A Photo-Crosslinkable Chitosan Hydrogel for Peripheral Nerve Anastomosis

Todd Rickett1, Zohreh Amoozgar2, Wenjing Sun3, Yoon Yeo1,2, Riyi Shi1,3
1Weldon School of Biomedical Engineering
2Department of Industrial and Physical Pharmacy
3Department of Basic Medical Sciences
Purdue University
West Lafayette, IN, USA
riyi@purdue.edu

Abstract—The predominant therapy for peripheral nerve transection is anastomosis by suture. However, sutures have been known to lead to tissue inflammation, granulomas, and poor functional outcomes. While adhesives offer a promising alternative, fibrin—the predominant bio-glue—can transmit disease. Here we examine a photocrosslinkable chitosan hydrogel for use in surgical therapies for peripheral nerve injury. Prepared by conjugating 4-azidobenzoic acid to amino groups of chitosan using carbodiimide chemistry, this formulation demonstrates a high potential of in-situ photocrosslinking. A 40 mg/mL solution gels under 40 s of UV illumination. This gel is demonstrated to be cytocompatible with neural cell populations and is not acutely toxic to nerve conduction ex vivo. Mechanical testing of nerves anastomosed by this hydrogel had tensile strengths comparable to conventional fibrin glues. These results show chitosan hydrogel to be biocompatible and mechanically suitable for use in nerve repair.

Keywords—chitosan; bioadhesive; peripheral nerve; nerve anastomosis

I. INTRODUCTION

Peripheral nerves are easily severed, which can lead to chronic pain, lack of sensation or even debilitating limb paralyses. Current clinical guidelines recommend using sutures to reattach the nerves stumps. However, the use of sutures in this operation often results in poor fascicular alignment, variable bond strength, and tissue granulomas—all of which can cause undesirable patient outcomes[1]. Replacing sutures with a suitable bioadhesive simplifies the surgical technique and reduces operating time three fold[2]. Most research has focused on the use of fibrin glues, which have gained market prominence. Despite rigorous sterilization procedures, these fibrin sealants cannot eliminate the risks of disease transmission, and their clinical use has met with variable results[1, 3]. There is not yet a suitable bioadhesive on the market for the surgical anastomosis of peripheral nerves.

Chitosan is a widely available, bioadhesive polysaccharide which has gained increasing interest in the biomedical community. Chitosan is reported to be biocompatible[4], and its degradation products are nontoxic and nonimmunogenic[5]. For these reasons, chitosan has been explored in a broad range of applications such as surgical adhesives[6], muco-adhesive oral drug/gene delivery[7], and tissue engineering[8]. Chitosan is a linear copolymer of glucosamine and N-acetylglucosamine, obtained by partial (>50%) N-deacetylation of the natural polymer chitin. The presence of primary amine groups provides a unique opportunity to conjugate functional groups to modify the physicochemical properties of chitosan. In particular, chitosan conjugated with 4-azidobenzoic acid (Az-chitosan) can readily form a hydrogel upon UV illumination[9].

Given the adhesive properties of chitosan and the potential for in-situ crosslinking introduced by 4-azidobenzoic acid conjugation, we hypothesize that this Az-chitosan hydrogel will enable nerve anastomosis with mechanical strength comparable to or superior to that achieved through conventional fibrin glues. We further postulate that Az-chitosan is safe for use with neural tissues. This paper addresses these concerns with cytocompatibility and electrophysiological studies of Az-chitosan exposure. Mechanical analysis was conducted through tensile testing of nerves anastomosed by bioadhesives.

II. MATERIALS AND METHODS

A. Materials

4-azidobenzoic acid was purchased from TCI America (Portland, OR, USA), and chitosan from Sigma (MW: 50-190 kD, deacetylation: 85%). Dimethyl sulfoxide (DMSO) was obtained from Mallinkrodt chemicals (Phillipsburg, NJ, USA). Solvents were purchased from VWR.

B. Chitosan-azidobenzoic acid conjugation

N,N,N',N'-tetramethylethylenediamine (300 µL, 1.98 mmol) was added to a solution of 4-azidobenzoic acid (80 mg, 0.49 mmol) in 1 mL DMSO, followed by 1 mL aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (159 µL, 0.9 mmol). The resulting mixture was vortexed for 30 s and added to a solution of chitosan (400mg, pH 3) in a 1:1 mixture of water and DMSO. The final pH was adjusted to 5, and the reaction mixture was stirred overnight at ambient temperature in darkness. The reaction mixture was purified by...
centrifugation at 10,000 rpm for 3 hours. The Az-chitosan in
the supernatant was purified by repeating alkaline precipitation
(pH 9) and redissolution in acidic solution (pH 3). The
purification cycle was repeated at least 5 times until there was
no detectable 4-azidobenzoic acid in the supernatant. Finally,
the pH was adjusted to 5 with hydrochloric acid, and the
resulting solution was lyophilized. Az-chitosan was analyzed
by $^1$H NMR spectroscopy (Bruker DRX500).

![Figure 1. Structure of Az-chitosan. Molecular diagram and $^1$H NMR with characteristic peaks.](image)

C. Preparation of Az-chitosan hydrogel

Dry Az-chitosan was dissolved in normal saline to 40
mg/mL. This precursor solution was gelled by illuminated with
a long wavelength UV lamp (Black-Ray, UVP, radiation range
315-400 nm, peak at 365 nm) for a period specified in each
experiment.

D. Measurement of gelation time

Five 100 μL drops of the gel precursor solution were placed
on a polyethylene dish. The dish with precursor drops was
illuminated with the long wavelength UV lamp for 3 to 60
seconds at a distance of 6 cm. After each illumination, the dish
was taken out to test the consistency of each drop. A plastic
pipet tip was passed along the diameter of the drop, and gel
formation was determined when clear division of the drop was
observed. Gelation time was defined as the point when 80% of
the drops had formed gels.

E. Cytocompatibility

To demonstrate biocompatibility of the chitosan hydrogel,
200 μL of Az-chitosan was plated in each well of a 12 well flat-
bottomed culture plate (Becton Dickinson Labware). Half of
each well was coated with Az-chitosan, while the remaining
area of exposed tissue culture polystyrene (TCPS) acted as a
control. Plates were sterilized and Az-chitosan was gelled
through 60 s of UV exposure at 6 cm. Neuronal medium was
prepared from F12 nutrient base (Gibco), 2% horse serum,
conalbumin, Penstrep, insulin and Vitamin C. Dorsal root
ganglia (DRG) were dissected from day 7 chick embryos by
conventional methods[10]. Cells were plated in neuronal
medium at a density of 2.5 x $10^4$ cells/well. DRGs were
cultured in an incubator at 37°C and 5% CO$_2$ for 48 hrs. Cells
were fixed with 4% paraformaldehyde, permeabilized with
0.1% triton-X-100, and stained with propidium iodide (0.05
mg/mL). Cells were imaged using a Nikon Diaphot 300
microscope, a Nikon mercury lamp, and a CCD (Diagnostic
Instruments). Images were analyzed using Image-J (NIH) to
measure process length and cell density.

F. Nerve isolation

All experimental protocols for animal handling were
approved by institutional review (PACUC# 04-049). Male
Sprague-Dawley rats (350-450 g) were anesthetized with
ketamine (80 mg/kg) and xylazine (10 mg/kg). Animals were
perfused transcardially with oxygenated Kreb’s buffer solution,
and sciatic nerves were excised. Nerves were stored briefly in
cold, oxygenated Kreb’s solution to permit biochemical
recovery from the surgical process. Sciatic nerves intended for
electrophysiological testing were split into tibial and peroneal
components.

G. Electrophysiological recordings

Electrophysiological testing was conducted using a double
sucrose gap recording chamber[11]. Briefly, isolated peripheral
nerves were placed in the center of the chamber, and both
compound action potential (CAP) amplitude and latency were
recorded continuously. After ~15 min. stabilization, the fluid
level was lowered and approximately 100 μL of Az-chitosan
hydrogel was applied directly to the center of the nerve. UV
illumination was initiated immediately and continued for 60 s
at ~6 cm distance. Once the nerve was coated with Az-
chitosan hydrogel, fluid levels were returned to normal.
Electrophysiological recording was continued for 15 min
following nerve coating. Instantaneous waveforms and time
histories of nerve conduction were recorded. Here, the CAP is
defined as the sum of all evoked potentials through the nerve
specimen. Latency was recorded as the time from stimulus to
recorded CAP peak. Equipment included Ag/AgCl electrodes
and a bridge amplifier (Neurodata Instruments). Analysis was
conducted with custom Labview software (National
Instruments). Latency and CAP results were normalized to
their values prior to Az-chitosan exposure.

H. Mechanical analysis

Rubber grips (Henkel Consumer Adhesives Inc) were
superglued to the ends of the sciatic nerves to increase nerve
friction. A control group of nerves were tested intact, but
experimental samples were transected with scissors and
reconnected by application of ~200 μL of Tisseel® fibrin glue
(Baxter) or Az-chitosan hydrogel. Az-chitosan samples were
gelled by 60 s UV illumination atop a Teflon® block
(Dupont). Samples were loaded into a 100Q250 mechanical
Conjugation of 4-azidobenzoic acid was confirmed by $^1$H NMR: Chitosan: 2.01 (s), 3.0 (s, broad), 3.2-4 (m); 4-azidobenzoic acid: 7.17-7.19 (d, $J=10$Hz), 7.81-7.83 (d, $J=10$Hz). Conjugation of 4-azidobenzoic acid to chitosan was calculated by comparing an integrated area of peaks in 7-8 ppm (4H, benzene) and that in 3-4 ppm (6H, chitosan). The percent of amino groups conjugated with 4-azidobenzoic acid was 2% (Figure 1). Gelation time was found to be 40 seconds.

III. RESULTS

Conjugation of 4-azidobenzoic acid was confirmed by $^1$H NMR: Chitosan: 2.01 (s), 3.0 (s, broad), 3.2-4 (m); 4-azidobenzoic acid: 7.17-7.19 (d, $J=10$Hz), 7.81-7.83 (d, $J=10$Hz). Conjugation of 4-azidobenzoic acid to chitosan was calculated by comparing an integrated area of peaks in 7-8 ppm (4H, benzene) and that in 3-4 ppm (6H, chitosan). The percent of amino groups conjugated with 4-azidobenzoic acid was 2% (Figure 1). Gelation time was found to be 40 seconds.

DRGs cultured atop these hydrogels (Figure 2) grew at a density of 519±213 cells/cm$^2$. This was not statistically different ($p=0.2543$) from the density of DRG cells on TCPS, found to be 463±191 cells/cm$^3$. Process lengths were slightly reduced ($p=0.04$) when grown on Az-chitosan (150.7±40.8 μm) when compared to TCPS (194.3±54.3 μm). As seen in Figure 2C, normal cell morphology was observed on both surfaces.

In comparing axonal conduction in nerve before and after coating with Az-chitosan, CAP amplitude did not change significantly ($n=5$, $P > 0.05$, Figure 3). Also, Az-chitosan coating did not demonstrate significant effects on latency ($n=5$, $P > 0.05$, Figure 3). Electrophysiological data showed no effect of Az-chitosan application for the acute period studied.

Mechanical properties of the adhesives and intact nerves are shown in Table 1. For mechanical analysis, the average cross-sectional area of rat sciatic nerves was calculated through microscopic imaging ($n=4$). An average area of 1.008±0.083 mm$^2$ was obtained with an average diameter of 1.132±0.046 mm comparable to literature[12].

III. RESULTS

Conjugation of 4-azidobenzoic acid was confirmed by $^1$H NMR: Chitosan: 2.01 (s), 3.0 (s, broad), 3.2-4 (m); 4-azidobenzoic acid: 7.17-7.19 (d, $J=10$Hz), 7.81-7.83 (d, $J=10$Hz). Conjugation of 4-azidobenzoic acid to chitosan was calculated by comparing an integrated area of peaks in 7-8 ppm (4H, benzene) and that in 3-4 ppm (6H, chitosan). The percent of amino groups conjugated with 4-azidobenzoic acid was 2% (Figure 1). Gelation time was found to be 40 seconds.

DRGs cultured atop these hydrogels (Figure 2) grew at a density of 519±213 cells/cm$^2$. This was not statistically different ($p=0.2543$) from the density of DRG cells on TCPS, found to be 463±191 cells/cm$^3$. Process lengths were slightly reduced ($p=0.04$) when grown on Az-chitosan (150.7±40.8 μm) when compared to TCPS (194.3±54.3 μm). As seen in Figure 2C, normal cell morphology was observed on both surfaces.

In comparing axonal conduction in nerve before and after coating with Az-chitosan, CAP amplitude did not change significantly ($n=5$, $P > 0.05$, Figure 3). Also, Az-chitosan coating did not demonstrate significant effects on latency ($n=5$, $P > 0.05$, Figure 3). Electrophysiological data showed no effect of Az-chitosan application for the acute period studied.

Mechanical properties of the adhesives and intact nerves are shown in Table 1. For mechanical analysis, the average cross-sectional area of rat sciatic nerves was calculated through microscopic imaging ($n=4$). An average area of 1.008±0.083 mm$^2$ was obtained with an average diameter of 1.132±0.046 mm comparable to literature[12].

TABLE I. NEURAL ADHESIVE MECHANICAL PROPERTIES

<table>
<thead>
<tr>
<th></th>
<th>Az-Chitosan</th>
<th>Fibrin Glue</th>
<th>Intact Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain (μm)</td>
<td>0.16±0.07</td>
<td>0.19±0.13</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Force (mN)</td>
<td>71.9±23.6†</td>
<td>69.5±31.6†</td>
<td>2430.7±989.1†</td>
</tr>
<tr>
<td>Stress (kPa)</td>
<td>71.3±23.4†</td>
<td>68.9±31.4†</td>
<td>2411.4±981.2†</td>
</tr>
<tr>
<td>Young’s Modulus (kPa)</td>
<td>897±412</td>
<td>528±247</td>
<td>22689±6965†</td>
</tr>
<tr>
<td>Work to Failure (mJ)</td>
<td>81.0±39.7</td>
<td>160.9±65.5</td>
<td>5292.7±3401.9†</td>
</tr>
</tbody>
</table>

* represents parameters calculated from the mean cross-sectional area. † indicates significant variation ($p<0.05$) from intact nerve. □ indicates significant variation ($p<0.05$) between adhesives.

IV. DISCUSSION

Az-chitosan was first described by Ono et al.[9], who demonstrated that the crosslinked chitosan gel was able to seal a pinhole incisions in pig intestine, aorta, and trachea ex vivo. The same excellent wound healing effect of the crosslinked chitosan gel was observed in the mouse skin incision model[13]. We prepared Az-chitosan with two modifications of the original method. First, instead of lactobionic acid-conjugated chitosan, we used chitosans with lower molecular weights (50-190kD, as compared to 800-1000kD[9, 13]) to prevent chitosan from becoming excessively hydrophobic after 4-azidobenzoic acid conjugation. Second, 4-azidobenzoic acid conjugation was increased by reacting in 50% DMSO in water. As a result, we were able to modify 2% of amino groups in chitosan with 4-azidobenzoic acid without making the modified chitosan excessively hydrophobic. The chloride salt form of Az-Chitosan dissolved in normal saline up to 55 mg/mL. A 40 mg/mL solution formed a hydrogel in only 40 s under UV illumination. This allows for the surgical coaptation of transected nerves much more rapidly than is possible through suturing techniques or the application and curing of fibrin glues.
Az-chitosan biocompatibility was further demonstrated through electrophysiology. Hydrogel was applied to the epineurium of intact peripheral nerves ex vivo. Measurements of both impulse latency and amplitude did not differ before and after (10 min) application of Az-chitosan. Given the acute sensitivity of neurons to changes in the extracellular environment, this data demonstrates that epineurial application of Az-chitosan is not acutely toxic.

V. CONCLUSIONS
Transected peripheral nerves are most commonly anastomosed with sutures, but bioadhesives are known to be less damaging[1] and can improve patient outcomes 15%. However, the market leading bioadhesives are made of fibrin prepared from human blood plasma and have been demonstrated to carry disease[3]. Using chitosan-based bioadhesive, we seek to reduce risks of pathogenicity. Conjugating chitosan with 4-azidobenzoic acid enables rapid crosslinking under UV illumination. This photo-crosslinkable chitosan hydrogel is not acutely toxic to neural cells and does not adversely influence CAP conduction. Mechanical analysis demonstrates that Az-chitosan hydrogel can reconnect nerves with mechanical properties comparable to conventional fibrin adhesive. These findings support the use of Az-chitosan hydrogel as a new bioadhesive for surgical repair of peripheral nerve.

ACKNOWLEDGMENT
The authors would like to acknowledge the financial support of the Showalter Foundation, The State of Indiana, the Indiana Spinal Cord and Brain Injury Research Fund, and the Indiana Clinical and Translational Sciences Institute.

REFERENCES


