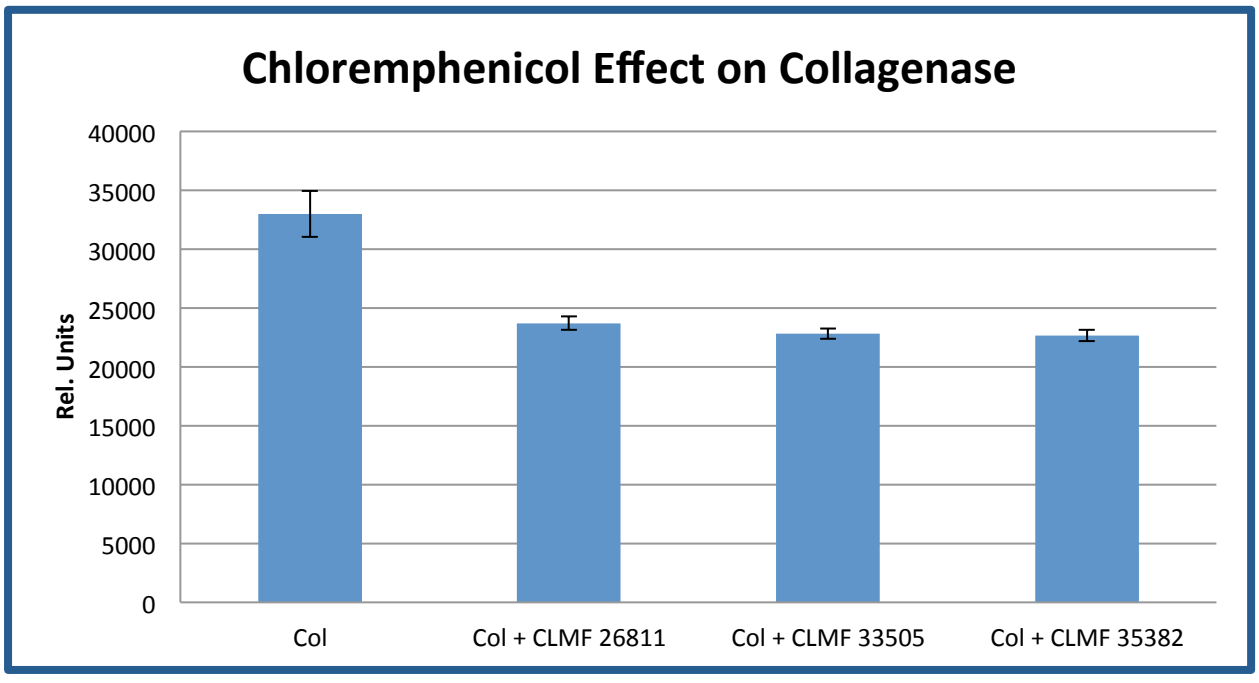

Figure 36: Chloremphicol vs Collagenase: Graph

From the data and graph above, chloramphenicol does have a negative inhibitory effect on collagenase. In EU-Collagenase chloramphenicol performs antibiotic actions but hose actions are acting against the active enzyme of choice. This data along with all of the analysis collected previously will be compiled into a secondary major report and sent to our German facility with recommendations on how to solve these problems.

The fourth stage of data collection came from the Ninhydrin Reaction Assays. All tests were performed using the Ninhydrin Assay Protocol in the "Materials and Methods" section. Each data table and graph set below includes different comparisons our team decided to observe. The first data set compares the added effect of adding an extra, separate surfactant to increase the proteins activity as well as the effect of using petroleum ether in place of petroleum benzene. This first set of data tested the effect of elongating the extraction period from 1 hour to 24 hours. That data is provided below.

Table 23: Extraction Comparisons w/ Altered Conditions: Data

Sample	Avg. Absorbency	Std. Deviation
Blank	0	0.020011122
Collagenase Std	2.51195	0.030476302
Benzene - 24 hr	0.34765	0.001555635
Ether - 24 hr	0.41905	0.043133514
Ether + Tween - 24 hr	2.03815	0.004666905
Ether + POL 407 - 24 hr	2.0054	0.016899852
Benzene - 1 hr	0.49625	0.021637468
Benzene + MeOH - 1 hr	0.1621	0.000212132
Ether - 1 hr	0.3101	0.015061374
Ether + Tween - 1 hr	1.93175	0.018667619
Buffer + Tween	-0.06575	0.004808326

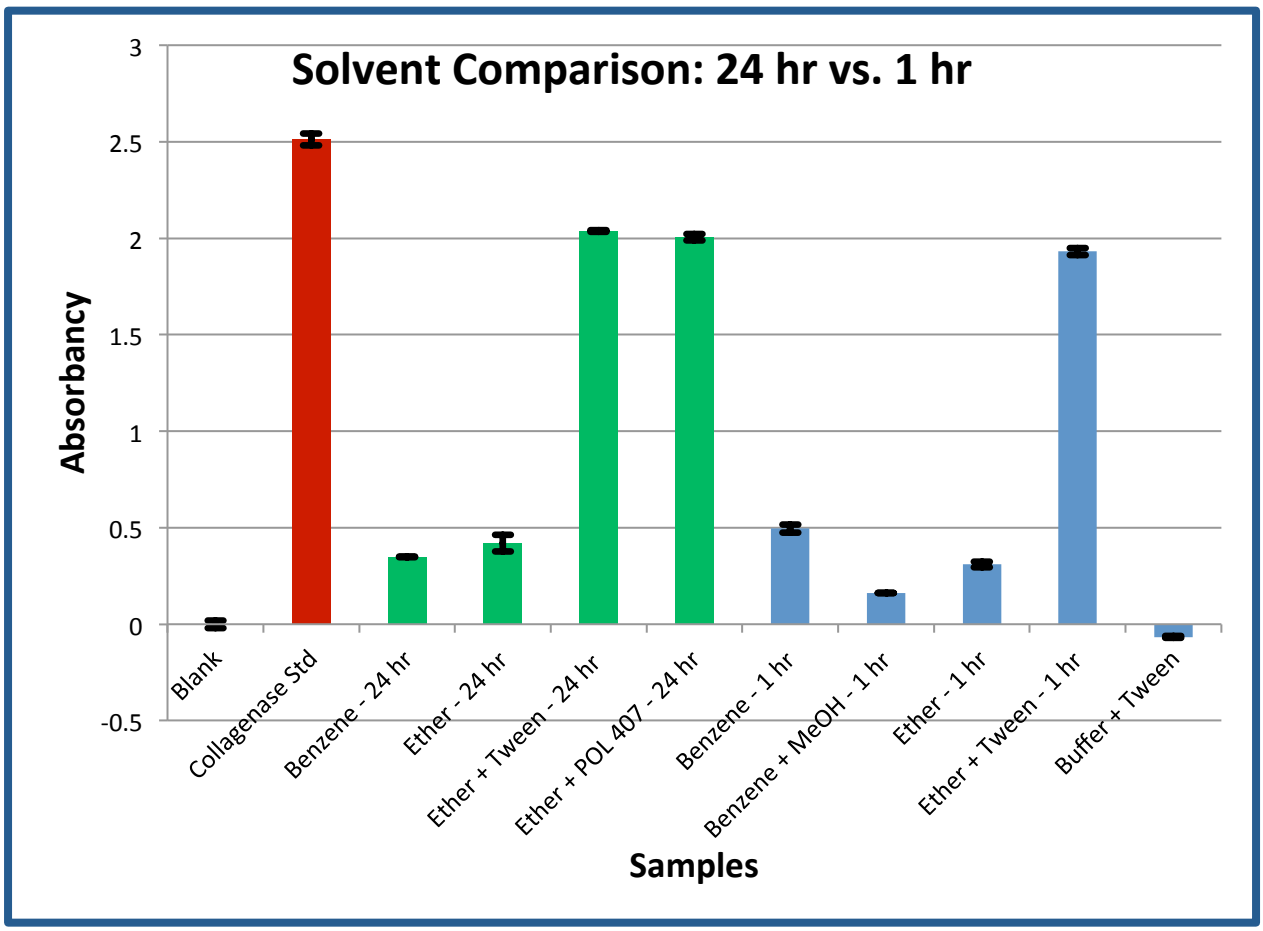

Figure 37: Extraction Comparison w/ Altered Conditions: Graph

The data in the above section shows that the extraction as designed produces a final enzyme activity drastically lower than the Reference Standard Solution for collagenase. When different surfactants are added, there is a significant increase in the activity of the active metabolites in EU-Collagenase. This data set also proved that the solubilization stage was statistically similar regardless of the duration. Following this test, we repeated the extraction protocol adding Brij at various periods during the extraction process. As mentioned previously, there are main steps in the extraction: Solubilization, Phase Change, and Extraction. For shorthand purposes, each sample has a 1, 2 or 3 next to it. If the sample name has a 1, Brij was added during the Solubilization. If the sample name has a 2, Brij was added during the Phase Change. If the sample has a 3, Brij was added with the Substrate during the Ninhydrin Assay. Any sample with more than one number has multiple additions of Brij 35. Doing this varied addition of the surfactant

shows the company possible ways of improving the protocol to enhance the activity of the final product. The data from that study is below.

Table 24: Brij 35 Varied Additions: Extraction Data

Sample	Avg. Absorbency	Std. Deviation
Blank	0	0
Collagenase Std	1.76385	0.078064589
Brij @ 3	0.10275	0.004384062
Brij @ 2 + 3	1.00295	0.033234019
Brij @ 1 + 2 + 3	1.0054	0.005868986
Brij @ 3 w/ MeOH	0.1003	0.002333452
Brij @ 3 (Non-CLMF)	0.2041	0.01251579
No Brij	0.22545	0.020081833
Brij in Benzene	0.00035	0.000424264

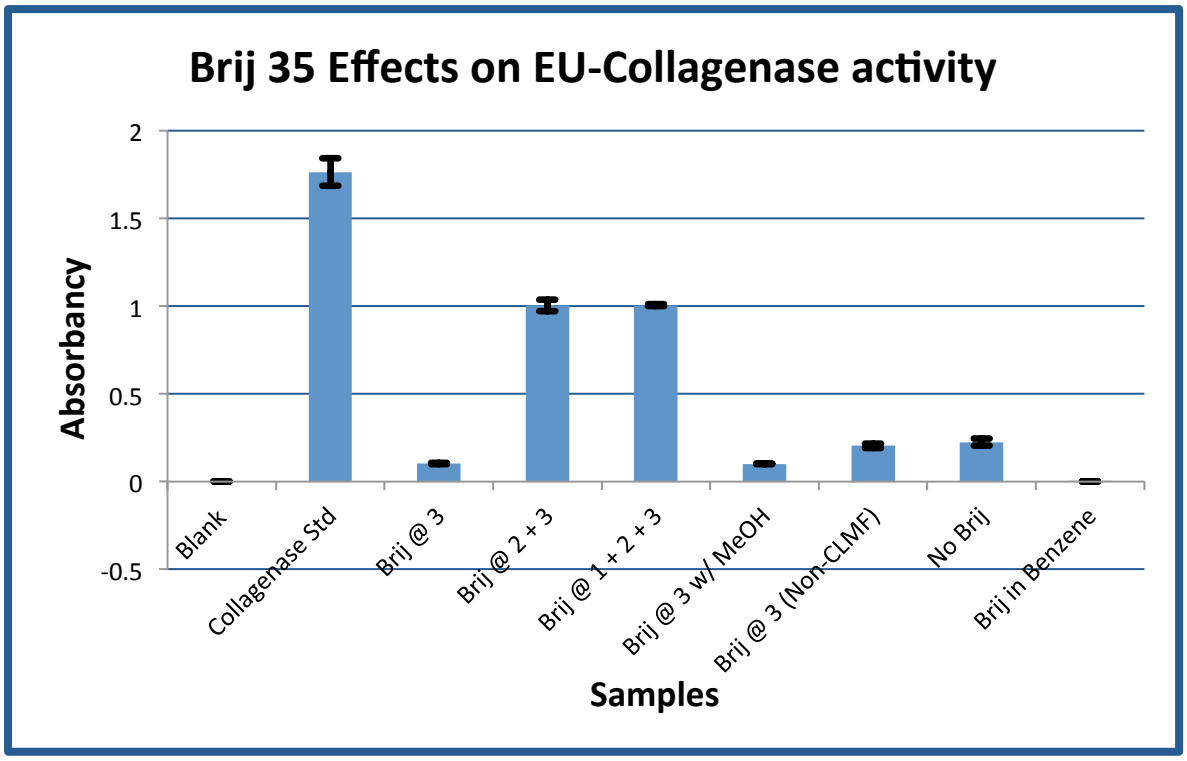

Figure 38: Varied Brij Addition: Extraction Graph

After this test was completed, a third round of Extraction needed to take place with specific samples from the first two assays. A direct comparison was made to observe the differences between Tween and Brij 35 as the preferred surfactant for use in this extraction. The data and graphs from this third set of testing are provided below.

Table 25: Surfactant Efficiency Comparison: Extraction Data

Sample	Avg. Absorbency	Std. Deviation
Blank	0	0
Collagenase Standard	2.00225	0.305187287
Brij @ 3	0.2033	0.042497118
Brij @ 2 + 3	1.3558	0.290691598
Brij @ 1 + 2 + 3	1.49165	0.212839141
Brij @ 3 w/ MeOH	0.24895	0.014707821
Brij @ 3 (Non-CLMF)	0.31325	0.004101219
Brij @3 (Tween 20)	1.20485	0.042002143
No Brij	0.2657	0.033304729
Brij in Benzene	-0.00385	0.002404163

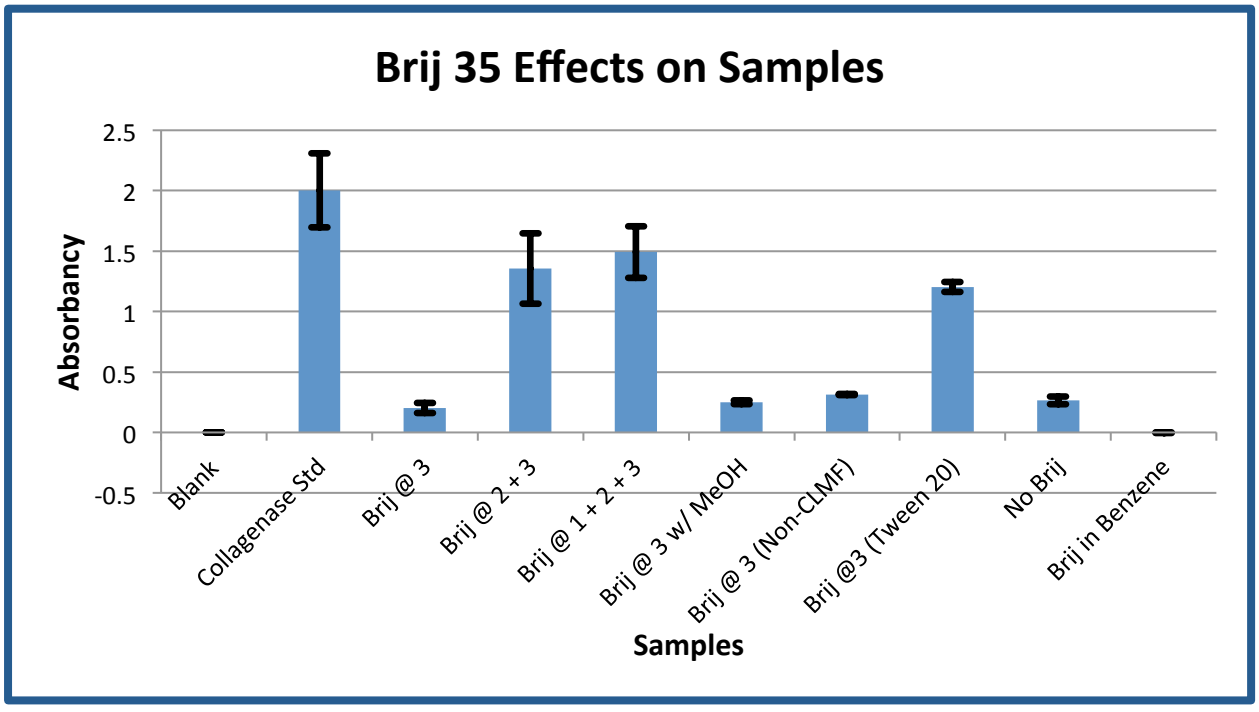

Figure 39: Surfactant Efficiency Comparison: Extraction Data

From these results, it is clear that adding Brij in the earlier steps of solubilization and Phase Change increase the activity of the final protein extraction. This will help S&N design a more efficient protocol for extracting the Collagenase from the ointment in Quality Control and Quality Assurance testing. After the direct comparison of the different surfactant additions were analyzed, the next major step in qualification of these protocols was the comparison between different concentrations of the Collagenase Standard Reference as well as the typical Brij 35 extraction protocol. This new Ninhydrin Assay would give us a clearer understanding of the possible saturation amount of these two solutions for future testing. The Collagenase Standard Reference was diluted to 0.53 U/mL, 0.18 U/mL, and 0.09 U/mL. The extraction solution containing the typical addition of Brij and a second amount was created that was 50% of the typical extraction sample, diluted 2-fold. This comparison data and graph are below

Table 26: Standard Saturation Analysis: Extraction Data

Sample	% Efficiency	Std. Deviation
Blank	0%	0%
Col Std Ref- 0.53 U/mL	100%	0.046527626
Col Std Ref- 0.18 U/mL	49%	0.020421244
Col Std Ref- 0.09 U/mL	24%	0.000848528
Brij Extraction - 100%	24%	0.005020458
Brij Extraction - 50%	18%	0.02920351

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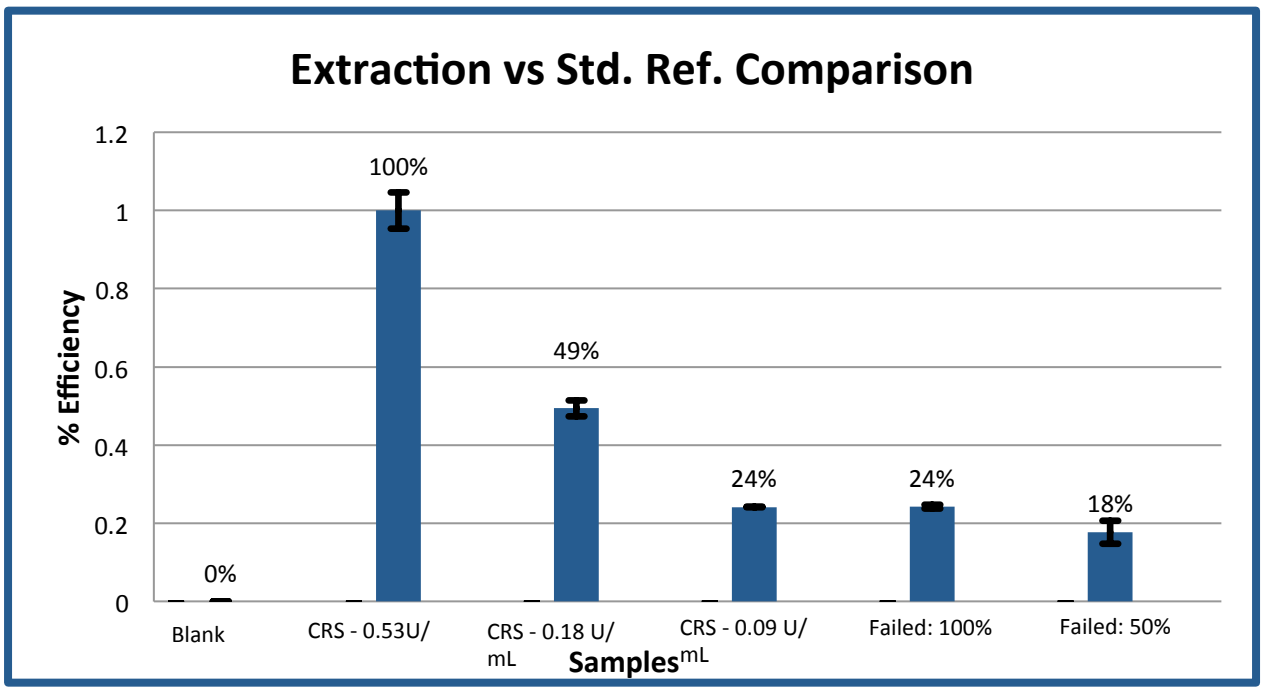

Figure 40: Standard Saturation Analysis: Extraction Graph

The data above shows that the typical extraction sample has a level of saturation keeping it from being accurately read. Although the 0.18 U/mL concentration of the Collagenase Reference Standard used in the protocol is not saturated and can be used without worry of saturation, the full extraction sample cannot be used in its current state for QC/QA purposes and needs to be redesigned.

Summary & Conclusions

EU-Collagenase, while being an efficient product has many flaws and downfalls that need to be addressed in the coming months. While the reference standards used are great for seeing the drugs effectiveness, the protocols designed are lacking in effectiveness. With a few changes we were able to increase the quality of the solubilization and increase the activity of the protein to match standard levels. Despite the failed batches of chloramphenicol, we were able to notice distinct difference in samples containing and not containing chloramphenicol. Chloramphenicol is an extremely effective antibiotic, but needs to be removed due to its deteriorative effects on the actions of the active metabolites, collagenase. Utilizing the data presented above, the best response to combat the negative effect of chloramphenicol is

to either replace it with a different antibiotic, or to simply remove it completely. Once it is removed from the product, the collagenase will become more potent and should create a more effective final product.

This study also provided ample data proving that clostripain, a secondary enzyme produced by our German facility's strain of Clostridium histolyticum, has inhibitory effects on collagenase. The German facility, according to our data, needs to take these effects into account when considering the future of this drug. Our recommendation is to add minute amounts of PMSF to the ointment to act as a negative inhibitor to clostripain. Since changing the strain used in the fermentation of collagenase would take the production through an entirely new series of safety qualifications, the simplest approach to this issue would be adding the PMSF. All of the data provided throughout this chapter was compiled into a PowerPoint presentation and sent to our German facility giving our recommendations. This practicum does not include the final decisions made on this product due to the nature of the information.

INTERNSHIP EXPERIENCE

Description of Internship Site and Overall Experience

Smith & Nephew Biotherapeutics is one of the many sites around the globe operating in the wound therapy field. Housed out of Hull, England, S&N mainly operates in the medical device industry acquiring pharmaceutical companies when they see fit. The Fort Worth site is one of the examples of this process and currently acts as the headquarters for US operations. Originally Healthpoint Biotherapeutics, S&N Biotherapeutics is transitioning into creating an in-house GMP facility within the Fort Worth facility. A portion of the work I completed while there was in preparation of that transition as well as to finalize a few of the protocols and manufacturing processes they would house there. S&N includes lab specializing the fields of Microbiology, Biochemistry, Formulation, Analytical Chemistry, Cell Biology, Medical Device, & various areas of QC/QA. I thought I knew what to expect being an intern at that large of a company, but I couldn't have been more wrong. The R&D team, from day one, told me I would be treated like any other associate scientist for the company, and they kept their word. Not only did I never grab coffee for another person, I was included at every level of the department's functions. From every monthly report meeting to being updated just like the other scientists, I was included at every step of weekly operations. The R&D group may be smaller than I would have expected, but the work and devotion I've seen come from each lab is remarkable and worthy of mention.

I can't even begin to describe the ease at which everyone in the department welcomed me. Within three weeks I started getting invitations to company happy hours as well as various group outings. Everyone here made conscious efforts to not inly include me, but to make me feel like an integral part of the team and family. From Friday R&D lunches to hearing everyone vent about someone/something over a pint at happy hour, this group of scientists deserve so much more than a quick mention in a practicum. On multiple occasions I was pulled aside just so they could tell me how much they wished they could hire

me. Due to budgetary and bureaucratic issues, they could not offer me a full time position, which is completely understandable. Despite this, I will still remember my experience here with great fondness.

If I ever have the opportunity to be invited back for a career opportunity within the R&D department at S&N, I would jump at the chance solely to experience this group of people in full. I wish them nothing but the absolute best that the future can offer and hope our lives intertwine in the future.

Journal Summary

Every day while at the internship site, an entry was made into a Daily Journal. Its contents include everything from daily activities to my thoughts on different developments. It documents everything I experienced while at Smith & Nephew and will hopefully show what its like being an Intern at an International Pharmaceutical company. While it could stand to have been done in a more professional manner, that's not fun to read. Attached below is my journal in full for any questions regarding my mental state, daily escapades, and reactionary responses to the industrial medical world.

APPENDIX

Daily Journal

Date	Daily Recording
Thursday – 12 August 2016	**Day 1:** Dr. Lei Shi (Stone) called me to come in a few days early to go ahead and finish some processing/onboarding to get my internship started a bit smoother. I was sent to the corporate building on Hulen to get my ID badge that would give me access everywhere. After a brief tour, Stone introduced me to every staff member (both lab and administrative) that he could find the way you force everyone to meet your new child. Since Aleska and the Senior Scientist, Jason Campbell, were absent this day, I really couldn't do much in respect to research or science. There were also substantial problems with getting me into the company's system. Since it's the summer, they're incredibly busy and short staffed due to everyone taking vacation at the worst possible times in respect to what I needed done. So, I read through some information literature surrounding the company's projects as well as annotated my way through some research articles published by this staff. Afterwards, I was given the option to either study there or go home. Since I have a puppy, I decided to read at home and left for the day.
Friday – 13 August 2016	**Day 2:** Today was substantially more interactive, fun, and informative. Jason Campbell was back and I got to get some hands on experience with some quality control and dosage testing for a product that they're working on using Thermolysin and collagenase. I performed a FAGLA Assay for the Thermolysin and created standards for analysis. Jason gave me a much more interactive and thorough tour of the entire facility showing me places that I would actually be using and helped me get more familiar with people that I would be working with, so it was far more helpful and useful this time. At lunch he grabbed a bunch of people that I'd be working with and they took me out for lunch at Pan Asia and it was just delicious. After coming back from lunch, Aleksa had returned from THE OLYMPICS IN BRAZIL and then we had more discussion about planning out a more detailed process that this internship would follow and some specific projects that I'll be working on. After that days testing was over, they gave me a few documents, manuals, and procedures to familiarize myself with. This day, everyone

	happened to be doing more digital record keeping, analysis of data, and administrative tasks. At 5, I went home and watched the Olympics where Michael Phelps basically won all the medals.
Monday – 15 August 2016	**Day 3:** Arrived to an almost empty office. Where is everyone? Turns out they have a meeting Monday mornings from 9-10 that I don't go to… Note to Self: Show up at 10 on Mondays. They still haven't gotten me fully into the system so we still have to wait to start the formal online training, safety orientation, and various necessities. So I do a lot of reading/sitting. Today, Jason is pulling me in on his rhPDGH-BB Pre-Formulation Study (Plate Derived Growth Factor). We're working on lengthening the shelf life of the active ingredient, *Becaplermin*. We prepared samples of "all kinds of different crap" to see how the drug degrades in different solutions to best gauge what type of ointment/cream the final drug should be infused in to guarantee the longest shelf-life. After this, I corresponded with Gwirtz about the Report as well as viewed some different examples of past reports. I have some work to do…
Tuesday – 16 August 2016	**Day 4:** So today I walked into my cubicle and there was a ridiculous stack of SOP's for me to start reading and getting acquainted with. I spent literally the entire day reading through SOP's and doing a bit of desk organization. That was the whole day.
Wednesday – 17 August 2016	**Day 5:** First half of the day was spent reading and acquainting myself with even more SOP's. Second half of the day, I prepared 2 different gels to test the effectiveness of two different wound treatments. One normal gelatin, one with 6 gram of hydrogen peroxides. I also prepared a solution that would resemble human skin in almost every way. Left it spinning overnight to fully dissolve and allow all components to go into solution.
Thursday – 18 August 2016	**Day 6:** Performed a few more gel matrix's with Aleksa, made the skin mixture and let it set in the forming apparatus. Started preparing my lab station for the TFF project that would start Monday. Was brought up to speed on a few more projects that will be outlined later in these entries. I also tried my hand at making my first emulsion that would be made into a lotion/cream. This lotion/cream could then be integrated into a product as a delivery mechanism for any topical drug, therapy, treatment, etc.
Friday – 19 August 2016	**Day 7:** Not only did we set up the entire apparatus for the TFF Purification of Collagenase, but we also went and had lunch as a department as a crazy, shady, hole-in-the-wall Chinese restaurant. It's like a family here. Today I also sat in

	with Paul Reinik and worked with him with a MRSA fermentation that he's been working on for something involving the army but I was told to not ask many questions about the legality, hahaha. Today was pretty light since they had some Suits here doing business stuff and keeping my advisors busy. I spent the remainder of my day watching the pressures and preparing the reagents I would be using for the TFF for next week.
Monday – 22 August 2016	**Day 8:** We started the first installment of the Collagenase TFF, and let it run literally all day. While that was going, I was briefed on a Navy contract that we're working on involved a "Super-bandage" that we're incorporating Snake-venom into. Cool, I know. Then went to lunch with the other Jason at Pie5. When we got back we basically maintained and cMC1ked on the TFF every 15 minutes or so to make sure it was maintaining a balanced operation. While maintaining that, I prepared 10 Liters of a Tris buffer that we'd be using for the third round of the TFF filtration. I also created a different emulsion cream to get a better stability index than the first time since it had too much mineral oil. Alex said it was Crap, but better than most first timers, haha. Alex seems impressed with how easily I pick things up, not only technique based, but also, theoretically when discussing the science and understanding behind new/upcoming projects. He was impressed enough to sit me down and rave about how impressed he was. Bahahaha
Tuesday – 23 August 2016	**Day 9:** So Today Aleksa, Jason, Sumith, and Stone are all in Boston until Thursday and I have half of the R7D facilities to myself and they told me to "have fun and see what progress I can do." So, I spent today, creating a sodium thiosulfate gel to test the absorbency of the Iodorsorb prototype, I'm creating yet another cream emulsion to find the perfect stability for a future product, and I'm going to be running the third batch of buffer through the TFF to get a final purified sample. Once I got the final sample, I loaded into a lyophilizer tray and threw it in the -80C freezer. Midway through the day, I found myself finished with the tasks Aleksa gave me and decided to spend the last hour or two cleaning the lab since Aleksa and Jason like mess. I emailed Aleksa and Jason to see what I can do tomorrow and will wait for their replies.
Wednesday – 24 August 2016	**Day 10:** Since everyone is in Boston, I spent the day running another round of the TFF. While it was running, I started another cream emulsion to find the perfect ratios of water to phospholipids to Tween. Mainly just trying to find the perfect stability to where it can remain stable at both room

	temp and 40 degree Celsius for more than 24 hours. I'm going to do what I can to see if I can shadow/annoy Paul today and get some hands on experience in the 'Ferm. Lab" as well as other micro techniques I haven't had yet. Oh, and I also read the results of yesterdays sodium thiosulfate gel and the iodine had completely been absorbed into the gel and out of the product.
Thursday – 25 August 2016	**Day 11:** So today we had visitors from the Netherlands whoa re cMC1king up on a project that I'm not really apart of, but they bought us all lunch, so that was great. Today, I ran the final round of TFF on the 2nd batch of collagenase. I made a second Sodium Thiosulfate gel to test the iodorsorb absorbency but this time I'm taking pictures every hour to make a timeline of the release of iodine from the gels. I'm starting my blood borne pathogen training after the R&D Meeting at 12. I started the outline for my proposal for the internship practicum. And kept an eye on the TFF hourly. I also started a lipid gel to mimic the effects of drugs on the skin to test the Iodorsorb Max and its dispersion of iodine to a wound compared to the previous prototype.
Friday – 26 August 2016	**Day 12:** So today, I monitored the Lyophilizer, made a few mote solutions for the biofilm project and made a few more gels to test the Iodosorb Lab vs. Max and went to lunch with the whole R&D department. Apparently our Director of R&D is Nazrene Jacobsen, who is a besties with my former PI and Mentor at UNT, Robert Benjamin. I then made my first Film emulsion, spun it down, and plated it onto one of those giant petri dishes.
Monday – 29 August 2016	**Day 13:** The Lyophilizer still isn't done so the collagenase project will be on hold until it is done. I made even more gels as well as made a second film emulsion this time including Chitosan and let it cool, settle overnight. Spent the day doing even more Iodosorb testing and a bit of literature work to prepare for this venom project.
Tuesday – 30 August 2016	**Day 14:** So today was slow. The Lyo still isn't done freeze-drying the collagenase material so we can't finish the testing until its fully dehydrated. We made two more cut-outs in the Lipid/HBSS/FBS/Na2SO3 gels to find out the timespan the iodine seeps out of the iodorsorb. We are also trying to incorporate a heat aspect to the Iodosorbs to mimic what you'd expect the Temp to be in the living mouse models. I was put in charge of studying Glutaraldehyde to figure what concentrations were best for testing the gels at 40C. So I read some literature where they said that a 2% by volume solution worked so we did that and the gels are now in the 40C incubator for an hour to find out if this isht will work.

	Oh, I was also trained on how to use an ultra-homogenizer, which was pretty neat.
Wednesday – 31 August 2016	**Day 15:** Today we were all required to take the yearly Blood-Borne Pathogen training, which took forever, I then was sent to Christi in Quality Control to take the Master Control training to get familiar with the online training and tracking databases for SOP's and stuff. I finally got word that I'll be getting my computer tomorrow. Also, the password that IT set up for me…doesn't work. Yay. I put the Glutaraldehyde gels in the 40C incubator and observed them hourly-ish and got the expected result. I also performed my first activity test analysis of the biomass vs. purified collagenase powders and got great results that showed that the purified collagenase had a significantly higher activity and efficiency than the original biomess, haha, mess. Although we still need to get the ratios perfect to get a pretty standard curve, we're on the right track.
Thursday – 1 September 2016	**Day 16:** The top tray in the Lyophilizer was done so we wee able to collect the weight, which came out to around 35.73g total of retentate collagenase. Also read the gels from the Glutaraldehyde gels and made new pores for a 2nd test for confirmation.
Friday – 2 September 2016	***Day 17:*** *DAY OFF – Labor Day Weekend Extravaganza*
Monday – 5 September 2016	***Day 18:*** *DAY OFF – Labor Day Weekend Extravaganza*
Tuesday – 6 September 2016	**Day 19:** Started the 3rd round of TFF and started working on drafts of my proposal. Emailed Simecka for a meeting about my practicum proposal. Started a power point for the Iodosorb gel timeline studies to use at the next progress report meeting. Tried to not stress. I got a lot of headway on my proposal and have about 11 pages so far and need to work a lot on the background and literature collection.
Wednesday – 7 September 2016	**Day 20:** Finished the third batch of TFF purification and kept it in a 1 liter bottle in the fridge to watch and see if the precipitate forms again on the bottom. We think the Tween separated something out of solution and we need to find out what it is. Today was mainly spent on my computer getting a few trainings done as well as working on my first power point presentation for the company to discuss and reveal the results from the Iodosorb Gel testing. I also did a LOT of work on the proposal and should be done in the next few days, going to need some of my crazy, great aunt's prayers. Finally heard back from Simecka and will be meeting with him tomorrow during my lunch break to discuss the best way to guarantee approval of the proposal and how to make it flow right considering I have multiple projects in one internship. Got to figure out the best way to make them all

	into a cohesive thesis. Discussed timelines with Aleksa and then got a pep-talk on why I should not marry a foreign woman when you yourself are foreign… What…
Thursday – 8 September 2016	**Day 21:** So today we finally were figure out how to isolate the precipitate from the collagenase TFF. We had to centrifuge it in the 250 mL tubes in the Micro lab. We spent an hour trying to use filter paper until everyone was like, screw that. So that's drying right now so we can try to solubilize it and figure out what it is later. I'm meeting with Simecka within the hour and hope to get some finalized, official information regarding the proposal. Later I'm meeting with Paul to run a few SDS-PAGE's to get verified weights of the final products in the first two TFF batches.
Friday – 9 September 2016	**Day 22:** Ran the SDS-PAGE's and used the gel imager to get a solid, banded picture of the gel. I ran the 2/3rd round of purification on the 4th batch of collagenase but won't have time to do the buffer round until Monday. We all spent a good 2-3 hours cleaning the lab in prep for the President of R&D coming on Tuesday. We then went to a baby shower for Jose in the Einstein conference room. Not too much happened today, but it was fine.
Monday – 12 September 2016	**Day 23:** Today I started the final buffer round of purification on batch 4 of the Collagenase purification. I did a lot of work on my proposal and I believe I've finished the first full draft. Sending to Aleksa this afternoon after rereading it a few more times. I met with Sumith and was assigned to help out with the Quality control testing for Regranex and to help pinpoint and figure out what a degradation compound in the final preparations is and isolate it out using silver-staining. He hopes I can identify it (since he's busy) and help them figure out a way to isolate it from the Regranex solution before it gets converted into a gel.
Tuesday – 13 September 2016	**Day 24:** Today was a day full of meetings and presentations. I continued the last buffer round of TFF as soon as I got in around 8 and was in the R&D Meetings with the new President of R&D for Smith & Nephew from London. After lunch I continued to monitor the TFF, finished it, spun it down, separated the precipitates and let it freeze in the -80 freezer, and then started a new batch of TFF this time not using tween. Finished off Lot 13-115

Wednesday – 14 September 2016	**Day 25:** Finished Batch #5 and started batch 6, centrifuged a sample of Batch 5 to see if any precipitate would come out without having put tween in the first round. Made a super specific and testy Acetate buffer with saline to a pH of 7.4 to dissolve the snake venom proteins into solution for use in the MC1/MC2/Chitosan film. Then went to lunch and to get a haircut. Wandering whom even notices when I get back. Made the first prototype venom film and finished batch 6 of TFF purification Batch 5 – Centrifuged Batch 6 – Non-Centrifuged
Thursday – 15 September 2016	**Day 26:** Started TFF batch 5 and 6 on Lyo, started and finished batch 7. Performed some centrifugation purification tests on some 50, 30, and 10 kD cartridges; those will be run on a gel tomorrow morning. CMC1ked on Chitosan/venom film: needs more time. Took lunch, got panda, bought some new shoes, went and got cash for laundry and still made it back with 15 minutes to spare. Placed batch 7 in freezer. Easy day.
Friday – 16 September 2016	**Day 27**: Walked into the lab to find that batch 4 had sucked the Lyo nozzle so far down the flask, it shot off the Lyo and busted all over the ground causing all my other samples to defrost and stop Lyophilization. I put those samples back into the -80C ASAP and cleaned up the mess. After contacting the manufacturer about their faulty tops, they agreed to send us 4 new tops and a new flask to replace the one we lost. Nice, of them, right? Wrong. We won't get the replacements for a while and I just have to wait. Monitoring the TFF's was basically my life for today, that and the traditional Friday R&D group lunch gave me food poisoning. Oh joy
Monday – 19 September 2016	**Day 28:** Collected and weight batches 3 and 6, started batch 10 on TFF, and centrifuged and froze batch 9 (TFF). Awaiting delivery of new flasks and rubber tops before starting batches 5,7,8, and 9 on Lyo. Not sure what they have planned for the rest of the day but I'm basically on standby until their meetings are done.
Tuesday – 20 September 2016	**Day 29:** So, today was super slow and uneventful. Finished batch 10 and 11, placed in freezer for overnight freeze and will put them on the Lyo in the morning. Waiting to start batch 12 for tomorrow since we're going to be doing a major flush and using a strong base to flush out the cartridges to get rid of any blockages, which we think is slowing down the process. We'll do a few flushes and then let it sit, with pressure in the system overnight. I worked on tweaking my

	proposal and admit to watching a little bit of YouTube while at my desk. Read through some articles and am awaiting the Silver staining project to begin.
Wednesday – 21 September 2016	**Day 30**: Started batch 12 on TFF and removed all of the venom film from the dishes for testing tomorrow on the clotting machine. Finished batch 12 & started 13. Super easy day. Silver Staining for the Regranex project keeps getting pushed back since Sumith is crazy busy.
Thursday – 22 September 2016	**Day 31**: finished the buffer stage of batch 13, started batch 14 on TFF. Ran the Silver stain gel and staining and washes of the PDGF samples for reformulation purposes for the Regranex Project. Started the buffer stage for batch 14 of TFF
Friday – 23 September 2016	**Day 32**: Finished batch 14, centrifuged it and it is now chilling on standby. After lunch I started the FALGPA Assays for Batch 3, 6, 9, 10, and the 50, 30, and 10 kD filter cartridges, as well as the mystery gunk from the TFF Precipitate.
Monday – 26 September 2016	**Day 33:** Today I spun down batch 15 and froze it while starting batch 16 of TFF. That's it. Nothing else. I watched Harry Potter and the Half Blood Prince at my desk while monitoring batch 16 since the TFF apparatus is slowing down, possibly because of clotting. I've been assigned to read up on the Fibrinogen Clotting apparatus for later this week for the tests of our films. I collected and weighed batches 5 and 13. And also froze 14 and 12.
Tuesday – 27 September 2016	**Day 34:** Performed a new FALGPA Assay and got BEAUTIFUL results. Since we still haven't received the new shipment of Tris, the TFF is on hold until further notice
Wednesday – 28 September 2016	**Day 35:** Continued a few TFF batches, started the venom emulsion for the coagulation tests, and made the fibrinogen solution for testing the venom coagulative properties. Added batches 14 and 15 to Lyo, cMC1ked the Lyo batches and they should be done tomorrow. Prepared some samples for tomorrow Silver Staining Trials, and planned with other Jason to figure out best way to filter biomass.
Thursday – 29 September 2016	**Day 36:** Finished batch 17, placed batch 16 and 17 in deep freeze. Collected batch 7,8,11 from Lyo, weighed and calculated info. Ran Silver Stain again with the NuPAGE reducing agent. Performed coagulation tests with the venom gel on the fibrinogen analyzer. Started some fresh Clostridium fermentation filtration to get rid of cell debris and compare 3 different methods of cell debris clearance. Long, busy, busy day. Tested the venom coagulative properties, twas a bust. We're tripling the concentration to see if we can get it to work

Friday – 30 September	**Day 37**: Started batch 18, ran gel for batches 5,7,8,9,10,11,13, as well as the Forced crash out sample. I also ran with these two samples from a live fermentation batch, one centrifuged and one filtered (both methods to get rid of cell debris). Tested the 3x concentrated venom solution and it didn't work as well as thrombin either, so we're back to the drawing board. Went to a sushi place for lunch and I will be regretting it after work.
Monday – 3 October 2016	**Day 38:** Finished batch 18, started batch 19. I ran a FALGPA test for batches 5,7,8,11, and 13. Collected batches 14 and 15 from the Lyo, weighted them out and calculated data. Trying a new standard solution and diluting the samples further. Basically got perfect results and have verified the best protocol for doing this assay for tff purified collagenase. I typed up a new protocol for this method and repeated with every batch sample I've collected so far and everything came out 100% beautifully. I updated my information in both data collections. Finished batch 19 and started batch 20. Froze both batch 18 and 19 in the -80C freezer to start Lyo tomorrow. Very successful and busy day
Tuesday – 4 October 2016	**Day 39:** Finished batch 20 and started batch 21, spent most of the day monitoring the TFF as well as catching up on my notebook for S&N. Very slow day since I'm now just waiting on the Lyo's as well as many reagents for continuing the Regranex tests, the venom trials, AND the Iodosorb studies.
Wednesday – 5 October 2016	**Day 40:** Spent the majority of last night emailing with Gwirtz and Simecka and about to pull my hair out, I'll be bald by Friday. I scheduled my advisory committee meeting for October 12th from 3-5pm in the Galen conference room at S&N. Finished batch 21 on TFF and started batch 22. For batch 22, I'm increasing the conc. from 6 to 15 mg/mL to see how it affects the purification process. Talked with Aleksa about how to deal with all the internship bureaucracy and thank god he has a level, and calm head about all of this. He destressed me and thankfully has 100% confidence in my abilities and said I've blown away their expectations for my time here (#Bless). Oh joy, the beaker with sample 12 just cracked and lost pressure when I tapped it to loosen the outer layer of powder from the frozen ball. I immediately took it off the lyo, put it into another beaker and restarted it. #Ouch
Thursday – 6 October 2016	**Day 41:** Continued batch 22 on TFF, finished it up, and spun it down and its chilling in the fridge. Started batch 23. CMC1ked on Lyos, still not done. I'm gonna take a half day since I'm really not feeling 100%
Friday – 7 October 2016	**Day 42:** So since I raised the conc. Off collagenase from

	6→15 mg/mL it's taking FOREVER to finish even one run of TFF. SO today, I worked a lot on my Lab notebook, monitored the Lyo and TFF all day and had some nice hot tea. Went to Pan Asia for lunch with R&D and it was just lovely.
Monday – 10 October 2016	**Day 43:** Still waiting on some reagents to continue the Silver Staining projects as well as waiting on more FALGPA to continue my activity tests. Also waiting on some thrombin and Fibrinogen top continue the clotting and coagulation studies with the venom. Just a lot of waiting and monitoring the TFF's and lyos today. Finally the fibrinogen and thrombin came in in the last two hours so I expect we spend time tomorrow working on that. Centrifuged the completed Batch 23 and started TFF batch 24
Tuesday – 11 October 2016	**Day 44:** Collected samples from batch 20, 21, and 22 from the Lyo, weighed composites and calculated yields, weighed out samples to run on Gel for tomorrow as well as weighed out samples to run activity tests on tomorrow morning during the gel run. Yay! I need sleep.
Wednesday – 12 October 2016	**Day 45**: So I collected Batch 22 from the Lyo, and weighed out samples to use for the FALGPA Assay as well as the Banding gel for tomorrow. I started a FALGPA Assay for batches 12, 16, 17, 18, 19, 20, 21, and 22. We made a standard curve solution set for the venom coagulation tests. We made a 5.56 mg/mL solution of bovine fibrinogen and its currently spinning for an hour or so because it is ridiculously picky to get into solution. Printed out an extra form I forgot for my Advisory Committee Meeting today at three o'clock with Gwirtz and Simecka. Meeting went well. Worked on some edits in my proposal for the rest of the afternoon
Thursday – 13 October 2016	**Day 46:** Started batches 23 & 24 on Lyo. Worked with Paul to isolate the supertanents from the fresh ferms and started their centrifugation and Filtration to remove all cell masses. Set up TFF for that sample
Friday – 14 October 2016	**Day 47:** Did a lot of work on my proposal, kept monitoring the Ferm filtration. Performed a total protein analysis on NP-Powder vs. Purified product.
Monday – 17 October 2016	**Day 48:** *DAY OFF - IPE*
Tuesday – 18 October 2016	**Day 49**: Lyo still isn't done. Emailed final draft of proposal to Gwirtz and Simecka. Completed a few trainings. Finished filtration of the ferm, ran a TFF on it, forced it to 600 mL's, threw in Deep Freeze and will start Lyo on it in the morning. Did a lot of literature reading about the media and the components in the final powder
Wednesday – 19 October 2016	**Day 50:** Prepared a thrombin solution for some coagulation tests for tomorrow and left at 4 pm. Did first venom trials!

	Finally found the perfect buffer to use to dissolve the fibrinogen. We used a Tris/HCl/NaCl buffer; we figured out that the Calcium was preventing the fibrinogen from going into solution.
Thursday – 20 October 2016	**Day 51:** CMC1ked on Lyo, halting the TFF work until we meet and discuss what next steps should be taken and where to go from here. I performed some Thrombin screening to figure out the lowest concentration of Thrombin needed to achieve full coagulation. Designed experiment to test the cooperative effects of Thrombin and venom on fibrinogen samples for further coagulative studies.
Friday – 21 October 2016	**Day 52:** I performed a fourth set of coagulation tests to observe the cooperative effect of additive amount of both venom and thrombin to get a better look at their interaction is what will be the wound bed. Slow day, Lyo is still working on the Ferm batch of collagenase.
Monday – 24 October 2016	**Day 53:** Collected The Ferm sample from the Lyo and calculated percent yields…not great. Hopefully its activity is crazy-high. Performed another batch of venom/coagulation tests, took some selfies, and planned out some experimentation for tomorrow. Started Batch 27 on TFF
Tuesday – 25 October 2016	**Day 54:** Monitored TFF, super slow. Read a lot of literature for my practicum, completed a few online company trainings. Very slow day. Made some Thermolysin test samples for Aleksa/Paul. (Capmul, Gentamicin Sulfate, Thermolysin, combo's)
Wednesday – 26 October 2016	**Day 55**: Performed a silver stain for the PDGF Regranex formulation study. Continued batch 27 on TFF. Got Lunch with older Jason.
Thursday – 27 October 2016	**Day 56:** Prepared an overnight incubation for the cellulose enzymes and Regranex for a gel tomorrow. Collected batch 27 from TFF, changed out filters, started batch 28, finished batch 28, centrifuged them both down, and threw them in the -80 freezer in Lyo trays. Planned out tomorrows gel.
Friday – 28 October 2016	**Day 57:** Started batch 27 & 28 on Lyo, started batch 29 on TFF, ran Regranex formulations gels(native & reduced), performed silver staining on the gels to maximize accuracy.
Monday – 31 October 2016	**Day 58:** Finished batch 28 on Lyo, started batch 29. Made a new MC1 film gel for testing on the fast-dissolving films. Went to campus to get signatures for some important firms.
Tuesday – 1 November 2016	**Day 59:** Still waiting to hear back about when to go get Gwirtz's signature to finish this whole process. This is taking way too long. I continued batch 30, spun down batch 29, and finished batch 30, started batch 31, Prepared 3 different MC1-based fast dissolving films. Films now chilling.

Wednesday – 2 November 2016	**Day 60:** Continued Batch 31 on TFF, Performed a new silver staining methodology using a different type of gel for the Native samples.
Thursday – 3 November 2016	**Day 61:** Worse than any Monday ever. Came in to batch 31 being ruined, long story, you know what you did. So I spent the day flushing out the entire system with a super strong base and performed another silver staining to get a successful result set.
Friday – 4 November 2016	**Day 62:** CMC1ked on some TFF stuff while it is being flushed out. Had a catered lunch, worked on my report all day, left at 1.
Monday – 7 November 2016	**Day 63:** Ran two failed FALGPA Assays before lunch, one w/ concentrations too high, one with concentrations too low, ugh. Running another set after lunch. Lyo condenser is broken so TFF's are on standby for now. Made one more fast-dissolving gel for Aleksa with 55% MC1 to reduce the Glycerol effects. Ran a successful FALGPA
Tuesday – 8 November 2016	**Day 64:** Came in, cMC1ked on Lyo, collected batch 26 from TFF cMC1ked on the Films in the 40 degree incubator, still sticky. Spent the majority of the day working on my practicum report
Wednesday – 9 November 2016	**Day 65**: Worked on my report, cMC1ked in on the Lyo. A lot of typing today
Thursday – 10 November 2016	**Day 66:** Cut out circles of films 1-3 and added 2 mL of saline to them to observe the reactions. Continued to monitor the Lyo. Did a lot more work on my report and my practicum.
Friday – 11 November 2016	**Day 67**: Prepared a 2nd round of testing for Iodosorb Gels by recreating Gel #'s 3-6 to re-observe the effects of Sodium thiosulfate on Iodosorb as well as the response of Iodosorb (both commercial and Max) to lipids, FBS, and HBSS in these new synthetic, skin-mimicking gels. I'll be doing those time-lapse studies starting Monday morning
Monday – 14 November 2016	**Day 68:** Prepared the gels for the Iodosorb tests by punching out holes for the Iodosorb. Monitored them every hour and took pictures for a time-lapse power point. Monitored the Lyo, as well as monitored the films drying in the back room. Worked on my practicum for a good chunk of the day when I wasn't distracted by election coverage news. Merry Trumpmas.
Tuesday – 15 November 2016	**Day 69:** Continued the time-lapse of the Iodosorb gels, and spent the majority of the day working on my practicum. Trying to finalize the abstract and get the acknowledgements completed.
Wednesday – 16 November 2016	**Day 70:** Continued the time lapse watching of the Iodosorbs, assembled the new 50 kD filter cartridge and connective

	apparatus. Kept monitoring the drying films in the back room and did a few more saline tests on the previously dried films. Took the second half of the day off
Thursday – 17 November 2016	**Day 71:** Ran a series of tests on the dried film prototypes. Ran a cycle of TFF on a 8 liters of ultra-pure water through the 50 kD to flush it out and left it filled until after the thanksgiving break.
Friday – 18 November 2016	**Day 72:** Attended a few meetings with the other Jason and helped Sumith run a last-minute Silver stain assay that took all day.
Monday – 21 November 2016	Thanksgiving Break - Week Off!!!
Tuesday – 22 November 2016	
Wednesday – 23 November 2016	
Thursday – 24 November 2016	
Friday – 25 November 2016	
Monday – 28 November 2016	**Day 78:** Ran the first test, batch 31, on the 50 kD cartridges, and finished it by 2 pm, ran it through the industrial centrifuge and stored it in the fridge. Will wait on Jason to get back from Vancouver to continue this process to get further oversight approval.
Tuesday – 29 November 2016	**Day 79:** Ran another last-minute Silver Staining Assay for Sumith and Stone for different batches of collagenase for the Santyl Production formulation changes
Wednesday – 30 November 2016	**Day 80:** Ran batch 32 on TFF on 50 kD, went to campus to deal with a form problem with Carla, Grabbed some groceries while I was out, did a load of laundry, got back right in time to finish the staining for some gels I put on earlier that morning.
Thursday – 1 December 2016	**Day 81:** Centrifuged Batch 32 from TFF, placed batches 26, 31, and 32 in Deep Freeze for Lyo tomorrow. Since all projects are on a temporary hold for various reasons, I took the second half of the day off to work on my practicum
Friday – 2 December 2016	**Day 82:** Came in at 8, started Lyo with newest samples, Went home XD
Monday - 5 December 2016	**Day 83:** Started batch 33 on TFF, completed saline expansion experiments on films 7, 10, 11, and 12. Met with Stone and Sumith about plans for the next three days of helping Sumith with the reformulation results. Updated the Iodosorb presentation as well as made a presentation covering the progress made with the films
Tuesday - 6 December 2016	**Day 84:** Turns out the Coomassie Blue never came so the gel stuff for Sumith is on hold. Spent an Hour in the Device Lab trying to set up the Tensile strength calculator machine thing. Spent a few hours online looking for adapter parts and various pieces necessary before we start. Kept waiting for the Coomassie to get here.

Wednesday - 7 December 2016	**Day 85:** Ran the 4 gels for Sumith and Stone and it basically just took all day. Went to a meeting with the Secant Group and they tried to convince us to buy one of their products but we saw through their little charade and decided not to and I spent the second half of the day staining the gels with the not Coomassie, Coomassie. And then went home when I changed the last one's water out and let them all rock overnight. Then spent the last few minutes talking crap with Paul about the Secant group and their mediocrity.
Thursday - 8 December 2016	**Day 86:** So I started and finished Gel 13, and put two samples on the counter to air dry and two into the -80C freezer to lyophilize either tomorrow or Monday. I then spent like 3 hours imaging al of the gels from yesterday and calibrating them band by band. And finally finished in time for lunch.
Friday - 9 December 2016	**Day 87:** Thawed lyo out and then restarted batch 26, and started Lyo-ing films 6, 9, and 13 (2 of each). Today is the holiday sweater day and Christmas potluck, but since no one told me, I'm neither wearing a sweater nor did I bring anything… But since I'm virtually done for the day, I may just continue my work offsite and continue my day in sweats and a hoodie. Sumith was supposed to come in today and we were going to spend the afternoon analyzing the collagenase samples from the failed batches from Curacao, but due to his surgery, we decided to take today off, so since literally everyone is gone but me and Aleksa, there's nothing else that can be done today… boo. I'ma go home, bye Felicia…
Monday – 12 December 2016	**Day 88**: Collected batch 26 from Lyo and took off films, 6, 9, and 13 from Lyo. R&D Meeting at 9:30. Started batch 34 on TFF, Conducted absorbency tests on films 6, 9, and 13 lyo'd. Worked on my practicum
Tuesday -13 December 2016	**Day 89:** Put the #14 films on the Lyo and put Batch 34 on Lyo that will hopefully finish before Friday. Created a new gel that will be soaked in glutaraldehyde to repeat the past gel tests will a better stability. Worked on my practicum and went to Campus for a meeting with GSBS and some other crap.
Wednesday - 14 December 2016	**Day 90:** collected the #14 films from the Lyo, and ran the absorbency tests on them. Made a new film concoction w/o MC1 or Chitosan to see how well the other components would hold up and used some polycadeomxers to make it hold together. Tested the stability of the newest Iodosorb gel mixture to higher heats since the glutaraldehyde had expired and was thrown out. We monitored the gels at various temperatures and every test above room temperature melted. Until we get a new batch of glutaraldehyde in, we can't

	continue this experiment.
Thursday - 15 December 2016	**Day 91:** Started the time lapse study on the Iodosorb Max at room temperature and monitored w/ pictures every hour. Went to get Spicy Chinese with the R&D Staff and did some various tests with Sumith and Jason to fill in the afternoon.
Friday – 16 December 2016	**Day 92:** Wore my slutty Santa hat to work and was told that I looked like a whore, so that happened. ☺ Turns out the Lyo sample may not be ready by the time I leave, we'll see. I kept monitoring the Iodosorb gels. Watched some Milo videos in my spare time. We had a meeting that was catered with Clinical to discuss the End of the Year Review and it was delicious. See you in 2017!!
Monday - 19 December 2016	Christmas Break - 2 Weeks Off!
Tuesday - 20 December 2016	
Wednesday - 21 December 2016	
Thursday - 22 December 2016	
Friday - 23 December 2016	
Monday - 26 December 2016	
Tuesday -27 December 2016	
Wednesday - 28 December 2016	
Thursday - 29 December 2016	
Friday - 30 December 2016	
Monday – 2 January 2017	
Tuesday - 3 January 2017	**Day 104:** Collected batch 24 from the Lyo. Started tensile-strength testing in the Blue System in the Device lab on the films. Saved the initial testing results on the desktop to use as a template for future tests. At 12 I started getting pretty bad cold symptoms, i.e. (coughing, sneezing, and congestion) so Aleksa told me to go home and get better, but alas I did not. I caught a bad viral/sinus infection. Yay.
Wednesday - 4 January 2017	**Day 105:** Sick-Day: Basically me dying.
Thursday - 5 January 2017	**Day 106**: Sick-Day: Basically me dying.
Friday - 6 January 2017	**Day 107:** Sick-Day: Basically me dying.
Monday - 9 January 2017	**Day 108:** Finally feeling like a human again. And yet I come in and I have 49 trainings to complete by tomorrow. Woo. I did those all day. That's it. Kill me. Finished the trainings and started the Tensile Strength testing for the completed films, but lyo'd and dried.
Tuesday - 10 January 2017	**Day 109:** Performed a FALGPA Assay, and total protein test and a gel electrophoresis on samples from every type of filtration we've used. This took basically all day to complete. Emailed Jason results even though they did not make me or him happy.
Wednesday - 11 January 2017	**Day 110:** Took a half-day off since I have to wait until Jason, Stone, and Aleksa get back from their various countries to decide which directions we need to go into next

	to further these experimentations. SO I got a few things done for my Practicum Report and then finished working at the Starbucks on Camp Bowie.
Thursday - 12 January 2017	**Day 111:** Redesigned layout for the lyo room because it was bothering me. Analyzed results from Wednesday since nothing made sense and coordinated with Paul to plan a side-by-side comparison test for the two developed(ing) methods.
Friday - 13 January 2017	**Day 112:** Started another comparison gel and will run the MC1k out of it to force ample separation between the 114 kD and 116 kD bands of collagenase. Went to lunch while the gel was de-staining in UP-H2O. Imaged the gel, analyzed the bandings and verified that the Double TFF sample was severely degraded and needs to be redone. Spent the rest of the day watching Mad-Dog Mattis' Senate Confirmation Hearing and working on my Practicum Report.
Monday - 16 January 2017	**Day 113:** Power was out when I showed up and was out up until 12 when I was told to just go home since we couldn't do anything. Apparently the backup generators only power the freezers and fridges to maintain storage of samples and schtuff. Oh, and the generators also power the offices of "Essential" personnel, so not me. Day off I guess.
Tuesday - 17 January 2017	**Day 114:** Worked on a lot of the analysis of the Tensile-Strength results and compiled all the data we had so far into a master spreadsheet for the Film experimentation. Aleksa gave me the current in-combat hemostatic bandages that we're hoping to put out of business. I looked up these products to figure out what they're made of and how they work. Broke into the Device Lab and copied my data results from last week.
Wednesday - 18 January 2017	**Day 115:** Spent an hour trying to get info for renewing my license but to no avail. Then I started the full analysis and graphing of all of the results I've gotten so far for these film preparations. Compared all of the raw data and created a presentation to show at a meeting on Thursday so Stone, Aleksa, and I can decide on which direction to go further on. Today just seems to be going very slow for some reason. Ugh.
Thursday - 19 January 2017	**Day 116:** Met with Stone, Aleksa, and Jason to plan what should be next in the film preparation and mapped out what's going to occur during my last few months. I made the 15th film in our progression and will start Lyo-ing them before the end of the day. Continued working on my practicum report as well as researching some stuff that we covered in our meeting this morning. Also, S&N provided everybody with endless popcorn since today is national popcorn day, yay… Started Film #15 on the Lyo.

Friday - 20 January 2017	**Day 117:** Tested Film 15 for all of the necessary data points, and based on its data; I made 8 more films with varying ratios of CMC vs Explotab in the films. Made each film have a dried and lyo'd counterpart from the same sample. Froze the plates meant for Lyo and started that lyo. Set out the rest to air-dry over the weekend. Went to Ume for lunch and it was good but not good enough for the price. Heads up. Kept working on my practicum and analyzing data from past experiments to get an idea of what direction to start in on Monday.
Monday - 23 January 2017	**Day 118:** Testing films 15-19 for water uptake data and for tensile strength. Had a lunch R&D Meeting for that covered a whole variety of stuff that's going on. Started a new batch of TFF where I'm going to resupply the final collection with more metallic salts to help increase activation of the final sample. Continued preparing more films off of the results from today's data. Got the go ahead from Hull to start full reformulation efforts on Regranex as well as the SECURA Skin Care Line. My work is going to triple this week. Yay me. I'm going to need all the booze.
Tuesday - 24 January 2017	**Day 119:** Was late getting here this morning because I had to go get my driver's license renewed and experienced the absolute best service EVER. Like if every DMV was like this one, the stereotype wouldn't exist! So, anyways, I started films 20-22 on the Lyo when I got here.
Wednesday - 25 January 2017	**Day 120:** Collected Film 20 and 21 from the Lyo, 22 wasn't ready yet. Performed all of the film tests on 20 and 21. Worked on my practicum and brainstormed with Jason and Aleksa on what direction we should take when continuing the TFF's
Thursday - 26 January 2017	**Day 121**: Collected film 22 from Lyo and ran film tests on it. Started back on some TFF runs. Start and finished batch 35 and started batch 36. Will have to continue it on Friday. Met with Stone, Jason, and Aleksa to discuss how we're going to wrap up my internship and how we're going to tie it in a big bow and the best ways to accomplish that.
Friday - 27 January 2017	**Day 122:** SPICY CHINESE FOR LUNCH TODAY. Ok, so I finished batch 36 on TFF and attempted to collect films 20-22 from the air-drying chamber
Monday - 30 January 2017	**Day 123**: Tested films 20-22 and tested them for water uptake, strength analysis and made a few changes to our formulation. Made films 23-26 for both Lyo and Air-drying. Incorporated Maltodextrin to see if I can induce a quicker dissolving time in saline. Went home and worked out.
Tuesday - 31 January 2017	**Day 124:** Mainly spent the majority of the day working on some data analysis as well as editing some sections of my

	practicum report. Simply having to wait for the Lyo at this point. Thankfully we finally got word from the Other lyo guy who should be back to fix our second Lyo on either Friday or Monday. Yay. Porch tonight!
Wednesday – 1 February 2017	**Day 125:** Performed an extra FALGPA assay for the latest samples of Collagenase TFF to get a better read on the specific progress. Input that data into all the spreadsheets and got a better feel for how these results are coming. Planned with the other Jason and Paul on how we're doing the industrial, manufacturing process for Friday, Saturday and Sunday, and then went and made sure we had everything for those days and mapped out how/where we're going to do everything. Sushi with Hanna/Paul after work!! Shinjuku Sushi is amazing
Thursday - 2 February 2017	**Day 126:** So basically, I'm still waiting for the lyo to finish the TFF Samples. I finished the testing of the Chloramphenicol and tested the dried films 23-26 on water uptake and tensile strength. Then I spent like 2 hours translating protocols from German…
Friday - 3 February 2017	**Day 127:** So, the 50-Liter firm was ready this morning so we started the testing series for it. First we collected 1 liter of straight ferm for testing and storage. Then we took another liter and centrifuged it in preparation for the ammonium sulfate process. Once we took the first two liters off, we ran the rest of the entire batch through a 500 kD TFF filter. This got rid of the bacteria. From that, we took 7 liters and ran the ammonium sulfate procedure and the other 22 liters we ran through the 50 kD TFF filter, froze and put it on the lyo. The 7 liter ammonium sulfate sample and the 1 liter ammonium sulfate sample were filled into dialysis tubing and dialyzed over the weekend with 2 water bath changes per day. Comin gin on the weekend isn't fun…
Monday - 6 February 2017	**Day 128:** OK, the Iroxel samples from Germany came in and the first testing series we did was a solution based assay to determine the effect of the different chloramphenicol's on the Collagenase. After a few meetings with not only the R&D Department but also the Formulation team, we finally decided on which series of tests each person would complete and how they should be done. I used FITC-labeled collagen for these tests. After the solubilizing, they were wrapped in foil and put on the rocker in the 37 degree incubator overnight.
Tuesday - 7 February 2017	**Day 129:** Tested the FITC-labeled collagen n the fluorescence analyzer. Theoretically, the more collagen that was chewed up, the more fluorescent tags will be active in the sample. So, the brighter the response data, the higher the

	collagenase activity.
Wednesday - 8 February 2017	**Day 130:** SO basically I spent all day in the lab trying to complete the clostripain characteristic profiles. I used the BAEE Assay to test the presence of chloramphenicol in the samples and the effects of PMSF on these samples. The first round didn't work because the phosphate buffer created aggregates with the Calcium Acetate/Tris buffer, making the solution cloudy an unreadable. Tried it with using Tris/Ca-Acetate for all samples but the lack of phosphates, I believe, is the cause of the lack of action on the substrate.
Thursday - 9 February 2017	**Day 131:** Casein-FITC Assay ALL DAY LONG Kill me
Friday - 10 February 2017	**Day 132:** Sumith's daughter ended up getting extremely sick and has to leave,. Stone ended up having to go to the hospital because his son had a 104 fever. So today I filled in for all of our roles. I was overworked like CRAZY and I deserve a drink. I performed the Calcium acetate extraction of the EU-Collagenase and then performed the Ninhydrin Assay, the BAEE Assay and the FITC-labelled assay on all of these samples. I'm getting intoxicated tonight. #Peace
Monday - 13 February 2017	**Day 133:** Collected some films samples and tested them on water uptake as well as tensile strength. Was supposed to do a Fluorescence analysis using a kit that Stone bought. Also ran a FALGPA using the same samples where I added methanol to see if it affected the active enzyme in EU-Collagenase. Also collected film 27 from Lyo and tested it.
Tuesday - 14 February 2017	**Day 134:** Continued testing the EU-Collagenase samples. Repeated from above. Same old, Same old. Worked with Aleksa to plan the reformulation of the Secura product line
Wednesday - 15 February 2017	**Day 135**: Started a 24-hour vs. 1-hour comparison test for the extraction processes. Met at the new building for the Quarterly Review and were fed lunch. Once back, I ran a new maintained the extraction process and got some work done on my practicum report.
Thursday - 16 February 2017	**Day 136:** Started the 1-hour extraction for comparison to the 24-hour. Once both rocked for 1 more hour, I added the calcium acetate buffer and once settled, tested the samples using a Ninhydrin Assay analysis. Once back to my desk, I analyzed the data and formatted it into graph forms for easy understanding and comprehension.
Friday - 17 February 2017	**Day 137:** Day Off
Monday - 20 February 2017	**Day 138:** Half Day off: Came back at 12 and spent the remainder of the day in meetings and completing online trainings that I had missed.
Tuesday - 21 February 2017	**Day 139:** Performed a series of Ninhydrin Assays trying to compare the effects of Brij 35 on the EU-Collagenase samples

Wednesday - 22 February 2017	**Day 140:** Ran a second Ninhydrin assay, this time slightly increasing the Brij 35 conc. in the substrates and including the Tween sample for comparison
Thursday - 23 February 2017	**Day 141:** Ran a gel containing the sample taken from the ring residue left over after EU-Collagenase extraction. Planned out the next phase of film trials for tomorrow.
Friday - 24 February 2017	**Day 142:** Prepared all twelve films for next week's trials and put them in the -80 freezers to freeze over the weekend. Lunch meeting to meet and discuss the new hire for Clinical/R&D position. And spent the rest of the day finishing the films and getting ready for happy hour.
Monday - 27 February 2017	**Day 143:** Put the new films in the Lyo. Started preparing the samples of the precipitate material from the ring buildup from the EU-Collagenase extraction procedures. Worked on my report as well as spent the majority of the day preparing for tomorrow's audit.
Tuesday - 28 February 2017	**Day 144:** No. Just no.
Wednesday – 1 March 2017	**Day 145:** Since yesterday we planned out a new *in-vitro* study for testing the efficiency for these films, I'll be running a few initial trials to test the effectiveness of this apparatus technique.
Thursday - 2 March 2017	**Day 146:** Spent the day trying to figure out how to re-run these gels so that we can actually get results. Had a meeting with Gwirtz and Simecka on campus for an Internship progress report and basic catch-up information.
Friday - 3 March 2017	**Day 147:** Spent the day in Frisco as a seminar at the UNT Frisco Campus meeting with companies from the area to start cooperative efforts with the university branch
Monday - 6 March 2017	**Day 148:** Started the *in-vitro* tests for the venom films and compared them to their non-venom counterparts. Aleksa and Stone are gone until Thursday to Baltimore for a Military/Medicine conference and I have to hold off on testing the Quik-clot material until Aleksa is back.
Tuesday - 7 March 2017	**Day 149:** Did a few more tests for the films, finalized the difference in venom vs non-venom coagulation. Finally, I started a new extraction of the failed samples of EU-Collagenase and did another Ninhydrin Assay to compare the effects of different std. Ref. Concentrations because we began to question whether or not the std. Ref was saturated and turns out, it was, so we got new data on the EU-Collagenase complications and Stone should be happy when he gets back
Wednesday - 8 March 2017	**Day 150:** Came in to find construction happening in my office and since I had no lab work to complete today since everything is on hold until Aleksa and Stone get back, I worked off site on my practicum report.

Thursday - 9 March 2017	**Day 151:** We have a Monthly Report meeting where they're serving lunch *insert heart-eyes emoji* Spent the entire afternoon running tests on the industrial-grade, currently-used hemostatic bandages. I ran them through absorption tests as well as the Franz Cell apparatus. They failed on all counts, which is fantastic.
Friday - 10 March 2017	**Day 152:** Spent the day with the representatives from Sartorius and spent the day testing different purification methods for the Collagenase Purification using depth-bed filters. Went to lunch with them at Beik and spent the rest of the day finishing the purifications with them assisting when they needed anything and filling in for Mr. Campbell when he had meetings. Left a bit early at 4.
Monday - 13 March 2017	**Day 153:** I started a simple test to see the long-term effect of the actives from each hemostatic bandage. I cut out equal sized pieces from each product, and placed them on the top of 10 mL of a the fibrinogen solution used previously and observed the effect of the actives on the fibrinogen over 24 hours.
Tuesday - 14 March 2017	**Day 154:** I came in and took pictures of the coagulative actions of the industrial films vs our films. Ours had substantial clotting in the solution, large opaque blobs in the middle of the solutions. Yay for venom.
Wednesday - 15 March 2017	**Day 155:** Came in and created a new batch of the venom films to resupply our stocks for future tests. Took Half the day Off- Spring Break!
Thursday - 16 March 2017	**Day 156:** Off- Spring Break!
Friday - 17 March 2017	**Day 157:** Off- Spring Break!
Monday - 20 March 2017	**Day 158:** Half Day at the Plant where I made new batches of Film #10, collected a bunch of data and documents from the Shared drives for my practicum and completed like 14 new trainings through MasterControl.
Tuesday -21 March 2017	**Day 159:** Off-Site Work on Practicum
Wednesday - 22 March 2017	**Day 160:** Came in and took the new batch of Film #10 off of the Lyo and packaged them near the other films for future directions S&N may take with the product. Collected some Data and files from the scratch drive and then headed to work Off-Site on the practicum
Thursday - 23 March 2017	**Day 161:** Off-Site Work on Practicum
Friday – 24 March 2017	**Day 162:** Off-Site Work on Practicum. Had an Interview for a Research Associate at UNTHSC with the Center for Alzheimer's and Neurodegenerative Diseases.
Monday – 27 March 2017	**Day 163:** Went to S&N to grab some pictures and specs on some of the machinery I've been using over the past few months as well as grab some protocols for my appendix and for in my practicum

Tuesday - 28 March 2017	**Day 164:** Worked on Practicum and got a haircut
Wednesday - 29 March 2017	**Day 165:** Spent the day working on the practicum.
Thursday - 30 March 2017	**Day 166:** Spent the day working on the practicum.
Friday - 31 March 2017	**Day 167:** Went in for the morning to discuss with the proprietary people about what I couldn't include in my practicum. Spent the rest of the day finalizing the practicum and sending the final drafts to my advisory committee.
Monday – 3 April 2017	**Day 168:** Spent the day working on the presentation for my defense.
Tuesday - 4 April 2017	**Day 169:** Spent the day working on the presentation for my defense.
Wednesday - 5 April 2017	**Day 170:** Spent the day working on the presentation for my defense.
Thursday - 6 April 2017	**Day 171:** Spent the day working on the presentation for my defense.
Friday - 7 April 2017	**Day 172:** Spent the day working on the presentation for my defense.
Monday - 10 April 2017	**Day 173:** Spent the day working on the presentation for my defense.
Tuesday - 11 April 2017	**Day 174:** Spent the day working on the presentation for my defense.
Wednesday - 12 April 2017	**Day 175:** Had my defense at 11 am
Thursday - 13 April 2017	Day 176:
Friday - 14 April 2017	Day 177:

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Figures:

Figure 1: TFF Concept Art: http://www.ivtnetwork.com/gallery/biotech-processes-images

Figure 2: TFF Apparatus: http://www.pall.in/main/laboratory/literature-library-details.page?id=34212