

LCC 4700 Undergraduate Thesis Writing

Sustained Delivery of Thermally Stabilized chABC by Lipid Microtubules

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ABSTRACT

Our knowledge of spinal cord injury repair is broadening with the developing technology for nerve regeneration and drug delivery. In this paper we discuss the current capabilities for spinal cord repair as well as those that are in development. We develop protocols for determining the thermal stability of chondroitinase ABCI and its ability to be implanted into a microtubule-hydrogel drug delivery vehicle as well as the release profile that results from this implantation. After the use of sodium dodecyl electrophoresis, we determined that the disaccharide trehalose has the capacity to thermally stabilize our therapeutic enzyme *in vitro*. We also determined that the microtubules are effective for sustaining the release of our enzyme while the hydrogel is effective for localizing its effects. The deactivation profile was experimentally quantified to allow for complete diffusion of our enzyme over the course of a two-week implantation. Our thermally stabilized enzyme and drug delivery system can be used for the purpose of facilitating nerve regeneration at the site of an injury.

1. INTRODUCTION

Spinal cord injury results in short-term and long-term loss of nerve control. In order to overcome injuries that sever nerves in the spinal cord, we can deliver specialized proteins to the site of injury that facilitate the redevelopment of these nerves. One such enzyme is chondroitinase ABCI (chABC). My research with Hyun Jung Lee, under the advisement of Dr. Ravi Bellamkonda, will improve the thermal stability of the enzyme chABC and determine the loading efficiency of the microtubule drug delivery vehicles that will be used to localize the enzyme at the injury site. The drug delivery vehicles were

implanted *in vivo* with a topical model to observe the cellular responses after spinal cord injury and quantify the functionality of the released chABC. The enzyme chABC was combined with brain-derived neurotrophic factor (BDNF) to facilitate the axonal recovery. We utilized the deactivation profile of the gel scaffolds to deliver the appropriate quantity of enzyme at the lesion site. We also attempted to produce longer microtubules by manipulating the protocol for their synthesis and holding the cooling process near the lipid melting point for longer durations. The quantification of the release profile facilitates the surgical implantation of our therapeutic drug delivery vehicle for the repair of spinal cord injury.

2. LITERATURE REVIEW

The field of clinical spinal cord repair is still in its infancy; while its history is very brief, it is nonetheless dynamic in its onset. This literature review is divided into two sections, the first focusing on overcoming the inhibitory molecules present at the site of injury and the second on focusing on the microtubule-hydrogel drug delivery pathway. In each, I provide a brief overview of the developments thus far followed by a statement of how the previous research will be built on in our lab.

2.1 Inhibitory Molecules for Nerve Regeneration

Chondroitin sulphate proteoglycans (CSPGs) are axon growth inhibitory molecules present in and around scar tissue (Lin 1970). After spinal cord injury, victims exhibit lower levels of sensory and motor function due to inhibitory molecules such as CSPGs that gather at the lesion site and prevent nerve regeneration. For this reason, there

has been a concerted effort by field researchers Anjana Jain, Young-Tae Kim, Robert McKeon and Ravi Bellamkonda to remove all CSPGs from a lesion site, effectively promoting nerve and functional regrowth. In July 2005, there existed no clinical strategy to promote regeneration in the injured spinal cord (Jain 497). Anjana Jain developed a clinical strategy in the form of microtubules in a BDNF-embedded agarose scaffold that conformally filled a spinal cord defect.

The agarose scaffold is ideal for neuronal applications because of its biocompatibility *in vivo*, its support of cell migration, its ability to be embedded with sustained release vehicles such as microtubules and its ability to bind protein to its backbone for spatial control (Jain 498). The mechanical properties of agarose can also be manipulated for optimal axonal outgrowth (Meilander 142). Jain embedded the agarose delivery vehicles with BDNF in an attempt to prove this method was capable of reducing the reactivity of the astrocytes and production of CSPGs that was responsible for hindering nerve regeneration.

After staining cross sections of the spinal cord injury with antibodies GFAP and CS-56 to identify reactive astrocytes and CSPG deposition, respectively, Jain found that the samples treated with BDNF exhibited significantly lower fluorescent levels of both, indicating BDNF's ability to significantly prevent the production of the inhibitory molecules. This breakthrough encouraged researchers to begin investigating the optimal ways in which the BDNF scaffolds could provide a supportive substrate for neurite expansion (Jain 502). The problem with this treatment lies in the fact that BDNF could only prevent the production of CSPGs, but it could not do anything to remove the CSPGs that were already present at the site of injury. Realistically, there is a significant duration

of time that elapses after spinal cord injury when the body delivers CSPGs to the lesion site before treatment is performed. In order to remove the CSPGs that are already gathered at the lesion site, there is a need for further research into not only CSPG prevention, but also CSPG removal.

The removal of these molecules is facilitated with the enzyme chABC. ChABC effectively cleaves the chondroitin sulphate glycosaminoglycan (GAG) chains responsible for growth inhibition. This enzyme promotes both axon regeneration and plasticity, but the problem lies in its ability to be used in vivo (Moreno-Flores 56). The enzyme is not biocompatible and denatures at body temperature, something that must be overcome before it is implanted at a site of injury.

To thermally stabilize the chABC, trehalose will be tested to see if it increases the lifetime of enzymatic activity at 37 degrees Celsius (Sakai 380). Trehalose is effective in enhancing the transgene expression mediated by DNA complexes (Tseng 1298). This characteristic is a direct result of its ability as a disaccharide to stabilize the structure of proteins. Trehalose stabilizes proteins while reducing immunosuppression and having minimal effects on the machinery of protein synthesis (Adamo 531). A study into plant physiology proved that the accumulation of trehalose in anhydrobiotic organisms allows them to survive severe environmental stress (Zentella 1473). The deactivation curve will be determined by measuring the enzymatic activity of chABC over a given number of days (Sakai 381).

Once the stabilization has been accomplished, we must develop an enzyme delivery pathway that allows the enzyme to be deposited at the site of injury over a long period of time. As of now, injection into test rats suggested the persistence of active

chABC for at least 10 days after injection (Lin 1990). The fact that chABC can significantly increase the critical length of nerve gap repair is motivation enough to try and harness its potential *in vivo* (Hattori 466).

2.2 Microtubule-Hydrogel Efficacy

Materials Science and Engineering researchers such as T.M. Allen, G. Gregoriadis, R. Santangelo, T.T. Hsu, J.K. Sherwood and M.V. Sefton began developing lipid-based systems to release therapeutic agents in the late 1980s. These systems include liposomes, cochleates, polymer-based systems such as ethylene-vinyl acetate copolymer and polylactides (Meilander 141). These systems are effective for drug delivery; however, they involve exposing the enzyme to organic solvents, and thus present the possibility of denaturing the protein and removing its ability to perform *in vivo*. To overcome this limitation, Nancy Meilander, Xiaojun Yu, Nicholas Ziats and Ravi Bellamkonda helped develop a microtubule-hydrogel system that utilized lipid microtubules embedded in agarose hydrogels.

These lipid microtubules were initially described by Yager and Schoen in 1984 as hollow and open-ended tubules with a lumen diameter of approximately 0.5 micrometers and walls formed of lipid bilayers (Schnur 1670). Meilander's group utilized these microtubules to provide slow release of the loading agent along with the hydrogel to localize the microtubules at the desired site and prevent their dispersion (142).

There are several different types of biocompatible and versatile lipid-based microtubule carriers for different therapeutic proteins and enzymes (Rawat 270).

Past research into local drug delivery has proven that d-alpha-Tocopheryl polyethylene glycol 1000 succinate is a novel additive to the poly(l-lactide) (PLLA) films (Dong 167). This addition proved to enhance facilitation of the release of the loading agent in vivo. Microtubules loaded with enzyme prove to be novel carriers for internal drug delivery. The size and concentration values of the loaded microtubules are factors that affect the long-term release profile of the enzyme (Wang 389).

Meilander studied the effect of protein molecular weight and found that it inversely affected the release rate. Further, she observed the effect of a higher initial protein concentration and observed that it increased the mass but not the percentage of the initially loaded protein released daily (141). These results still leave researchers with the task of utilizing these microtubules loaded with therapeutic agent to facilitate nerve regeneration. This healing process can be accomplished by combining the microtubule-hydrogel system with a porous scaffold that propagates and directionalizes nerve growth.

The purpose of porous scaffolds is to mimic the fibrous architecture of type I collagen, where nerve growth is optimal (Chen 2066). Some methods utilize a phase separation technique that we have not tried in the lab. This method allows the scientist to manipulate spherical pore size, interfiber distance, and fiber diameter while promoting cell seeding. However, we will utilize salt leaching, where the interconnectivity between pores is low and difficult to control and the fiber diameters are far greater than the typical fibers of the extracellular matrix (Chen 2067). The most simple and practical, this crude method of scaffold production will suffice to obtain the data sets for my research.

Research into cell proliferation within the PLLA scaffold has been done in the form of incubating a specimen over the course of 1 and 2 weeks (Ren 507). The

researchers also observed the cell morphology on the scaffold-cultured cells using SEM, which gave them insight into how effective cell growth is inside of a porous scaffold. These scientists observed cells attached to the pore surface and conglomeration of cells in some areas of the scaffold. The cells were concentrated in the gaps of the scaffolds, giving incentive to do away with these gaps and focus on the creation of a more homogenous porous polymer. A homogenous polymer will form microfibrillar structures within the pore walls of the PLLA foam that may act as additional soft anchorage sites for cells (Prabaharan 427).

3. MATERIALS AND METHODS

3.1 Analysis of Enzymatic functionality

The enzymatic functionality of chABC was examined using a molecular weight separation technique, SDS-PAGE. The substrate protein that was incubated with the chABC was decorin, with a known molecular weight of approximately 100 kDa. When digested by chABC, the decorin only exhibited its core weight of approximately 40 kDa. This discrepancy allowed for the determination of chABC's activity. The samples affected with chABC were cured with cross-linking sulfur molecules to produce a high net negative charge. These samples were then be loaded into an electrophoresis gel and run through a voltage gradient to allow for separation based on molecular weight. The results of the experiment allowed for the quantification of each sample's molecular weight and served as an indication of chABC's enzymatic functionality.

The procedure involved diluting protein samples 1:1 with Tris-SDS sample buffer containing 5% mercaptoethanol to denature the protein and incubating at 95 degrees

Celsius for four minutes. The gel electrode was placed into the electrophoresis chamber filled with Tris running buffer. Twenty microliters of each sample were loaded into each lane and electrophoresed at 200 Volts for one hour. Diluted chABC in PBS with and without trehalose was prepared to determine the difference in enzymatic activity with the addition of trehalose. The absorbance of fresh chABC with chondroitin sulfate C was considered the standard at 100% activity. This data will allow for the production of the deactivation curve.

3.2 Synthesis of lipid microtubules and loading agent

Hollow, open-ended lipid microtubules were synthesized using 1,2-bis-(triscosa-10-12-diynoyl)-sn-glycero-3-phosphocholine. The lipid was dissolved in 70% ethanol and cooled from 55 to 21 degrees Celsius, heated to 33 degrees Celsius and cooled again to 20 degrees Celsius for 48 hours. The microtubules self-assembled during this cooling procedure and after 2 weeks of incubation at room temperature, 50 mM trehalose were mixed and incubated overnight. After centrifuging and drying overnight, the microtubules were embedded with the loading agent based on the loading efficiency. To measure the functionality of the fresh microtubules, the microtubule/trehalose mixture, without pre-incubation at 37 degrees Celsius, was combined with decorin. The samples were centrifuged after 6 hours of incubation and the supernatant was analyzed with dimethylmethylene blue.

The protocol for synthesizing the microtubules was manipulated in an attempt to produce larger microtubules. The cooling process was lengthened at certain points and the resultant microtubules were observed to determine average length. Larger

microtubules would facilitate the capability for more of the therapeutic enzyme to be loaded and ultimately released at the lesion site.

3.3 Kinetic Analysis of chABC: Deactivation curve

In order to measure the kinetic activity of the enzyme, a protocol involving dimethylmethylene blue (DMMB) was used. A sample of diluted chABC mixed with trehalose was incubated at 37 degrees Celsius. For two weeks, a sample of the incubated solution was extracted every other day and mixed with a 200 $\mu\text{g/ml}$ decorin solution. After incubation, the DMMB was added to the sample and the absorbance was recorded at 520 nm. As a control, the absorbance of the enzyme without decorin was recorded and subtracted from the other absorbance reading. This procedure was repeated with chABC and no trehalose, whose absorbance was compared with the trehalose-stabilized enzyme. The deactivation curve was calculated by determining the percentage of the destabilized enzyme's absorbance in relation to the trehalose-stabilized enzyme.

3.4 Release profile of chABC from gel scaffolds

SeaPlaque was dissolved in PBS to produce an agarose concentration of 1.2%. After cooling the solution to 37 degrees Celsius, the gel was mixed with the volume of microtubules embedded with chABC/trehalose and put in a 48-well plate. The final concentration summed to 0.6%. The mixture gelled and incubated for two weeks. The supernatant was taken during incubation to determine the amount of released protein. Every other day, the PBS was sampled and replaced. The supernatant was mixed with

decorin and incubated at 37 degrees Celsius, at which point the sample was analyzed with the dimethylmethylene blue to quantify the release profile of the gel scaffold.

3.5 Fabrication of gel scaffolds and topical delivery

The microtubules, loaded with chABC/trehalose, was mixed with 1.2% filtered and sterilized SeaPlaque to obtain a final concentration of 0.6% gel. After gelling, a 15 microliter volume of the gel scaffold was implanted into each animal. The gelled scaffold of the microtubule-hydrogel complex loaded with the thermally-stabilized enzyme, was delivered into a dorsal hemisection animal model. A two-millimeter cubed scaffold was implanted on top of the lesion site and 0.7% SeaKem was used to cover the scaffold.

3.6 Immunohistology and Quantitative Analysis

To determine the enzymatic functionality of the chABC, the cross sections of the lesion site was stained with 3B3, 2B6, GFAP and CS56. Positive areas of astrocytes, digested CSPGs and intact CSPGs were thereby observed. Immunostained images were analyzed with custom-built software in MATLAB. The software generated line profiles radial to the defined interface and produced an output of the intensity of the stained signal along the profile. This output could be used to determine the relative intensity as a function of distance from the lesion interface.

3.7 Diffusion Profile

In order to visualize the diffusion profile of the microtubules, the loaded enzyme had to be conjugated with rhodamine. The chABC and rhodamine were dissolved in deionized (DI) water and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), which acts as a coupling agent for the conjugation. The reaction was allowed 24 hours at room temperature without light and then dialyzed against DI water for two hours. The dialysis removes the unused reactants from the solution and leaves the conjugated rhodamine-chABC complex.

The diffusion profile of the microtubules was thereby qualitatively viewed by way of the rhodamine staining. The stained enzyme was loaded into the microtubules and allowed to diffuse through the surrounding gel medium. Pictures were taken on the hour for three hours to determine the efficiency of the microtubule diffusion.

4. RESULTS

4.1 Enzymatic functionality of chABC

The enzymatic activity of chABC was tested using SDS-PAGE. This protein assay allowed for the comparison of the decorin substrate when exposed to chABC in its active and inactive states. The enzymatic functionality of chABC is shown in Figure 1.

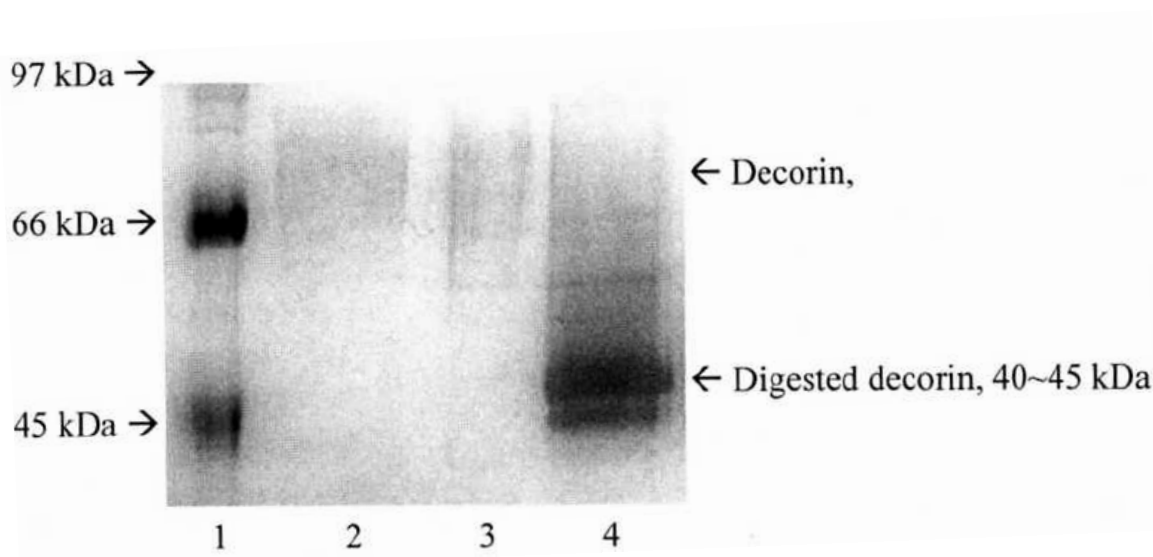


Figure 1. Enzymatic functionality test of chABC. The lanes represent (1) the ladder, (2) intact decorin (3) decorin reacted with pre-incubated chABC, and (4) decorin reacted with fresh chABC.

In Lane 2, a sample of intact decorin was electrophoresed. Lane 3 exhibits the electrophoresis of decorin reacted with chABC pre-incubated at 37 °C for 24 hours. Lane 4 exhibits the electrophoresis of chABC incubated for 4 hours at 37 °C. The substrate protein, decorin, was reacted with fresh chABC for 4 hours at 37 °C, producing a clear band in Lane 4 at 45 kDa, indicating digestion of the substrate. Not surprisingly, Lanes 2 and 3 are very comparable, displaying that the pre-incubated chABC produced the same results as the intact decorin, indicative of chABC's loss of enzymatic functionality when exposed to body temperature.

4.2 Thermal stability of chABC with trehalose

Trehalose at 20 mM, 50 mM, 100 mM, 250 mM, 500 mM and 1 M was reacted with chABC to examine the ability of the sugar-enzyme complex to retain its function when exposed to body temperature. The trehalose-chABC samples were pre-incubated at

37 °C for 2 weeks and allowed to react with decorin at 37 °C for 4 hours. The results of the electrophoresed samples are displayed in Figure 2.

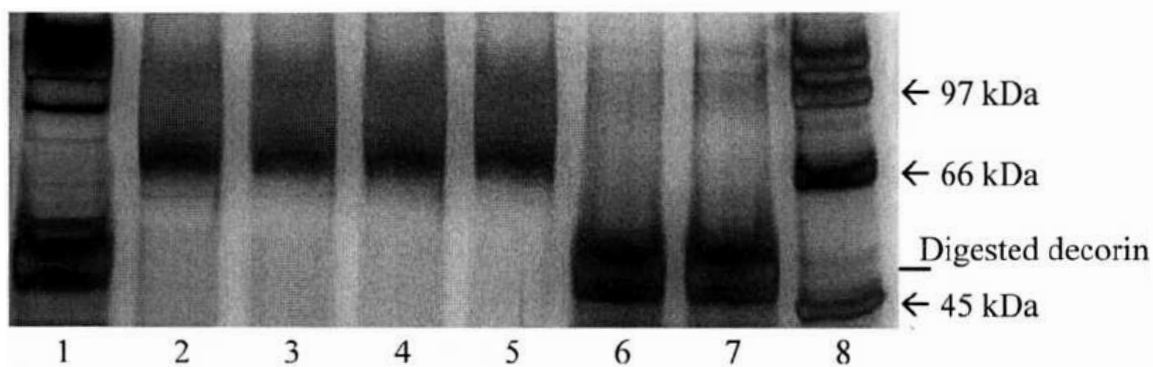


Figure 2. Enzymatic functionality test with various concentration of trehalose after incubation at 37 °C for 2 weeks. The lanes represent (1) decorin with fresh chABC, (2) 20 mM, (3) 50 mM, (4) 100 mM, (5) 250 mM, (6) 500 mM, (7) 1 M trehalose incubated with decorin, and (8) the ladder.

After running the samples through the gel, there is clear indication that decorin was only digested in Lanes 1, 6, and 7. This is telling of the concentration of trehalose needed to fully stabilize the enzyme and allow it to retain its functional ability through a 2-week incubation period. Only chABC incubated with 500 mM and 1 M trehalose could prevent the denaturing of chABC at body temperature.

4.3 Deactivation Curve

The enzymatic activity of chABC was analyzed every two to three days by determining the percent of decorin digested. Figure 7 shows the deactivation profile of chABC both with and without trehalose.

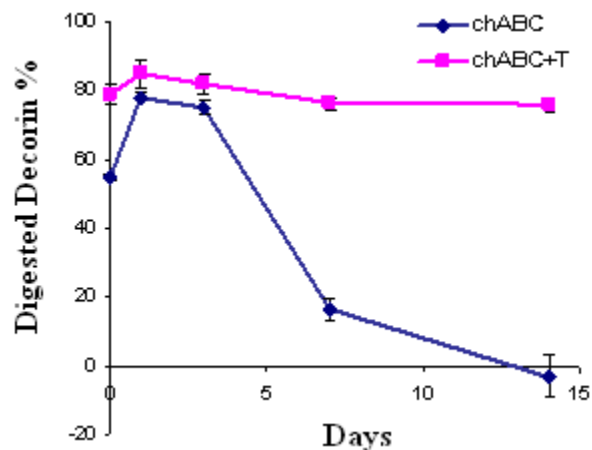


Figure 7. Deactivation profile of chABC enzymatic activity by DMMB assay. With the exception of day 1, all paired T-tests between the control (chABC without trehalose) and chABC with trehalose produced a P-value less than 0.05, indicating the significance of trehalose on protein stabilization.

Comparing the two curves, it is evident that trehalose has a significant impact on the deactivation curve of the enzyme. It took 14 days for the chABC without trehalose to lose all of its enzymatic activity. The time until the enzyme-trehalose complex reaches complete deactivation is currently unknown.

4.4 Microtubule Synthesis

After lengthening the cooling process used in microtubule synthesis, the resultant microtubules were examined at 10x magnification and imported to an image analysis software package. Each microtubule was measured and the average length was calculated. The microtubules did not exhibit an average length any greater than those produced from the original protocol. Figure 3 displays a histogram of the microtubule population for both the original procedure and the new extended time procedure.

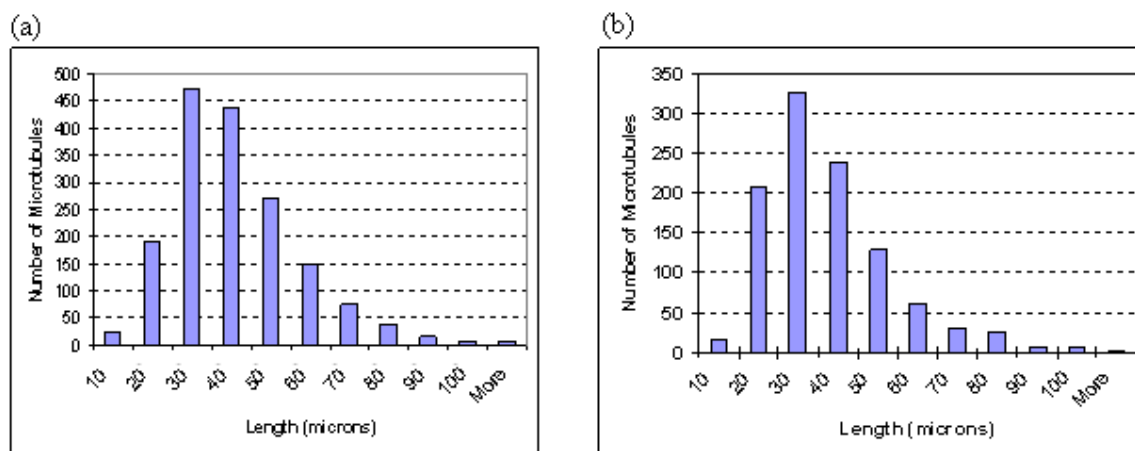


Figure 3. Histogram of microtubule population for both original and new protocol. (a) Histogram of the lengths of microtubule produced from the original protocol. The majority of microtubules were between 30 and 40 microns long. (b) Histogram of the lengths of microtubules produced from the extended time protocol. The majority of microtubules produced were also between 30 and 40 microns long.

It is evident from comparing these charts that the new protocol did not significantly increase the length of the microtubules. The distribution of lengths is very similar for both protocols.

4.5 Lipid microtubules as a vehicle for controlled delivery

The lipid microtubules were loaded with the thermally-stabilized chABC to examine its ability as a carrier for sustained delivery. The chABC released from the microtubules was combined with decorin and tested using SDS-PAGE. The resultant electrophoresis produced bands at 45 kDa. The functionality test of the chABC released from the microtubules is displayed in Figure 4.

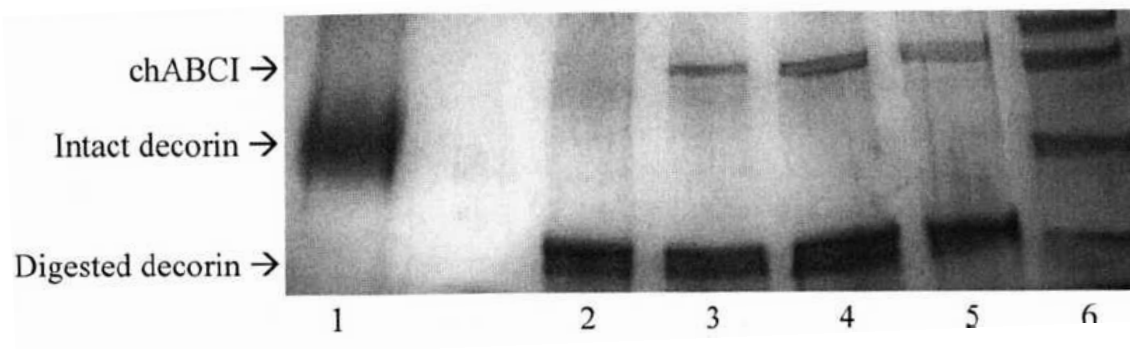


Figure 4. Functionality test of chABC released from microtubules. The lanes represent (1) intact decorin, (2) fresh chABC and decorin, (3)(4)(5) fresh microtubules and decorin, and (6) the ladder.

The functionality test shows that the chABC released from the microtubules was still in its active form, since its substrate produced bands that indicate its digestion. This is a promising result since it shows that the drug delivery system does not affect the potency of the enzyme.

This functionality test was used in conjunction with a control DMMB assay. In the control, chABC-trehalose diffused from microtubules was compared with penicillin-trehalose diffused from microtubules, which produced a deactivation curve as follows:

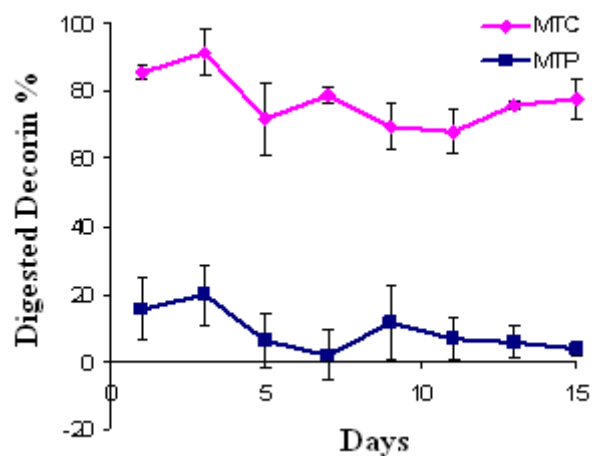


Figure 5. Control deactivation curve for chABC against penicillin. It is evident that the enzymatic ability of the trehalose with chABC-loaded microtubules is far more dramatic than that of trehalose with penicillin-loaded microtubules.

The hydrogel-microtubule delivery system was also implanted into a test rat to validate its applicability *in vivo*. The results from the procedure proved biocompatibility of the scaffold; a schematic of the delivery model is displayed in Figure 6.

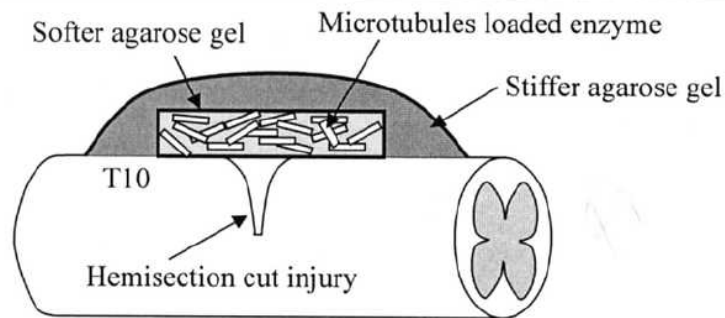


Figure 6. Schematic of the modeled injury with the microtubule-hydrogel system of enzyme delivery. The agarose gel scaffold is implanted on top of the lesion and coated with the stiffer agarose to prevent migration of the scaffold.

4.6 Immunohistology and Quantitative Analysis

The cross sections immunostained with the antibodies CS-56 and GFAP recognized the intensity of CSPGs and reactive astrocytes, respectively. The immunostained samples were photographed for image analysis and used as the input for custom MATLAB software that utilizes line profiles to relate relative intensity to distance from the lesion site interface. Figure 8 displays the initial step for the image analysis in which a box is sampled at the lesion site interface.

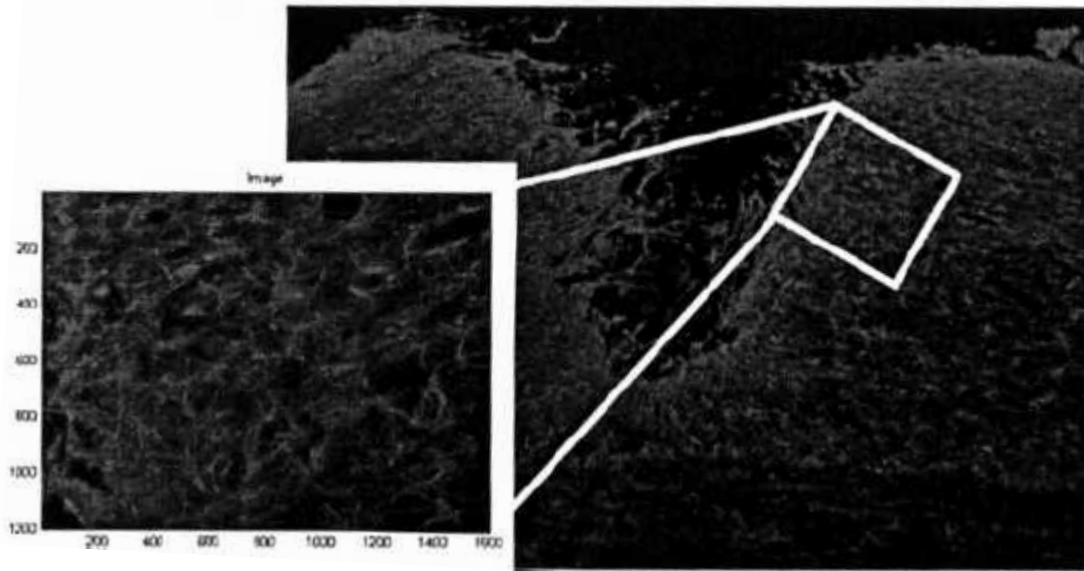


Figure 8. Immunostaining of GFAP for reactive astrocytes. The box is expanded here to display the analysis to be performed by the MATLAB code.

The biological remnants of CSPGs and reactive astrocytes were shown to exhibit high intensity at the lesion interface, which exponentially decreased as a function of distance from the interface.

4.7 Rhodamine-conjugated Diffusion Profile

The microtubules were allowed to diffuse their loaded enzyme stained with rhodamine and pictures were taken every hour. The results are below:

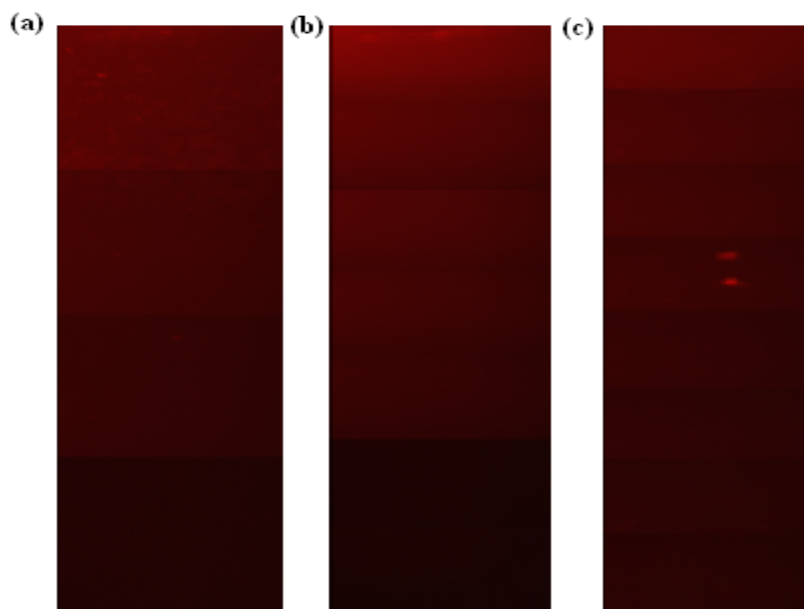


Figure 9. Diffusion profile of the rhodamine-stained enzyme released from the microtubules. (a) Diffusion profile after one hour. (b) Diffusion profile after two hours. (c) Diffusion profile after three hours.

These results make it difficult to see a significant difference between the hours of diffusion. However, it is easy to view the difference in intensity at the top of the diffusion profile between hours one and two. Although its intensity is more distributed throughout, the profile for the third hour spans the entire length of the medium, indicative of the diffusion effect for the rhodamine-stained enzyme.

5. ANALYSIS

5.1 Enzymatic functionality and kinetic analysis of chABC

The functionality of chABC is exhibited in the fact that the sample of decorin combined with the pre-incubated chABC in Lane 3 produced bands similar to the intact decorin in Lane 2. The electrophoresis indicates that the decorin was not digested by the pre-incubated chABC, inferring chABC lost its enzymatic ability during its exposure to

the 37 °C incubation. This lack of biocompatibility needs to be compensated for before the enzyme is used *in vivo*.

5.2 Thermal stability of chABC with trehalose

Even after 2 weeks of pre-incubation at 37 °C, chABC combined with 500 mM or 1 M trehalose produced bands of digested decorin. The incubations with 20 mM, 50 mM, 100 mM and 250 mM trehalose failed to produce electrophoresis bands around the 45 kDa characteristic of digested decorin. Therefore, if the enzyme is to be used *in vivo*, it must be incubated with a concentration of the stabilizing protein that is higher than 250 mM. The results for Lanes 6 and 7, 500 mM and 1 M trehalose, are strikingly similar, telling that increasing the trehalose concentration above 500 mM threshold will have minimal effects on chABC's enzymatic ability.

5.3 Microtubule Synthesis

Increasing the cooling time in an attempt to produce longer microtubules produced minimal results that were actually opposite of what was expected. The majority of the microtubules were still between 30 and 40 microns, but the fraction of microtubules between 20 and 30 microns increased while the fraction of those between 40 and 50 microns actually decreased. The new protocol that called for a slower cooling process did not result in longer microtubules. We will therefore not consider it in the experimental method for microtubule synthesis.

5.4 Lipid microtubules as a system for controlled delivery

The diffused chABC reacted with decorin and produced bands at 45 kDa, making it known that chABC does not lose its enzymatic activity even when diffused from the lipid microtubules and hydrogel. When diffused from a nanoparticle, chABC loses its enzymatic ability, making these microtubules a technological advancement in topical drug delivery for the enzyme. This loss of enzymatic ability is due to the toxic process that must be used to synthesize the nanoparticles. These results allow us to consider the sustained delivery system as a safe and effective way to localize the enzyme *in vivo*.

The topical delivery model on the test rat showed the functionality of the microtubule-hydrogel system. The stiffer agarose that covered the gel scaffold effectively stabilized the system and was fortunately accepted by the rat's anatomy. These clinical results are the initial steps to performing this type of operation on human patients.

5.5 Deactivation Curve

Starting at day 2, the paired T-test between the deactivation profile of chABC and chABC with trehalose produced a P-value of 0.01. This shows that the stabilizing effect of trehalose significantly increases the enzymatic activity of the enzyme. The 14 days that it took for the naked enzyme to lose its enzymatic ability is rivaled by the 80% digested decorin that the stabilized enzyme was able to maintain after the same duration. These results are promising for the *in vivo* maintenance of chABC.

5.6 Immunohistology and Quantitative Analysis

The aggregation of CSPGs and reactive astrocytes at the lesion interface tells us where the BDNF scaffold with chABC-loaded microtubules needs to be localized. In order to achieve maximum results, the microtubule-hydrogel system need only be implanted above the lesion site to deliver its therapeutic at the interface, where it will resist the gathering of the CSPGs present in the immunohistology analysis.

5.7 Rhodamine-chABC Conjugated Diffusion Profile

The rhodamine-stained enzyme indicates the even distribution of the chABC throughout the medium. This is indicative of the fact that the enzyme will spread throughout the agarose at the lesion site with even concentrations at all points of the interface.

6. DISCUSSION

The problem of biocompatibility has been overcome with the use of trehalose as the protein stabilizer. This sugar shows promise in making chABC immune to the denaturation that occurs at 37 °C. The enzyme can therefore be used *in vivo* for the removal of CSPGs from the site of injury, making the only remaining problem that of sustained delivery to the lesion site.

The microtubule-agarose partnership allowed for the loading of the enzyme as well as its active-state diffusion. The resulting release profile can be manipulated by either changing the size of the microtubules or the concentration of the loading agent. Since microtubule size was unsuccessfully manipulated, we should rely on enzyme concentration to produce the desired enzyme immersion at the lesion interface. Pending

on further research into the ideal enzyme quantity for the treatment of spinal cord injury, this concentration can be considered variable.

In order to prove the adaptability of the carrier system *in vivo*, the enzyme-loaded microtubules suspended in the gel scaffold will need to be implanted by way of the topical delivery model. If this can be reproduced consistently, the biocompatibility of the system will be validated clinically.

As a result of the analyses, we have determined that the thermal stability of chABC when treated with the stabilizing trehalose is enhanced when compared to the quickly-denaturing enzyme without trehalose. This overcomes the initial critical limitation of the clinical treatment for spinal cord injury. Further, the steady release of the enzyme from the microtubule-hydrogel partnership was demonstrated as a possibility and can be sustained over a two-week period with minimal invasiveness to the patient.

The overall biocompatibility of the drug delivery system will be marketable to medical distributors and the general public upon approval from the necessary government agencies. With this system of therapy, the treatment of spinal cord injury will be minimally invasive, requiring only one operation on the patient. The sustained delivery of chABC combined with the BDNF-embedded scaffold will allow for the digestion of gathered CSPGs and the prevention of others from aggregating. By clearing the lesion site of these fatty acid chains, the treatment will facilitate the redevelopment and maturation of axons into the extracellular space.

The potential of such a technology is groundbreaking for the field of spinal cord injuries. If successfully integrated into hospitals as the primary treatment for nerve damage, the partnership between chABC and BDNF will facilitate an ample environment

for nerves to develop. This environment will need to be further analyzed to optimize this development, something that will prove to be the next frontier.

Further research is needed for a predictable manipulation of nerve regrowth. Engineers will need to be able to localize growth factors that will encourage nerves to develop once the enzyme has performed its task of clearing the site of inhibitory molecules. The microtubule-agarose system will serve well to create an environment that has the potential to promote nerve regeneration. However, to guarantee the nerves grow in the right direction and through the right space will require a nerve-friendly scaffold. A scaffold will change the mere potential for regrowth into a surety for regrowth with the proper orientation and destination. Ideally, this scaffold will be embedded with the growth factors necessary to initiate growth from the surrounding nerves and to reconnect the nerve ends for functional recovery of the axon potential. The combination of a biocompatible scaffold that orients the direction of nerve growth with the microtubule-agarose drug delivery vehicle will allow modern medicine to bypass the permanent sensory and motor loss previously associated with spinal cord injury.

7. ACKNOWLEDGEMENTS

I want to thank my graduate student advisor, Hyun Jung Lee, for providing the materials necessary to obtain the procedures, methods and results

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