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Exosome Level Retention for Spinal Cord Therapy in Canines

A Major Qualifying Project Report submitted to the faculty of
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Abstract

Spinal cord injuries in canines can lead to serious and debilitating physical issues resulting in loss of neurological function. Currently, exosomes are a new biological method being explored for therapeutic applications. These extracellular vesicles created by cells can be found in most biological fluids. While only 30-130nm in size, they contain miRNA, proteins, and lipids that could aid in cell proliferation and survival. The purpose of this project was to design, create, and test an exosome delivery vehicle that could successfully release exosomes for spinal cord injuries. The exosomes were derived from the mouse fibroblast 3T3 cell line and concentrated by tangential flow filtration and ultracentrifugation. The exosomes were loaded into fibrin and methacrylated chondroitin sulfate plugs and incubated in cerebrospinal fluid. Material degradation and subsequent exosome release were characterized by nanoparticle tracking analysis and gel electrophoresis. NTA revealed fibrin degraded faster and more particles were released. Gel electrophoresis revealed high molecular weight proteins present after material degradation and release into fluid. In conclusion, this project shows exosomes can be loaded into a degradable device and released for therapeutic purposes.

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Executive Summary

Introduction: Spinal cord injuries in canines can be debilitating and can cause serious problems for the dogs. They can be caused by various diseases and injuries but are most often caused by disc herniation. This can have numerous effects on the animal including paralysis [1]. Therapeutic treatment with exosomes gathered from mesenchymal stem cells has proven somewhat effective in treating spinal injuries but the problem is the exosomes do not last long enough in the system and thus prolonged treatment and multiple injections are required. To fix this the team hopes to create a device to facilitate the slow release of exosomes in the spinal cord.

Literature Review: Spinal injuries in canines are primarily caused by disc herniation due to physical stress [2]. As a result of these injuries dogs can suffer from many side effects and treatment of these spinal injuries often result in the animal not improving, or improving slightly but retaining permanent spinal damage. Treatment with certain exosomes derived from mesenchymal stem cells has proven somewhat effective in the treatment of these Exosomes are small extracellular vesicles that can contain proteins, RNA and lipids and aid in cell to cell communication [3]. They are generally readily absorbed by nearby cells and can deliver their cargo to various cells [4]. THis makes them an ideal transport for therapeutics. However since they are so easily absorbed they would need protection in the body to ensure they are not all absorbed immediately. To do this the team looked into various promising hydrogels. The team looked into fibrin, methacrylated chondroitin sulfate (CSMA), and silk. All are biocompatible and degrade naturally in the body and thus were believed to be good models.

Project Strategy: The device needs to be biocompatible, biodegradable, and it needs to have the exosomes maintain integrity in the injected device while limiting the rate at which they are released from the device. The device should also remain in the body for the duration of physical therapy, and be as cost effective as possible.

Methods and Alternative Designs: For our experiments exosomes were seperated from 3T3 cells cultured in serum free media via tangential flow filtration (TFF). After seperation they were loaded into fibrin and CSMA hydrogels and two negative controls were prepared. The samples were allowed to degrade and then were tested for exosomes and proteins that may be found within exosomes. Some alternative designs that were considered include cell encapsulation and drug delivery methods for transporting exosomes. Drug delivery ran into problems with sustained release whereas cell encapsulation ran into problems with how to keep the cells alive in-vivo and control there exosome release.

Design Verification: Our results were very promising, our nanoparticle tracking analysis showed that the fibrin plugs released most of their exosomes when they completely degraded, where CSMA which hardly degraded had released significantly less exosomes. Gel electrophoresis protein analysis also showed that the exosomes still had high molecular weight proteins remaining.

Final Design: For the final design the team predicts that cost may be a limiting factor. Improvement in the production and isolation of exosomes will have a net positive effect on practically all social and economical impacts. If this treatment is considered for human patients in the future the economic and social impacts may change and may need to be reevaluated.

Discussion: Based on the initial results the therapy could prove extremely effective. Our results showed that based on the hydrogel exosomes can be released slowly or quickly based on the needs of the patient. The team also demonstrated that exosomes are capable of surviving for extended periods in an active state when implanted into hydrogels.

Conclusions and Recommendations: The team suggests that more study be done on exosomes. Though initial results seem promising in-vivo testing needs to be done to determine efficacy of exosomes if this treatment is intended to be used on humans. More testing with exosome and hydrogels in canine models may prove effective and could be good indications for human models.

Chapter 1: Introduction

Spinal cord injuries in canines can be serious and debilitating. Although very common, the injury can lead to permanent loss in neurological abilities. Spinal cord injuries can be caused by mechanical forces, such as shearing, contusion, and laceration, or originate from a disease such as degenerative myelopathy (DM). During a spinal cord injury, blood and extruded disc material build up in the spinal canal which causes compression and ischemia, or lack of blood to the spinal column. As a result of the initial trauma, a series of alterations ultimately leads to apoptotic cell death, demyelination, necrosis, loss of spinal cord function, and the formation of glial scar tissue [5].

Certain spinal cord injuries, like intervertebral disk herniation, can cause complete paralysis of the lower body, and are usually treated with corrective surgery with varying success [1]. In one study with dogs, 14% of the dogs treated for intervertebral disk herniation were euthanized within 3 weeks of surgery [1]. Already more than 1 in 10 of the animals treated had no improvement and were euthanized. 58% of the dogs regained the ability to walk with Deep Pain Perception (DPP), or the sensation of pain in the muscles, tendons, joints, and skin. However, out of that group, 41% lost control of their bowel movements and 32% lost control of their bladder [1]. Current treatment of spinal cord injuries in dogs only cures slightly more than half of the dogs treated, and from that number, many still experience complications after surgery. Other options besides surgery exist as well but they also have many complications. Corticosteroids are another option for treatment, however the drugs may show no effect or show negative side effects such as diarrhea, vomiting, and pneumonia to name a few [2]. While these

treatments have moderate success, there are still evident gaps in technology as seen by the setbacks in canine recovery.

The use of exosomes as possible therapeutic agents is still a new and emerging field, but shows great potential in a variety of applications. Exosomes are extracellular vesicles (EVs) created by all cells that can be found in most biological fluids such as saliva, blood, and urine. While only 30-130nm in size, these tiny "cargo" nanoparticles are packed with miRNA, proteins, and lipids that could aid in the formation of new blood vessels, cell proliferation and cell survival [3][4]. They are capable of crossing the blood-brain barrier (BBB) and primarily assist in cell-cell communication [3]. Their unique contributions could provide a possible therapeutic for canine spinal cord injury for which there is currently no cure. Administration of exosomes could potentially promote recovery as well as reduce apoptosis and inflammation by delivery of protein regulators such as miRNA and siRNA in order to moderate expression in proapoptotic (BAX) and antiapoptotic mediator proteins (Bcl-2) as well as proinflammatory (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-10) [6][7].

In a recent trial done by Professor Andrew Hoffman and Dr. Raymond Page, a dog with DM was injected with a dose of exosomes. The initial dosage reduced symptoms in the dog and tissue began to repair. However, the dog relapsed and it was determined that further treatment with exosomes would not be effective enough to completely heal the animal [8]. Since the initial treatment was effective it was hypothesized that large concentrations of exosomes must be maintained until all of the damaged tissue is repaired. Because DM has a similar pathology to some forms of human ALS, canines are an ideal large animal model for such testing and a successful therapy could have implications for humans in the future. By creating a

device/repository that would facilitate the slow release of exosomes to maintain effective levels that could be easily replenished, the team hopes to make exosomes an effective form of therapy.

The team's goal is to create a device that helps facilitate exosome based therapy in dogs and potentially humans. Current methods of repairing spinal cord injuries are accompanied by a plethora of side effects and require an intense post-care regimen. Exosome therapy shows potential as a valid way of treating external traumas, however, current methods of delivering exosomes are not efficient enough to be considered an effective form of treatment. The current methods do not deliver enough exosomes to make a significant change in the patient's condition and have not been able to maintain sufficient exosome concentrations due to their rapid consumption by off-target cells in the body. The team's project will address this issue, in that the device created will release and maintain enough exosomes to improve the patient's condition.

In order to accomplish the goal of making exosomes a more effective method of therapy the team will be proposes an injectable biocompatible device that will act as an exosome repository. With local administration, the device will keep the number of exosomes within the wound site at a high level so that the therapy is effective. This device will be placed within the body via local administration (injection at the wound site) and last for an extended period of time, in order to reduce the frequency of the procedure. Once in the body the device will release the exosome therapy as necessary. The device will facilitate exosome release by slow degradation. Administration of the treatment should be relatively easy and not burden the daily life of the animal or its owner.

Spinal cord injuries are traumatic and treatment options are limited. Dogs who suffer from it often do not fully recover. The team's device would ideally improve the dog's health and

restore function. The device could have the potential to eventually move on to testing in human models upon further research and improve the lives of humans as it would in dogs.

Chapter 2: Literature Review

2.1 Spinal Injury

Spinal cord injuries in canines are most often caused by intervertebral disc herniation, which is the result of disc material pinching on the spinal column "causing mechanical injury to the vasculature and neural and supporting tissue of the spinal cord [2]." There are other spinal diseases that can affect spinal column damage, some are acute like disk herniation while others are chronic, but the resulting spinal damage is very similar [5]. This damage of spinal tissue can lead to permanent problems in canines, including paraplegia, or paralysis of the lower body, uncontrollable bladder and bowel movements, and loss of feeling and Deep Pain Perception (DPP) [1]. Current treatment methods involve surgically moving the herniated disc back into position to alleviate stress on the spinal column. While this has proven to be moderately effective it is far from perfect. In one study 7 out 18 dogs with paraplegia and loss of DPP caused by intervertebral disc herniation were surgically treated and though they regained the ability to walk, they did not regain bowel or bladder function, or DPP [2]. In another study 14% of the dogs treated for intervertebral disk herniation were euthanized within 3 weeks of surgery [1]. While surgically removing pressure from the disc on the spinal cord does prevent the loss and damage of further nerve tissue it does not promote neural healing and thus, the more serious the nerve damage, the less likely corrective surgery will help. Though some methods have been used to help promote growth in spinal column for disc herniation, like applying oscillating electrical fields to promote neural regeneration, these have shown only slight effectiveness [2]. There is a great clinical need for a therapy to help promote neural regeneration after the disc has been moved off of the spinal column. While moving the herniated disc off of the spinal column

prevents any further damage, a treatment that could promote regrowth of damaged neural cells in the spinal column could help reduce symptoms caused by the disc herniation. Though it is probable and "agreed amongst those in the spinal cord injury research community that there will not be one solely effective therapy" for spinal cord injury, so when moving forward one has to consider therapeutic methods in conjunction with surgery and other treatments [2].

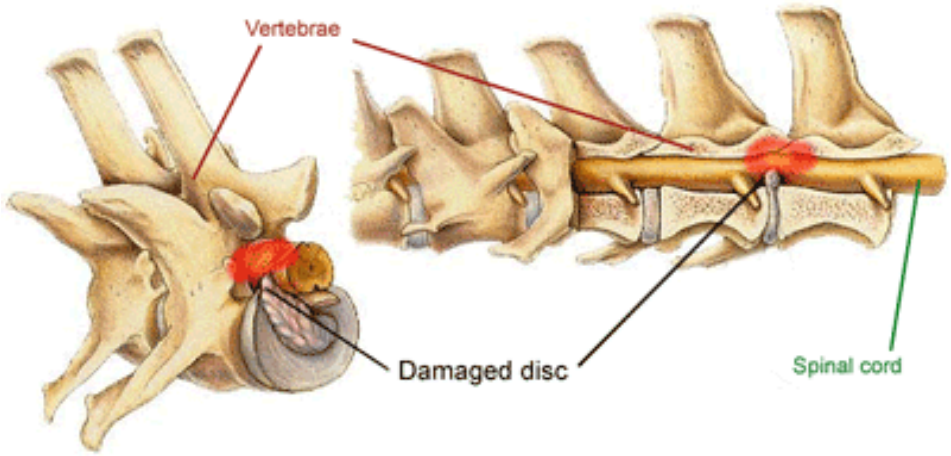

Figure 1: Herniated Disc in Canine [9]

2.2 Exosomes

The team used exosomes as a treatment method for damaged spinal tissue. Exosomes are small extracellular vesicles created by all cell types. Though all of their exact functions are unknown, it is believed that they play a role in cell to cell communication [3]. It is also known that exosomes "play pivotal roles in normal physiological and pathological conditions through

long-range signaling via blood, cerebrospinal fluid, and breast milk to modulate target cell behavior" [3]. They hold and transport numerous types of biological material, like miRNA, proteins, and lipids depending on which cell type they are derived [4]. Exosomes are created by the plasma membrane folding in on itself to form a pouch, in a process called invagination [4]. The resulting vacuoles are then treated by the "endosomal sorting complex required for transport (ESCRT)", to begin turning the vacuoles into early exosomes [4]. After exosomes are generated and transported they are absorbed by the cell, and are either "processed to lysosomes for degradation (degradative multivesicular bodies (MVBs)) or fused with plasma membrane (exocytic MVBs) for the release of intraluminal vesicles (ILVs) into the extracellular space where they are referred to be as exosomes" [4]. As a result after transportation all exosomes are either digested or recycled into the cell membrane where they can be used to generate new exosomes. Therefore exosomes produce no negative byproducts. Exosomes in general are taken up by nearby cells, though they do have surface proteins to help the cell identify exosomes before they are absorbed [4]. However tumor derived exosomes containing mRNA and miRNA have been absorbed by cells in the body, and it is believed that RNA from tumor derived exosomes could play a role in angiogenesis, cell proliferation, and cell survival [4]. As a result exosomes could be cultured to potentially aid in the cell healing process, and these cultures could use fast growing and effective tumor cells to grow a large quantity of exosomes for treatment in a short amount of time. It is important to note though that exosome composition can change as a result of stimuli and environmental conditions. These stimuli can have an effect on exosome release and their packages, for instance inflammatory proteins and molecules, such as lipopolysaccharides, TNF-α, and IFN-γ, strongly affect the composition of exosomes released by

dendritic or mesenchymal stem cells (MSCs)" [3]. Exosomes also differ from other extracellular vesicles in that they have a high lipid content that can differ based on their cell of origin [4]. The lipid content is important as it contributes to biophysical properties such as their rigidity and delivery efficacy [4]. A multitude of different lipids have been found on exosomes including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), lysobisphosphatidic acid, ceramide, cholesterol, and sphingomyelin [4]. Exosomes may also contain nucleic acids, such as mRNA, miRNA, non-coding RNAs (ncRNA) and even DNA [4]. Though the exact function of the DNA in exosomes is unknown the RNA factors are believed to regulate gene expression after delivery to cells [4].

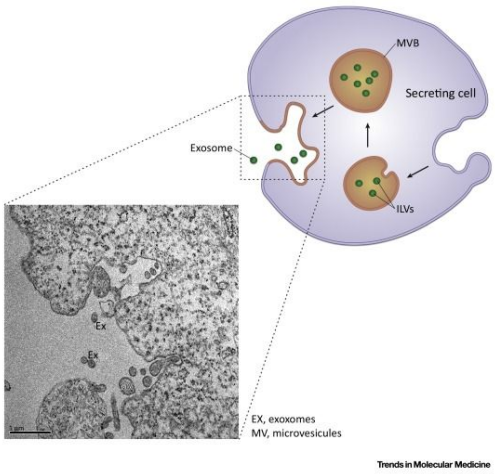

Figure 2: Exosome Production via Invagination [3]

2.3 Delivery

For effective delivery of exosomes, viable implantable materials must be considered. In general, materials need to be biocompatible, nonimmunogenic, nontoxic, and have good mechanical, thermal, and chemical stability. Ideally the material should also have modifiable

degradation and release behavior and optimal processing to sustain drug viability during drug-loading while providing continual stability for the encapsulated drug [10]. Specifically this material needs to be compatible with the spinal cord environment and cerebrospinal fluid (CSF). Exosome integrity must remain intact and the material cannot change internal spinal conditions including pH. The material also needs a robust half life for the purpose of sustained degradation and release for an appropriate therapeutic administration. Current research into exosomal effect on neuron healing and exosome circulation suggests that they will be able to survive in the environment given an effective delivery mechanism [3][4]. However it is unknown if the cerebrospinal fluid could have an effect on exosome production, delivery or cell binding capabilities. Silk, methacrylated chondroitin sulfate (CSMA), and fibrin have all been previously used in other drug delivery efforts, but none have been experimented with exosomes. Their characteristics are evaluated for the purpose of this study.

2.3.1. Silk

During the initial concepts of the project silk was a top choice. The silk fibroin being considered is a naturally derived protein from spiders, scorpions, mites, and flies, but the most beneficial form of silk originates from the silkworm, *Bombyx mori* [11]. It is produced in the posterior region of the worm's gland [12]. Silk consists of a combination of heavy (390kDa) and light polypeptide chains (25kDa) linked by disulfide bonds at the C-terminus. Together, these chains give silk an overall high molecular weight (2.3MDa) [12][13][10]. The heavy chain is an amphiphilic alternating block copolymer that has a hydrophobic region and a smaller hydrophilic region as well as hydrophilic regions at the C- and N- terminals [12][10][14]. Silk is mainly comprised of glycine, alanine, and serine [15]. Silk has been looked into for a sustained drug

delivery device as it has many desirable properties for drug delivery *in-vivo*. It exhibits surface degradation and produces non-inflammatory byproducts. It can be easily purified to remove immunogenic properties.Additionally, it can be sterilized with many standard methods, and has surprising mechanical strength, good elasticity, and is completely biocompatible [12]. This material is also economically advantageous, evidenced by the textile industry where nearly 1,000 metric tons of silk are produced annually. [11]. Silk has been used in the delivery of small drug delivery molecules for the purpose of drug delivery and by managing the silk properties, such as crystallinity and number of coating layers, the release of the drugs was effectively regulated [12].

2.3.2 Methacrylated Chondroitin Sulfate (CSMA)

Polymers are commonly used in biomedical devices for a variety of reasons. They are easy to manipulate, are easily produced, and generally have good mechanical properties of materials being put into the body. In order to fully understand which material was the best suited for holding the exosomes, the team also considered the polymer, methacrylated chondroitin sulfate (CSMA). This is a polymer in which the main branch consists of chondroitin sulphate(CS) which is then functionalized with methacrylate groups. Chondroitin sulfate is a glycosaminoglycan and located in the ECM [16]. Chondroitin sulfate is a good adhesive and has been shown to be biocompatible. It is FDA approved for wound healing and enhanced re-epithelialization. Furthermore, it can be taken orally for osteoarthritis or delivery of proteins or peptides [17]. As a gel, it has a good compressive strength as CS in its natural role is designed to absorb shock. This feature could provide support and protection and a delivery vehicle. Additionally, its compressive modulus, along with other mechanical features such as swelling

ability, can be tailored by modifying the macromer composition [18]. It is currently being used in cartilage repair, but with some modifications, such as the methacrylated functional group, it can be used in other parts of the body. CS has been shown to have anti-inflammatory properties, improve healing, and even aid in water and nutrient absorption. Methacrylate groups were added to allow for proteins and biomaterials to bridge together which is essential when putting something in the body. The device will be anchored and so won't move into undesired parts of the body [19]. Chondroitin sulfate is also beneficial because it can degrade within the body. It works as a degradable scaffold which is ideal for loading exosomes for drug delivery purposes [20]. That being said, the main use of CSMA is in cartilage repair and scaffolds for chondrocyte seeding. The implant will be put into an area without cartilage, so these differences will be considered when implanting this device into a non- cartilaginous location [20]

2.3.3 Fibrin

Fibrin is a naturally occurring protein network consisting of fibrinogen and thrombin, two proteins essential for blood clotting in response to injury. It gels enzymatically as thrombin cleaves fibrinogen converting it to insoluble fibrin. Fibrin is essential *in vivo* as a tissue adherent, holding together and supporting a variety of tissues, in addition to its role in the blood clotting cascade and wound healing. In response to an injury, a fibrin mesh is the first thing to form at the wound site adhering platelets and red blood cells to form a blood clot to staunch bleeding. Following the necessary healing, the fibrin mesh is dissolved by fibrolytic enzymes in a process called fibrinolysis, pointing to the biodegradative properties of this material [21]. In addition, its degradation properties are modifiable with the addition of aprotinin which can slow degradation

rate [22]. Being a material naturally produced by the body fibrin is also inherently biocompatible, non-toxic, and non-inflammatory. Adverse immunogenic effects can also be mitigated if the fibrin is able to be sourced autologously. Fibrin has seen use in clinical applications as a tissue sealant or glue to control surgical bleeding, speed up wound healing, and secure sutures across a wide range of surgical disciplines [21]. More recently it has also been implicated in gel form as a novel slow-release system for a variety of factors including cells, growth factors, and synthetic drugs [21]

2.4 Limitations

While exosomes' potential for therapy has garnered a lot of attention, it is still a new and growing field of study and therefore leaves some unknown properties. While various studies have shown promising results, there is still ambiguity pertaining to a defined therapeutic dosage as most studies are in the early stages of characterizing exosomes. In addition, there is still little known about exosome viability when isolated from the cell and its durability in drug delivery devices and in various conditions in the body. Exosomes' absorption can also be influenced by environmental factors and not all of the positive and negative effects of the environment on exosomes is known [3]. Without knowledge on what promotes exosome production, absorption, migration or viability, the ability to influence cell repair or other cellular processes is limited. Lack of perfect exosome filtration is another challenge to overcome before becoming clinically relevant in the future. Currently exosome filtration is not perfect and a large concentration of exosomes is required for effective treatment. The financial feasibility regarding the vast number of cells and resources surrounding this therapy need to be evaluated.

2.5 Gold Standard

Currently most treatment methods for damaged spinal cords primarily caused by disc herniation is decompressive surgery. This surgery moves the ruptured disc back into place to alleviate pressure on the spinal column and prevent further tissue damage [2]. While somewhat effective in treating animals that have paraplegia induced from mechanically ruptured discs it does not promote regrowth of damaged tissue and thus is not as effective for degenerative diseases that could lead to disc rupture or other spinal injuries. The effectiveness of the surgery itself is also in question, with many cases of dogs not regaining motor function, DPP or bladder or bowel control [1] [2]. In one instance 9 dogs comprising 14% of the group being tested were treated with decompressive surgery and were euthanized within three weeks of the surgery, 7 of which had ascending myelomalacia, which is the softening of the spinal cord, often caused by disk herniation [1]. Of the tested group 37 dogs, or 58% regained the ability to walk and DPP, 7 (11%) regained the ability to walk without DPP and 11 (17%) showed no improvement and remained paraplegic with no DPP [1]. It is also important to note that even the 58% of dogs that were deemed successful many of the dogs, (15 and 12 or 41% and 32% respectively) did not regain bowel or bladder control respectively [1]. So current surgery has a fairly low success rate among dogs with most of the dogs showing some degree of problem, some of which can be fatal. Most therapeutic methods are also fairly ineffective and in the case of herniated discs, they still require decompression surgery to alleviate pressure on the spinal column. Therapies for the spinal column involve "1) preventing secondary injury, 2) promoting regeneration and/or sprouting of remaining axons, 3) enhancing the purposeful function of remaining neural circuitry, 4) replacing destroyed spinal cord tissue, and 5) a combination of these approaches"

[2]. Though many methods have had limited results in these factors. Preventing secondary injury can be done by preventing unnecessary stress on the injured area during healing to prevent the disc from re-rupturing. One factor used to help promote enhancement of neural circuitry involves using 4-aminopyridine to block certain voltage-gated potassium channels to help improve action potential [2]. Oscillating electric fields have also been used to help promote axon regrowth in dogs with spinal injuries [2]. However these methods have only proven partially effective and can have negative side effects on the subject [2]. It is important for our treatment to consider possible side effects and attempt to improve upon typical treatment methods and consider other possible means of failure, not related to our treatment, like secondary injury.

Chapter 3: Project Strategy

3.1 Initial Client Statement

The initial client statement was decidedly vague, and rightfully so, to allow for an optimal degree of innovation. As exosome therapies are a novel treatment technique with little to no published research and clinical trials to rely upon there was a myriad of potential paths this project could have followed. The initial client statement read:

"Design an implantable system capable of retaining exosomes in their biologically active state with a porous biocompatible matrix."

This original statement left many avenues open for exploration such as the material the implantable system would be comprised of, how the system would be placed within the body, the duration of the therapy, how the treatment would reach the desired site, which condition/s this system would focus on treating, as well as a multitude of other design functions.

3.2 Design Requirements - Technical

3.2.1 Objectives

As stated previously, exosome therapies are a relatively unexplored method of disease or injury treatment. As such, there are no models for which to base the function of this therapy on and a slew of design requirements that were previously unconsidered at the conception of this

project have emerged. However, there have been a few that have been apparent from the beginning, inherent properties of any biomedical device designed for implantation within the body, namely biocompatibility, sterility, and non-immunogenicity. It is important, and a simple matter of ethics, that any biomedical device proposed as a treatment option possesses these three characteristics so as not to cause additional complications or trauma to a patient already struggling to cope with their condition.

It would also be beneficial if the device and its implantation procedure were minimally invasive and could last within the body for the full duration of the treatment. The device should not have to be constantly replaced or refilled as it causes inconvenience to the animal and its owner and could lessen the willingness to continue with the treatment process. Exosomes should be loaded, transported, and delivered to the damaged area. The device should not trigger an immune response and must successfully deliver the therapy before being metabolized or cleared.

Table 1: Objectives

Objective	Reason
Biocompatible	The device is being implanted in the spinal column. The device cannot be toxic as this is incompatible with regulations and negates the purpose of a therapy
Biodegradable	Degradation eliminates the need for device removal. Slow release allows for reduced frequency of procedure and sustained therapy
Exosomes maintain integrity in the injected device	miRNA, proteins, and other therapeutic cargo cannot be compromised while in the device. The exosomes phenotype must remain intact during loading and release.
Determine optimal material to use for the device	Based on prior literature and characterization of biocompatibility, degradation, and testing of exosome particles, suggestions for an optimal material can contribute to future

	designs and prototypes
Create an in vitro model showing what the device can do	Demonstrate the feasibility of the devised model to provide the groundwork for exosomes as a sustained therapy option

Based on the given objectives the team created a pairwise comparison chart to rank importance. Features (horizontal) that are more important get a score of 1 and features of equal importance get a score of 0.5. Facilitation of exosome protection and release were considered the most important objectives along with the device biocompatibility to allow for safe implantation in the spinal column.

Table 2: Pairwise Comparison Chart

	Degrades over approx. 6	Exosomes maintain integrity	Biocompatibility	Holds 10^{10}-10^{15} exosomes [10]	Facilitate release of exosomes	**Total**
Degrades over approx. 6	**X**	0	0	0.5	0	**0.5**
Exosomes maintain integrity	1	**X**	0.5	1	0.5	**3**
Biocompatibility	1	0.5	**X**	1	0.5	**3**
Holds 10^{10}-10^{15} exosomes [10]	0.5	0	0	**X**	0	**0.5**
Facilitate release of exosomes	1	0.5	0.5	1	**X**	**3**

3.2.2 Functions

Many of the design requirements that are critical to consider appear as functions of the material that is chosen for use in this device. Therefore, it is of paramount importance that extreme caution be exercised in the selection of the ideal material for this application.

Polymers, both natural and synthetic, are options for the materials to be used for this device. The materials being evaluated include fibrin, silk, and CSMA.

One of the most important design requirements is the degradation characteristics of the chosen material. The device will ideally degrade over a period of six months, releasing the exosomes in a controlled level throughout the entire degradation process. Surface degradation is beneficial to this application as it would allow for a constant, regulated stream of exosomes to be released as opposed to the burst release that is often seen with bulk degradation of a material. Degradation by surface erosion would also help with the calculation of a more accurate release rate if exosomes are being evenly released.

It is also crucial that the material chosen for this implantable device not negatively impact the integrity of the exosomes. The exosomes suspended in the device should be released in their proper phenotypical state, with their surface, shape, and therapeutic cargo fully intact. If the shape or surface properties of the vesicles are altered it could result in complications with cellular uptake and if the therapeutic cargo is altered or degraded in any way it could completely destroy the therapeutic efficacy of this treatment method. Therefore, it is important that the exosomes are released in the same phenotypic state in which they were loaded.

Table 3: Functions

Function	Reason
Degrades over 6 months	The device will not need to be taken out of the body once all of the exosomes have been release, a new one can simply be put in its place.
Release exosomes	Exosomes are the method of therapy and must be released into the body to be effective.
Exosomes are released in their normal phenotypic state	In order for the exosomes to work they must remain intact while they are in the device and when they are released from the device.

3.2.3 Constraints

Throughout the design process there were several constraints the team needed to consider, with the biggest being the time and budget allotted for the project. The team was given roughly eight months and $1000 dollars to design and test an in vitro model of the exosome delivery device. The materials necessary to do this are extremely expensive and limited how much the team could test. The team also had to be very careful with their materials, so as to not waste them, therefore wasting money. Reliably culturing cells and collecting their produced exosomes is not always successful and the number of cells lines that are able to be acquired may be limited. Separating and identifying exosomes is also fairly difficult due to their size. While separating extracellular materials, transmission electron microscopy can be used to identify them, if available to us. Even if the team was able to identify them, separating them from the other extracellular materials could be problematic. Implanting a device into the spinal column is also very dangerous and many careful steps have to be taken to ensure that the material entering

the cerebrospinal fluid does not affect the pH or other chemical properties necessary to its function.

In addition to testing constraints there are several other constraints that need to be considered. The temperature of the body will be a significant constraint. The team wants the device to be delivered via hypodermic needle, however it must stay in place once administered. This means the device must start out as a liquid and turn into a gel. For this to happen it must have thermosensitive qualities, meaning that the temperature of the environment in which the substance is placed will affect the physical state of the material. Seeing as the temperature of a dog is 39 degrees celsius, with very little leeway, the temperature could be a significant factor in this design. Another constraint to be considered is the physical space within the spinal cord. Because of the fragility of the cord, the team cannot insert a device that disrupts its current properties too heavily. This means that the size and shape of the device have to be dictated by the space available and the shape of the spinal cord. The final constraint the team will consider is regarding the byproducts released by the materials used. The pH of the spinal cord will affect the amount of acidic byproducts that can be safely released by the device. If the spinal cord does not circulate fluid well enough, or is easily manipulated by a disruption in the natural pH, the device will have to release as few acid byproducts as possible. If this factor in not taken into consideration the device could be toxic to the animal, which goes against the overarching objects of the device. Overall the team realizes that working within the body means there are a lot of constraints to consider. Biological functions are difficult to alter in a way that doesn't have negative impacts on the body, which means the team's device will have to work in symbiosis with the rest of the body's functions.

Table 4: Constraints

Constraint	Reason
Spinal Cavity	The spinal column is delicate and there is limited space (1-1.5 cm). Device must not disrupt the properties of the spinal column
Regulations	Limited regulations
pH	Many polymers give off acidic byproducts which could be detrimental to the cells and effectiveness of the device, and so in choosing a material the amount of acidic byproduct given off could limite material options
Financial	More than half of pet owner unwilling to pay more than $1000 for treatment

3.3 Design Requirements - Standards

The initial device is intended for use on dogs, but could be applied to human treatment following additional testing and research lying outside the scope of this project. Depending on the patient, standards can be drastically different. Implantable medical devices for both human and veterinary use are managed by the United States Food and Drug Administration (FDA). In veterinary treatments the devices do not need to be pre-approved before being sold on the market [23]. The FDA may intervene if it deems the device overly harmful or dangerous but no application or approval needs to be filed for a medical device if it is explicitly going to be used on animals [23]. For medical devices to be used on humans, however, the FDA has many more standards. It classifies devices into types based on the risk to patients, and considering the team's would likely be implanted in or near the spinal cord, the team's device would most likely be a type III, requiring pre-market approval from the FDA. A good example of an implantable

device near the spinal cord is the Senza Spinal Cord Stimulation (SCS) System (Premarket Approval Application (PMA) Number: P130022), which has been approved by the FDA and did require premarket approval [24]. Though not related to drug delivery this is a medical device that implants a small battery beneath the patient's skin with two small wires hooked up to the spinal cord to deliver small electrical shocks to reduce pain in the patient. The battery on this device can be recharged by piercing the skin with a specially designed charger. Relating this to our device, the team would most likely need to reload a new polymer loaded with exosomes into the site and the SCS shows that, if proper precautions are taken, reloading through injection into a device under the skin that transports exosomes to the wounded area could be possible. The device also offers insight into the potential risks expected with a therapeutic spinal implant. The SCS has minimal risks apart from the initial surgery. Almost all of the risks involve a problem with the surgery and not the device itself, such as infection, the leads falling out of place or not initially being placed properly, and pain in the implantation site [24]. The most serious case of failure involves paralysis, but the fact that the device was approved likely means this is minimal and they reference in the approval report that “anticoagulation therapies may increase the risk of procedure-related complications such as hematomas, which could produce paralysis” [24]. Based on the SCS for a spinal device to be approved the surgery has to be the riskiest part of the procedure and post-surgery the devices problems should be minimal or easily fixed. However our device will be fairly different from the SCS and will involve other materials the team will need to look into, such as exosomes and hydrogels. Since the SCS involves a potentially harmful surgery the team is looking into injectable hydrogels to avoid the need for surgery if possible.

The fact though that an implantable, rechargeable device was placed in the body for spinal therapy provided a lot of good comparisons for what would be expected in the team's device.

To ensure that our device is created with the highest quality possible, the team have several standards from the International Organization of Standards that they would like to follow. The first is ISO 12189:2008 which "specifies methods for fatigue testing of spinal implant assemblies(for fusion or motion preservation) using anterior support."[25]. The team felt that this was a relevant standard because how their device reacts once implanted into the spinal cords is important and it should be done in a safe and approved way. The second standard the team will consider is ISO 80369-6:2016 which "specifies requirements for small-bore connectors intended to be used for connections in neuraxial applications." [26]. This is significant to the team because the device will be going into the spinal cord and affecting the nerves in the body. This is a dangerous area to deal with and so it must be considered with caution and care. The third standard the team will consider is ISO 10993-6:2007 which "specifies test methods for the assessment of the local effects after implantation of biomaterials intended for use in medical devices."[27]. This is an important standard because how the implant reacts with the body is crucial to its success. The implant just can't solve the problem at hand, it also has to work with the whole body. It should not cause problems while solving one.

3.4 Revised Client Statement

The client statement, in its revised version, has gained focus as a feasible design route was uncovered through carefully conducted research and a number of sponsor interviews. This amended statement now reads:

"Design an injectable polymer hydrogel to act as an exosome repository for the treatment of canine spinal cord injuries. This repository will facilitate local administration of an exosome based therapy to promote cell re-growth and development in the insult area. Further, it will be biocompatible and fully degrade within the body over a period of about 6 months, maintaining therapeutic levels for this entire time period."

This evolution of the client statement lends focus to the number of important design attributes that were intentionally ignored in the initial version. It is now clear which condition this therapy will concentrate on treating as well as how it is planned to be administered, which class of material the system will be made from, how it proposes to accomplish its function, and the duration of a viable dose.

3.5 Management Approach

In order to stay on track with the project the team had specific goals to accomplish each term. In A-term the team's goals were to do as much research as possible. This included looking into what is already on the market, as well as information on exosomes, polymers, and the

variety of diseases and injuries that could potentially be cured through exosome treatment. The team also met with Professor Andrew Hoffman, a project advisor from Tuft's Cummings School of Veterinary Medicine, to gather a pretext as to how he hoped for the device to be utilized as well as to explore any knowledge he may have on the current state of exosome therapies as they are readily tested in his laboratory. The team also planned out preliminary designs with the prospect of testing during B-term as well as formulated a list of tests that would need to be completed in order to obtain data about the transport and degradation properties of exosomes that could have implications on final design parameters. They noted what devices or procedures they would need to be trained on, in order to successfully perform these tests.

In B- term the team continued to research materials as they tested to understand as much about what they were testing as possible as well as make sure they were not overlooking any valuable options. They then began prototyping their device and modelling how it would work via an in vitro model. They performed tests, that were planned out in A-term, on the many different aspects of their project to ensure that the individual aspects would be able to come together in the following term. They continued talking with Professor Hoffman to relay data and get his opinion on how to make things work better.

In C-term the team brought all of their knowledge and piece testing from B-term together to test their prototype. The team tweaked the device as necessary and eventually created a working in vitro model.

In D- term the team processed all of the data collected from their experiments. They completed the paper analyzing all of their data. They put a presentation together detailing their

project and presented it for their advisors and peers. The detailed agenda for all four terms can be seen in the Gantt chart.

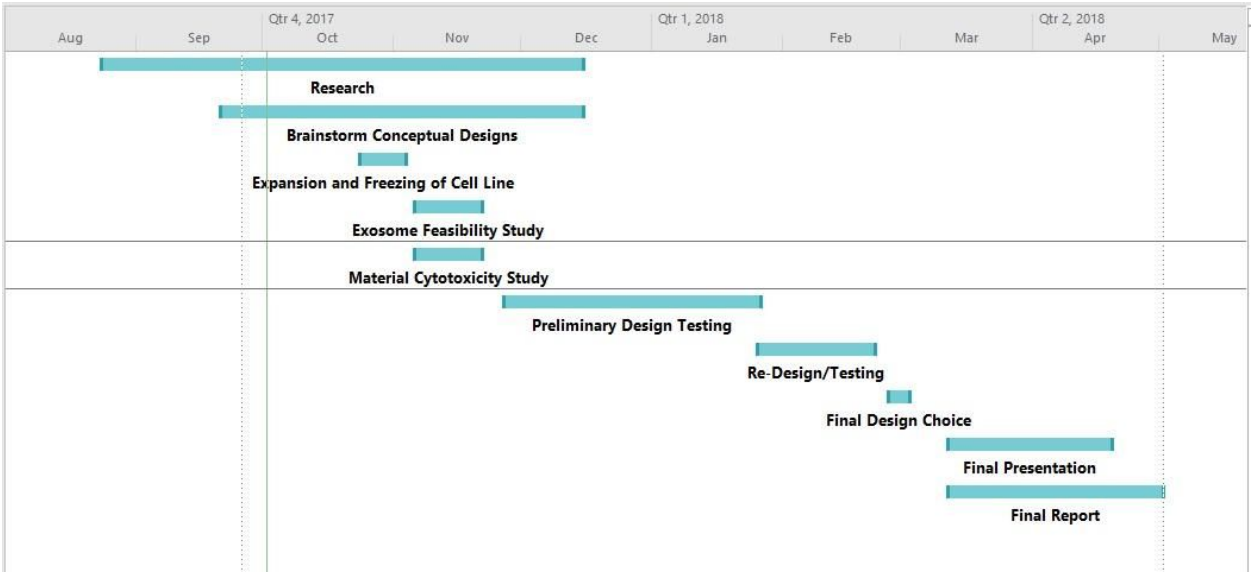

Figure 3: Gantt Chart

In addition to managing time the team will also have to manage their finances. As stated the $1000 budget it not a lot. For this project the team will be spending $250 dollars on the required shared lab equipment fee. The cell lines could either be an expensive cost or be free. There is potential that the team's sponsor and advisor have cells that can be used. If that is the case, this will not be a factor, however if the team does not receive free cells they are budgeting $500 for them. The remaining $250 will go towards purchasing the materials that will go into the device such as the polymers, self assembly peptides, and materials for the actual setup of the in vitro prototype. This is not a lot of money to accomplish this task, and in order to stay within the budget the team will most likely need the cell donation to make the $1000 budget reasonable.

Chapter 4: Methods and Alternative Designs

4.1 Needs Analysis

The device needs to be able to administer large concentrations of exosomes into the wounded area. The device should not be harmful to the surrounding tissue on which it is implanted. The chance of it causing infections or moving post administration needs to be minimal and so some support structure may need to be implemented to provide stability.

4.2 Specifications

In order for our device to be a practical form of therapy it will need to hold 10^10 exosomes. This is the minimum number of exosomes needed so that the device can be considered a feasible mode of treatment. The device will be able to release them over time and they should not run out in too short a period of time. The team hopes to make a device to allow them to last for approximately 6 months. The device itself will need to fit within the spinal cord and therefore must be less than 1-1.5cm in diameter. Because this device is being implanted into the spinal cord, size needs to be taken into consideration. There is very little room in the cord to begin with, and adding too much foreign material can cause the cord trauma.. The acidity of the implant needs to be taken into consideration as well. Ideally the pH will be close to 7.33, the typical pH value of native cerebrospinal fluid. This means that the team needs to consider the acidic byproducts given off during degradation of the device and how they affect the system. A material that releases too many byproducts is not able to be used as it might do more harm than good. With that being said, the material chosen should have a slow degradation rate, lasting

roughly 6 months. This is a practical time frame because having a treatment occur twice a year is not too intrusive for the animals and their owners. Additionally, this is a pivotal time during postoperative monitoring in which the vet determines whether the dog will recover or the procedure failed. The overall cost of the device is also necessary to consider. Seeing as only about half of pet owners are willing to spend $1000 on their pet, the team would like for the device to cost $1000 or less.

Table 5: Specifications

Specification	Reason
Capacity	The ideal minimum number of exosomes held by the device should be approximately 10^10-10^15 exosomes to maintain a therapeutic dose over the given timeframe [8].
Size	The device must fit in the spinal cord without interrupting its natural dimension of 1-1.5cm diameter [8].
pH	The byproducts of the device should not alter the pH of the spinal cord - 7.33 [8]
Temperature	The hydrogel can polymerize or be in a gel state at no less than 39 degrees celsius which is the internal body temperature of a dog [8]
Pressure	The device should not apply excess pressure to the spinal cord of the animal. It should not disrupt the natural pressure within the spinal column of 5-15 mmHg [8].
Degradation	The device should degrade over a 6 month period. This is an optimal timeline for patient convenience, comfort, and cooperation [1].

4.3 Feasibility Study/ Experiments

A preliminary test was conducted prior to the design process as proof of concept for material properties. When the team was trying to determine the best material for the project, they attempted a degradation experiment using silk, fibrin, and CSMA. The test yielded inconclusive results as the plate became contaminated. Additionally, the degradation could not be quantified due to technological challenges with diameter imaging. Upon further discussion, exosome evaluation was the priority of this project and degradation profiles could be reflected in particle release results. Therefore, the team did not redo the test. In addition, due to time constraints, biocompatibility testing was also bypassed. Previous literature had already proven both materials were biocompatible and the team proceeded based on these assumptions.

One of the most important procedures conducted was the exosome isolation from a mouse fibroblast cell line, 3T3. This procedure is repeated as needed to extract exosomes for preliminary and design experiments. Cells are expanded on 25x182cm^2 cell culture plates at 70-80% confluency. Cell culture follows the procedures in appendix A, which includes cell passaging, culture, freezing cells for storage and creation of complete media. Once they have reached this confluency 50% serum free media 50% regular complete media is applied for 24 hours. This allows the cells to acclimate to the serum free environment somewhat. Serum free media is media without fetal bovine serum additive which enabled exosomes to be produced. Other nutritional and growth factors are needed to replace fetal bovine serum and ensure cell survival in serum free solution. After 24 hours the cells are transitioned to 100% serum free media to encourage ECM formation and cell-cell interactions. Serum free media consists of

IMDM (VWR, cat #45000-366), 10ng/ul hrPDFG-AB (prospecbio, cat # CYT-342), 5ng/ml human rFGF2, and 1x pen-strep [28]. Exosomes are concentrated via tangential flow filtration (TFF). An example of a TFF machine is shown in figure 5. The media is pooled and filtered through a 0.22 um filter into sterile flask. Exosomes are concentrated through a Sartorius VivaSpin 250, with 100,000kDa MWCO filter insert. The filtration is driven by pressure (2-4 barr or lowest possible). The filtration leaves a retentate volume of 1-2ml that must be washed off the innermost leaf of the membrane with PBS to obtain the exosomes. Ultracentrifugation was performed by Optima L-90K Ultracentrifuge (Beckman Coulter) at 100k*g x 2.

Figure 4: Tangential Flow Filtration Setup

Fibrin and CSMA were loaded with exosomes in cerebrospinal fluid (CSF) and released particles were assessed using gel electrophoresis and Nanoparticle Tracking Analysis (NTA). Controls were loaded with exosome-free media collected from TFF and PBS.

Fibrinogen from human plasma (Calbiochem, cat. # 341576) at a concentration of 70mg/ml was combined with thrombin from bovine plasma (Calbiochem, cat # 605157) at 40 U/ml, 40mM CaCl2, and exosomes. Solutions gelled at room temperature for about 10 minutes forming a plug. The fibrin plugs were a 50:50 mixture of fibrin and exosomes (25µl of each). However, the plugs did not come out equal sizes. 3 fibrin plugs loaded with exosomes were evaluated [29].

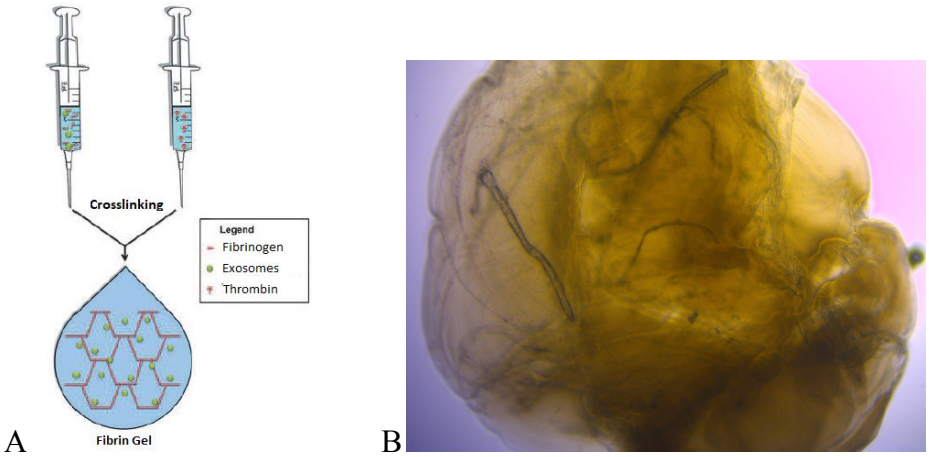

Figure 5: A) Fibrin gel preparation schematic [30]. B) Fibrin Plug Loaded with Exosomes at 4X magnification

The CSMA gels were a 50:50 mixture of CSMA and exosomes (25 ul of each) with a final concentration of 10% CSMA. They also have 0.1% irgacure 2959. They took about 20 minutes to UV crosslink. 3 CSMA plugs loaded with exosomes were evaluated.

The CSMA control gels were a 50:50 mixture of CSMA and either the control media or PBS (25ul of each). The fibrin gels were 50:50 mixture of fibrin and either the control media or pbs. These gels were closer to the desired size but there was slight variation between gel sizes.

Experimental and control plugs (see Figure 6) were incubated at 37 C and 5% CO2 in CSF for 3 weeks and assessed for degradation and exosome release.

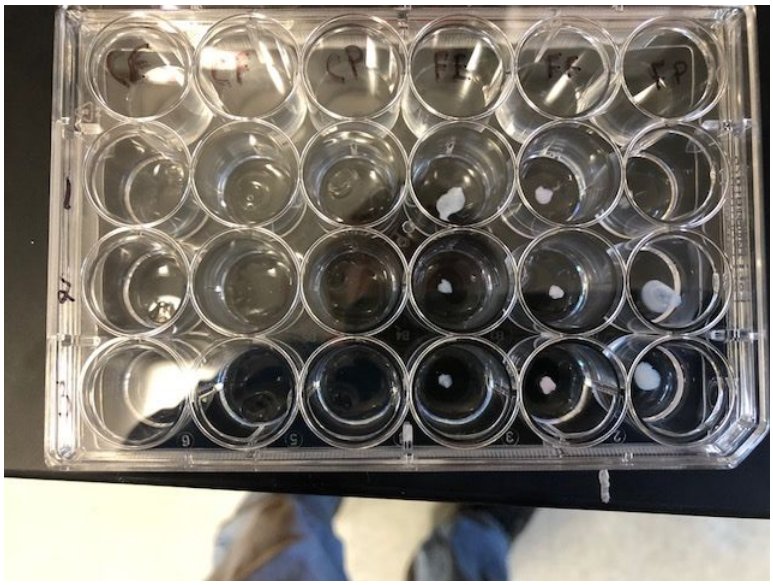

Figure 6: CSMA and Fibrin Plugs

Nanoparticle tracking analysis (NTA) was performed by Nanosight NS300 (Malvern) to evaluate the particle size and distribution (see Appendix B). Nanoparticle tracking analysis (NTA) is a laboratory technique that allows for visualizing and analyzing the size distribution of small particles with sizes ranging from 10-1000 nm. It accomplishes this by establishing the viscosity of the liquid and temperature of the particle samples and relating it to the rate of Brownian motion within the samples to particle size. Particle movement is calculated by the Stokes-Einstein equation. Samples of concentrated exosome media and CSF of both

experimental and control plugs after 3 weeks of degradation were run through the tracking analysis.

Gel electrophoresis (Appendix B) was used to evaluate protein size in exosomes released from fibrin and CSMA, pure CSF, and exosome-free control media. Gel electrophoresis is a technique used for separating DNA, RNA, and other proteins based on their size. This is accomplished by loading protein samples into a porous gel material and applying an electrical field to the gel. This electrical field causes the proteins to migrate through the gel at a speed that is inversely proportional to their size, meaning that smaller proteins will travel a greater distance towards the bottom of the gel than larger proteins given the same subjection to the electrical field. A darker relative stain correlates to a higher concentration of proteins of that size.

4.4 Conceptual Designs

The primary design option was a hydrogel loaded with exosomes at a large enough concentration to produce necessary exosome levels. The hydrogel, with the loaded exosomes, would be placed in the body near the wound site. The hydrogel would be designed to allow exosomes and their cargo to diffuse out but protect the exosomes viability. The hydrogel should degrade over 6 months, so that a new one does not need to be inserted often. By virtue of the degradation process exosomes are released.

Figure 7 illustrates a potential experimental setup to test an in vitro version of the device. The setup recreates the internal environment of a dog. The team will create a simulation of a spinal cord and place it into a saline bath that simulates a dog's body. The team will then implant the device into the "spinal cord". A pump will ensure that the fluid within the "cord" moves as it

would in a dog. A filter will also be attached to catch all of the exosomes released from the device. The team will be able to see how many exosomes are obtained over time. The main function of this setup is to see how the device would actually work in the body of a dog and if the project is feasible at that level. Ideally the team will do many other experiments prior to this one to prove that the individual pieces work on their own, this will determine if they work together.

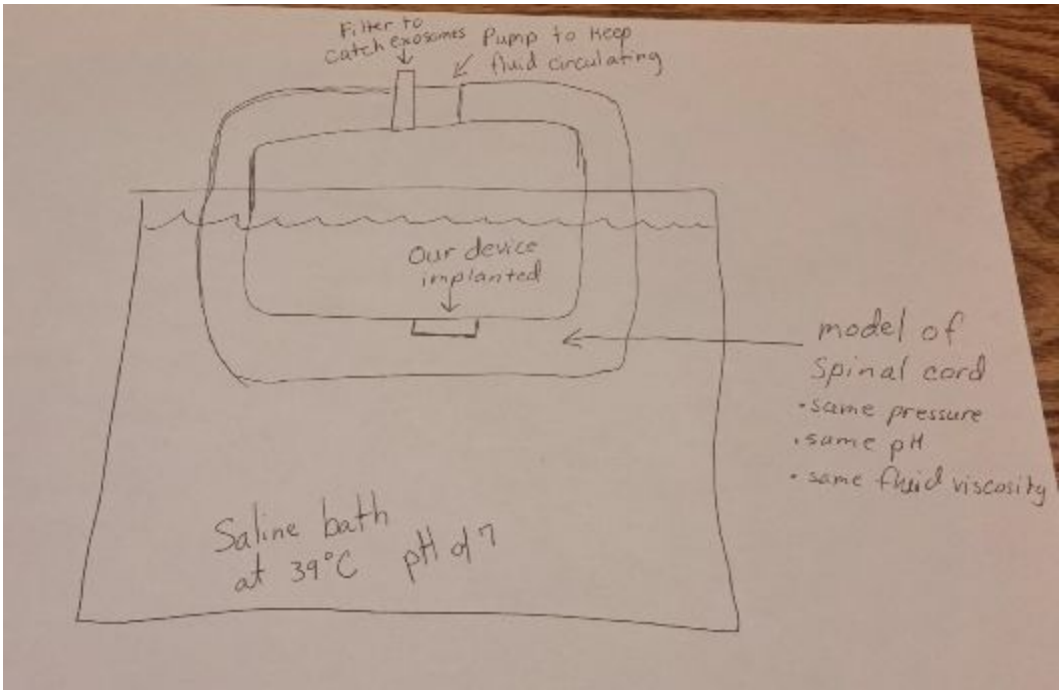

Figure 7: Conceptual Design

4.5 Alternative Designs

Several concepts that could potentially be working alternatives are drug delivery pumps and cell encapsulation devices. The drug delivery pump would function similar to glucose pumps for diabetic patients with a small surface patch, a subdermal hydrogel mold, and tubes or vessels connecting to the treatment area. The patient can be given concentrations of exosomes that they themselves can put in the pump to manage their own treatment and maintain effective levels.

Another idea was creating a device similar to a “Pain Pump” known as SynchroMed™ II in which a targeted drug delivery system delivers pain medication directly to the fluid around the spinal cord, in the intrathecal space. The system includes a drug pump implanted in the abdomen, connected to a thin, flexible catheter tube also implanted under the skin. The team proposes the exosomes be administered in a similar fashion to the way SynchroMed™ II delivers pain medication for patients dealing with chronic pain such as cancer pains [31].

If the half-life of exosomes proves too short for a feasible therapy in isolation, an alternative design option is cell encapsulation. This consists of a device loaded with mesenchymal stem cells that will facilitate the release of exosomes in their biologically active state. The team is also looking into using self-assembling peptides. They are able to hold a high concentration of drugs and can fit into the spinal cord well, so that there is no worry about the integrity of the cord being disturbed. They can be used to target certain sites within the body, which would be useful for facilitating exosome delivery to the wound site.The team also considered smart materials that could respond to external stimuli. Changes in properties such as swelling (interpenetrating polymer network) or gelation can occur based on temperature, pH, etc.

Silk was a front runner in deciding a material, however, for the team’s situation it was not a feasible choice. The silk took too long to make and loading it with exosomes was more complicated that with the materials that were actually chosen. Although the team was able to perform a few preliminary tests with the silk, they were not able to obtain it for the final tests and therefore it was no longer considered for this projects.

Chapter 5: Design Verification

5.1 Nanoparticle Tracking Analysis

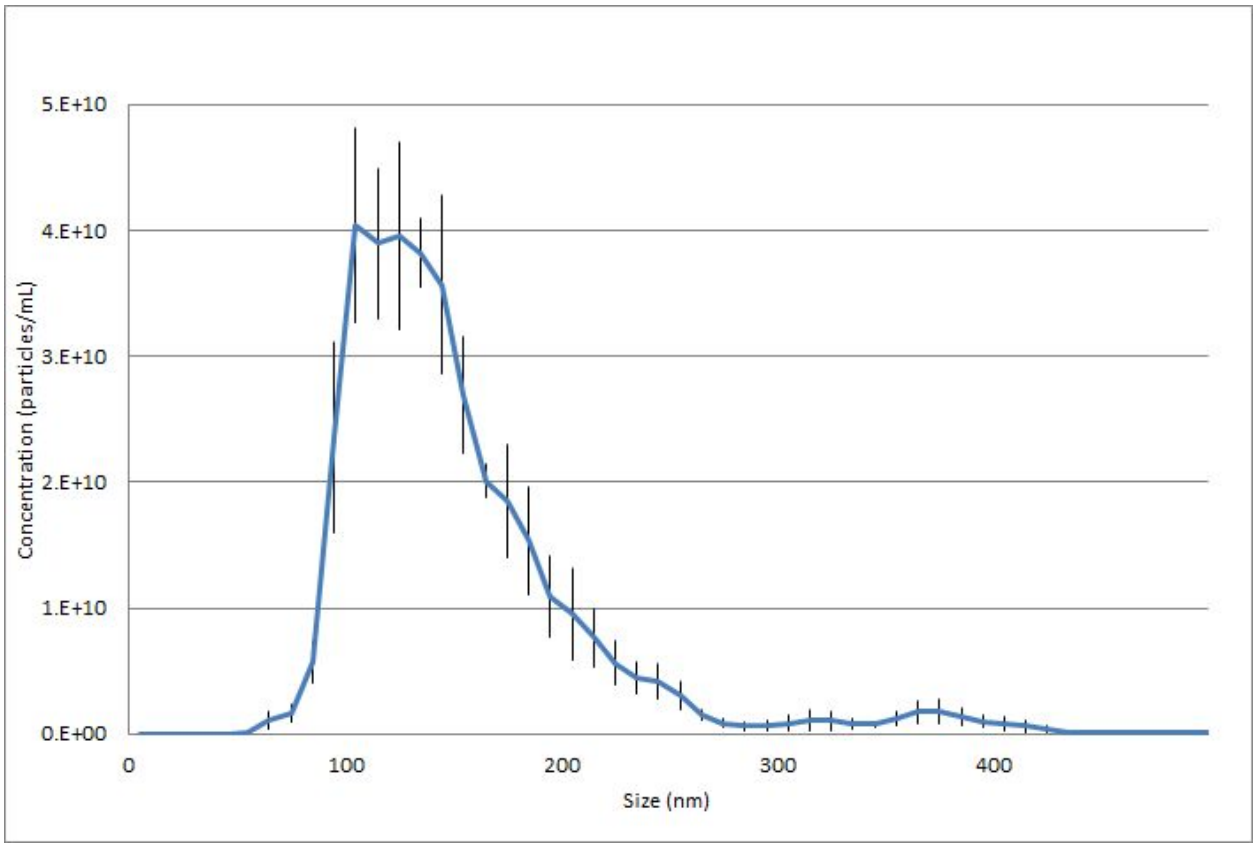

Figure 8: Particle Size Distribution of 3T3 Exosome Media

NTA was first employed after the culturing of the 3T3 cells in serum-free media to ensure that exosomes were present in the culture media prior to the TFF and ultracentrifugation processes that concentrated the exosome sample. As is apparent from the graph of particle size from the initial NTA results shown in Figure 8, most of the particle sizes within the media sample were about 100-150 nm in diameter with an average particle size of 148.8nm and mode

of 122.4nm. This falls within the parameters of normal exosome size distribution. Other larger and smaller particles were attributed to cellular debris accumulated during cell culture procedures.

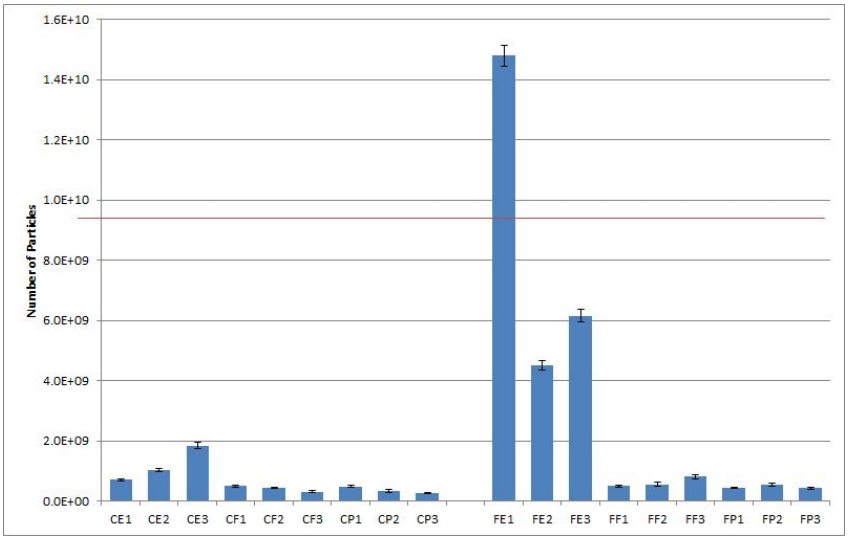

Figure 9: NTA Particle Count

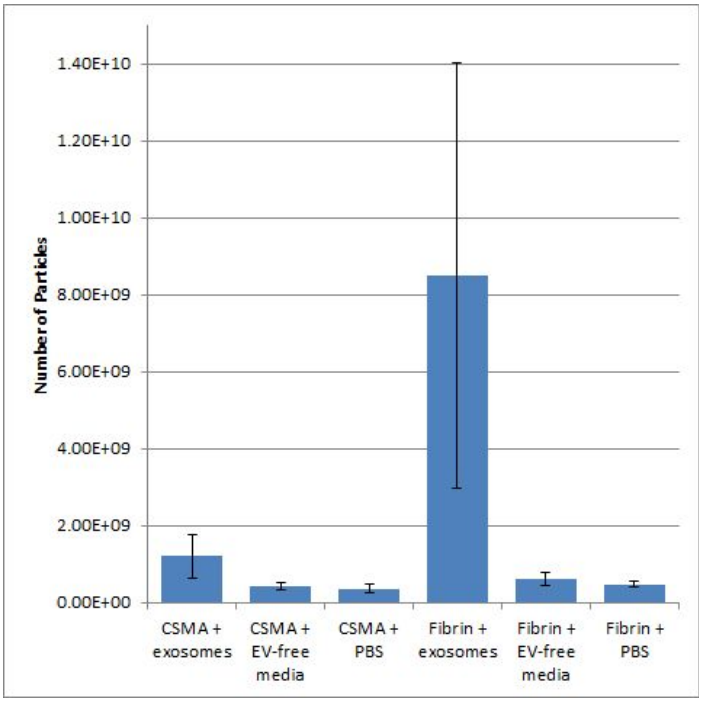

Figure 10: Average NTA Particle Count

NTA was again utilized when analyzing fluid samples after allowing both the fibrin and CSMA hydrogel plugs to incubate in cerebrospinal fluid for 3 weeks to determine the number of particles released from each of the samples following degradation. Absolute values of particles rather than concentration was assessed due to evidence of evaporation of the CSF and varying volumes upon tracking analysis. Figures 9 and 10 show the amount of particles released for each type of plug. In Figure 9 the first nine bars represent plugs made with CSMA and the second nine are plugs that were made with fibrin. The first three bars of each material are the experimental plugs filled with exosomes, followed by three filtrate controls, and then three PBS controls, from left to right. The red line in the figure represents the amount of exosomes originally loaded into the plugs . After analyzing Figure 10 it is clear that the fibrin control plugs released the most particles. The CSMA plugs also released particles, however not as many as the fibrin. The controls of both materials released the least amount of particles.

5.2 Gel Electrophoresis

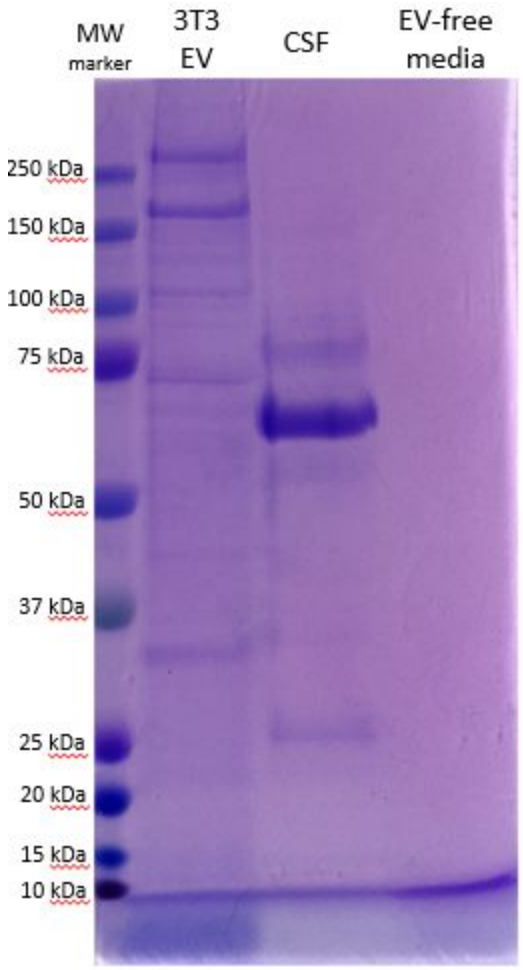

Figure 11: Coomassie Gel

Figure 11 shows the stained proteins separated by gel electrophoresis. Four lanes were run for analysis of this sample. The far left lane was the ladder, which is proteins of known size that would allow the team to estimate the size, and thus the the type of proteins separated from the 3T3 exosome sample. The second lane was loaded with the actual 3T3 exosome samples, while the third and fourth lanes were loaded with excess equine cerebrospinal fluid from earlier experimentation and an exosome-free cell culture media sample respectively. High molecular

weight proteins (approximately 175kDa and 260kDa) were present in the samples containing released exosomes (3T3 EV). The exosome-free control media contained small molecular weight proteins (<15kDa).

Chapter 6: Final Design

6.1 Economics

The team expected this treatment to act as a therapy after the initial surgery to move a herniated disc back into place. As a result the price of this treatment will be higher than current methods and thus with the higher cost it must produce higher rates of success compared to traditional methods. With a higher rate of success in post operation the team expects to see less re-injury and need for additional surgery. Assuming the exosomes prove effective in treating damaged spinal tissue and potentially other damaged tissues the market for stem cells and exosomes would greatly expand. Expanding said market could drive the cost of production of both of these products down. Additionally the hydrogel market could be effected based on what type of hydrogel proves most effective. Ideally mass production and improved methods of producing hydrogels, and stem cell derived exosomes could drive the price of treatment down and ensure that the treatment could reach more patients.

6.2 Environmental Impact

Although the product being created does not have a significant impact on the environment, the process of producing it does. Although the team completed several initial experiments, there is still considerable amounts of testing that need to be completed on the product before it can move into being produced on a large scale. These preliminary benchtop experiments require the use of many materials including culture plates, pipettes, filters, and a

variety of other materials. The act of producing and throwing away these materials does impact the environment in that there is energy needed to create them, sanitize them, and eventually disposed of them. When looking to improve production methods for exosomes and hydrogels the team advises that considerable efforts be made to minimize the use of materials, especially non-degradable plastics and biohazardous material that could be harmful to the environment. This would not only be environmentally sound but also economical as reducing the amount of materials you use can help limit cost. All dangerous lab equipment, including biohazards and sharp objects should also be disposed of properly.

6.3 Societal Influence

Based on the efficacy of the final design this treatment could alter how spinal injuries are treated and greatly reduce healing periods post surgery in both canine and human models.

6.4 Political Ramifications

Given the fact that exosome therapies are a relatively novel and not fully understood mode of treatment much more research and analysis needs to be completed before definitive statements can be made on the impact that therapies of this kind would have on the global market. Following preliminary exploration and experimental trials it would seem that this mode of treatment displays the potential to be considered a major breakthrough for the treatment of a number of diseases and injuries in both the veterinary and human realms. Once the therapeutic process has been fully characterized and it is understood which types of injuries and diseases can be appropriately treated with exosome therapies, there is a possibility that former modes of

treatment could be rendered obsolete and for this technology to dominate the global market for treatment of these specific ailments.

6.5 Ethical Concerns

Since the miRNA required for this specific spinal therapy is only produced in mesenchymal stem cells, the treatment would require the culturing of these cells and facilitating their production of exosomes containing the correct miRNA. The mesenchymal stem cells can be found in the umbilical cord and bone marrow. In both cases the amount of cells available to harvest is limited. There are many ethical concerns with using umbilical cord blood because it is in such short supply and the cost of banking the blood is high. Because of this not everyone can afford to keep the blood, which could potentially limit the people who have access to this treatment. There could be a class divide between the people who can afford treatment and those who can't. Obtaining bone marrow is also difficult because it is in short supply as well and the procedure to obtain it is painful. If the patient is not healthy enough to provide their own bone marrow, they will need to look to donors and being put on a donor list has its' own set of politics of who gets treatment first.

6.6 Health and Safety Issues

As with any biological device there are many risks associated with this device and treatment. Every part of this process, from manufacturing to administration, needs to be sterile. Contamination could easily occur while the cells are growing, so it is essential that they are monitored throughout the entire process to ensure all batches are free of it. To reduce the chance of contamination everything should be done in a sterile environment. This will greatly reduce the

risk of a batch of cells needing to be disposed of because of they are deemed a biological hazard. Another factor to consider is the organism from which the cell are sourced. The cells need to be practical for growing on the large scale meaning they should be durable and grow relatively fast. They should be sourced from an organism similar to the one receiving the treatment so that they have a better chance at being effective. The source should also be healthy. If the cells are sourced from a sick patient the treatment might not be effective, or even worse the treatment could cause harm to the patient, resulting in a new medical issue that needs to be addressed.

6.7 Manufacturability

In order to produce the exosomes necessary for this treatment on a large scale, bioreactors will be necessary. In order to create a therapeutic amount of exosomes for an individual, many cells are necessary. Because of this cells are going to need to be grown in large batches so that enough exosomes are produced to be distributed on a large scale. The growth of exosomes will probably be the most difficult part of manufacturing this treatment, because of the large quantity necessary to produce a small amount of exosomes and the fact that the speed of cell proliferation can not be sped up as much as other manufacturing functions. The timing of the cell development needs to be closely watched to ensure that there are enough cells to produce the exosomes but not too many so as to have the cells be too crowded. The team worked with 3T3 cells, an immortal cell line, which have an incredibly high rate of proliferation but in practice would likely have to work with mesenchymal stem cells, a primary cell line. To get our effective dosage of exosomes the team needed to passage 3T3 cells which typically had 4 population doublings per passage. To reach twenty five plates at 80% confluency the team had

approximately 40 passages. So the total number of population doubling for the 3T3 cells was 160. The mesenchymal stem cells would likely need to achieve a similar population doubling number for an effective dosage. However since mesenchymal stem cells double much slower (especially with cell aging), and cannot double indefinitely (Hayflick's Limit) the time to reach the effective dosage would be significantly longer with more passages and likely needing a higher initial population of cells. In addition cells would need to be replenished.

Once the exosomes have been produced there is also the matter of isolating them. On a small scale, isolating exosomes from 500mL of media took several hours. Isolating the exosomes on a large scale will require large filters that are very expensive. The isolation process will take many hours and need to be monitored to ensure that the filter does not clog. It is possible that filtration will need to happen in several steps which will add time and money to the process. In addition, cell media will be an expensive aspect when considering scaled up manufacturing.

6.8 Sustainability

Sustainability of the device is based on the ability to culture or acquire large amounts of mesenchymal stems cells. Since the miRNA that helps promote spinal cord regeneration is only evident in these mesenchymal stem cells their culturing is paramount to the sustainability of the project. Though not as easily cultured or available as 3T3 cells, mesenchymal stem cells can be cultured from donors or from samples taken from an umbilical cord. However the cells do not expand indefinitely and typically have a population doubling limit (PDL) of 10 [32]. As a result new cells would be needed to start new populations and a single cell line could not be continued

for very long to continually produce exosomes. As a result for sustainable exosome production multiple donations of cells would likely be required.

Chapter 7: Discussion

3T3 cells were the initial cell type chosen for experimentally producing exosomes as they are easy to culture, easy to obtain and everyone in the group has had experience culturing them. In the final design of this product however it is important to note that exosomes derived from mesenchymal stem cells are the only exosomes that have shown to have therapeutic properties on damaged spinal tissue. The 3T3 cells and their exosomes were used as a model given the availability of the cells, growth time and the teams experience culturing them. The team acquired a population from Prof. Ambady and began culturing them, subculturing when the population reached about 80% confluency. Culturing cells to successfully express exosomes proved more difficult than initially anticipated due to a lack of certain materials and methods. A large number of 3T3 cells had to be grown, specifically 25x182cm^2 plates each at about 80% confluency at the recommendation of Professor Hoffman. This number would yield an effective concentration of exosomes. These plates were cultured on complete media with 10% FBS to maximize the rate at which they grew. However once at the appropriate amount of plates and confluency the cells would need to be transferred to serum free media to get the cells to release exosomes. After the exosomes were produced they needed to be separated from the media. While TF was the most effective method for separating the exosomes, the team did not have access to TFF and a significant amount of time was spent trying to get access to a filtration device on campus before Professor Hoffman offered to run the TFF at his lab. Since the team could not proceed without TFF the cells were routinely subcultured in complete media for longer than anticipated while the team was researching and waiting on permission to use TFF.

After exosomes were seperated with TFF the solution they were placed into their hydrogels, fibrin and CSMA, along with hydrogels loaded with exosome free serum free media and PBS. The serum free media was guaranteed exosome free as it had no interaction with the cells and was fresh. All of the plugs then underwent nanoparticle tracking analysis to identify if exosomes survived and released in an active state from the plugs. The team was looking for intact, rounded particles in the analysis of the test group. The PBS loaded hydrogels and EV free serum free media were done as negative controls to show that the hydrogel materials or materials from the serum free media were not giving false positives. After obtaining the results from the nanoparticle tracking analysis the team concluded that for short term release the fibrin is the best choice, but that is not to say that the CSMA is not a valid choice for our device as well. When looking at the fibrin plugs loaded with exosomes in Figure 9 it is obvious that the first bar in the largest. This is due to issues creating the fibrin plugs which resulted in plugs of varying sizes. The largest bar corresponds to the largest plug. This being said, the red line on the graph indicated the average amount of exosomes put into the plugs. Assuming all of the hydrogels had been made equal size the team would expect that all of the bars would be touching the red line if they had released all of their exosomes. The sum of the nanoparticles recorded in the graphs is slightly less than the sum of the exosomes the team placed in the plugs. Since the plugs did completely degrade the team can attribute the loss of particles a factor of particle rupture, errors in loading of exosomes or variation in the observed sample as well as issues with evaporation that altered the volume in the wells. However the fact that the amount of recorded nanoparticles was very close to our estimated loading shows that the amount of exosomes put into the plugs is almost equal to the amount that came out. This is a good result because it means that the

therapeutic amount inserted will almost be the amount delivered. These results were obtained three weeks after the plugs were placed in the media, however within four days the fibrin had dissolved. This means it is good for delivering the treatment fast and that the exosomes may be able to survive for a fairly long period of time, assuming they are not absorbed or phagocytosed. However, this also means that the likelihood of using only fibrin for an extended treatment period is not high. It's release profile is far too quick and the exosomes need to be released slowly at a high concentration to ensure proper treatment. There is the option to alter the ratios of the components of the fibrin to make it last for longer or add aprotinin. CSMA degradation can also be more closely monitored and modified with the addition of chondroitinase. Chondroitinase has the added benefit of treating the glial scar tissue. In conjunction with exosomes, this could be another area explored for enhanced therapeutic effect. Because of CSMA's slow degradation, the team believes in combining fibrin and CSMA to create a plug that will deliver the initial therapeutic dose quickly due to the quickly degrading fibrin and then have a slower long term release mechanism due to the CSMA. This is thought to be the ideal method because the patient won't have to wait a long time to feel the positive effects of the treatment and they won't need to have the treatment constantly administered so it is not significantly frequent.

Chapter 8: Conclusions and Recommendations

Based on the results gathered and previous research done by Professor Hoffman the team believes that the treatment of nerve damage in the spinal cord, within canines, with an exosome loaded hydrogel is possible and could prove profitable. There are still many challenges and this research is still in its infancy. In order for any treatment to be profitable an initial investment must be made towards the production of exosomes from mesenchymal stem cells and their long term production must be cost effective. It should also be noted that research of exosome treatment for humans and for other areas in the body may be possible and the team would suggest research into the effect of exosomes on damaged human spinal tissue and other degenerated tissues. The limited restrictions on medical devices for veterinary models means that if the device was approved and proven effective on canines the research could extend to aid humans. If this research proved effective on humans the initial $1000 budget would likely increase due to an increased demand and willingness to treat human patients as opposed to canine patients. In the event that the treatment did prove effective on human patients it is recommended that other more expensive hydrogels, like silk, be tested more thoroughly. These hydrogels may prove to be more effective but were largely excluded from this project due to the budget constraints.

Overall the team feels as though the project was successful in accomplishing the goals they intended to accomplish. There is still a great deal of work that needs to go into this area of research, but this project was a good start. Future work could lead to exosome therapy becoming a mainstream therapy that is adopted by medical professionals everywhere.

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Appendix A

Cell Culture protocols

Cell culture and passaging

1) Aspirate media and wash cells two times with DPBS(-), aspirating DPBS off each time.

2) Trypsinize cells for 5-10 minutes by adding 2 mL of trypsin per 5mL of solution and placing them on a hot plate at 37 C to lift them off the plate.

3) Add 3mL fresh complete media per 5mL of solution to stop the trypsin.

4) Move cell suspension into a conical tube and centrifuge for 5-10 minutes.

5) Aspirate the supernatant

6) Resuspend pelleted cells in fresh media and count or split as desired.

7) Pipette desired amount of cells onto new plate filled with media and transfer to the incubator, cells should be kept in an incubator at 37 C, 5% CO2 and 95% humidity.

Creation of complete media

For 10% FBS complete media mix, for 5% or 2% you would alter the amount of FBS and Dmem:

1) 1% penicillin streptomycin at 100x
2) 1% Glutamax at 100x
3) 10% Fetal Bovine Serum
4) 88% Dmen Basal Media

Freezing Mammalian Cells for Storage and Thawing Frozen Cells

1) Trypsinize the cells following normal subculture protocol
2) Resuspend the cells at double the intended final concentration in complete media (i.e. if you wanted a final concentration of 1x10^6 cells/mL resuspend 2x10^6 cells/mL in complete media)
3) Mix 20% DMSO with 80% complete media (i. e. 1mL DMSO and 4mL complete media) in a tube to create a freezing solution
4) Shake the cells to ensure they are uniformly distributed
5) Using a micropipette quickly transfer 1mL (assuming cells are measured in number of cells desired per mL) of cell suspension to a separate tube labeled freezing mixture
6) Using another micropipette slowly introduce 1mL of freezing solution to the cells in the freezing mixture tube while gently mixing them
7) After the cells and solution have mixed transfer the amount of cells you wish to freeze into cryovials, you may need multiple cryovials based on the amount of cells you intend to freeze.
8) Transfer the tubes to a freezer and keep them at -80 C.
9) When the cells are ready to be thawed remove them from the freezer.
10) You may thaw them faster by holding them in a gloved hand or by placing them in warm water with the cap above the water and the vial not floating
11) Once thawed use a pipette and mix the contents of the vial with 90% complete media (i.e. 9mL of complete media if 1mL of cells frozen) in a conical tube
12) Mix the tube by inverting it a number of times
13) Place the tube in a centrifuge and spin at 200G for 5-10 minutes

14) Aspirate the supernatant
15) Resuspend the cells at the desired concentration in complete media, it is recommended that after this the cells are checked under a microscope to determine if a significant amount died during freezing
16) Transfer to the incubator, cells should be kept in an incubator at 37 C, 5% CO2 and 95% humidity.

Appendix B

Characterization Protocols

Gel electrophoresis preparation protocol

A BCA protein assay was used to determine protein concentration in solution as directed by Thermo Scientific [33]:

1) Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL) (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041). Note: If sample size is limited, 10µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000µg/mL.
2) Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3) Cover plate and incubate at 37°C for 30 minutes.
4) Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader

Protein amount (6 mg) was normalized across samples. The gel electrophoresis test performed involved using a pre-made kit. The pore-made gel (4-12%) was removed from the wrapping and placed into the electrophoresis chamber. MOPS Buffer was added to both the front and back parts of the machine. The samples were then loaded into the wells in the gel, and the cap of the machine was placed on it, so that the electrical current could be applied. Run the gel at 200V for

40 minutes or until good protein separation. Once done the gel was rinsed with deionized water and then dyed with coomassie overnight for a distinct protein profile.

Nanoparticle Tracking Analysis

Nanosight NS300 (Malvern) was used to calculate the distribution and size of particles. The machine is flushed out with PBS to ensure there are no previous samples in the tubing. The desired testing sample is then loaded into the machine's tubing. Loaded samples should be dilute (10^7-10^9 particles/ml). Flow mode (ml/min) is set so particles are in the field of view for 5-10 seconds. The viscosity is the value of liquid suspension - PBS. A lower detection threshold was used to maximize tracked particles but not so low that the machine picked up noise. This also ensures that the particles are not so bright that you cannot see how they are moving. The sample was set to flow and a video camera captured the particles moving under Brownian motion identifying and calculating individual particles for total concentration and size. The data was exported to Excel.

Appendix C

Bill of Materials

- T182 Culture Plates
- 150mm Cell Culture Plates
- 15mL Conical Tubes
- 6 Well Plates
- 12 Well Plates
- 24 Wells Plates
- DMEM
- IMDM
- Horse Cerebral Spinal Fluid
- Fetal Bovine Serum
- PDGF
- FGF
- PBS
- Silk
- Chondroitin Methacrylated Sulfate
- Fibrinogen
- Thrombin
- CaCl2
- 3T3 Cells
- 1mL Serological Pipettes
- 5mL Serological Pipettes
- 10mL Serological Pipettes
- 25mL Serological Pipettes
- Pasteur Pipette Tips