Rapidly Photo-Cross-Linkable Chitosan Hydrogel for Peripheral Neurosurgeries

Todd A. Rickett,[†,‡] Zohreh Amoozgar,[†,§] Chad A. Tuchek,[‡] Joonyoung Park,[§] Yoon Yeo,[*,‡,§] and Riyi Shi[*,‡,||]

Weldon School of Biomedical Engineering, Department of Industrial and Physical Pharmacy, and Department of Basic Medical Sciences, Purdue University, West Lafayette, Indiana 47907, United States

Received August 26, 2010; Revised Manuscript Received October 18, 2010

Restoring continuity to severed peripheral nerves is crucial to regeneration and enables functional recovery. However, the two most common agents for coaptation, sutures and fibrin glues, have drawbacks such as inflammation, pathogenesis, and dehiscence. Chitosan-based adhesives are a promising alternative, reported to have good cytocompatibility and favorable immunogenicity. A photo-cross-linkable hydrogel based on chitosan is proposed as a new adhesive for peripheral nerve anastomosis. Two Az-chitosans were synthesized by conjugating 4-azidobenzoic acid with low (LMW, 15 kDa) and high (HMW, 50−190 kDa) molecular weight chitosans. These solutions formed a hydrogel in less than 1 min under UV light. The LMW Az-chitosan was more tightly cross-linked than the HMW variant, undergoing significantly less swelling and possessing a higher rheological storage modulus, and both Az-chitosan gels were stiffer than commercial fibrin glue. Severed nerves repaired by Az-chitosan adhesives tolerated longitudinal forces comparable or superior to fibrin glue. Adhesive exposure to intact nerves and neural cell culture showed both Az-chitosans to be nontoxic in the acute (minutes) and chronic (days) time frames. These results demonstrate that Az-chitosan hydrogels are cytocompatible and mechanically suitable for use as bioadhesives in peripheral neurosurgeries.

Introduction

Peripheral nerves are commonly damaged by mechanical trauma, iatrogenic injury, and mechanical insult. Such wounds can cause pain, numbness, dysaesthesia, and loss of mobility or dexterity over acute and chronic time frames.[1] The most severe form of nerve injury, neurotmesis, separates proximal and distal nerve trunks. If anatomical continuity is not restored, there is little chance of nerve healing or functional recovery.[2] Therefore, reconnecting severed peripheral nerves is crucial for improving patient outcomes. Surgical treatment of transected nerves usually involves microsurgical suture techniques to anastomose the divided ends with epineurial or perineurial sutures.[3] However, this conventional treatment has several noteworthy limitations. Microsuture placement extends operating times and further traumatizes injured nerves. Chronic implantation can lead to inflammation, foreign body reactions, and scarring that discourage regeneration and may impair nerve function.[4] Functional outcomes after standard therapy vary, but are frequently poor. Kim et al. reported that 28% of patients receiving suture repair of the ulnar nerve described post-treatment outcomes as fair, poor, or absent.[5] Additionally, in the Jaquet et al. study of conventional surgical treatments for upper extremity nerve injuries, less than 50% of patients reported good motor function.[6] Therefore, the development of alternative treatments with increased efficacy is necessary.

Bioadhesive alternatives to sutures for nerve anastomosis, such as coaptation with cyanoacrylates or fibrin-based surgical glues significantly reduce operating times.[7] Cyanoacrylates provide excellent bonding strength between nerves, but potentially cause inflammation.[8,9] Furthermore, cyanoacrylates are known to release formaldehyde and cyanoacetate, inducing histotoxic effects when used for internal applications.[10] Fibrin glues bond quickly, but have mixed results in nerve anastomosis. Some human studies have reported positive clinical outcomes,[11] while other groups have indicated an extremely high rate of dehiscence and low bonding force postoperatively.[12,13] As a product derived from human blood plasma, commercial fibrin glues can also function as pathogen vectors.[14]

As the existing techniques have considerable drawbacks, there is a clear need for an improved bonding agent in peripheral neurosurgeries. Ideally, such an adhesive would be prepared from nonhuman biomaterials that are safe for chronic implantation, bond rapidly, provide a mechanically stable environment to support nerve healing, and preferably disappear once the anatomical continuity is restored. Additionally, the presence of such an adhesive should not impact nerve function.

Chitosan is a biopolymer of natural origin, possessing many of the aforementioned properties. Prepared through the alkaline *N*-deacetylation of chitin, chitosan is a linear polymer consisting of $\beta(1-4)$-linked D-glucosamine and *N*-acetyl-D-glucosamine. Widely regarded as biocompatible,[15] chitosan degrades into byproducts known to be nontoxic and nonimmunogenic.[16] Chitosan has been investigated for many biomedical applications including tissue culture scaffolds,[15] drug/gene delivery vectors,[17] wound healing,[18] and surgical adhesives.[19] The adhesive properties of chitosan may be attributable to strong intermolecular forces such as hydrogen bonding and ionic attraction between polycationic chitosan and the anionic part of collagen ubiquitous in mammalian tissues.[20]

For maintaining nerve continuity under stress, cohesive forces (chitosan−chitosan interaction) are as important as adhesive

* To whom correspondence should be addressed. Tel.: +1 765-496-3018 (R.S.); +1 765-496-9608 (Y.Y.). Fax: +1 765-494-7605 (R.S.); +1 765-494-6545 (Y.Y.). E-mail: riyi@purdue.edu (R.S.); yyeo@purdue.edu (Y.Y.).
† These authors contributed equally to this work.
‡ Weldon School of Biomedical Engineering.
§ Department of Industrial and Physical Pharmacy.
|| Department of Basic Medical Sciences.

10.1021/bm101004r
Published on Web 12/03/2010

forces (chitosan−tissue attraction). Introducing covalent cross-links between the chitosan chains is one approach for improving gel cohesion. Photo-cross-linkable hydrogels have been widely explored for tissue engineering,[21] wound healing,[22,23] drug delivery,[24,25] and bioadhesive applications[19] for the unique ability to form covalent cross-links by photoactivation, a relatively mild chemical reaction that can be performed in contact with tissues. A variety of natural and synthetic polymers have been modified to photo-cross-linkable macromers[26] via (meth)acrylation[27,28] or conjugation with aryl azide.[29] Hydrogel networks of these macromers are formed by photoactivation of the coexisting photoinitiators ((meth)acrylated macromers) or reactive groups built in the macromers (aryl azide-modified macromers). Photo-cross-linkable chitosan (Az-chitosan), a polysaccharide backbone grafted with 4-azidobenzamide, can be created by reacting chitosan with 4-azidobenzoic acid.[29] The product dissolves in water, producing a viscous solution that rapidly forms a hydrogel under ultraviolet (UV) irradiation. Gelation of Az-chitosan occurs via photolytic conversion[30] of aryl azide to reactive nitrene, which undergoes ring expansion and reaction with amines to form inter- and intramolecular networks.

We hypothesize that Az-chitosan hydrogels are safe and effective adhesives for peripheral nerve anastomosis, and we predict that varying the molecular weight will impact their physical properties. Hydrogels were prepared with chitosans of two different molecular weights to examine the effects of polymer chain length on their gelation time, mechanical stability, swelling behavior, and toxicity. The chemical, mechanical, and biological evaluation of the Az-chitosan hydrogels warrant further study into this line of neurosurgical adhesives.

Materials and Methods

Materials. 4-Azidobenzoic acid (ABA) was purchased from TCI America (Portland, OR, U.S.A.), and low molecular weight chitosan (MW: 15 kDa; deacetylation degree: 87%; "LMW chitosan") was purchased from Polysciences (Warrington, PA, U.S.A.). High molecular weight chitosan (MW: 50−190 kDa; deacetylation degree: 83%; "HMW chitosan"), bovine serum albumin (BSA), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), horse serum, PenStrep, triton X-100, and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Solvents were purchased from VWR (West Chester, PA, U.S.A.). The commercial bioadhesive, Tisseel fibrin sealant, was purchased from Baxter Healthcare Corp. (Deerfield, IL, U.S.A.). Superglue (ethyl-cyanoacrylate) was obtained from Henkel Adhesives (Dusseldorf, Germany). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Green Island, NY, U.S.A.), as were AlexaFluor-488 conjugated phalloidin and a Live/Dead viability/cytotoxicity kit (L3224). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, U.S.A.), and nerve growth factor (NGF, GF-022-5) was obtained from Austral Biologics (San Ramon, CA, U.S.A.). The anesthetics ketamine and xylazine were purchased from Fort Dodge Animal Health (Fort Dodge, TX, U.S.A.) and Lloyd Laboratories (Shenandoah, IA, U.S.A.), respectively.

Synthesis of Az-Chitosan. Az-chitosan was synthesized according to a previously reported method.[29] TEMED (300 μL, 1.98 mmol) was added to a solution of ABA (80 mg, 0.49 mmol) in 1 mL of dimethyl sulfoxide (DMSO), followed by addition of 1 mL aqueous solution of EDC (159 μL, 0.9 mmol). The resulting mixture was vortexed for 30 s and added to a solution of chitosan (either LMW or HMW, 400 mg) in a 1:1 mixture of water and DMSO. The final pH was adjusted to 5 using 1 M HCl, and the reaction mixture was stirred overnight at ambient temperature in darkness. ABA precipitates in the reaction mixture were removed by centrifugation at 10000 rpm for 3 h. ABA-chitosan conjugate (Az-chitosan) in the supernatant was purified by alternating alkaline precipitation (pH 9) and redissolution in acidic solution (pH 3). To minimize salt accumulation, the alkaline precipitates were removed from the residual liquid as much as possible prior to redissolution in the acidic solution. The purification cycle was repeated at least five times until there was no detectable ABA in the supernatant by UV/vis spectroscopy (DU 650, Beckman Coulter Inc., Brea, CA, U.S.A.). Without further purification, the pH was adjusted to 5 with 1 M HCl, and the resulting solution was lyophilized. Az-chitosan was analyzed by ^{1}H NMR spectroscopy (DRX500, Bruker Scientific Instruments. Billerica, MA, U.S.A.). The salt content in the lyophilized Az-chitosans, irrespective of molecular weight, was consistently 8 wt %, as determined from the lost weight after further dialysis of Az-chitosan in water.

Az-Chitosan Photopolymerization. Az-chitosan was prepared as 40 mg/mL solution in normal saline. The Az-chitosan solution was gelled by illumination with a long wavelength UV lamp (Blak-Ray B-100 AP/R, UVP, Upland CA, U.S.A., radiation range 315−400 nm, peak at 365 nm). The effective irradiance of this lamp was measured at a 90° angle at distance of 6 cm using an IL1700 Radiometer (International Light Technologies, Newburyport, MA, U.S.A.). Readings were repeated at 5 min intervals to establish an average irradiance. Proper protective equipment was utilized at all times when working with the UV light.

To measure the gelation time, five or six drops (each 100 μL) of the gel precursor solution were placed on a polyethylene dish. The dish with precursor drops was illuminated with the long-wavelength UV lamp at a distance of 6 cm. After UV illumination for 15, 20, 25, 30, 35, 40, 45, or 50 s, the dish was taken out to test the consistency of each drop. A plastic pipet tip was passed along the diameter of the drop. Samples were considered to have gelled when the pipet tip was able to divide the drop into two separate halves. The gelation time was defined as the range of time taken for all 5−6 drops of precursors to form gels.

Rheological Analysis of Az-Chitosan. The rheological properties of gels were studied using a stress-controlled AR-2000 Rheometer (TA Instruments, Leatherhead, Surrey, U.K.) with standard parallel steel plates (20 mm diameter). The gel was formed by placing 450 μL of Az-chitosan precursor solution in a template made of a silicon sheet (900 μm thickness) on the Peltier plate, followed by UV illumination. Gels of this thickness are typically formed in less than 1 min, but the gels were irradiated for 10 min, simply to ensure that the gelation was complete. Fibrin glue was also analyzed for comparison. Fibrin glue was prepared per the manufacturer's instructions. The precursor solutions were extruded from a dual-barrel syringe through a common outlet. The extruded fibrin glue (450 μL), which turned to a gel instantly, was placed on the Peltier plate. A gap width of 900 μm was used for all experiments. A solvent trap (a circular well) filled with water was placed around the samples to prevent their dehydration during the experiment.[31] The water in the solvent trap evaporates and creates an environment saturated with water vapor inside the enclosure. All measurements were performed at 25 °C. A stress sweep (σ, 0.07−1000 Pa) was performed on the gel at 0.1 Hz in a dynamic oscillatory mode to determine their storage (G', Pa) and loss (G'', Pa) moduli. The G' results were expressed as mean values of all data points on the linear viscoelastic region.

Nerve Isolation. Adult male Sprague−Dawley rats (350−500 g, Harlan) were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) in an institutionally approved protocol (PACUC #04-049). Transcardial perfusion with cold, oxygenated Krebs' buffer solution euthanized the animals while removing blood. Sciatic nerves were excised from the sacral plexus to their split in the posterior thigh. Nerves for mechanical testing were stored in refrigerated (4 °C) Krebs' buffer. External connective tissue was removed from specimens intended for electrophysiology, which were maintained in cold, oxygenated Krebs' buffer to allow for biochemical recovery.

Tensile Analysis of Nerves Anastomosed with Az-Chitosan. The methods for evaluating adhesive efficacy in peripheral nerves under tension were modified from a previously described protocol.[8] Trial and error were used to develop a procedure for efficiently coapting peripheral nerves with force profiles that could be monitored with high precision. Briefly, nerves were severed at their midpoint and a coating of ~100 μL of either LMW or HMW Az-chitosan was applied, and the samples were gelled by 60 s UV illumination at a distance of 6 cm. A second coating was applied to the reverse surface and hydrogels were again irradiated for 60 s. Control groups of nerves were anastomosed either with two applications of ~100 μL Tisseel fibrin glue or two quadruple square knot sutures of 10−0 monofilament nylon (N-2770, Monosof Covidien Synature, Mansfield, MA, U.S.A.).

Samples were loaded into a 100Q250 (Test Resources, Shakopee, MN, U.S.A.) computer-controlled vertical mechanical testing system. The force sensor was zeroed to the weight of each specimen, and the nerve's unstressed reference length was measured. The load cell recorded the tension on the system at 64 Hz while nerves were stretched at 5 mm/min (~0.1% strain/s) until failure. A Windows XP (Microsoft, Redmond, WA, U.S.A.) computer with Wincom software (ADMET, Norwood, MA, U.S.A.) was used to export data from the testing device.

For data analysis, standard engineering definitions were used. The ultimate tensile strength was obtained as the maximum force value recorded by the load cell prior to failure. Strain was calculated as the change in position for the grips divided by unstressed length of the nerve. Engineering stress values (in kPa) were estimated for each tension reading recorded by the load cell (in N) divided by the average cross-sectional area for unloaded rat sciatic nerves (in mm^2). As nerves are soft tissues and easily deformed, it was necessary to use this representational value obtained by imaging sectioned sciatic nerves from comparably sized rats and measuring their area with ImageJ (U.S. National Institutes of Health, Bethesda, MD, U.S.A.). The Young's modulus for each nerve-adhesive was calculated as the slope of the linear portion of the characteristic stress−strain curve.

Swelling Ratio. Az-chitosan was prepared as 40 mg/mL solution in normal saline. A total of 100 μL of the gel precursor solution was transferred to a cylindrical template (diameter: 4 mm; height: 6 mm) and cross-linked by UV illumination. Gels of this thickness typically formed in 3 min, but the gels were irradiated for 5 min to ensure complete gelation. The gels were then transferred to plastic dishes with known weights, dehydrated under vacuum overnight, and weighed (W_d). Subsequently, the Az-chitosan disks were rehydrated in distilled water, and their weight changes were monitored periodically until the weights no longer changed: the hydrogels were recovered at regular time points, blotted with tissue paper to remove excess water, and weighed (W_t). The mass swelling ratio (Q_M) at equilibrium was defined as the ratio of the wet weight of a fully swollen gel to the dry weight (W_t/W_d).

Acute Toxicity. Conduction through peripheral nerves is highly sensitive to changes in the extracellular media: toxic materials are known to impact conduction within minutes.[32,33] To assess the potential acute toxicity of the Az-chitosan hydrogels, electrophysiological conduction through intact ex vivo nerves was evaluated before and after exposure to the adhesive. These tests were performed using a double sucrose gap recording chamber, as has been previously described.[8] Briefly, isolated sciatic nerves were sealed in the chamber and conduction was established at 25 °C. Stimulus was applied using a Neuro Data PG4000 digital stimulator, and recording was accomplished using a bridge amplifier (Cygnus Technologies, Delaware Water Gap, PA, U.S.A.). The compound action potential (CAP) amplitude and latency were monitored continuously and allowed to reach steady state. After 10 min of stable conduction was recorded, the fluid level was lowered. A syringe was used to apply ~100 μL of either the LMW or HMW Az-chitosan on the nerves. This was gelled through 60 s UV exposure at a distance of 6 cm. Prolonged UV exposure was avoided to prevent dehydration of the nerves, which can change their mechanical properties. Fluid levels were restored, and recording was reestablished. Conduction through the nerve was allowed to re-equilibrate, and an additional 10 min of recording was conducted. Data analysis was performed using a custom Labview interface (National Instruments). Results from CAP and latency were normalized to their average values prior to adhesive exposure.

Cell Survival. The potential toxicity of Az-Chitosan was evaluated through week long cell culture. A ~100 μL aliquot of either HMW or LMW Az-chitosan was placed in four wells of a 24-well flat-bottomed culture plate. Solutions covered most of the well surfaces and were gelled through 60 s of UV exposure at 6 cm. Rat PC-12 cells were plated at 20000 cells/well on surfaces of LMW ($n = 4$), HMW Az-chitosan ($n = 4$), or unmodified tissue culture polystyrene (TCPS, $n = 4$). Cells were cultured in standard media prepared from 84% DMEM, 12.5% horse serum, 2.5% FBS, and 1% PenStrep with 100 ng/mL NGF at 37 °C in 5% CO_2. Cells were maintained in culture for 1, 4, or 7 days, and medium was replaced every other day with gentle suction to avoid removing loosely adherent cells. At the end of their culture period, cell viability for each plate was assessed using a Live/Dead viability/cytotoxicity assay. Cells were incubated in 2 μM calcein AM and 4 μM ethidium homodimer in PBS for 40 min to stain the cytoplasm of live cells and the nuclei of dead cells, respectively. Microscopy was conducted immediately using a Nikon Diaphot 300 microscope. For each culture surface, 12 images were obtained for each dye at random positions within the wells using a Diagnostic Instruments 11.2 color mosaic CCD camera and exported using ImagePro (MediaCybernetics Inc., Bethesda, MD, U.S.A.). Images were cropped to exclude clumps of cells. To prevent observational bias, cell number was assessed using a macro in ImageJ. For automated analysis, cells were defined as fluorescing objects with areas between half the average size of ethidium stained cells (representing dead cells) and 2 times the average size of calcein-stained cells (representing live cells). Cell survival was quantified as the number of cells fluorescing at 488 nm divided by the sum of cells fluorescing at 488 and 540 nm. Average survival values for LMW and HMW Az-chitosan hydrogels were normalized to the average survival on the control TCPS surface for each time point.

Cell Process Extension. Cell process extension was evaluated as a measure of the capacity of neural cells to grow on these materials. For these experiments, cells were cultured as described above. After 1, 4, or 7 days, cells were removed from culture, fixed with 4% paraformaldehyde, and permeabilized with 0.1% triton X-100. AlexaFluor-488 conjugated phalloidin and propidium iodide stained cytoskeletal actin and nucleic acid, respectively. Cells were imaged as above using a fluorescence microscope at 20×. Four images were acquired at random locations within the well. Cell process length was quantified using ImageJ to measure 15 processes for each surface and time-point. Only neurites that were larger than the cell nuclei, did not touch other cells, and did not extend outside the imaged boundary were measured. For branching neurites, only the longest process was measured.

Statistical Analysis. Results are reported as average values ± the standard deviation. A two-tailed paired Student's t test was used to compare electrophysiological values before and after adhesive exposure. All other experiments used one-tailed ANOVA to compare means between groups. For all tests, statistical significance was ascribed to p-values ≤0.05, and post-hoc comparisons were performed using Tukey's test.

Results

Synthesis of Az-Chitosan. Schemes of Az-chitosan synthesis and gelation are shown in Figure 1A. Conjugation of ABA to chitosan was confirmed by ^{1}H NMR: 4-azidobenzamide, 7.17−7.19 (d, $J = 10$ Hz), 7.81−7.83 (d, $J = 10$ Hz); chitosan, 2.01 (s), 3.00 (s, broad), 3.20−4.00 (m; Figure 1B). The percentage of chitosan amine converted to 4-azidobenzamide was calculated by comparing an integrated area of peaks in 7−8 ppm (4H, benzene) and those in 3−4 ppm (6H, chitosan). For both HMW and LMW Az-chitosan, the conversion rate was 2%. Az-chitosan solution (1 mg/mL) showed a consistent extent of UV absorption at 277 nm, irrespective of the molecular

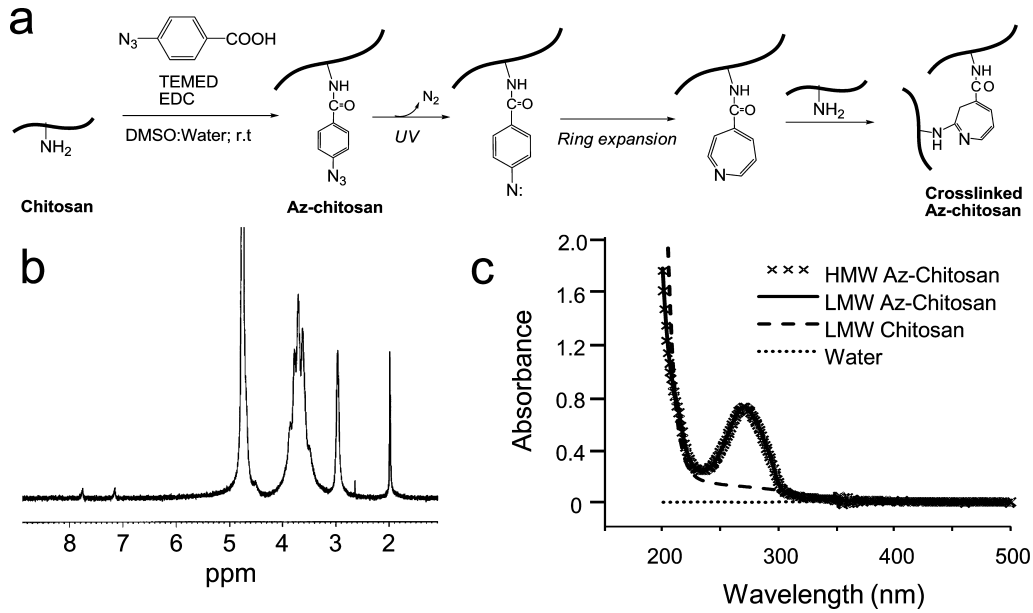

Figure 1. Schematic of the conjugation of chitosan with ABA and the photo-cross-linking of Az-chitosan chains (A). The ^{1}H NMR spectra (B) were identical for LMW and HMW Az-chitosan. Similarly, the UV/vis absorbance spectra (C) of LMW and HMW Az-chitosan in water (1 mg/mL, pH 5) overlapped. Water and LMW chitosan spectra are shown for comparison.

weights of unaltered chitosan (Figure 1C), corroborating the NMR results.

Az-Chitosan Photopolymerization. All experiments were conducted at a distance of 6 cm, at which the irradiance was observed to be 2.4 ± 0.2 mW/cm^2 (n = 6). A total of 100 μL of solution formed a gel less than 1.2 mm thick. HMW Az-chitosan solution (40 mg/mL) formed a hydrogel in 40−50 s (n = 5). The LMW chitosan (40 mg/mL) formed a gel in 20−40 s (n = 10).

Rheological Analysis. Fibrin glue showed a constant G' of 56.3 ± 20.6 Pa in the stress range of 0.07−100 Pa (σ) at the frequency of 0.1 Hz (Figure 2). At higher stress levels, the gel detached from the surface of the rheometer. On the other hand, HMW Az-chitosan hydrogel (40 mg/mL) demonstrated a constant storage modulus (G') of 330.8 ± 114.6 Pa, 6 times higher than that of fibrin glue ($p < 0.01$), in the stress range of 0.07 to 1000 Pa. LMW Az-chitosan was even stronger than HMW Az-chitosan, showing G' of 877.1 ± 122.5 Pa, 3-fold higher than HMW Az-chitosan ($p < 0.01$) and 16-fold higher than fibrin glue ($p < 0.01$).

Tensile Analysis of Nerves Anastomosed with Az-Chitosan. Tensile analysis results are shown in Figure 3. The force necessary to separate anastomosed nerves was highest for those repaired with nylon suture (n = 8), which tolerated 257.9 ± 173.0 mN. The strength of all the adhesives was significantly reduced compared to suture but was similar between groups. Fibrin glue (n = 8) tolerated 69.5 ± 31.6 mN, the HMW Az-chitosan (n = 8) bore 68.9 ± 24.6 mN, and the LMW Az-chitosan (n = 8) failed at an average load of 88.8 ± 15.8 mN. The mean strain at failure was not significantly influenced by the method of repair, straining to 19.23 ± 12.70, 14.28 ± 6.04, 15.00 ± 4.10, and 21.61 ± 14.58% for the fibrin glue, HMW Az-chitosan, LMW Az-chitosan, and nylon suture groups, respectively. Methods of failure differed between the experimental groups. For the nerves anastomosed with fibrin glue, failure occurred when nerves separated from the adhesive. Samples joined by Az-chitosan always separated within the gel. Under tension, sutures either tore through the nerve tissue or separated at the knot.

For calculations of stress and Young's modulus, the average cross-sectional area of comparably sized rat sciatic nerves was

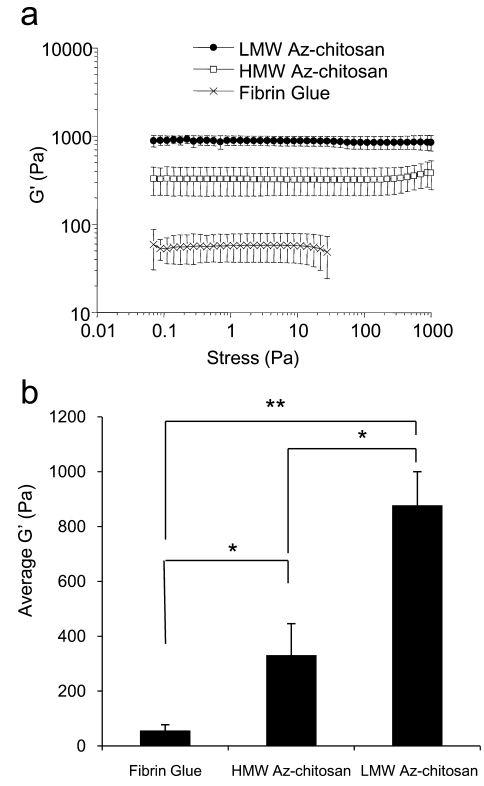

Figure 2. Result of the stress sweep test (A) of fibrin glue (n = 6), HMW Az-chitosan (40 mg/mL; n = 3), and LMW Az-chitosan (40 mg/mL; n = 3) gels at 25 °C with a frequency of 0.1 Hz. The average storage modulus (G', Pa) of each hydrogel (B) was calculated as a mean of all data points on the linear viscoelastic region; *$p < 0.05$; **$p < 0.01$.

observed to be 1.008 ± 0.083 mm^2, similar to the dimensions reported by other groups.[34,35] The engineering stresses calculated using this value are, thus, averages experienced by the nerve trunks prior to dehiscence. Maximum stress values were estimated to be 69.0 ± 31.6 kPa, 68.3 ± 24.4 kPa, 88.1 ± 15.7 kPa, 255.8 ± 171.7 kPa for the fibrin glue, HMW Az-chitosan, LMW Az-chitosan, and nylon suture. Derived from force values,

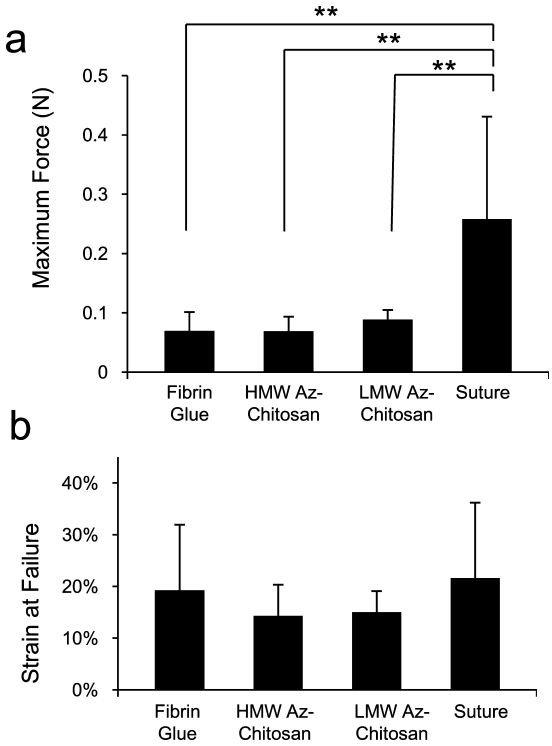

Figure 3. Average maximum forces (A) tolerated prior to failure by nerves anastomosed with fibrin glue (n = 8), HMW Az-chitosan hydrogel (n = 8), LMW Az-chitosan hydrogel (n = 8), or nylon monofilament sutures (n = 8), and mean strain at failure (B); $^{**}p < 0.01$.

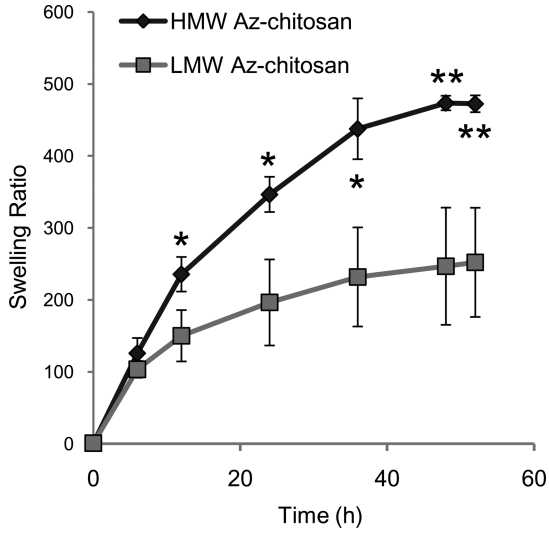

Figure 4. Kinetics of swelling for the HMW and LMW Az-chitosan hydrogels (n = 3). Between HMW and LMW chitosan at each time point, $^{*}p < 0.05$; $^{**}p < 0.01$.

the stresses followed similar trends, with the nylon suture significantly outperforming all of the adhesives. The Young's modulus was obtained from stress−strain plots, and the average values of fibrin glue (527.8 ± 246.9 kPa), HMW Az-chitosan (908.9 ± 400.0 kPa), and LMW Az-chitosan (915.6 ± 385.1 kPa) were significantly less than that of nylon suture (2578.7 ± 2153.4 kPa).

Swelling Ratio. To compare the cross-linking density of HMW Az-chitosan and LMW Az-chitosan hydrogel networks, the swelling ratio of each gel was determined. Both gels reached equilibrium swelling in 48 h (Figure 4). HMW gels swelled to a significantly greater extent than LMW gels ($p < 0.05$), absorbing 470 times their dry weights in water.

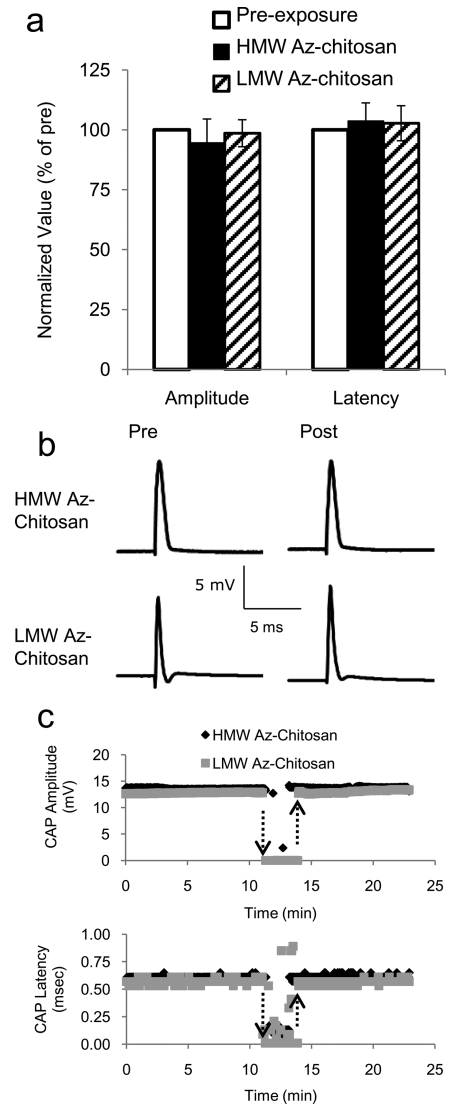

Figure 5. Mean compound actional potential amplitude and latency values (A) for intact nerves exposed to HMW (n = 9) or LMW (n = 10) Az-chitosan for 10 min normalized to their pre-exposure average values. Representative waveforms (B) for all sample types. Representative time histories (C) for CAP amplitude and latency before, during, and after HMW or LMW Az-chitosan hydrogel application. Dashed arrows represent the lowering of the fluid level (down arrow) for adhesive coating with a resultant loss of conduction and the return of signal (up arrow) when the chamber was refilled with Krebs' solution after Az-chitosan application.

Acute Toxicity. As measured, the CAP latency evaluates the speed of conduction through intact nerves, while the amplitude corresponds to the relative number of axons carrying a signal. No significant changes were observed acutely in electrophysiological conduction through intact rat sciatic nerves after application of either Az-chitosan (Figure 5A). For the HMW gels (n = 9) average CAP amplitude after adhesive application was 94.63 ± 9.93% of the value pre-exposure, while latency was increased by 3.73 ± 7.56%. Similar trends were observed for the LMW Az-chitosan (n = 10), with postexposure conduction average 98.62 ± 5.64% of the amplitude and 102.79 ± 7.32% of the conduction time of the average values preceding exposure. The CAP waveforms (Figure 5B) showed no distinguishable differences before and after adhesive application. Time histories (Figure 5C) indicated that the signal was irregular during Az-chitosan application and UV irradiation, but the differences in CAP conduction before and after application were

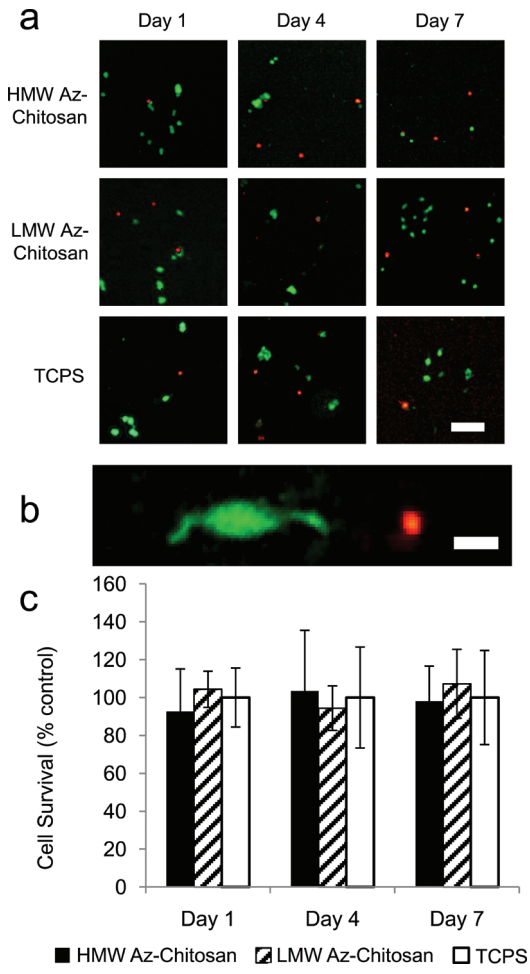

Figure 6. Representative figures (A) of PC-12 cells cultured on HMW Az-chitosan, LMW Az-chitosan, and TCPS stained green (live, B left) or red (dead, B right) from days 1, 4, and 7. Substrates had no significant effect on average cell survival when normalized to TCPS control (C). For each surface and time point, $n = 12$. Scale bar in A is 100 μm, and in B it is 30 μm.

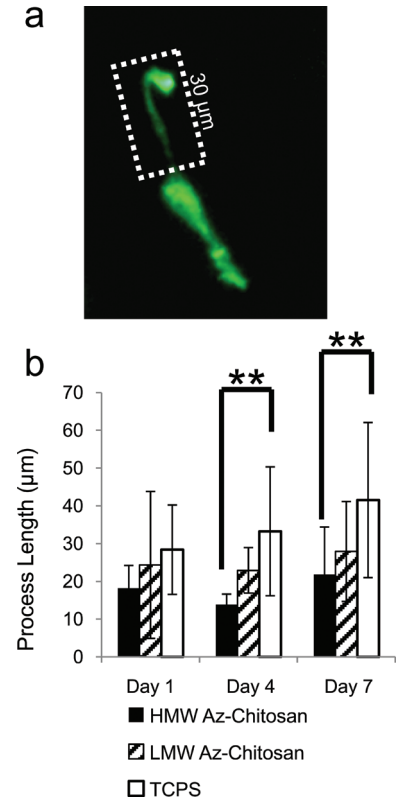

Figure 7. Length of processes (A) extended by PC-12 cells cultured on surfaces of HMW Az-chitosan ($n = 15$ cells), LMW Az-chitosan ($n = 15$ cells), or TCPS ($n = 15$ cells) was measured from the cell body to the longest point. Average process values (B) for days 1,4, and 7; $^{**}p < 0.01$.

negligible. Following photopolymerization, a clear coating of Az-chitosan gel was observed to encapsulate the nerve trunks.

Cell Survival. Cultures of PC-12 cells grew normally in wells coated with the Az-chitosan gels, and the substrate did not have any significant effect on cell dispersion (Figure 6A) or morphology (Figure 6B). Cells were frequently found to grow in clusters, causing overlap that interfered with automated image analysis techniques. Survival ratios were thus calculated from image sections containing well dispersed cell populations. For each substrate and time point, values were normalized to the survival of cells growing on the control tissue culture polystyrene (Figure 6C). For the HMW gels, cell survival was found to be 92.6 ± 22.5, 103.4 ± 32.1, and 98.2 ± 18.4% of that on the TCPS for days 1, 4, and 7, respectively. Cultures on the LMW Az-chitosan grew at 104.3 ± 9.5, 94.4 ± 11.8, and 107.2 ± 18.2% of the TCPS cell survival for the same time points.

Cell Process Extension. Normal cell morphology (Figure 7A) was observed on all substrates. Average measured values for PC-12 process length are shown in Figure 7B. Cells growing directly on the flat TCPS surfaces were easy to visualize, with observed process lengths of 28.4 ± 11.8, 33.1 ± 17.1, and 41.5 ± 20.5 μm for days 1, 4, and 7, respectively. Cells were observed on multiple focal planes within the Az-chitosan wells, complicating the imaging and analysis of the PC-12 process length. Therefore, the measurements obtained by two-dimensional imaging techniques may underestimate the true values. Process length values for the HMW Az-chitosan were 18.2 ± 6.0 μm on day 1, 13.9 ± 2.8 μm on day 4, and 21.8 ± 12.6 μm on day 7, which is significantly reduced compared to PC-12 cells cultured on TCPS for days 4 and 7. Cells growing on/in the LMW Az-chitosan gels extended process of mean length 24.3 ± 19.4, 22.9 ± 6.0, and 27.9 ± 13.2 μm for days 1, 4, and 7, respectively, which is shorter than those on TCPS, but not significantly so.

Discussion

Dehiscence is a critical problem for surgical treatments of peripheral nerve transection. Severed peripheral nerves not restored to continuity do not demonstrate histological recovery,[2] and nerves repaired with commercial fibrin glues have demonstrated an increased risk of separation compared to sutures.[36] Chitosan is known to be biocompatible, biodegradable, and nonimmunogenic. Efforts to develop a new chitosan based surgical glue can capitalize on its natural bioadhesive properties. Chitosan cohesion could be improved by introducing covalent bonds to increase intermolecular forces between the polysaccharide chains. We predict that this chitosan-derived bioadhesive will be comparable or superior to fibrin glue for peripheral nerve anastomosis. In this work, we evaluated chitosan modified with ABA, which covalently polymerized upon photoactivation. We also investigated the effect of varying the chain length of chitosan on the chemical and mechanical properties of the Az-chitosan hydrogels.

^{1}H NMR spectra indicate that the absolute number of aryl azide groups in the system that can cross-link with other amine

groups in chitosan was consistently 2%, irrespective of the molecular weights. A thin drop of the solution (<1.2 mm) formed a gel in less than 1 min. It is possible that thicker gels take longer to form due to the attenuation in light penetration. We have not determined the minimal time requirement for thicker gels, but it did not take more than 3 min to form even the thickest gels (6 mm) used in this study. For all chemical and mechanical analyses, Az-chitosan hydrogels were cross-linked longer than 3 min to ensure complete gelation. The electrophysiological and tensile tests used ex vivo nerve, which dehydrates quickly. Accordingly, UV exposures were curtailed to 1 and 2 min, respectively. We do not expect that prolonged irradiation would have further increased Az-chitosan cross-linking. Upon irradiation, aryl azide decomposes rapidly (half-life in water ~8 s at 254 nm[37] or 34 s at 365 nm[38]) into a short-lived intermediate nitrene (half-life < 30 μs).[39] Therefore, the cross-linking reaction is likely to be completed as soon as the photolysis begins.

Despite the similar proportion of reactive side groups, the two formulations showed different physical properties: LMW Az-chitosan gelled significantly faster than the HMW formulation. Moreover, LMW Az-chitosan experienced less swelling than HMW Az-chitosan (Figure 4). These two results suggest that LMW Az-chitosan and HMW Az-chitosan differ in the network (cross-linking) density. When calculated based on the equilibrium swelling ratio (Q_M) according to the simplified Flory−Rehner equation,[40] the cross-linking densities of HMW Az-chitosan and LMW Az-chitosan were 2.0×10^{-7} and 6.4×10^{-7} mol/cm^3, respectively (see the Supporting Information for calculation).

Differences in the cross-linking density were also reflected in the ability of the gels to resist shear and longitudinal stresses. Rheometry indicated that the average G' for LMW Az-chitosan was three times that of HMW Az-chitosan. Both gels significantly outperformed conventional fibrin glue, which could not tolerate shear stresses greater than 28 Pa. The rheological results signify that LMW Az-chitosan is significantly stiffer than the HMW gels, which are in turn more rigid than Tisseel fibrin glue. The differences between the Az-chitosan groups are consistent with the calculated cross-linking densities. Of the three adhesives, LMW Az-chitosan appears the most resistant to lateral movement, requiring significantly more energy to deform the gel. For applications involving shear stress, such as sealants or dental implants, either Az-chitosan formulation should outperform the fibrin glue.

To our knowledge, this molecular weight effect on cross-linking density of photo-cross-linkable chitosan hydrogels has not been reported previously. In physical networks of chitosan, it has often been reported that higher molecular weight chitosan forms a network with higher tensile strength[41] or viscosity,[42] apparently contrasting with our observation. This difference may be explained by the network formation mechanism. Unlike physical networks based on random interactions among chains that increase with the length, the gelation of Az-chitosan solution relies on a chemical reaction between amines and a small number of aryl azide, which likely requires a specific orientation of the polymer chains. We speculate that the shorter LMW Az-chitosan chains are relatively less challenged in doing so and are, thus, able to cross-link more densely than HMW Az-chitosan with the same number of aryl azide groups.

Simulation of repaired nerves experiencing longitudinal strain directly evaluated the adhesive's resistance to acute dehiscence. As expected, microsutures imparted significantly superior strength when compared to any of the adhesives (Figure 3A). Nerves severed and repaired by fibrin glue tolerated 19.2 ± 12.7% strain and 69.5 ± 31.6 mN force. These values were larger than those reported by Menovsky et al., who found that nerves repaired with Tissucol fibrin glue failed at 26.5 ± 11.8 mN (2.7 ± 1.2 g).[13] This variance illustrates that commercial fibrin glues can vary greatly in bond strength.

In our longitudinal strain experiments, the LMW Az-chitosan tolerated 28.9% more force than the HMW gels prior to failure, though this difference was not significant. Both Az-chitosan gels performed similarly to the fibrin glue. Likewise, Ono et al. reported similar adhesion between fibrin and 50 mg/mL lactose-conjugated Az-chitosan.[29] The discrepancy of gel performance between the rheometry and the tensile experiments may be attributed to the basic mechanics of the tests. While rheometry applies shear forces to material samples in isolation, the tensile experiments involve multiple material interactions, not only between the polymer chains in the network (cohesion) but also between the polymer and the nerve (adhesion), which act in opposing directions. Therefore, the results of tensile analysis measuring the net effect of nerve-gel adhesion and gel cohesion should not be compared directly to the rheological analysis, which only reflects mechanical properties of the gel itself.

Of note, during longitudinal strain, all Az-chitosan samples separated within the hydrogel, rather than at the tissue interface, as was observed for the fibrin glue. The same failure mechanism has been previously reported for chitosan adhesives.[29,43] This suggests that adhesive interactions dominate over cohesive interactions in Az-chitosan and vice versa in fibrin glue. Future efforts could seek to improve cohesion within the Az-chitosan gel by adding additional moieties for covalent cross-linking between gels or introducing interpenetrating networks to stabilize the chitosan matrix.

Az-chitosan did not demonstrate toxic effects on nerve conduction or cell survival. Coating intact nerves with either HMW or LMW Az-chitosan did not significantly alter the CAP amplitude or latency (Figure 5A). Compared to the TCPS positive control, PC-12 cell survival in culture was not acutely or chronically impacted by culture with either HMW or LMW Az-chitosan, suggesting that these gels are not cytotoxic (Figure 6).

On the other hand, it is noteworthy that a large portion of the cells were only loosely adherent to the gels. Poor cellular attachment also influenced the extension of cell processes, for which the cells cultured on TCPS extended significantly longer processes than those growing on HMW Az-chitosan (41.5 ± 20.5 vs 21.8 ± 12.6 μm for day 7, $p < 0.01$). This may be a simple cellular response, as PC-12 cells preferentially extend neurites on stiffer surfaces.[44] For these studies, the rigid TCPS surfaces served as a positive control, allowing us a standard for comparison in gauging process extension. The observed difference in cellular response may also be attributed to the effects of three-dimensional culture, as cells growing within the gel or on an uneven surface would have their process lengths underestimated by two-dimensional imaging. LMW Az-chitosan showed a similar trend, but the average difference in neurite length between cells growing on LMW Az-chitosan gels and TCPS was not statistically significant. These results indicate that both Az-chitosans were compatible with PC-12 cells, although they did not seem to actively promote cell adhesion on the surface compared to TCPS. In some aspects, the inhibition of nerve process extension through the gel may be considered a positive effect, as it may reduce aberrant regeneration of neurites escaping the anastomotic site.

Lauto et al. suggested that UV reactive adhesives were unsuitable for use in vivo due to the possible mutagenic effects of radiation exposure.[45] The effects of radiation vary with the wavelength, sensitivity of the organism, intensity, and length of exposure. For Az-chitosan gelation, we used a 100 W long wave UV source with a peak emission at 365 nm. Applied at a distance of 6 cm, irradiance was observed as 2.4 ± 0.2 mW/cm^2. Cumulative exposure for 60 and 120 s are calculated to be 144 ± 15 and 287 ± 30 mJ/cm^2, well below the threshold limit value of 1000 mJ/cm^2 recommended by the American Conference of Governmental Industrial Hygienists for UV-A exposures less than 1000 s.[46] Overall, our studies demonstrated that exposure to Az-chitosan formulations did not impact nerve conduction, reduce PC-12 cell survival, or prevent neurite extension, and thus, both formulations appear suitably nontoxic for use as bioadhesives.

Conclusions

There is a clear need for alternative surgical adhesives. In this study, we report on the potential application of chitosan based adhesives cross-linked by UV illumination for peripheral nerve anastomosis. These were synthesized through conjugation of ABA to chitosan backbones of low or high molecular weight, producing solutions that rapidly formed hydrogels when photoactivated. Rheology showed that the LMW Az-chitosan tolerated shear stress significantly better than the HMW formulation, which outperformed the fibrin glue. These results indicate that the Az-chitosan bioadhesives might be effectively employed as surgical adhesives for functions involving shear stresses. When used for nerve anastomosis ex vivo, these gels provided mechanical stability comparable to a commercial fibrin glue. Lacking the potential for pathogen transmission, the experimental Az-chitosan glue has a clear advantage for this application. Electrophysiological tests showed no acute toxicity to nerve function, and cells cultured on the surfaces survived at levels similar to controls. Az-chitosan is thus demonstrated to be cytocompatible and nontoxic. Further experiments will attempt to improve gel mechanical properties, and to evaluate the efficacy of these Az-chitosan adhesives in vivo.

Acknowledgment. This work was supported by grants from the Indiana Clinical and Translational Sciences Institute-PDT (PHS NCCR Grant No: 5TL1RR025759-02), the Showalter Foundation, and the State of Indiana. We would also like to acknowledge Gary Leung and Wenjing Sun for assistance in electrophysiological recordings, Dr. Ernest Blatchley for assistance with measuring irradiance, Judy Grimmer for help in cell culture, Melissa Tully and Dr. Jianming Li for critical review of the manuscript, and Michel Schweinsberg for graphic design.

Supporting Information Available. The calculation of cross-linking densities. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Marchettini, P.; Lacerenza, M.; Mauri, E.; Marangoni, C. Painful peripheral neuropathies. *Curr. Neuropharmacol.* **2006**, *4*, 175–81.
(2) Jiao, H.; Yao, J.; Yang, Y.; Chen, X.; Lin, W.; Li, Y.; Gu, X.; Wang, X. Chitosan/polyglycolic acid nerve grafts for axon regeneration from prolonged axotomized neurons to chronically denervated segments. *Biomaterials* **2009**, *30*, 5004–18.
(3) Bednar, M. S.; Light, T. R. Hand Surgery. In *Current Diagnosis and Treatment in Orthopedics*, 4th ed.; Skinner, H. B., Ed.; Lange Medical Books/McGraw-Hill: New York, 2006; Chapter 10.
(4) DeLee, J. C.; Smith, M. T.; Green, D. P. The reaction of nerve tissue to various suture materials: a study in rabbits. *J. Hand Surg. Am.* **1977**, *2*, 38–43.
(5) Kim, D. H.; Han, K.; Tiel, R. L.; Murovic, J. A.; Kline, D. G. Surgical outcomes of 654 ulnar nerve lesions. *J. Neurosurg.* **2003**, *98*, 993–1004.
(6) Jaquet, J. B.; Luijsterburg, A. J.; Kalmijn, S.; Kuypers, P. D.; Hofman, A.; Hovius, S. E. Median, ulnar, and combined median-ulnar nerve injuries: functional outcome and return to productivity. *J. Trauma* **2001**, *51*, 687–92.
(7) Whitlock, E. L.; Kasukurthi, R.; Yan, Y.; Tung, T. H.; Hunter, D. A.; Mackinnon, S. E. Fibrin glue mitigates the learning curve of microneurosurgical repair. *Microsurgery* , *30*, 218–22.
(8) Rickett, T.; Li, J.; Patel, M.; Sun, W.; Leung, G.; Shi, R. Ethyl-cyanoacrylate is acutely nontoxic and provides sufficient bond strength for anastomosis of peripheral nerves. *J. Biomed. Mater. Res. A* **2009**, *90*, 750–4.
(9) Landegren, T.; Risling, M.; Persson, J. K. Local tissue reactions after nerve repair with ethyl-cyanoacrylate compared with epineural sutures. *Scand. J. Plast. Reconstr. Surg. Hand Surg.* **2007**, *41*, 217–27.
(10) Toriumi, D. M.; Bagal, A. A. Cyanoacrylate tissue adhesives for skin closure in the outpatient setting. *Otolaryngol. Clin. North Am.* **2002**, *35*, 103–18, vi−vii.
(11) Narakas, A. The use of fibrin glue in repair of peripheral nerves. *Orthop. Clin. North Am.* **1988**, *19*, 187–99.
(12) Cruz, N. I.; Debs, N.; Fiol, R. E. Evaluation of fibrin glue in rat sciatic nerve repairs. *Plast. Reconstr. Surg.* **1986**, *78*, 369–73.
(13) Menovsky, T.; Beek, J. F.; van Gemert, M. J. CO_2 laser nerve welding: optimal laser parameters and the use of solders in vitro. *Microsurgery* **1994**, *15*, 44–51.
(14) Kawamura, M.; Sawafuji, M.; Watanabe, M.; Horinouchi, H.; Kobayashi, K. Frequency of transmission of human parvovirus B19 infection by fibrin sealant used during thoracic surgery. *Ann. Thorac. Surg.* **2002**, *73*, 1098–100.
(15) Kim, I. Y.; Seo, S. J.; Moon, H. S.; Yoo, M. K.; Park, I. Y.; Kim, B. C.; Cho, C. S. Chitosan and its derivatives for tissue engineering applications. *Biotechnol. Adv.* **2008**, *26*, 1–21.
(16) Muzzarelli, R. A. A. Biochemical significance of exogenous chitins and chitosans in animals and patients. *Carbohydr. Polym.* **1993**, *20*, 7–16.
(17) Bowman, K.; Leong, K. W. Chitosan nanoparticles for oral drug and gene delivery. *Int. J. Nanomed.* **2006**, *1*, 117–128.
(18) Kozen, B. G.; Kircher, S. J.; Henao, J.; Godinez, F. S.; Johnson, A. S. An alternative hemostatic dressing: comparison of CELOX, HemCon, and QuikClot. *Acad. Emerg. Med.* **2008**, *15*, 74–81.
(19) Ono, K.; Ishihara, M.; Ozeki, Y.; Deguchi, H.; Sato, M.; Saito, Y.; Yura, H.; Sato, M.; Kikuchi, M.; Kurita, A.; Maehara, T. Experimental evaluation of photocrosslinkable chitosan as a biologic adhesive with surgical applications. *Surgery* **2001**, *130*, 844–850.
(20) Taravel, M. N.; Domard, A. Relation between the physicochemical characteristics of collagen and its interactions with chitosan 0.1. *Biomaterials* **1993**, *14*, 930–938.
(21) Fujita, M.; Ishihara, M.; Morimoto, Y.; Simizu, M.; Saito, Y.; Yura, H.; Matsui, T.; Takase, B.; Hattori, H.; Kanatani, Y.; Kikuchi, M.; Maehara, T. Efficacy of photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 in a rabbit model of chronic myocardial infarction. *Surg. Res.* **2005**, *126*, 27–33.
(22) Ishihara, M.; Nakanishi, K.; Ono, K.; Sato, M.; Kikuchi, M.; Saito, Y.; Yura, H.; Matsui, T.; Hattori, H.; Uenoyama, M.; Kurita, A. Photocrosslinkable chitosan as a dressing for wound occlusion and accelerator in healing process. *Biomaterials* **2002**, *23*, 833–840.
(23) Obara, K.; Ishihara, M.; Ishizuka, T.; Fujita, M.; Ozeki, Y.; Maehara, T.; Saito, Y.; Yura, H.; Matsui, T.; Hattori, H.; Kikuchi, M.; Kurita, A. Photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing in healing-impaired db/db mice. *Biomaterials* **2003**, *24*, 3437–3444.
(24) Ishihara, M.; Obara, K.; Nakamura, S.; Fujita, M.; Masuoka, K.; Kanatani, Y.; Takase, B.; Hattori, H.; Morimoto, Y.; Ishihara, M.; Maehara, T.; Kikuchi, M. Chitosan hydrogel as a drug delivery carrier to control angiogenesis. *J. Artif. Organs* **2006**, *9*, 8–16.
(25) Obara, K.; Ishihara, M.; Ozeki, Y.; Ishizuka, T.; Hayashi, T.; Nakamura, S.; Saito, Y.; Yura, H.; Matsui, T.; Hattori, H.; Takase, B.; Ishihara, M.; Kikuchi, M.; Maehara, T. Controlled release of paclitaxel from photocrosslinked chitosan hydrogels and its subsequent effect on subcutaneous tumor growth in mice. *J. Controlled Release* **2005**, *110*, 79–89.
(26) Drury, J. L.; Mooney, D. J. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **2003**, *24*, 4337–4351.

(27) Lee, J. I.; Kim, H. S.; Yoo, H. S. DNA nanogels composed of chitosan and Pluronic with thermo-sensitive and photo-crosslinking properties. *Int. J. Pharm.* **2009**, *373*, 93–99.
(28) Quick, D. J.; Anseth, K. S. DNA delivery from photocrosslinked PEG hydrogels: encapsulation efficiency, release profiles, and DNA quality. *J. Controlled Release* **2004**, *96*, 341–351.
(29) Ono, K.; Saito, Y.; Yura, H.; Ishikawa, K.; Kurita, A.; Akaike, T.; Ishihara, M. Photocrosslinkable chitosan as a biological adhesive. *J. Biomed. Mater. Res.* **2000**, *49*, 289–295.
(30) Hermanson, G. T. Heterobifunctional cross-linkers. *Bioconjugate Techniques*; Academic Press: San Diego, 1996; pp 228−286.
(31) Sato, J.; Breedveld, V. Evaporation blocker for cone-plate rheometry of volatile samples. *Appl. Rheol.* **2005**, *15*, 390–397.
(32) Logan, M. P.; Parker, S.; Shi, R. Glutathione and ascorbic acid enhance recovery of Guinea pig spinal cord white matter following ischemia and acrolein exposure. *Pathobiology* **2005**, *72*, 171–8.
(33) Chaplan, S. R.; Guo, H. Q.; Lee, D. H.; Luo, L.; Liu, C.; Kuei, C.; Velumian, A. A.; Butler, M. P.; Brown, S. M.; Dubin, A. E. Neuronal hyperpolarization-activated pacemaker channels drive neuropathic pain. *J. Neurosci.* **2003**, *23*, 1169–78.
(34) Meyer, R. S.; Abrams, R. A.; Botte, M. J.; Davey, J. P.; Bodine-Fowler, S. C. Functional recovery following neurorrhaphy of the rat sciatic nerve by epineurial repair compared with tubulization. *J. Orthop. Res.* **1997**, *15*, 664–9.
(35) Layton, B. E.; Sastry, A. M. Equal and local-load-sharing micromechanical models for collagens: quantitative comparisons in response of non-diabetic and diabetic rat tissue. *Acta. Biomater.* **2006**, *2*, 595–607.
(36) Temple, C. L.; Ross, D. C.; Dunning, C. E.; Johnson, J. A. Resistance to disruption and gapping of peripheral nerve repairs: an in vitro biomechanical assessment of techniques. *J. Reconstr. Microsurg.* **2004**, *20*, 645–50.
(37) van der Horst, G. T.; Mancini, G. M.; Brossmer, R.; Rose, U.; Verheijen, F. W. Photoaffinity labeling of a bacterial sialidase with an aryl azide derivative of sialic acid. *J. Biol. Chem.* **1990**, *265*, 10801–10804.
(38) Carrico, I. S.; Maskarinec, S. A.; Heilshorn, S. C.; Mock, M. L.; Liu, J. C.; Nowatzki, P. J.; Franck, C.; Ravichandran, G.; Tirrell, D. A. Lithographic patterning of photoreactive cell-adhesive proteins. *J. Am. Chem. Soc.* **2007**, *129*, 4874–4875.
(39) DeGraff, B. A.; Gillespie, D. W.; Sundberg, R. J. Phenyl nitrene. Flash photolytic investigation of the reaction with secondary amines. *J. Am. Chem. Soc.* **1974**, *96*, 7491–7496.
(40) Leach, J. B.; Bivens, K. A.; Patrick Jr, C. W.; Schmidt, C. E. Photocrosslinked hyaluronic acid hydrogels: Natural, biodegradable tissue engineering scaffolds. *Biotechnol. Bioeng.* **2003**, *82*, 578–589.
(41) Chen, R. H.; Hwa, H.-D. Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membrane. *Carbohydr. Polym.* **1996**, *29*, 353–358.
(42) Zhou, H. Y.; Chen, X. G.; Kong, M.; Liu, C. S.; Cha, D. S.; Kennedy, J. F. Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system. *Carbohydr. Polym.* **2008**, *73*, 265–273.
(43) Lauto, A.; Stoodley, M.; Marcel, H.; Avolio, A.; Sarris, M.; McKenzie, G.; Sampson, D. D.; Foster, L. J. In vitro and in vivo tissue repair with laser-activated chitosan adhesive. *Lasers Surg. Med.* **2007**, *39*, 19–27.
(44) Leach, J. B.; Brown, X. Q.; Jacot, J. G.; Dimilla, P. A.; Wong, J. Y. Neurite outgrowth and branching of PC12 cells on very soft substrates sharply decreases below a threshold of substrate rigidity. *J. Neural. Eng.* **2007**, *4*, 26–34.
(45) Lauto, A.; Mawad, D.; Foster, L. J. R. Adhesive biomaterials for tissue reconstruction. *J. Chem. Technol. Biotechnol.* **2008**, *83*, 464–472.
(46) *Ultraviolet Radiation: TLV Physical Agents*; American Conference of Governmental Industrial Hygeinists: Cincinnati, OH, 2001; pp 1−6.

BM101004R