Injectable Hydrogels Derived from Apoptosis Decellularized Rat Peripheral Nerves

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Abstract

Spinal cord injury (SCI) has devastating effects on one’s quality of life, which can greatly impact patient’s physical and psychological state. Traumatic SCI can result in permanent nerve damage causing partial or complete paralysis and other health concerns that may affect organs such as heart, liver, and bladder. Currently, there are no therapies in the clinic to restore function after SCI. The lack of functional restoration is the result of inhibitory factors present at the injury site, which limit regeneration of adult neurons. Enhancement of regeneration may be accomplished via local delivery small therapeutic molecules to limit the effect of inhibitory factors and/or by mimicking native ECM properties to promote cell proliferation and differentiation. Therefore, researchers have been trying to design and fabricate biomaterial scaffolds to obtain controlled delivery of therapeutics along with the ability to provide chemical, physical and biological cues to promote regeneration. One technique to obtain ECM-mimicking biomaterial systems is to decellularize the harvested tissue or organ to remove the immunogenic cellular components while maintaining the native composition to provide pro-regenerative cues. However, majority of decellularization protocols require harsh chemicals that disrupt the native microarchitecture and remove proteins and bioactive factors. Therefore, in this project we utilized an alternative decellularization protocol by harnessing the potential of apoptosis or programmed cell death using anti-cancer drug, Camptothecin. During apoptosis, cell detach from ECM and form small apoptotic bodies and easily permits the removal of cellular debris. In this study, rat peripheral nerves were decellularized and immunohistochemical analysis was performed to confirm cell removal and ECM matrix preservation. Decellularized nerves were then digested in pepsin/hydrochloric acid solution and then further neutralized to create ECM hydrogels. Thermal gelation was performed at 37℃ for 15 minutes, which lead to development of a stable hydrogel. Furthermore, we observed that the pre-gel solution can be stored for up to 4 weeks in 4℃ or -20℃ before neutralization eliminating the need for immediate use. This study focused on the development of injectable hydrogel derived from apoptosis-induced decellularized rat peripheral nerves as a potential biomaterial to promote nerve regeneration after SCI.

Introduction

Severity of Spinal Cord Injuries (SCI)
Currently, there are over 288,000 Americans living with a spinal cord injury (SCI) and an additional 17,700 new injuries each year [1]. SCI’s injuries can occur when there is a direct impact to the vertebrae, damaging the region, causing permanent nerve damage, and resulting in paralysis. Although, a loss of function and mobility is dependent on the location of the injury, the most common and traumatic type of SCI is when there is direct impact to the cervical. This can potentially cause tetraplegia, which results in paralysis beneath the neck, shown in Figure 1. In general, those affected with the burden of any SCI have an estimated lifetime cost of $1.5 million for medications, hospitalization fees, hiring caregivers or

Figure 1: Types of spinal cord injuries. Image from myhealth.com
nurses, and treating other health related conditions that can occur after SCI, such as kidneys failure, and bladder dysfunction. Furthermore, SCI’s are more common in young adults leading them to live the majority of their life with this debilitating condition. Unfortunately, there is no effective treatment for SCI, despite multiple therapeutic strategy attempts and clinical trials. A possible explanation for this lack of treatment can be caused by the many obstacles that prevent nerve regeneration after injury and the low efficacy of therapies due to poor delivery methods.

Glial scar formation as an obstacle to nerve regeneration

After injury, permanent nerve damage occurs due to obstacles that prevent nerve regeneration. An example of a common inhibitor to nerve regeneration is the formation of glial scar. Astrocytes are the most abundant glial cell in the central nervous system and reactive astrocytes are the major component of glial scar. Changes to the local microenvironment after SCI induces astrocytes to undergo dramatic morphological changes, including the increased expression of extracellular matrix (ECM) components [2]. A major component that is secreted by glial scar is chondroitin sulfate proteoglycans (CSPGs). CSPGs are known to inhibit neurite growth by creating a physical and chemical barrier to axonal regrowth [2]. At first glance, glial scar may seem favorable as it reduces inflammation and prevents unwanted debris at the site of injury. However, after injury a proliferation of astrocytes migrate to the site of injury and form a dense meshwork of glial cells which blocks nerve regeneration. Following SCI, the blood-spinal cord barrier is damaged, thus increasing permeability between spinal cord tissue and blood vessels temporarily [2]. This allows the transport of pharmaceuticals for possible treatment to promote axonal regeneration. Chondroitinase ABC (ChABC) is a promising enzyme that is known to inhibit CSPGs and glial scar formation. If ChABC is present after SCI, it will enzymatically digest CSPG sidechains, preventing the proliferation of astrocytes, thus avoiding the formation of glial scar.

Importance of Controlled Release Delivery Systems

Currently, there are treatments that can aid in reducing the inflammation in the spinal cord after injury and that can promote nerve regeneration. Although some therapeutics have shown promising results in experimental models, reduced efficacy and effectiveness of therapies have occurred due to poor delivery methods in clinical trials [3]. Conventional delivery of therapeutics involves multiple injections that can cause pain and discomfort to the patient and present a risk for infections. As shown in Figure 3, delivery via injections can result in a high dosage of the therapeutic over a small period of time and possibly exceeding the toxicity level in an extreme case. However, controlled release systems can provide an initial burst of the therapeutic that eventually plateaus over a longer time period. Thus, controlled release systems can promote less dosing frequency and fewer risks for infections.

There is a need for a sustained release delivery system for SCI treatment due to the fact that nerve regeneration can take from few weeks to even months. Combining a controlled and sustained delivery system with an effective biomaterial to promote nerve regeneration after injury

![Figure 2: Conventional vs. controlled release drug delivery](image-url)
can result in a potential treatment to permanent nerve damage. Locally delivered, tunable drug-releasing hydrogels present a potential solution to developing a novel controlled release drug delivery system for SCI treatment. Due to hydrogels’ high versatility, the mechanical properties and degradation rates can be tuned accordingly to specific drug delivery systems and drug targets. In the case of promoting nerve regeneration after SCI, the hydrogel would possess similar mechanical properties to native nerves and a slow degradation rate of up to 4 weeks for a controlled therapeutic release.

ECM Hydrogels and Decellularization Approaches for SCI Treatment

Potential biomaterials for promoting nerve regeneration after SCI must be biocompatible to prevent immune rejection and acquire physiological properties that promote adhesion and cell growth on the site of injury [3]. Biologic scaffolds that mimic the native tissue properties are highly desirable for tissue regeneration [4,5]. Specifically peripheral nerve scaffolds consist of native ECM pro-regenerative proteins. The ECM consists of molecular and mechanical cues that can contribute to promoting tissue function and repair [4,5]. Thus, using naturally derived nerve scaffolds to promote nerve regeneration is promising due to its biocompatibility, biodegradable nature, and preserved ECM properties. ECM hydrogels present a common approach to meet this criteria. Hydrogels acquire favorable characteristics in controlled delivery systems due to their tunable nature [3]. ECM hydrogels include ECM proteins and pro-regenerative agents such as, collagen and laminin. To ensure the prevention of immune rejection, it is important to remove all cellular components prior to injection. The process of decellularization can remove cellular components via cell death, which can be induced via necrosis or apoptosis.

Common decellularization techniques consist of inducing necrosis with the use of harsh detergents or chemicals. During necrosis the cell membrane quickly becomes permeable and causes the release of intracellular remnants throughout the tissue matrix, which may be difficult to remove [5,6]. In addition, the use of harsh detergents for decellularization can damage the structural and biomechanical properties of the ECM scaffold [5,6]. Here, we use apoptosis to induce decellularization. Apoptosis, also known is programmed cell death, causes the formation of small apoptotic bodies that are easier to remove while preserving the matrix architecture. Moreover, by inducing apoptosis, the use of potentially harmful chemicals is neglected. Thus, by inducing apoptosis on rat peripheral nerves, we fabricated a novel biocompatible and biodegradable biological scaffold hydrogel to obtain a controlled and sustained release of regenerative proteins to promote nerve regeneration after SCI, as shown in Figure 4.

Here we designed an injectable, decellularized peripheral nerve hydrogel as a biomaterial platform to promote nerve regeneration following SCI.

**Figure 3: Apoptosis induced decellularized nerve hydrogel schematic**
Methods and Materials

Peripheral nerves were harvested from adult Sprague-Dawley rats and epineurium was carefully removed using fine forceps. Rat sciatic and radial nerves were harvested, which are located near the rat’s hamstring and front legs, respectively as shown in Figure 5 in red.

Decellularization Process

Once the epineurium was removed, the nerves can be stored in -20°C until use. If the nerves were stored in -20°C before beginning the decellularization process, the nerves were thawed for 15 minutes. Decellularization was initiated by transferring thawed or fresh nerves into 15 mL conical tubes (1 nerve/tube) and treating with 5 µM of an anti-cancer drug, Camptothecin, in a nutrient rich media, DMEM-F12, containing 1% PSA at 37°C under constant agitation for 24 hours, shown in Figure 6. After, the media was removed and replaced with 4X PBS to create an osmotic imbalance for the removal of apoptotic bodies for another 24 hours under agitation in room temperature.

After the hypertonic wash with 4X PBS, the nerves were followed by a 1X PBS wash for 15 minutes under agitation at room temperature. Once PBS was removed from the tubes, the nerves underwent a treatment of 1 mL of 75 U/mL of DNase to remove the nucleic material. Nerves were treated with DNase for 24 hours at room temperature without agitation, assuring that the nerves were completely immersed in the solution.

Once the DNase treatment was completed, three one-hour 1X PBS hypertonic washes were performed at room temperature and under agitation. After completion of PBS washes, nerves were treated with 0.2 U/mL of Chondroitinase ABC (ChABC) enzyme to remove proteoglycans. The nerves were immersed in this solution for at least 16 hours at 37°C without agitation. Finally, the nerves were washed three times in three hour intervals with 1X PBS to ensure the removal of any residuals or apoptotic bodies.
After the decellularization process, the nerves were either further processed for digestion and gelation or for an immunohistochemical analysis to verify decellularization.

**Immunohistochemical analysis**

To verify that the process did remove the majority of cellular components, the nerves were also prepared for immunohistochemistry. Immediately after the 3 three-hour 1X PBS washes, the nerves were fixed in 1.5 mL of 4% PFA for 1 hour in room temperature. The solution was removed and the nerves were washed with 1X PBS three times for 15 minutes. 1X PBS was removed and the nerves were stored in 7 mL of 30% sucrose in ddH₂O in 4°C for 3-4 days. Then, the nerves were fixed in OCT (optimal cutting temperature) compound media in 4°C for 2 days. After initially fixed in OCT, the nerves were positioned as desired for cyrosectioning using forceps. Once the nerves were positioned accurately, they were placed in the -80°C freezer for 20 minutes or until completely frozen. Nerve/OCT blocks can be stored in -20°C until further use. Cross-sectional and longitudinal cryosections were obtained for antibody staining.

Acellular and fresh nerve sections were stained with primary antibody neurofilament (RT97) (neurons), S-100 (Schwann cells), DAPI (nuclei), collagen, laminin followed by corresponding secondary antibodies to provide images to observe the removal of neurons, Schwann cells, and nuclei as well as the preservation of collagen and laminin, respectively.

A blocking buffer containing 0.3% Triton X100, 3% goat serum in 1X TBS was created. One set of fresh and acellular nerve sections were covered with RT97 (1:200 in blocking buffer) and S-100 (1:400 in blocking buffer) and another set was covered with primary antibody collagen produced in mouse (1:500 in blocking buffer) and laminin produced in rabbit (1:500 in blocking buffer). Both sets were kept in 4°C overnight in the dark. The samples were later washed with 1X TBS three times for 10 minutes covered at room temperature. Both sets of fresh and acellular nerves were covered with corresponding secondary antibodies (1:500 in blocking buffer) at room temperature for 24 hours covered and in the dark. 1X TBS washes were performed again (three times for 10 minutes) covered at room temperature. The samples were counterstained with DAPI in ddH₂O (1:1000 in ddH₂O) covered at room temperature for 5 minutes. The slides were rinsed with 1X PBS and covered with Fluoromount-G media and covered with coverslip. This was left overnight at room temperature to dry. Once the samples were dried, the slides were sealed with nailpolish and dried again overnight covered at room temperature. After the samples were all properly stained, images were taken to observe the removal of cellular components and preservation of ECM proteins.

**Digestion and gelation**

After the decellularization process, the nerves were flash-frozen with liquid nitrogen and lyophilized for at least 72 hours and later stored at -20°C until further use to create a precursor solution for nerve digestion. Based on previous experiments, a 30 mg/mL decellularized nerve precursor solution provided stable hydrogels. A 1 mg/mL pepsin/HCl precursor solution for hydrogel construction was created. A 10 mg/mL stock solution was initially created with 10 mg/mL porcine pepsin (Sigma P7012-1G) in 0.1 M HCl, which would be further diluted (1:10). If the nerves were stored in -20°C, the nerves were placed in a desiccator for 15 minutes or until the nerves reached room temperature. The appropriate amount of lyophilized nerves were weighed
and minced into 1-2 mm pieces using micro-scissors. The grinded nerves and 1 mg/mL pepsin solution were collected in a scintillation vial and wrapped the cap of the vial with Parafilm to prevent evaporation. The digest solution was placed on a stir plate to mix for 64 hours at room temperature.

After 64 hours of digestion, the precursor solution was neutralized with 1 M NaOH. To bring the digest solution to a neutral pH, a miniscule amount (approximately 1 µL) of 1 M NaOH was added to the vial and was mixed on the stir plate for approximately 1.5 minutes. After mixing, 0.1 µL of the pre-gel solution was pipetted out and placed on pH paper to test neutrality. This process was repeated until the pH was approximately 7.4. It is important to note the amount of volume being added and removed to obtain an accurate measurement of the final volume. Once the desired pH was reached, 1/9th of the current volume of 10X PBS was added to achieve the final dilution. Figure 7 represents a completed digestion. Finally, 35 µL of the precursor solution was added to a cylindrical silicon mold for hydrogel formation. The molded solution was placed in 37°C for 15 minutes, 30 minutes, 45 minutes, and 1 hour to observe gelation.

Results and Discussion

Immunohistochemical Results

Immunohistochemical results confirm successful cell removal and ECM preservation. The majority of nuclei and Schwann cells were removed while collagen and laminin remained prominent, as shown in Figure 8.
Based from Figure 8, it is concluded that decellularization via apoptosis provides successful removal of the majority of nuclei and Schwann cells. However, after decellularization, axons are still present. Although, the axons were not removed, their presence should not promote immune rejection based from previous experiments. Furthermore, it is observed that regenerative proteins, such as collagen and laminin were preserved throughout the decellularization process. Collagen I and IV were tested as they are the most prominent forms of collagen. Collagen and laminin were present in both the fresh and acellular nerve, however the majority of nuclei was removed from the decellularized tissue. This qualitative data provides promising and successful results towards our apoptosis decellularization system.

We also analyzed the individual effects of the chemicals within the apoptosis decellularization process, such as Camptothecin, DNase, and ChABC. The decellularization process was repeated in a similar fashion as previously discussed, however, only incorporating either Camptothecin, DNase, or ChABC while using 1X PBS as a control. Figure 9 shows the effects of the individual components on cell removal.
Figure 9: Effects of individual apoptosis components on cell removal.

Only using Camptothecin removes the majority of nuclei and Schwann cells. Only incorporating DNase removes nuclei and only using ChABC does not promote noticeable cell removal. These results are expected since DNase is known to only remove nucleic material and ChABC is known to remove CSPGs.

Digestion and Gelation Results

Gelation of the precursor solution was observed after a short incubation time of 15 minutes at 37°C, as shown in Figure 10.

Figure 10: Successful thermal gelation of apoptosis decellularized rat peripheral nerves in 1X PBS at various time intervals.

Figure 10 above demonstrates successful gelation of the apoptosis decellularized rat peripheral nerves after 15 minutes, 30 minutes, 45 minutes, and 60 minutes of incubation. No significant differences were observed between each time interval. Each gel was immersed in 1X PBS to test for stability and each observed promising and successful results. The precursor solution in 37°C incubation for 15 minutes created a stable, stiff, and well-formed hydrogel.
The precursor solution before neutralization was also tested for its ability to be stored at 4°C and -20°C for up to 4 weeks. Figure 11 and Figure 12 below represent the hydrogel formation after one and four weeks of storage at both temperatures, following the same neutralization and thermal gelation procedure as previously reported.

Figures 11 and 12 demonstrate the capability and success of storing the precursor solution up to 4 weeks at various temperatures. This provides the opportunity for the solution to be transported to different facilities over time and to create a bulk of precursor solution for future gelation experiments.

Conclusion

We developed a novel detergent-free decellularization method largely effected by the induction of apoptosis using Camptothecin. Images from the immunohistochemical analysis prove successful removal of cellular components, such as Schwann cells and nuclei while preserving the important pro-regenerative matrix proteins, such as collagen and laminin. After 15 minutes of incubation at 37°C, durable and stable ECM hydrogels were formed. Furthermore, the precursor solution was found to have the ability to be stored in 4°C and -20°C for up to 4 weeks. Future experiments will focus on hydrogel characterization, such as analyzing mechanical properties, degradation rates, and drug release kinetics. Finally, we plan to test the biocompatibility and efficacy of the hydrogel in a rat SCI model. This forward-looking detergent-free decellularization method presents the opportunity to develop native biomaterials from nerve scaffolds that can be use in a controlled release delivery system to reduce the formation of glial scar and ultimately promote regeneration following spinal cord injury.
References


