A Photo-Crosslinkable Chitosan Hydrogel for Peripheral Nerve Anastomosis

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***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 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 cytocompatability 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 Mallinckrodt 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

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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).

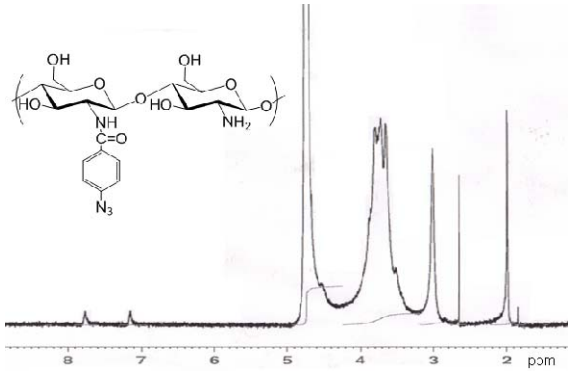

Figure 1. Structure of Az-chitosan. Molecular diagram and ^{1}H NMR with characteristic peaks.

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

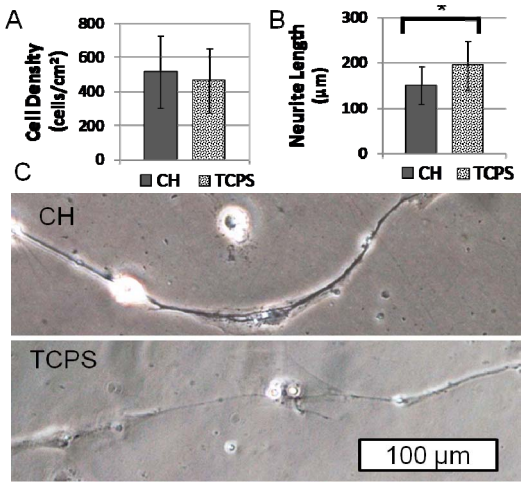

Figure 2. Az-chitosan cytocompatibility. (A) Cell density was similar on Az-chitosan hydrogel and TCPS. (B) DRG process lengths were slightly shorter growing on Az-chitosan. (C) Representational light micrographs of DRGs growing on the two surfaces show similar morphology. (* $p<0.05$)

testing system (Test Resources, Inc). The unstressed nerve was measured, and the system was zeroed to this unstressed reference length. Nerves were stretched until failure at 5 mm/min. Data was exported to a PC through Wincom® software (ADMET). Rat sciatic nerves (n=4) were imaged and analyzed by ImageJ to measure a representative cross-sectional area for tensile analysis.

Standard engineering definitions were used for analysis. Strain was calculated as the change in nerve length divided by the unstressed span. The force recorded by the load cell divided by the calculated average cross sectional area of rat sciatic nerve provided stress. Young's modulus was calculated as stress divided by strain for the linear portion of each stress-strain curve. Work to failure integrated from the load-strain curve and indicates the energy necessary to rupture a bioadhesive bond or intact nerve.

I. Statistical analysis

Results are reported as means±standard deviation, except for electrophysiological data which is reported as means±standard error of the mean. One-tailed ANOVA with pairwise comparisons between groups was used to compare means: statistical significance was ascribed to p-values less than 0.05.

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=10Hz), 7.81-7.83 (d, J=10Hz). 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^2. 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

	Az-Chitosan ***(n=8)***	**Fibrin Glue** ***(n=8)***	**Intact Nerve** ***(n=7)***
Strain	0.16±0.07	0.19±0.13	0.16±0.03
Force (mN)	71.9±23.6†	69.5±31.6†	2430.7±989.1†
Stress * (kPa)	71.3±23.4†	68.9±31.4†	2411.4±981.2†
Young's Modulus*(kPa)	897±412 †□	528±247 †□	22689±6965 †
Work to Failure (mJ)	81.0±39.7 †□	160.9±65.5 †□	5292.7±3401.9 †

* 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.

Mechanical tests on nerves anastomosed with this experimental adhesive demonstrate that the resultant bond is comparable to fibrin glue, but significantly weaker than intact nerve. The recorded loads and calculated values for stress did

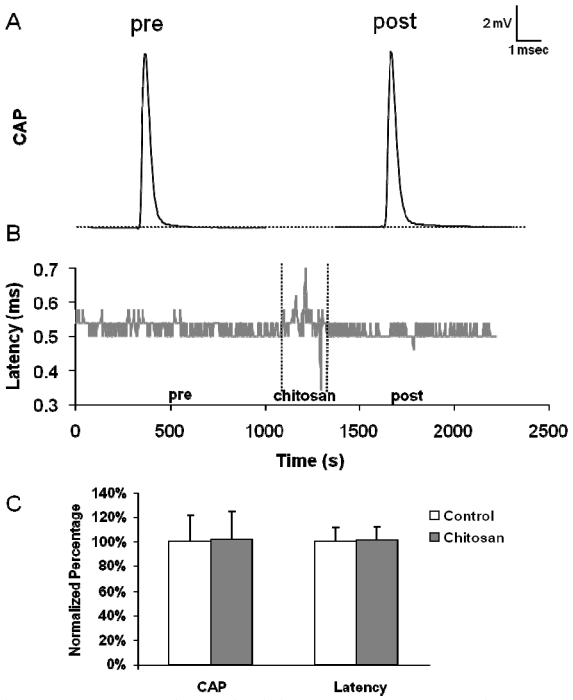

Figure 3. *Ex vivo* electrophysiology. (A) Representative CAP amplitudes before and 10 min. after application of Az-chitosan. (B) Representative time-lapse recording of latency of conduction. (C) Comparisons between normalized CAP amplitude and latency in pre- and post- Az-chitosan application.

not vary significantly between the two bioadhesives. Moreover, the Young's modulus of the Az-chitosan adhesive was 70% stiffer than the fibrin glue, better mimicking natural nerve. However, the fibrin absorbed more energy prior to rupture. Az-chitosan anastomosed nerves withstood 16% strain before structural failure, a value comparable to the 16% and 19% strains recorded for intact nerve and fibrin, respectively. It is known that sustained functional deficits are incurred by as little as 10% strain[14]. Therefore, these results indicate that nerves coapted with Az-chitosan are capable of withstanding *in vivo* levels of force.

The use of sutures has been linked to granulomatous inflammation and poor fascicular alignment[1]. While chitosan is generally considered biocompatible, this formulation had not been tested in neural cell culture. In this study, short term exposure to Az-chitosan was not found to inhibit neuronal adhesion or survival when compared to standard tissue culture polystyrene. This demonstrates that neural cells are capable of growing on or in the hydrogel. A small but significant decrease in the length of DRG processes was observed on Az-chitosan. This indicates that while cells can attach and extend processes on the hydrogel, using Az-chitosan as a neural adhesive could discourage aberrant regeneration. Thus, it is expected that applying the adhesive coating around the severed nerve stumps will act to inhibit neuronal process extension outside the nerve trunk without affecting cell viability. Consequently, the Az-chitosan coating will likely encourage proper regeneration and improve functional outcomes.

Az-chitosan biocompatibility was further demonstrated through electrophysiology. Hydrogel was applied to the epineuria 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%[2]. 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.

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