Transdural Drug Release for Treatment of Central Nervous System Injury

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Abstract

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Traumatic spinal cord and brain injuries may lead to a devastating loss of neurological function. After the traumatic, primary insult, a secondary injury phase ensues, which significantly increases the extent of the injury. Inflammation is a dominant component of secondary injury, which includes the immune response in which free radicals and proinflammatory cytokines are released that induce the death of surrounding neurons. Dexamethasone, a corticosteroid, produces neuroprotective effects by inhibiting inflammation and reducing cytokine release. However, the clinical application of large systemic doses of steroids is limited by side effects, such as sepsis and pneumonia. There is a need to develop a localized delivery method that minimizes these side effects and improves efficacy by delivering directly at the injury site. In this thesis, we present a

delivery method that utilizes clinically used collagen dural substitutes as a vehicle to provide dexamethasone directly to the location of injury without introducing new surgical procedures or devices. This thesis will also explore multiple methods of delivery, such as controlled release from a polymer matrix in a microneedle format as well as coating the dural substitute. We quantified dexamethasone concentrations using high-performance liquid chromatography (HPLC) coupled mass spectrometry (MS). We conducted in-vitro studies using a customized trans-well insert that allowed for the simulation of transdural delivery into activated BV-2 microglia cells. After 24 hours of treatment, we used biological assays to determine nitric oxide, IL-1b, IL-6, TNF-α, and MCP-1 concentrations. With both systems, we were able to achieve sustained release for up to 24 hours. In-vitro, we achieved a significant reduction in nitric oxide concentrations using a polymer-coated dural substitute.

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Chapter 1. INTRODUCTION

1.1 THESIS AIMS AND ORGANIZATION

This thesis aims to develop a localized drug delivery method for the treatment of traumatic spinal cord injury. The goal is to improve drug delivery to the injury site post-injury that reduces the need for systemic dosing of steroids and their detrimental side effects. A secondary goal is to reduce the extent of the secondary injury phase by reducing the presence of proinflammatory cytokines and the release of nitric oxide from microglial cells. This work describes the process to achieve these goals by developing a polymer hydrogel system to encapsulate our model drug, dexamethasone. One approach is to create a strategy to manufacture microneedle arrays formulated out of this hydrogel. The goal for the microneedles is to pierce the dura and deliver a drug to the sub-dural space without the need to open the dura. A second approach outlined in this thesis is coating a collagen-based dural substitute in the hydrogel, utilizing the substitute as a backing and allowing for it to be easily sutured into the dura after an injury. To properly quantify dexamethasone release, we developed a liquid chromatography-mass spectrometry method that allowed for the accurate analysis of samples. Finally, we evaluated these systems using a novel transwell insert that simulated the ability to deliver drugs across a membrane and into the subdural space.

Chapter 2. BACKGROUND

2.1 INTRODUCTION

In this section, I introduce the motivation for the project and the need we are trying to address. I will also provide background information on multiple aspects and tools used throughout my project.

2.2 SPINAL CORD INJURY AND TREATMENT

2.2.1 *Incidence and Cost of Spinal Cord Injury*

In the United States, the incidence rate of spinal cord injury (SCI) is approximately 54 cases per million, resulting in 17,900 new cases per year. Currently, about 296,000 persons are living with SCI [1]. The cost of living with SCI depends on the severity level, with AIS D scores costing $379,698 initially then $46,119 every subsequent year. A 25-year-old patient that suffers a minor SCI injury can expect to pay $1,724,594 over their lifetime in medical costs directly related to the injury. This number rises as higher forms of paralysis occur, with a high tetraplegic patient paying $1,163,425 in their first year after injury and paying $202,032 every subsequent year. A 25-year-old patient suffering a high tetraplegic injury has a lifetime cost of $5,162,152 [1].

2.2.2 *The Secondary Injury Phase*

Traumatic spinal cord and brain injuries may lead to a devastating loss of neurological function. After the traumatic primary insult, a secondary injury phase ensues, which significantly increases the extent of the injured area. Inflammation is a dominant component of the secondary injury phase which includes the immune response in which free radicals and proinflammatory

cytokines are released that induce the death of surrounding neurons. This secondary injury phase is very unpredictable and adds complexity to the diagnosis and treatment of the injury. Improvements to reduce or control the secondary injury phase would drastically improve treatment and recovery in patients [2]–[4].

Clinically, systemic doses of steroids have shown anti-inflammatory effects that reduce the secondary injury phase at the injury site. With large dosages circulating the body, there are many side effects such as pneumonia, sepsis, or hypoglycemia [5], [6]. Therefore, a localized delivery method could provide therapeutic results with minimal side effects. One of the most clinically used steroids for this treatment is dexamethasone. A dosage of 8 μg/mL over a 24-48 hour window has shown improvement in reducing inflammation in activated microglial cells [7].

2.3 Delivery Methods

The motivation for the project is to reduce the secondary injury phase by localizing the delivery of anti-inflammatory steroids directly to the injury site. To meet this need, we developed a hypothesis that we could use a biodegradable microneedle array. The array could pierce the dura and delivery the steroids into the subdural space directly at the injury site. The microneedle array would be made of a biodegradable polymer that would provide a controlled release for 24-72 hours and maintain a level of 8 μg/mL of dexamethasone. We began by developing a polymer that could achieve this target window. This preliminary work was conducted by Jessica Johnson and is outlined in this paper.

2.3.1 *Applications of Microneedles*

The field of transdermal microneedle arrays is rapidly growing. Microneedle patches allow for localized drug delivery with applications for burn victims, child vaccinations, and diabetic patients [8]–[10]. To date, there are no known applications of microneedles for transdural drug delivery.

2.3.2 *Hydrogels and Controlled Release from Polymers*

To achieve a biodegradable microneedle array that can pierce the dura, we chose a hydrogel mixture. The field of hydrogels is vast, so we focused on work being done for transdermal applications. Specifically, poly-ethyl glycol diacrylate (PEG-DA) was shown to encapsulate different drugs and provided the structural support to pierce the dermis [8]–[10]. This monomer wass mixed with the cross-likening agent vinylpyrrolidone (VP). This solution was then polymerized under ultra violet light to create a polymer network that encapsulates dexamethasone.

2.4 QUANTIFICATION

2.4.1 *Ultraviolet-visible absorption (UV-Vis) Spectroscopy*

Ultraviolet-visible absorption spectroscopy is a prevalent method to quantify a wide variety of molecules. It has inexpensive and quick operation without extensive sample preparation before receiving results. UV-Vis works by emitting light within the UV to visible light spectrum through the sample, and then a sensor measures the amount of light transmitted (T). Using formula 1.1, the absorbance (A) of the sample can be calculated. Using Beer's Law, the concentration of the sample can be determined [11], [12]. Comparing the results to a known, linear standard curve, the

unknown concentration of the sample can be computed. This method can be helpful when samples include very distinct analytes that have different absorbances. This method struggles when the sample matrix has a variety of chemicals that may have similar structures. To help separate the absorbances, an analyte such as dexamethasone can be fluorescently tagged to distinguish it from the rest of the matrix.

Equation 1.1 Transmittance to Absorbance

$$A = -\log(T)$$

2.4.2 *Liquid Chromatography - Mass Spectrometry*

Liquid Chromatography coupled mass spectrometry (LCMS) is a highly robust system for quantifying the amount of analyte in solution. It uses two different techniques, first separating different compounds from solution using liquid chromatography. In this phase, the samples are mixed with a gradient of solvents; usually, one is aqueous, and the other is organic. This solution is then run through an HPLC column wherein the molecule's physical and chemical attributes determine its affinity to the column and determine the speed at which it travels through the column. This separation allows molecules with similar absorbance or structure to be quantified using MS or UV-Vis at varying times [13].

After the sample has been separated, it can then be quantified using the MS. This is done by a process known as electrospray ionization. The machine charges the solution in a capillary tube which charges individual droplets. These droplets are then sprayed through a sensor and as they land on and evaporate, they impart a charge onto the sensor. From this, a mass to charge ratio (*m/z*) can be measured [14]. The MS machine used in this project also includes a collision gas,

argon, which is added into the stream that causes the molecules to fragment, introducing daughter ions that can also quantify to help determine a specific analyte.

The machine outputs a chromatogram (figure 1) which includes time on the horizontal axis. The retention time of a specific molecule, such as dexamethasone, should remain consistent across all trials. The intensity of the signal is on the verticle axis. We can solve the area under the curve to understand total intensity at a given time point. When compared to a standard linear curve, the concentration of analyte in the solution can be determined.

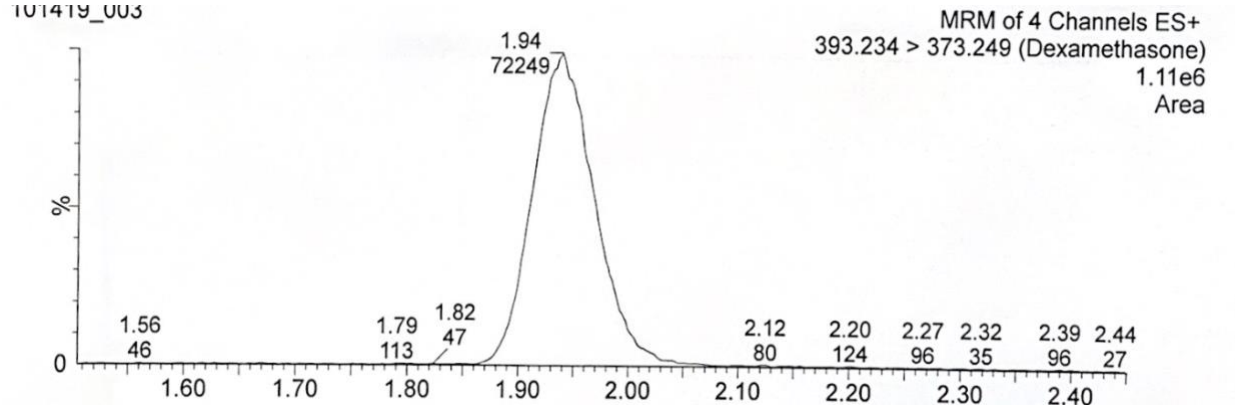

Figure 1: Example chromatogram of dexamethasone.

2.5 IN-VITRO MODEL

2.5.1 *Microglial Cells*

Microglial cells are a type of macrophage cell located within the central nervous system (CNS). Their primary role is to remove dead or diseased neurons within the CNS. Microglial cells are one of the primary drivers of the inflammatory response present after traumatic injury. They release nitric oxides and cytokines that drive inflammation within the tissue [15], [16]. Because of these properties, we use them as the cell lines within our in-vitro studies.

2.5.2 *Nitric Oxide*

Nitric Oxide (NO) is naturally produced by the CNS and is used for several cellular functions. In the CNS, it helps with cognitive function, maintenance of the synapsis, and other bodily functions. Microglia cells, when under stress, produce excess NO, which becomes toxic to the surrounding neurons. This excess causes cells to become cytotoxic and undergo apoptosis [16]–[18]. Because of NO's role in progressing the secondary injury phase, we have chosen it as a marker to determine the decrease in inflammation within our in-vitro model.

2.5.3 *Cytokines*

Cytokines are a type of protein secreted by cells as a signaling agent. They have many functions, but a primary factor of cytokine production is to elicit an inflammatory response. In the CNS, interleukins (IL) and tumor necrosis factor (TNF) are overproduced when the tissue is damaged. This leads to inflammation within the tissue and drives many neurons to cell death. For our studies, we look at our system's ability to reduce IL-1b, IL-6, TNF- α, and MCP-1 as these have been linked as crucial cytokines in the inflammation immediately post-injury [19], [20].

Chapter 3. POLYMER DESIGN

3.1 CHAPTER OVERVIEW

In this chapter, I introduced the polymer composition used throughout my project. This work is based on preliminary work done by Jessica Johnson. The solution is created as a monomer mixture of vinylpyrrolidone (VP) and poly(ethylene glycol) diacrylate (PEG-DA) [9]. This monomer solution can be polymerized under UV light using the photoinitiator, irgacure2959,

which caused the VP to begin cross-linking with itself and the PEG-DA molecules, making a dense network that can be used to encapsulate our drug, dexamethasone.

3.2 Polymer Composition

Polymer

The monomer solution was prepared in a 95/5 *w/v* ratio of VP to PEG-DA. 1.018mL VP, 312.5uL PEG-DA-700, 9.38mg of irgacure2959, and 469uL of PBS were added to a 15mL falcon tube (Table 1). The tube was then covered in aluminum foil and vortexed for 1 minute. The tube was then stored in a 2 °C refrigerator.

Table 1: Volumes of chemicals used to create VP-PEG-DA monomer solution.

	Molecular Weight (g/mol)	Ratio	Moles (mol)	Weight (g)	Volume (μL)
VP	111.14	95	0.0095	1.0583g	1018
PEGDA700	700	5	0.0005	0.35g	312.5
Irgacure2959				.00938g	
PBS				.469g	469

The dexamethasone-loaded monomer solution was prepared by adding 200 μL of VP to 20 mg of dexamethasone in a 5 mL Eppendorf tube. The solution was vortexed for 1 minute. 2 mL of

VP-PEG-DA monomer solution was added to the tube and vortexed for 1 minute. The tube was then covered in aluminum foil and stored in the refrigerator.

3.3 Preliminary Release Data

Initial testing of the gel produced excellent results from both gel and microneedle formats. The release was consistent and surpassed the 8 μg/mL goal of the project. With these results, we moved forward to the transdural release outlined in chapter 6.

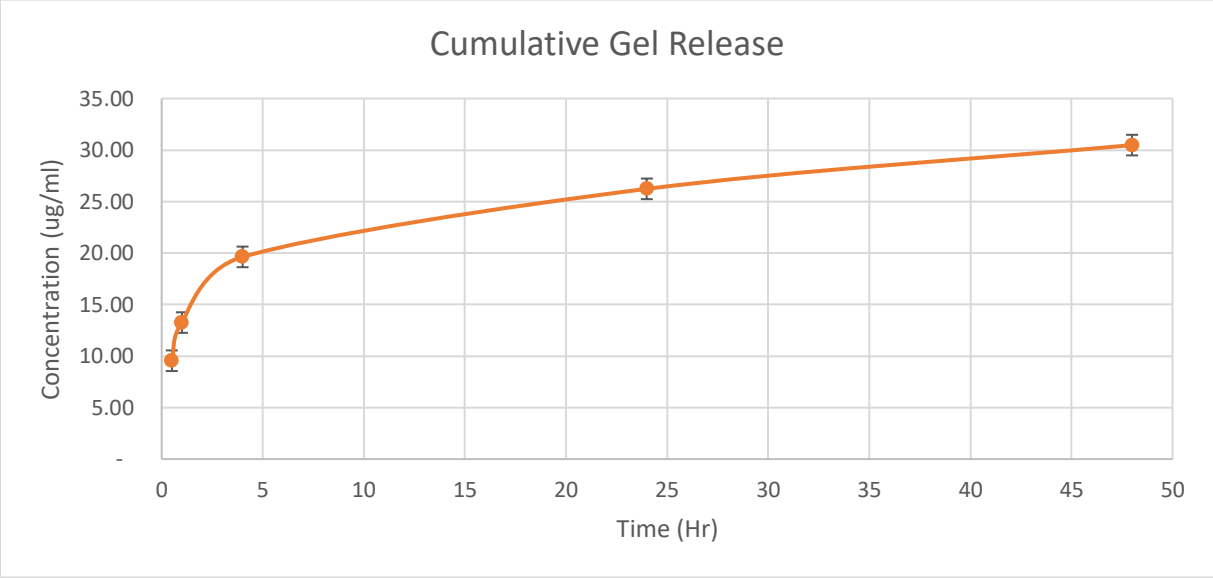

Figure 2: Cumulative release of F-Dexa from PVP-PEG-DA gels placed in a microcentrifuge tube for 48 hours (n = 5)

Chapter 4. MICRONEEDLE FABRICATION

4.1 Chapter Overview

In this chapter, I outline the process of how I designed and manufactured microneedles of various sizes. At the core of the process is a three-stage molding technique. A positive mold of the microneedle array is created using 3D printing tools and is then cast into polydimethylsiloxane

(PDM) to create a negative mold. With this mold, the monomer-drug solution can be vacuum loaded in and cured under UV light. This mold is re-usable and has been shown to sustain over 50+ castings before mechanical failure.

4.2 MICRONEEDLE DESIGN

There were many iterations of the microneedle design throughout this work. The initial design focused on sizes that would fit future in-vivo work in rodents [21]. An array of 15 x 15 conical needles, each with a height of 300μm and a base diameter of 100μm, sat on top of a 200μm base layer that connected the array. Each needle is 300μm away from the center point of the neighboring needle. These designs were drafted in Fusion360 (Autodesk) and exported as an STL file. Later iterations of this design were done to fit better the in-vitro transdural experimental design outlined in chapter 6. For this design, multiple ideations were done to produce an array that could pierce the dura substitute and deliver the correct payload. These needles were conical and had a height ranging from 0.5 – 5mm but a consistent base diameter of 1mm. These needles sat on top of a base layer with a height of 1mm. Spacing varied with arrays of 5 x 5 or sparse arrays with only a single needle on all four corners of the base layer. This was done to test a theory that the dense array of needles acted as a "bed of nails" and therefore were unable to pierce on the individual level.

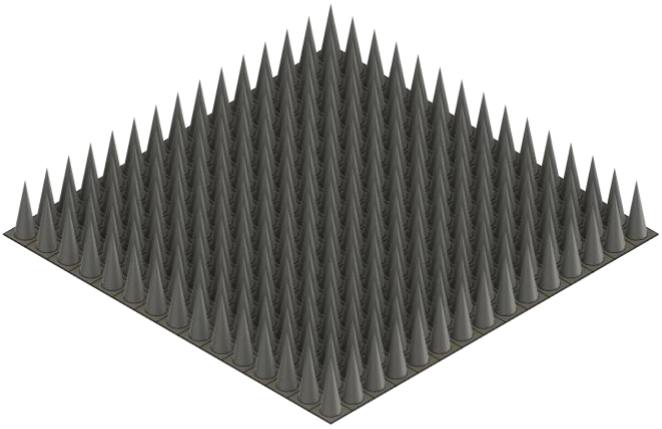

Figure 3: 3D model of the microneedle array. 15x15 array

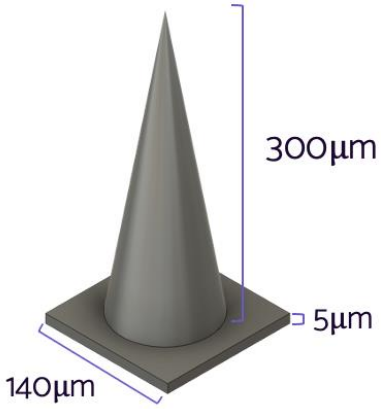

Figure 4: Individual needle dimensions. Height: 300um, base diameter: 100um.

4.3 MICROFABRICATION

To fabricate the microneedles, we developed a three-stage molding process. The process began by manufacturing a positive mold. We chose to use 3D printers to make the positive molds, as this gave us the flexibility to change design quickly compared to milled or prefabricated metal molds. After the initial design was printed, we then cast it into PDMS. After curing, this negative mold was vacuum loaded with our monomer solution and cured under UV light. The resulting polymer array was extracted from the mold and used for experimental testing.

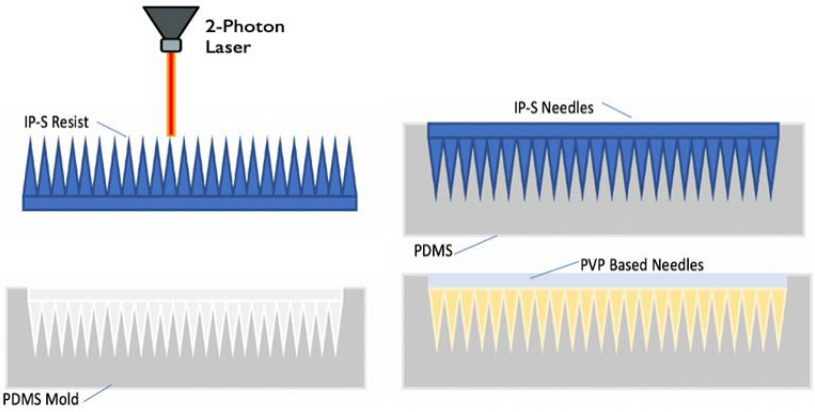

Figure 5: Diagram of the proposed molding process.

4.3.1 *Nanoscribe*

The positive microneedles were fabricated using a three-dimensional photolithography tool called Nanoscribe (Nanoscribe, Gmbh.). The machine uses a two-photon polymerization laser to polymerize IP-S resist on a plasma-treated silicon wafer. The microneedles were printed in a 15x15 array. The dimensions of the conical needles were 300μm in height with a 100μm diameter base. The needles were printed on a 50μm base layer with a center to center spacing of 140μm. After polymerization, the needles were developed in SU-8 Developer for 20 minutes. Needles were then cleaned with isopropanol solution to remove any un-exposed IP-S resist.

4.3.2 *PDMS Molding*

The positive molds were then cast into PDMS. The silicon wafer was placed into a small container made of aluminum foil. A 10:1 ratio of PDMS base polymer solution was mixed with a curing agent (SYLGARD184, DOW). For this application, 10g of the base were mixed with 1g of curing agent. The resulting mixture was mixed for 5 minutes and placed into a vacuum chamber for 10 minutes to remove any bubbles. The mixture was then poured into the foil container until the entire array was covered with 4-5mm of mixture. The container was then placed into the

vacuum chamber for 10 minutes to remove any air bubbles. The container was then placed into an oven for 2 hours at 80 ºC. The mold was then allowed to cool, and the foil was removed. The silicon wafer was gently peeled from the PDMS mold, making sure not to leave any positive microneedles in the mold.

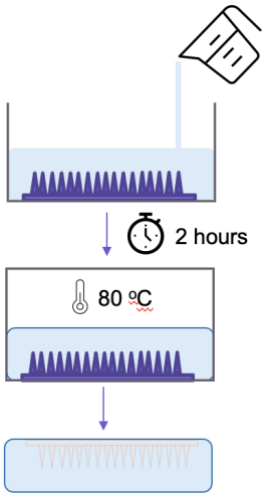

Figure 6: PDMS molding process. First, the solution is poured over the molds. Second, the container is placed into an oven for 2 hours at 80 ºC. Finally, the positive mold is removed.

4.3.3 *UV Curing*

With the negative mold complete, the polymer could be loaded. 10uL of VP-PEG-DA monomer solution was pipetted onto the mold and placed into the vacuum chamber for 5 minutes. The mold was then inspected under the microscope to verify that all microneedle wells were filled with monomer solution. The mold was then placed in a foil-wrapped glass dish. A small Kimwipe was soaked in DI water and put into the dish. A glass lid was placed onto the container. The dish was then placed under UV light. The light emitted UV light with a wavelength of 365nm for 30 minutes. After curing, the polymer microneedle array was carefully removed from the mold using forceps and stored in an Eppendorf tube.

4.3.4 *Resin Printer*

For features larger than 350μm, a UV resin printer was used to print positive molds (ASIGA, MAX UV). This printer had a resolution of 24μm per layer. The printer used 385nm UV light to polymerize a proprietary monomer solution (PlasCLEAR, ASIGA). After printing, the print was placed into an isopropanol bath for 5 minutes to remove any uncured resin. The array was then cured under 6000 flash cycles of UV in the range of (300nm – 700nm). This method was used to print an array with conical needles ranging from 0.5 – 5mm in height and a base diameter of 1mm on top of a base layer of 1mm height.

4.4 Discussion and Reflection

Overall, the final manufacturing system was efficient and could produce multiple polymer microneedle arrays. During the development of the system, we found that printing microneedle onto quartz wafers caused poor adhesion. This would cause microneedles to come off the slide while printing and during the PDMS molding stage. There were also issues with the slide surface being unlevel, which caused prints to fail. An example can be seen in figure 7. These issues were resolved by working closely with the Nanoscribe team, refining our print method, and switching to a silica wafer. These changes allowed for better adhesion to the slide, allowing up to five negative PDMS molds to be made.

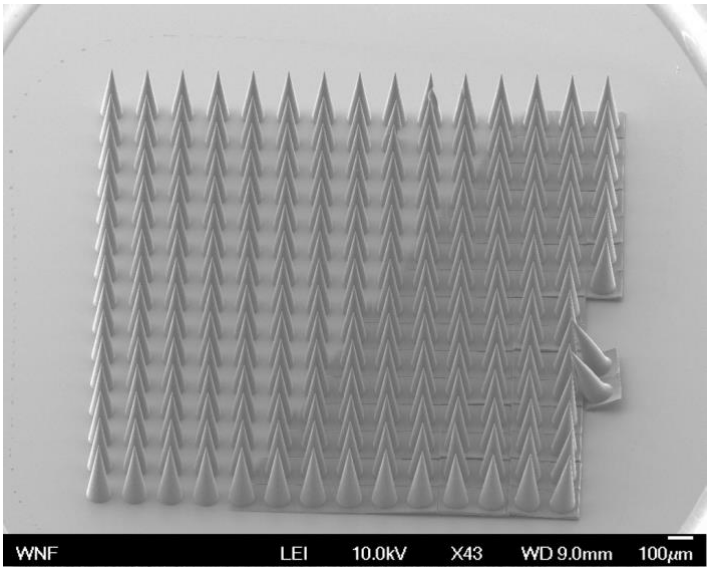

Figure 7: SEM image of failed Nanoscribe print.

After resolving the adhesion issues, we focused on the loading and polymerization of the monomer solution. The vacuum chamber was adequate for loading our monomer solution. We had issues with more viscous solutions, as there was not enough force to consistently load each microneedle well. We also had concerns about the sharpness of each microneedle. Therefore we examined the arrays at various stages in a mold's lifecycle to evaluate the consistency. These images can be found in figure 4. The tips of the needles started to thin out or break off. There is also a slight curve in the needles, which we believe is caused by the extraction of the arrays from the mold. To avoid this, we began ensuring all arrays were extracted vertically and not at an angle.

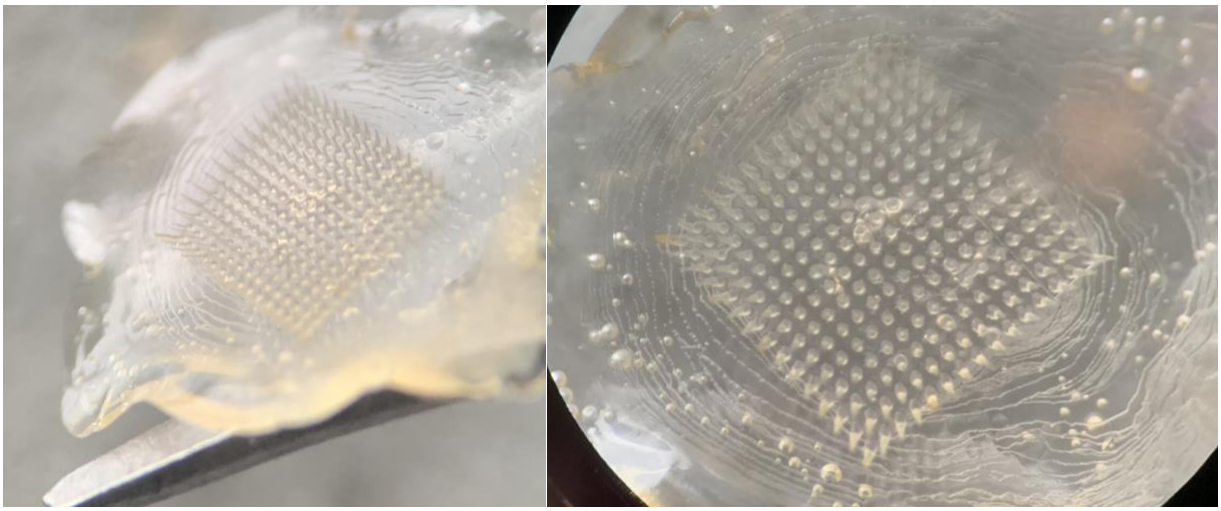

Figure 8: Image of finished PVP-PEG-DA microneedle array. Side view (left), top view (right).

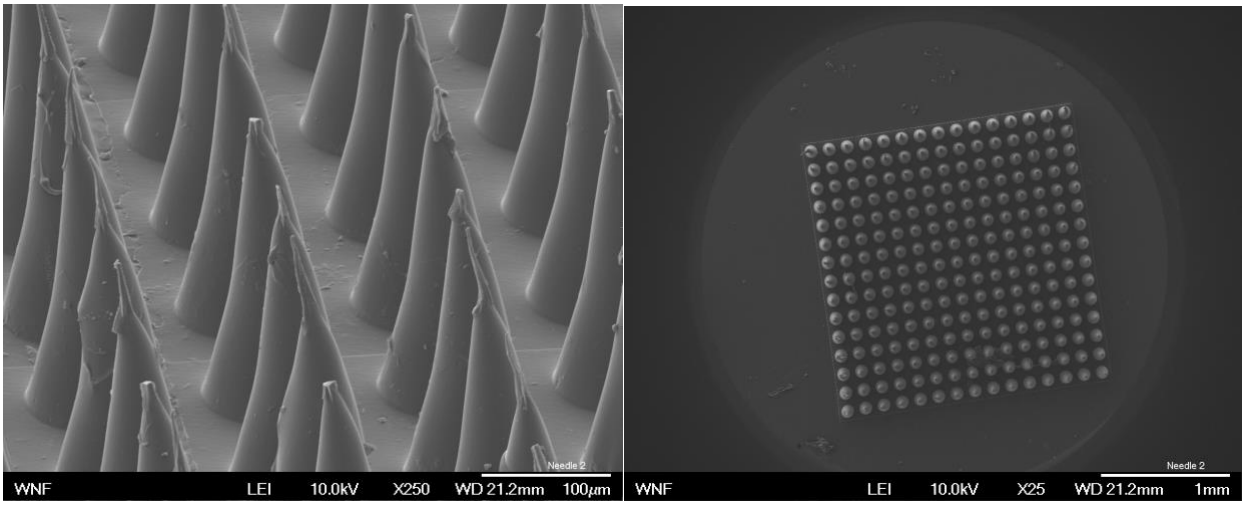

Figure 9: SEM image of PVP-PEG-DA microneedles. Side view (left), top view (right)

Chapter 5. DEVELOPMENT OF A QUANTIFICATION METHOD USING HPLC-MS

5.1 CHAPTER OVERVIEW

In this chapter, I outline the process of developing a high-throughput HPLC-MS method for the quantification of dexamethasone from a variety of mediums. We developed this method in partnership with the Mass Spectrometry Facility in the Department of Chemistry at the University of Washington.

5.2 METHOD DEVELOPMENT

5.2.1 *Equipment*

A Waters Quattro Micro quadrupole tandem mass spectrometer was used with a 1525u LC pump and a 2777 autosampler. For LC separation, a Zorbax Extended C-18 μm HPLC column from Agilent was used. The final method uses 1% Acetic Acid/5% Acetonitrile/94% H_2O for mobile phase A and methanol for mobile phase B. Argon gas was used as the collision gas with nitrogen gas used in the cone.

5.2.2 *Method*

5.2.2.1 Liquid Chromatography

Table 2: Mobile phase gradient for LC-MS method.

Time (min)	Flow (ml/min)	%A	%B	Curve
0	0.2	70	30	
4	0.2	5	95	6
6.5	0.2	5	95	6
7	0.2	70	30	6

The total run time was 10 minutes, and the analyte retention time was found to be 3.71 minutes.

5.2.2.2 MS Method

The MS was tuned with a capillary voltage of 3.5kV, cone voltage of 22V, extractor at 3V, and RF lens set to 0.2V. Source temperature was set to 100 ^{o}C and desolvation temperature to 350 ^{o}C. Parent peak was set at 393.2 with a daughter peak of 373.5. Parent scan was set to 56 and a mass of 288.21, a span of 1, and a gain of 1. The daughter scan was set to 288.3, with a mass of 172.3, a span of 1, and a gain of 6.

5.2.2.3 Sampling

Before each run, the machine was tested by running standard five and evaluating the peak of previous runs. The start of each run was the standard curve in ascending order. A MeOH wash sample was run before and after each standard curve. Samples were run in groups. All samples from a variable at a given time point were run, followed by a MeOH wash. After all groups in a time point were run, the standard curve was rerun to monitor drift. After all samples were run, the standard curve was rerun.

5.3 Discussion and Reflection

Switching to LC-MS provided a more accurate system of quantification compared to UV-Vis. Early methods included ammonium acetate for mobile phase A, which caused precipitation during the run, causing the system to shut down due to overpressure. We resolved this problem by switching to the 1% AA/5% ACN/94% H_2O mobile phase A. We can use this method for various mediums such as PBS and future in-vivo work with processed tissue samples. The system can be improved by including an internal standard to each sample, such as flumethasone. Flumethasone has a very similar chemical structure and can account for any loss during the processing and quantification of the samples.

Chapter 6. EXPERIMENTAL STUDY OF THE CONTROLLED RELEASE OF DEXAMETHASONE FROM PVP-PEG-DA MICRONEEDLE ARRAYS

6.1 Chapter Overview

In this chapter, I describe the process of testing the microneedle's ability to deliver dexamethasone into a solution. I introduce two testing methods, one where I completely submerge the array in the solution, which allows for degradation from all sides and gives insight into the total loading capacity of the microneedle arrays. The second experimental design includes a transwell insert that simulates the microneedles' trans-dural drug delivery aspect. In this setup, I place a piece of collagen dural substitute in a custom well-insert, and the microneedles are pressed

into this dura. The solution below these inserts is then sampled, and the transdural delivery can be accurately quantified using the methods described in chapter 5.

6.2 Experimental Design: In-Solution

6.2.1 *Experimental Protocol*

Microneedles were manufactured based on the process outlined in chapter 4. Microneedle arrays were placed into a 1.5mL Eppendorf tube with 1mL of PBS. Tubes were then placed in a holder in an incubator at 37.8 ^{o}C for 24 hours. At the 30-minute, 1 hour, 4 hour, and 24-hour time point, tubes were removed from the incubator, and the entire 1mL content was transferred using a pipettor to new Eppendorf tubes. PBS was replenished, and tubes were placed back into the incubator. The tubes of PBS solution that were removed were then placed into a refrigerator until all samples were collected. After the 24-hour time point, all samples were filtered through a 0.2µm PVDF filter before being added to 1.5mL autosampler vials. Samples were then quantified using the HPLC-MS method outlined in chapter 5.

6.2.2 *Results*

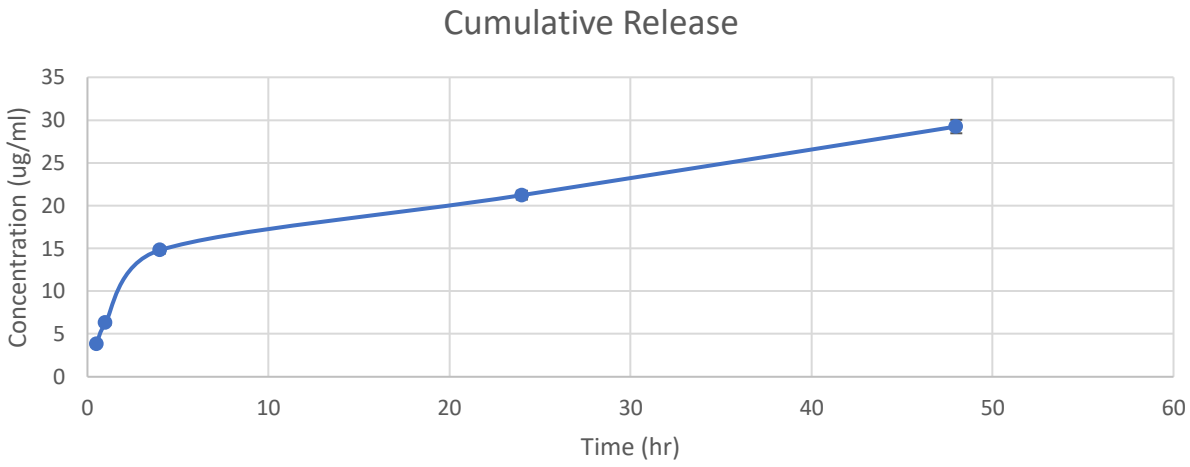

Figure 10: Cumulative release from microneedle arrays within a microcentrifuge tube over 48 hours (n = 5).

Initial results were promising, with the microneedles sustaining a controlled release over 48 hours. The arrays released 30% of their loading capacity and surpassed the 8 μg/mL goal. With the arrays being able to encapsulate and exceed the target range, we introduced a new experimental method to better replicate the microneedle arrays' transdural delivery.

6.3 Experimental Design: Transdural

6.3.1 *Transwell Inserts*

To more accurately simulate the trans-dural delivery of the microneedles, a novel transwell insert was developed with Joyce Huang. This transwell insert was designed and printed with a 3D printer using PLA. A collagen dural substitute can be glued into these inserts, which allow for suspension directly above the solution in a 12-well plate. The microneedles can then be pressed into the dura from above, replicating the insertion into the actual dura and the delivery of dexamethasone into the subdural space.

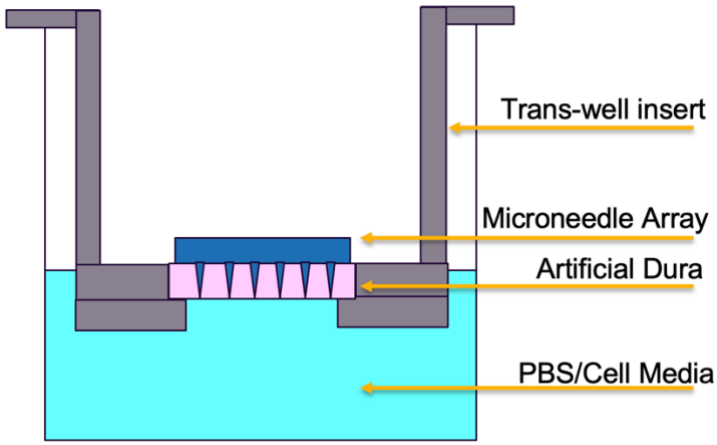

Figure 11: Transwell inserts design.

6.3.2 *Experimental Protocol*

The experiment was done using 12 well plates. Each well was filled with 1.5mL of PBS. Microneedles were manufactured in the method outlined in chapter 4. A 1cm x 1cm square of the dural substitute was cut and glued into the transwell inserts using cyanoacrylate. The glue was allowed to cure for 5 minutes before the dura was hydrated using 1mL of PBS. The PBS was allowed to hydrate for 5 minutes. The microneedle arrays were then placed onto the dura and, using thumb pressure, pressed into the dura. The well inserts were then placed into the 12-well plates. The lid was placed on top, and the plates were put into the incubator for 24 hours. At the 30 minutes, 1 hour, 4 hours, and 24 hours' time points, the plates were removed from the incubator. All 1.5mL of PBS was removed and stored in separate Eppendorf tubes. The PBS was replenished, and the plates were returned to the incubator until the next time point. The tubes of PBS taken from the plates were stored in the refrigerator until the 24 hours of release were complete. Each sample was filtered using a 0.2μm PVDF filter before being added to autosampler vials. The samples were then quantified using the method outlined in chapter 5.

6.3.3 *Issues with Design*

The design had a few critical issues. The microneedle arrays had difficulties piercing the dura, with very little penetration for the 300μm needles. To improve this, we altered the design to needles ranging from 0.5mm to 5 mm. We tested each one for its ability to pierce the dura, settling on 4mm. Another issue arose when placing the needles into the dura; the arrays quickly pulled themselves out of the dura and did not remain inside the dura for the duration of the test.

6.3.3.1 Introduction of Locking Mechanism

To fix the issue of the microneedles not staying within the dura, we introduced a new locking mechanism. This lock was a small plate that could be 3D printed and pressed into the well insert after the array was placed. This plate stayed in place due to friction for the duration of the experiment. An image of this locking mechanism can be seen in figure 4.

Figure 12: Transwell inserts locking mechanism. 3D model (left), photo of lock in place (right)

6.4 Design Alterations

6.4.1 *Design Changes*

One of the most significant issues we found with the microneedle arrays was the "bed of nails" phenomenon, where we could see individual needles pierce when tested, but the entire array would not pierce. The needles were too close together and did not have the height to pierce through the dura properly. To reduce this effect, we changed the design of the array to be much larger and more spread apart. We found that 4mm was the ideal length to cross the entire dural substitute. A few design iterations can be found in figure 13.

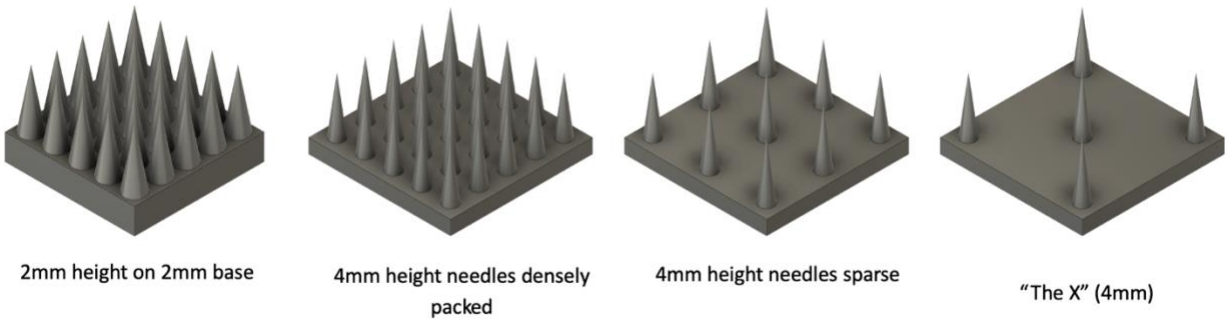

Figure 13: A few design iterations that were tested on both abilities to pierce the dural substitute and how far the needles penetrated the dural substitute.

6.4.2 *Results*

After multiple design alterations, the one that provided the best results was an array with 4mm microneedles spread out over a 10mm x 10mm surface. This design is labeled as 4mm spares in figure 13. When tested on only the dural substitute, the needles pierced and left indents in the dura. The arrays still slowly pushed out of the dura, so we used the well lock to hold them in place during the experiment. We used the same manufacturing method and experimental design as in previous sections.

Table 3: Description of variables used for transdural drug delivery experiments.

Variable Name	Description
Drug Needle (DN)	Dexamethasone loaded microneedle array
Drug Gel (DG)	Dexamethasone-loaded gel without needles placed on top of the dura. Dexamethasone and polymer volumes are equal.

Blank Needle (BN)	Microneedle array without dexamethasone.
Blank Gel (BG)	Polymer gel without needles.
Epidural (ED)	Dexamethasone solution that was pipette on the top of the dura. Volume and dexamethasone concentrations were the same.
Subdural (SD)	Dexamethasone solution pipetted into the well. Same volume and concentration as arrays.
Control	Only PBS in the wells, no treatment.

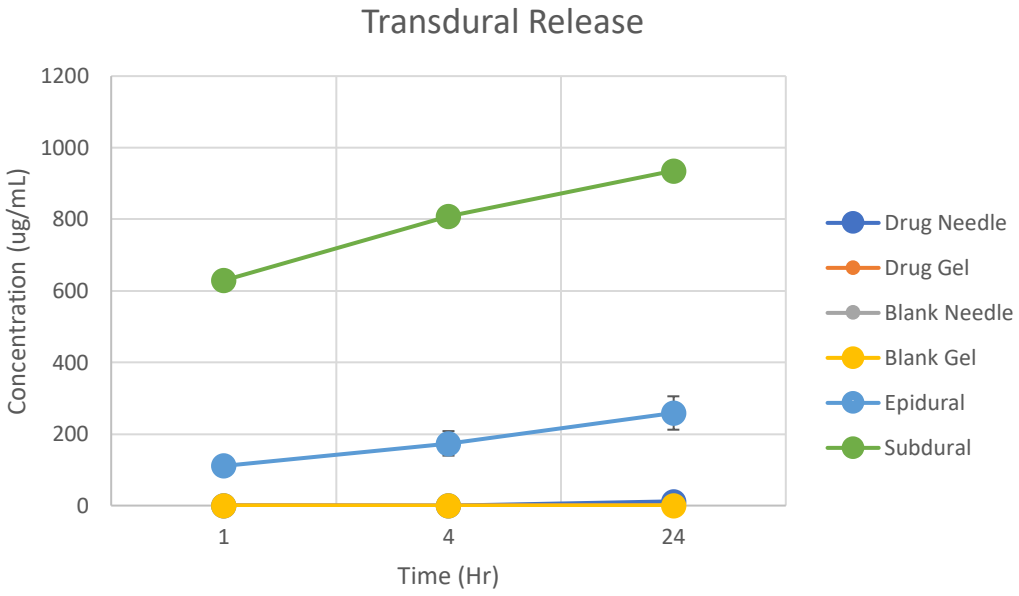

Figure 14: Transdural release from 4mm sparse arrays (n = 6)

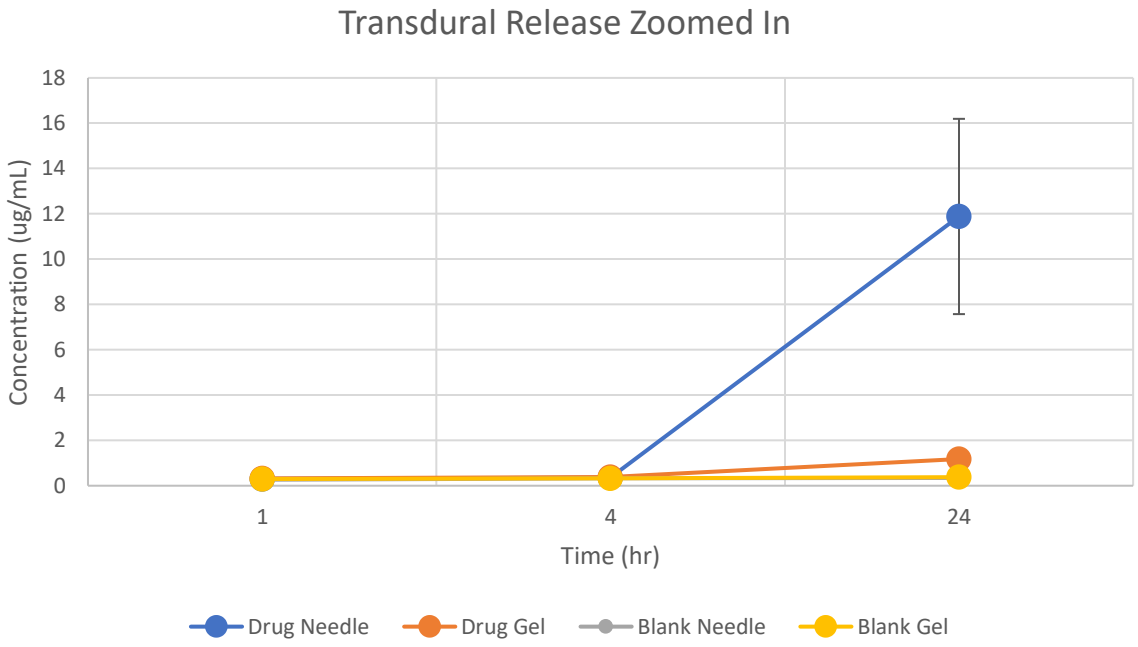

Figure 15: Transdural release from 4mm sparse arrays zoomed-in (n = 6).

6.5 Discussion and Reflection

Results of the microneedle study were mixed. We found improvements in needle stability with increased sizes but still could not overcome the "bed of nails" effect. Although the increased dimensions improved the rigidity, the needles still failed to pierce the dura completely and were still pushed out of the dura over time. The results did show that we were able to deliver some payload across the dura within our therapeutic target but only after 24 hours. The needles also underperformed, just delivering dexamethasone solution epidurally and passively diffusing across the dura. One issue with our experimental system is that the collagen dural substitute is much more fibrous than biological dura and that the needles may perform better in ex-vivo dura samples.

Chapter 7. POLYMER-COATED COLLAGEN DURA SUBSTITUTE FOR THE DELIVERY OF DEXAMETHASONE

7.1 Chapter Overview

In this chapter, I outline the design changes I made after issues with the microneedle arrays. Instead of developing a polymer microneedle array for transdural drug delivery, I leverage the surgically available collagen dural substitute as a backbone for the polymer. I hypothesized that the same polymer solution could coat this dural substitute and allow for controlled drug delivery at the injury site. I introduce a few different techniques, from creating the entire dural substitute out of the polymer, coating the dural substitute, and even soaking the substitute directly in a solution of dexamethasone.

7.2 Experimental Design: Intradural Release

7.2.1 *Changes to Setup*

After many setbacks in the microneedle design, I switched focus to utilizing the dural substitute already used in most SCI cases as the delivery vehicle. Instead of manufacturing the microneedles, the dural substitute would be soaked in the VP-PEG-DA-Dexamethasone monomer solution and polymerized under UV light. We also altered the transwell inserts to handle these design changes. Since we did not need to press the microneedles into the dura, the dura could be placed on the bottom of these inserts. The dural substitute could then be glued in the cutout on the bottom of the inserts, leaving them flush with the insert to have the same effect where only a single surface of the dura was in contact with the solution below.

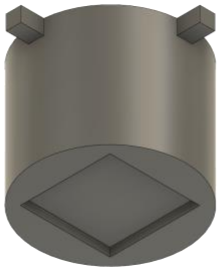

Figure 16: 3D model of the altered Transwell inserts to test intradural drug release.

7.2.2 *Experimental Protocol*

We used the same polymer formulation from chapter 3. We cut out a 1cm x 1cm square of the dural substitute. For each condition, 150μL of the monomer solution was pipetted onto the dural substitute and allowed to soak for 5 minutes before curing under UV light for 5 minutes. Once cured, cyanoacrylate was coated into the divot of the transwell insert. The polymer-coated dura was placed into the divot and held in place for 1 minute. For the control, 150μL of PBS was pipetted onto the dural substitute and allowed to sit for 5 minutes before being added to the transwell insert. As another control, a solution of 20mg of dexamethasone was dissolved into 200μL of vinylpyrrolidone. The solution was vortexed for 1 minute then 2mL of PBS was added to the solution. This solution was then vortexed for 1 minute until homogeneous. 150μL of Dexamethasone in PBS was pipetted onto the dural substitute. This condition is called *Drug-Soaked Dural Substitute*.

7.2.3 *Results*

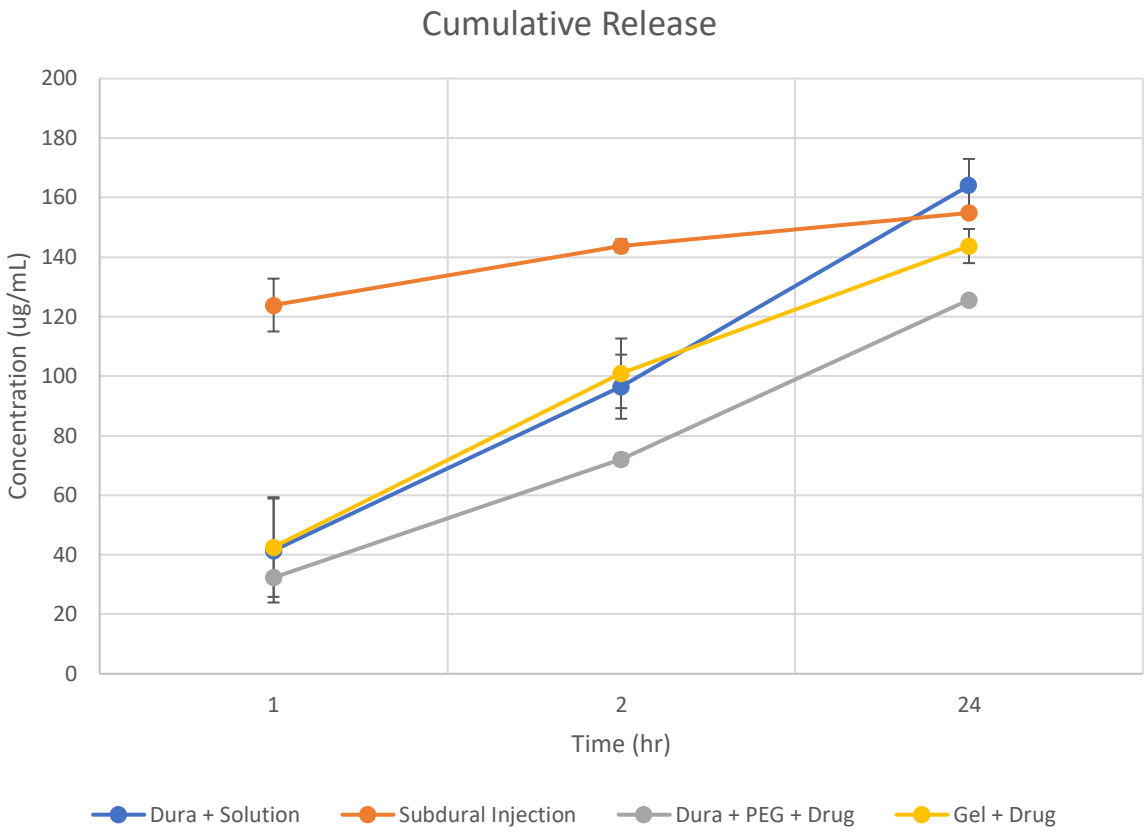

Figure 17: Cumulative release from coated dura over 24 hours (n = 4)

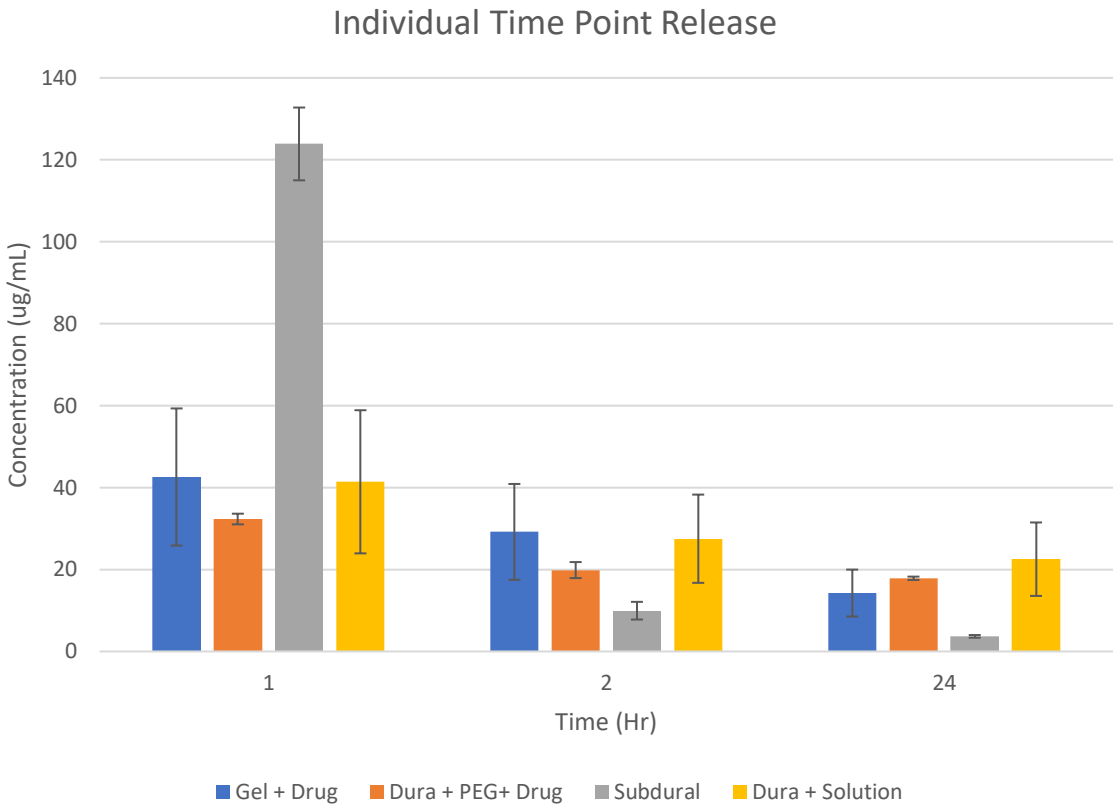

Figure 18: Individual release from coated dura at each time point (n = 4)

7.3 In-Vitro Studies

To study the bioactivity of our solution, an *in-vitro* neuroinflammation model was used. In this model, activated BV-2 microglial cells were seeded into 12-well plates, and the transwell inserts were added. After 24 hours, multiple biological assays were run to determine the cytokine presence, nitric oxide concentration, and cell survival rate.

Table 4: Description of variables used for in-vitro experiments.

Variable Name	Description
Unactivated (UA)	Cells are unactivated and have no treatment

Activated (A)	Cells are activated but receive no treatment
Drug-loaded Polymer	Dexamethasone encapsulated PVP-PEG-DA is coating the dural substitute.
Blank Polymer	PVP-PEG-DA coated dural substitute without and dexamethasone.
Subdural	An injection of dexamethasone in PBS into the activated cell media.
Drug-Soaked Dural Substitute	Dural substitute is allowed to soak in a solution of dexamethasone and PBS.

7.3.1 *Experimental Protocol*

We used a similar protocol as outlined in 7.2.2. We did all actions in a cell culture hood for this protocol, and components were thoroughly sterilized using 70% ethanol. A sterile package of dural substitute (DuraMatrix, Stryker) was used for these experiments. BV-2 microglial cells were seeded at a density of 100,000 cells per well two days before the start of the experiment. The cells had 1.5mL of Dulbecco's modified eagle media (DMEM, Gibco) with 4.5g/L D-glucose, 584mg/L L-glutamine, and 110mg/L sodium pyruvate (Invitrogen). The DMEM media include 5% fetal bovine serum (Invitrogen) and 2% anti-mycotic/anti-biotic (Invitrogen). After being seeded, the cells were incubated for 48 hours at 37 ºC to adhere to the wells properly. On the day of the experiment, a mixture of DMEM was made with the addition of 0.005% interferon-gamma (Ifn-γ, Invitrogen) and 1% lipopolysaccharide (LPS, ThermoFisher). In each treatment group except activated, the media was aspirated, and 1.5mL of activated media was added to each well. The unactivated group received 1.5mL of regular DMEM. The cells were then incubated for 1 hour. During this time, the treatment groups were prepared in a similar method as in 7.2.2, except the process took place within a sterile cell hood. After 1 hour, the well inserts were added to each

well. The unactivated, activated, and subdural groups also received a well-insert but did not have a dural substitute glued to the insert. The plates were returned to the incubator for 24 hours.

Free radical nitric oxide was quantified using a Griess reagent kit (Invitrogen) that detects nitrates in cell media. The assays were performed following the protocol provided in the Griess reagent kit. Luminex Magpix Multiplexing assay (Millipore) was used to quantify proinflammatory cytokines. The assay was performed following the protocol provided in the kit. A lactate dehydrogenase (LDH) assay was performed following the protocol provided in the kit to measure cytotoxicity. For each assay, every sample was run as a duplicate, and the average value between the two was used when reporting the results.

7.3.2 *Statistical Analysis*

Statistical analysis was performed using SPSS Statistics 25 (IBM). A one-way ANOVA with posthoc Bonferroni analysis was performed on all the biological experiments.

7.3.3 *Results*

7.3.3.1 Griess

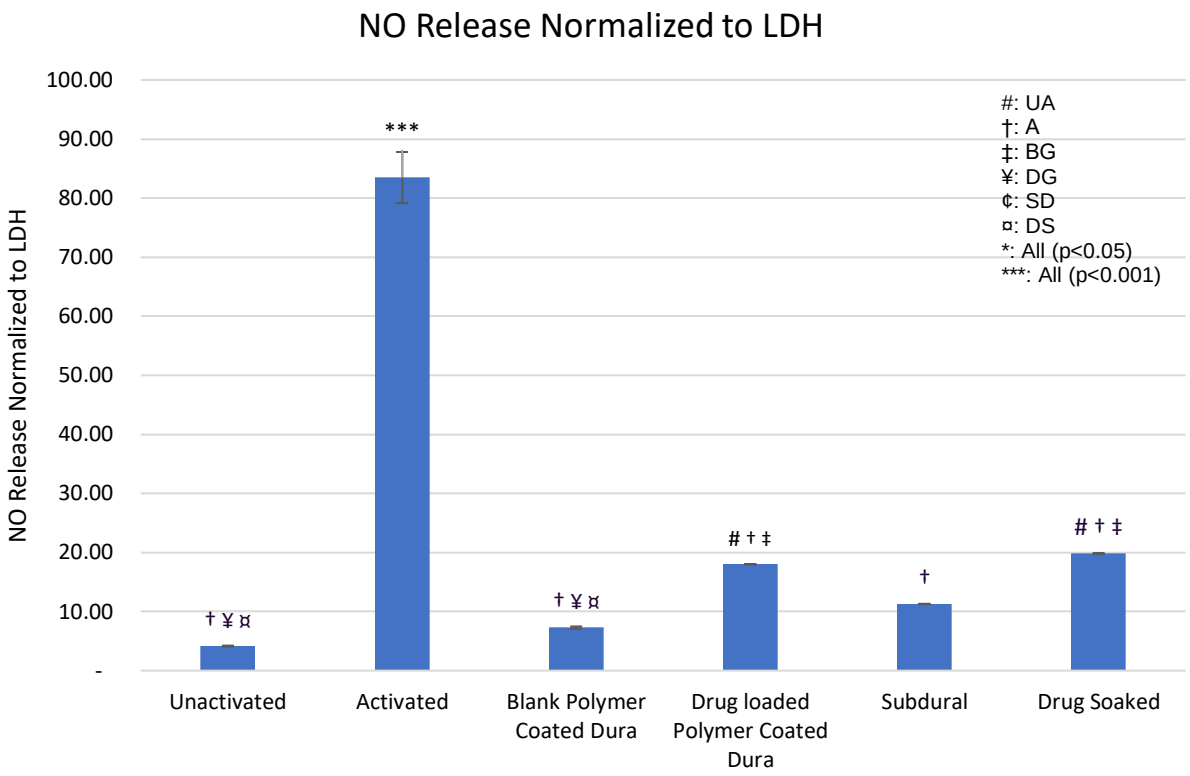

Figure 19: Nitric oxide concentration in solution normalized to LDH cell count (n = 4)

7.3.3.2 Luminex

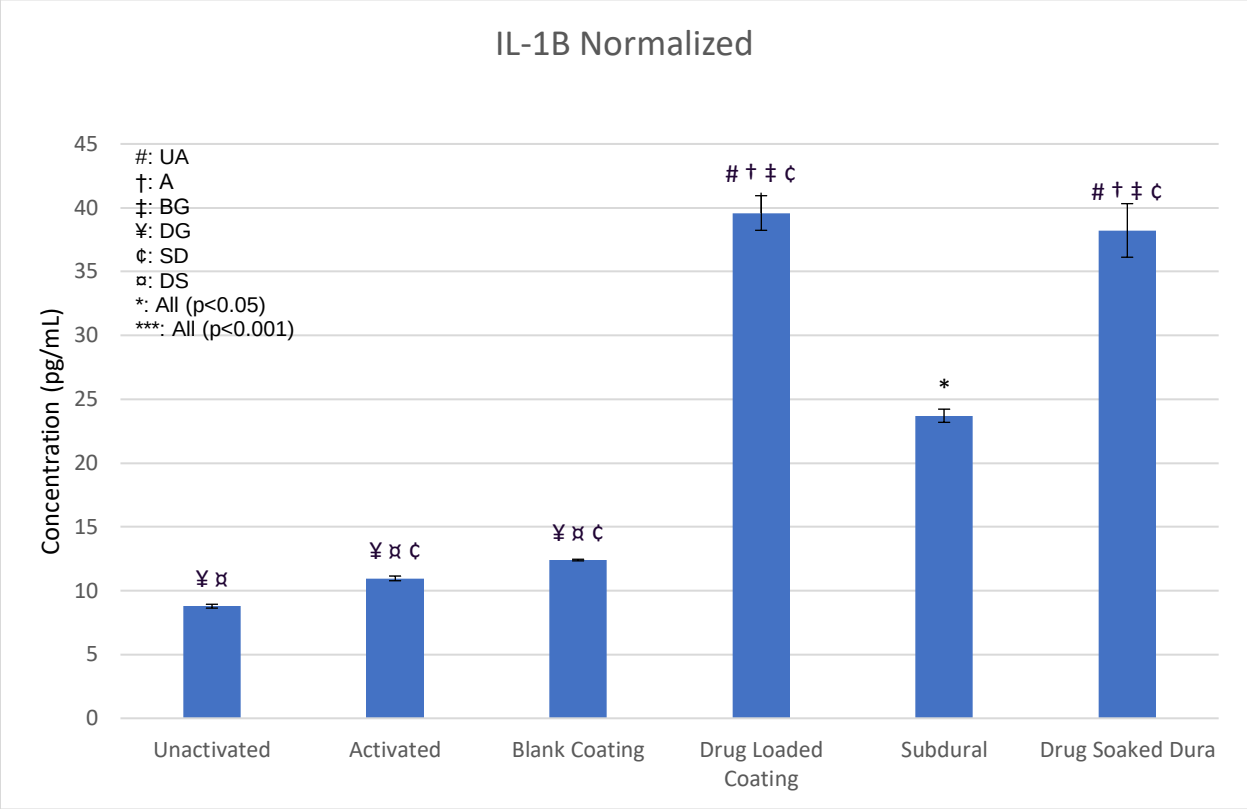

Figure 20: IL-1B concentration in solution normalized to LDH cell count (n = 4).

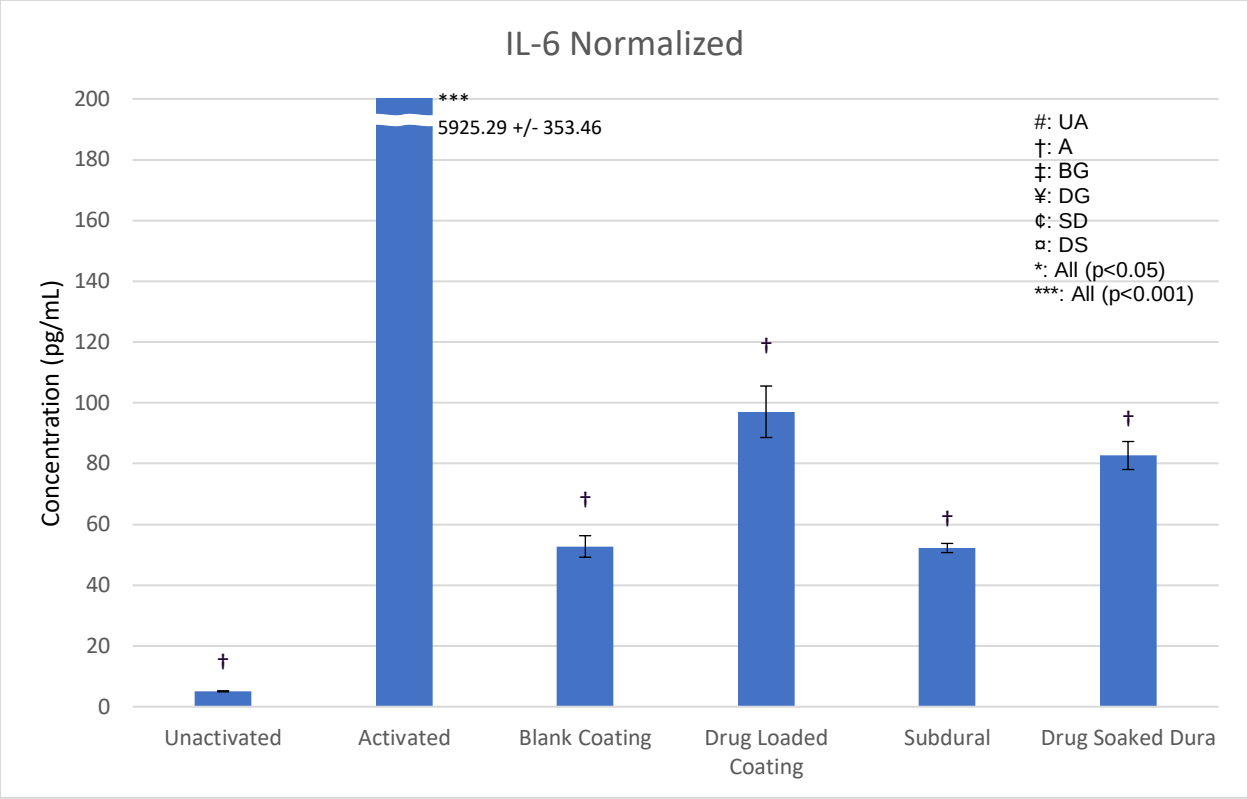

Figure 21: IL-6 concentration in solution normalized to LDH cell count (n = 4).

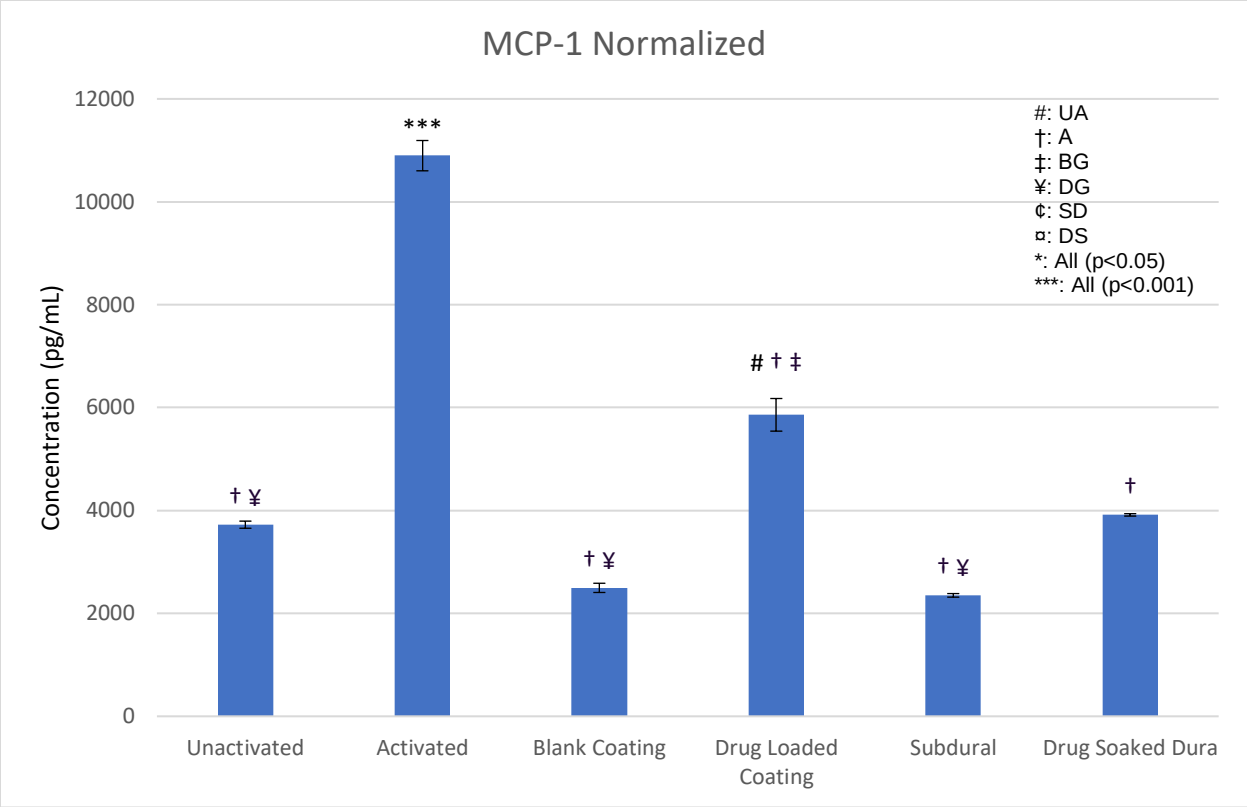

Figure 22: MCP-1 concentration in solution normalized to LDH cell count (n = 4).

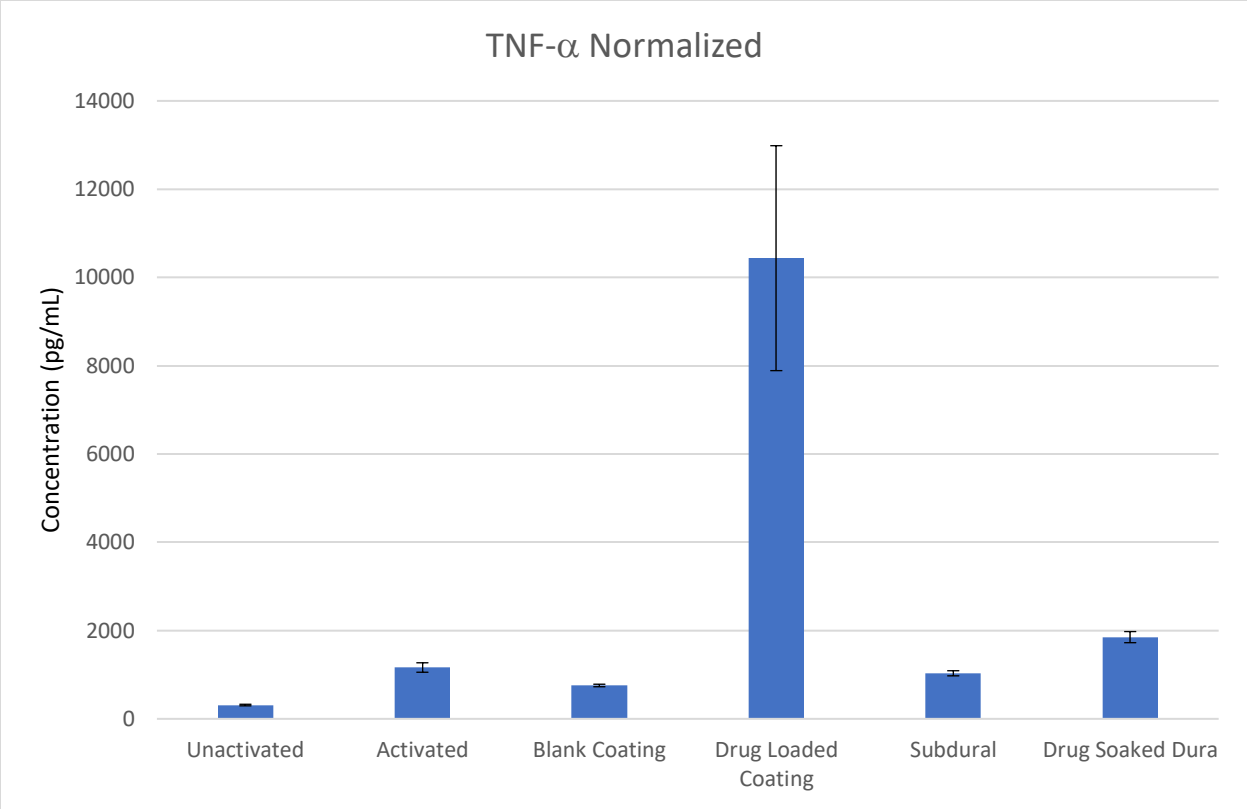

Figure 23: TNF-a concentration in solution normalized to LDH cell count (n = 4).

7.4 Discussion and Reflection

The biological assays had mixed results. There were clear signs of proper activation for NO release, and all experimental groups reduced NO concentration in the solution. The variable that reduced NO concentration the most was the blank polymer-coated dura. These were not the results that we were expecting. This could indicate that the PVP-PEG-DA polymer has some anti-inflammatory properties on its own. The Luminex kit also had mixed results. IL-1b and TNF-α both had minimal presence in the activated groups. In the TNF-α study, dexamethasone-coated dura had the highest concentration of TNF-α after normalization, which opposes predicted results Because of the improper count in the activation groups, these two studies should be repeated to

verify the results. For the IL-6 study, there was a clear sign of activation, with every group having significantly lower concentrations of IL-6; but between the groups, there was no significant value. Because of this, we are not able to conclude which method had the most significant reduction in IL-6 release over 24 hours.

Based on the results from the release and the biological studies, we found that soaking the dural substitute may be the simplest and most effective solution for localizing dexamethasone at the site of injury. This method would be easy to implement in practice and would not require the addition of any new polymers or foreign bodies when suturing in these substitutes in surgery.

Chapter 8. CONCLUSIONS AND FUTURE DIRECTIONS

8.1 FUTURE DIRECTIONS

Looking forward, we can use this work as a framework of methods to optimize a localized delivery system for the treatment of spinal cord injury. The polymer solution, PVP-PEG-DA, might not be suited for delivering small molecules such as dexamethasone but might be well suited for the delivery of larger peptides and proteins such as IL-10. Utilizing the polymer for this purpose is currently an ongoing project within the lab.

To verify the results from the in-vitro study, the experiment should be repeated. To reduce variability and guarantee optimal results, future studies should use a new BV-2 cell line. Another essential aspect that should be controlled for is including two additional groups in the study. In these groups, the dural substitute should just be suspended in solution. We hypothesis that the dural substitute may, both with and without polymer, may cause the cytokines in question to adhere to the surface. This may lead to the decreased presence in the polymer groups as the cytokines may be present but are sticking to the polymer or dura removed from the solution.

Finally, one flaw in our experimental setup is the use of the collagen dural matrix. The dural substitute is quite tough and does not pierce easily, even with metal tools. Because of this, our microneedle design may be underperforming, and alterations to adapt to this system may not translate well to in-vivo studies. Because of this, the microneedle system should be tested using ex-vivo dura samples. The microneedles may be able to pierce and stay within a biological tissue better than they do in the dense, collagen matrix.

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