

Biocompatible, Ion-Conductive Hydrogel-Filled Nerve Conduit for Peripheral Nerve Regeneration

Joyce Huang, Jing Tian, Tzu Chun Chung, Sam Denton, Youchao Teng, Kun-wei Yeh, Stephanie K. Seidlits, Nasim Annabi, and Song Li*



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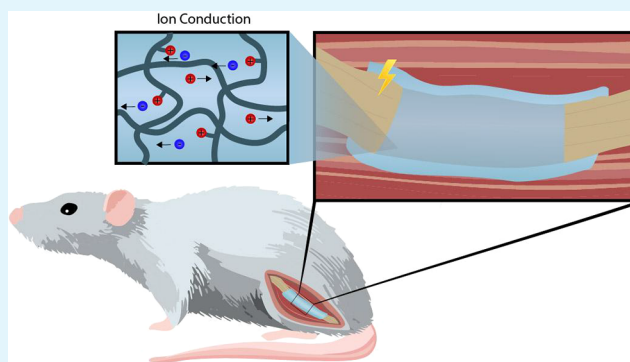


Supporting Information

ABSTRACT: Peripheral nerve injury can result in a loss of sensation and muscle control. Native axon regeneration rates are insufficient to bridge a large gap due to severe damage, leading to a permanent loss of function. Contemporary use of autografts as a treatment, while effective, is limited by donor-site morbidity. Conductive nerve guides can provide mechanical support for regenerating axons, while electrical conductivity provides bioelectrical cues. However, conventional materials used to provide electrical conductivity to hydrogels are not biodegradable and can induce inflammation, which can further impede regeneration. To address these issues, a biodegradable conductive hydrogel containing choline-based bioionic liquid (BioIL) was designed to bridge large nerve gaps and support native axon regeneration.

Choline, a small molecule precursor of various biomolecules, combined with a gelatin-based hydrogel, creates a biodegradable and resorbable hydrogel. Conjugation of BioIL to a gelatin methacryloyl (GelMA) hydrogel, followed by saline submersion, imparted an ionic conductivity to the hydrogel. Ion-conductive GelMA/BioIL hydrogels supported the myelination function of Schwann cells and the axon outgrowth from dorsal root ganglia *in vitro*. Implantation of GelMA/BioIL-filled poly(L-lactide-co-caprolactone) nerve conduit in a rat model of peripheral nerve injury improved the recovery of motor and sensory function. The engineered GelMA/BioIL hydrogel increased the number of regenerated axons, improved the myelination of regenerated axons, and reduced the atrophy of downstream muscle. These results support the use of a BioIL-functionalized hydrogel in creating effective, biocompatible nerve conduits for peripheral nerve regeneration.

KEYWORDS: biomaterials, ion-conduction, peripheral nerve injury, hydrogel, ionic liquids, nerve regeneration



1. INTRODUCTION

Peripheral nerve injuries (PNIs) occur as a result of trauma, compression, or vascular deficiency, leading to loss of sensation and paralysis. While the peripheral nerves have some ability for axonal regeneration, the rate at which regeneration occurs can be insufficient to bridge large defects. Motor neurons can regenerate at a rate of 1–2 mm/day.¹ However, an extended period of denervation of the distal nerve stump and muscle can lead to apoptosis of the nerve² and atrophy of muscle tissue,³ making reinnervation of the target organ challenging. Therefore, medical intervention is necessary to restore nerve function in many cases of PNI. Current practice for the treatment of large-gap PNI is the use of an autograft. However, autografts are severely limited by supply and can lead to donor-site morbidity.

As alternatives to autografts, various studies have investigated the use of biomaterials. Biomaterial nerve conduits have been engineered to provide structural support as well as mechanical and biochemical cues for axon regeneration.^{4,5} In addition to mechanical and biochemical cues, neurons and

Schwann cells respond to bioelectrical cues.⁶ For example, electrical stimulation of stem-cell-differentiated neurons on conductive poly(3,4-ethylenedioxythiophene) (PEDOT) substrates led to greater neurite extension.⁷ While less commonly studied, electrical stimulation of Schwann cells has been reported to drive Schwann cell migration, as well as elevate nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) release.⁸ Furthermore, neuronal cells have also been found to respond to the substrate conductivity alone. PC12 neurons cultured on conductive reduced graphene oxide (rGO)/gelatin methacryloyl (GelMA) hydrogels showed a greater number of neurites as well as longer neurite extension without the use of exogenous electrical stimulation.⁹ PC12

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neurons cultured on conductive hydrogel sheets also showed increases in transcription of neural markers.¹⁰ Schwann cells seeded on increasingly conductive graphene hydrogels demonstrated increased transcription of BDNF, NGF, and ciliary neurotrophic factor (CNTF).^{10,11}

Due to the electroactivity of neurons and glial cells, conductive biomaterials have been a subject of particular focus for peripheral nerve regeneration *in vivo*. A variety of conductive biomaterials have been investigated for use in nerve conduits. The materials used largely fall into categories of metal, carbon-based materials, and conducting polymers (e.g., polyaniline, polypyrrole, polythiophene).^{11–13} While bulk electroconducting materials are mechanically incompatible with biological tissues due to stiffness and brittleness, composites with biomaterials can improve their mechanical properties. Conductive biomaterial composites can serve to transmit native electrical signals from the proximal stump through the gap, thus providing electrical stimulation for axon growth. Additionally, neuron response to conducting materials alone can also contribute to accelerating nerve regeneration. Nerve conduits fabricated from rGO/GelMA increased sciatic nerve function recovery with similar improvements to a gold standard autograft, without the use of exogenous electrical stimulation.⁹ Polypyrrole (PPy) and silk fibroin (SF) nerve conduits similarly demonstrated increased functional recovery compared to nonconductive scaffolds in a rat sciatic nerve model.¹⁴

While carbon-based materials and conducting polymers provide high electrical conductivity, their functional translation is limited by processability and biocompatibility concerns.^{15–17} In addition, the hydrophobicity of conducting polymers may make the manufacturing process difficult.¹⁸ Furthermore, significant steps must be taken to purify conducting polymer products, as manufacturing impurities left in the material can be highly cytotoxic.¹⁹ While researchers have made great strides in the modification of conducting polymer and carbon materials for improved biocompatibility, the difficulty in manufacturing may ultimately limit translation. In addition to cytotoxicity concerns,^{17,20–22} carbon materials and conductive polymers are not easily biodegradable.^{16,23,24} Endocytosis of these materials during the erosion process can induce inflammatory responses, including upregulation of pro-inflammatory cytokines.^{21,25} While inflammation is necessary for initial Wallerian degeneration, failure to transition from a pro-inflammation environment impairs necessary axon regeneration.²⁶

To address the biocompatibility, processability, and degradability concerns with the use of these materials, ionic liquids have been used to impart materials with conductivity without the use of electronic materials. Ionic liquids offer the same mechanism of conduction (ion flow) that neural cells experience. In addition, an ionic hydrogel using ionic liquid (VPIImBF₄) was demonstrated to increase the expression of neural markers in PC12 neurons encapsulated in the hydrogel. Bioionic liquids (BioIL) based on choline have attracted attention as a promising material for use in neural tissue engineering applications due to their biocompatibility.^{27,28} Choline, a precursor of various biomolecules, can be metabolized by neurons as well as other cells in the body.²⁹ In our previous work, BioIL was conjugated to a polymer backbone to create conductive hydrogels.³⁰ The BioIL-functionalized hydrogels demonstrated excellent biocompatibility as well as the ability to transmit bioelectrical signals and

support the culture of electroactive cells. GelMA-based BioIL hydrogels are also biocompatible for the culture of neural cells.³¹ The use of a resorbable small molecule to impart conductivity to a gelatin-based polymer creates a more easily biodegradable conductive hydrogel.

Here, we have developed a BioIL-functionalized hydrogel-filled nerve conduit that utilizes the ion-conductive properties of BioIL to aid in the regeneration following PNIs. The conductive hydrogel conduits were tested in a rat model of sciatic nerve injury, and their effects on peripheral nerve regeneration were evaluated *in vivo*.

2. MATERIALS AND METHODS

2.1. Hydrogel Synthesis

Acrylated BioIL and GelMA were synthesized as described in a previous publication.³⁰ Hydrogel precursors were produced by dissolving 10% (w/v) GelMA, 0.05% (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and up to 50% (v/v) acrylated BioIL in deionized water. Hydrogel precursors were cross-linked using 3.5 mW 405 nm light for 4 min. Hydrogels for characterization were cross-linked in either cylindrical (5.5 mm diameter and 3 mm height) or rectangular molds (12 mm long, 5 mm wide, and 1.25 mm thick). Hydrogels were then submerged in 1× phosphate-buffered saline (PBS) overnight prior to characterization.

2.2. Conductivity and Structural Characterization

Ionic conductivity of the hydrogel was measured by using a potentiostat (PalmSens4). Rectangular hydrogels prepared as described in Section 2.1 were first incubated in water or saline (1× or 5×) overnight. Following incubation, swollen hydrogels were used for the conductivity measurements. Hydrogels were sandwiched between two gold-plated electrodes. Ionic conductivity of the hydrogels was measured by using electrical impedance spectroscopy. A sinusoidal potential of 0.01 V, from 10 kHz to 0.1 Hz, was applied to the hydrogel. Ionic conductivity of hydrogels was calculated from the first semicircle intercept of Nyquist plots.³² Three ($n = 3$) samples each were tested for GelMA/BioIL hydrogels and GelMA hydrogels.

The polymer chain structure of the hydrogels was analyzed by using wide-angle X-ray scattering (WAXS). The WAXS images were acquired in transmission mode at Brockhouse X-ray Diffraction and Scattering Beamlines of a Canadian light source. The images were acquired at a wavelength of 0.81931 Å and a detector distance of 201 mm.

2.3. Hydrogel Degradation

Cylindrical hydrogels were frozen and lyophilized for dry weight measurement prior to degradation. Following measurement, samples were submerged in 10% fetal bovine serum (FBS) and incubated at 37 °C. The FBS solution was refreshed every 2 days. At each time point (days 1, 7, 14, and 28), three samples were removed from incubation, frozen, and lyophilized. Dry weight of the lyophilized samples was measured and compared to the original weight of the same sample to evaluate percentage degradation.

2.4. Mechanical Characterization

Mechanical properties of the hydrogel were measured by using tensile methods in a mechanical tester (Instron 5943). Stress and displacement were recorded with Bluehill software. Rectangular hydrogels were synthesized as previously described and incubated in PBS overnight prior to testing. Samples were fixed to the device, and tensile force was applied at a rate of 2 mm/min. Material strain was calculated by the device displacement and sample dimensions. The modulus of the material was obtained by linear curve fitting of the linear region of the stress/strain curve (approximately 0 to 10% strain). At least three samples were tested for each hydrogel formulation.

2.5. *In Vitro* Cell Viability and Immunostaining

Hydrogels for *in vitro* cell culture were synthesized using a photo-cross-linkable precursor as described previously in the nerve conduit fabrication section. Briefly, 10% GelMA was dissolved with 0.05% LAP in deionized water. Acrylated BioIL was added to precursors for the fabrication of conductive gels. All reagents were filter sterilized prior to fabrication. Gels with 0.5 mm thickness were shaped by cross-linking while the precursor solution was compressed between two pieces of glass. Four samples for each GelMA and GelMA/BioIL hydrogels were fabricated for cell studies. Gels were then washed in PBS overnight. S16 Schwann cells were seeded on top of the gels and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS and 1% penicillin/streptavidin. Cell viability at days 1, 3, and 7 was visualized using a Live/Dead kit (Invitrogen, R37601). The viability of cells was then quantified using ImageJ.

S16 Schwann cells cultured on GelMA or GelMA/BioIL were stained to visualize the expression of myelination markers and cytoskeletal and adhesion structures. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min, followed by blocking in 5% normal donkey serum for 1 h, and permeabilized with 0.05% Triton-X 100. Samples were then incubated with primary antibodies for S100 (1:100, Invitrogen MA1-26621) and P0 (1:4000, Aves Laboratories PZO-0020) overnight at 4 °C. Primary antibodies were washed following incubation and incubated with secondary antibodies at room temperature. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1:2500) for 10 min. Immunostained samples were imaged using fluorescence microscopy (Zeiss). Relative concentration of P0 was quantified for each cell using CellProfiler.

2.6. Focal Adhesion Quantification

Focal adhesion structures of each cell on the hydrogels were quantified through immunofluorescent images using Fiji software. Briefly, cells were manually segmented using phalloidin images of the cells. The puncta of paxillin (PAX) staining were emphasized through the application of a high-pass filter. The puncta were then identified through the application of a constant threshold and segmented by the application of a watershed function. Binarized puncta were then quantified using the Analyze Particle function to assess the puncta number and average size.

2.7. Dorsal Root Ganglia Isolation

Dorsal root ganglia were isolated from E17 Sprague–Dawley rat embryos following published protocols.³³ Briefly, spinal structures were dissected from embryos, and then dorsal root ganglia were isolated from the spinal cord by dissection. The DRGs were then cultured on a poly-L-lysine/laminin (Sigma-Aldrich)-coated plate in C medium (MEM supplemented with 10 mL of 0.2 g/mL D-glucose, 50 mL of FBS, 5 mL of 200 mM L-glutamine, and 50 μ L of 2.5S NGF) before transfer to hydrogels. DRGs were cultured on hydrogels for 2 days before fixation and immunostaining for visualization. Samples were immunostained with β -tubulin (1:200, BioLegend PRB-435P) using a process as previously described.

2.8. Nerve Conduit Fabrication

The outer layer of the nerve conduit was fabricated through electrospinning. Poly(L-lactide-co-caprolactone) (PLCL) (70:30, Purac Biomaterials) was mixed with poly(D,L-lactide-co-glycolide) (PLG) (50:50 Polysciences) to create a biodegradable conduit with sufficient mechanical support while maintaining flexibility.³⁴ The polymers were mixed in a 1:1 ratio in hexafluoroisopropanol with 15% w/v. The mixture was loaded into a charged injector with 17 kV applied electrostatic charge and 2000 μ L/h flow rate. The charged polymer jet was collected on a grounded 3 mm rod rotating in 970 rev/min.

Hydrogel-filled nerve conduits were fabricated by injection of photo-cross-linkable hydrogel precursor into an electrospun scaffold. Hydrogels were then photo-cross-linked for 4 min similarly to hydrogels used for characterization. Hydrogel-filled conduits were then washed in sterile PBS for 12 h prior to implantation.

2.9. *In Vivo* Sciatic Nerve Regeneration Model and Nerve Conduit Implantation

All surgical procedures were conducted in accordance with the guidelines established by the UCLA Institutional Animal Care and Use Committee under protocol number ARC-2016-101-03. Adult female Sprague–Dawley rats (Charles River Laboratories) weighing 200–250 g were anesthetized using inhaled isoflurane. An incision was made through the skin and gluteal muscle of the left hindlimb to expose the sciatic nerve. A critical nerve gap of 10 mm was created 5 mm proximal to the trifurcation of the sciatic nerve and repaired using hydrogel or empty nerve conduits (each $n = 3$), secured with 8–0 Nylon monofilament sutures. Following implantation, the overlying muscle layers and skin were closed with 4–0 absorbable sutures to complete the surgical procedure.

2.10. Sciatic Nerve Functional Analysis

Gait functional tests were conducted at 3 and 8 weeks postsurgery. During the assessment, rats were placed in a transparent walking corridor made of Plexiglas, measuring 80 cm in length, 6 cm in width, and 12 cm in height. Video recordings of the rats walking were captured from the floor plane of the corridor. Recordings continued until five or six satisfactory walks of at least four continuous steps were obtained. The sequential footprints of the hindlimbs were manually identified from the recorded videos. The toe spread and print length of each rat were measured for both the injured and normal (uninjured) hindfoot. Subsequently, the sciatic function index (SFI) was calculated based on these metrics using the following equation

$$\text{SFI} = 118.9 \left(\frac{\text{ITS} - \text{NTS}}{\text{NTS}} \right) - 51.2 \left(\frac{\text{IPL} - \text{NPL}}{\text{NPL}} \right)$$

SFI = sciatic functional index

ITS = injured toe spread

IPL = injured print length

NTS = normal toe spread

NPL = normal print length

Each parameter was averaged from a minimum of 20 footsteps.

The electrophysiology test was conducted 8 weeks postsurgery, just prior to euthanasia. To assess electrophysiological function, compound muscle action potential (CMAP) measurements were taken from the tibialis anterior (TA) muscle using an established protocol.³⁵ During the nonsurvival surgery, the animal was anesthetized, and the sciatic nerve was exposed following established procedures. Two stimulating electrodes were positioned around the site of the injury to the sciatic nerve. Additionally, two recording electrodes were placed at both the center and the ends of the TA muscle. A stimulus of 0.2 ms duration was applied to the sciatic nerve via stimulation electrodes. The stimulation ranged from 1 to 20 mA, and the maximal response waveform was recorded as the CMAP.

2.11. Histological Analysis

Tissue collection was conducted 8 weeks after surgery, following functional testing. After isolation, the gastrocnemius muscle and sciatic nerve tissue from the defect location were immediately snap-frozen in liquid nitrogen. Tissue was embedded in an OCT embedding compound (Tissue-Tek) and submerged in isopentane precooled by liquid nitrogen. For the analysis of inflammatory cells, nerve samples were also harvested at 2 weeks postimplantation.

Frozen tissues were sectioned into 10- μ m thick samples using a cryostat. H&E staining was performed on gastrocnemius muscle samples to visualize muscle fiber atrophy. Samples were first fixed in 4% PFA for 10 min and then washed in PBS and water for 3 min each. Samples were then stained in hematoxylin for 30 s, quickly rinsed in water, and then dehydrated through increasing concentrations of ethanol (70, 95%) each for 30 s. Samples were then stained in Eosin for 30 s, followed by further dehydration through 95 and 100% ethanol before clearing through xylene. Stained slides were then dried and mounted with DPX mountant.

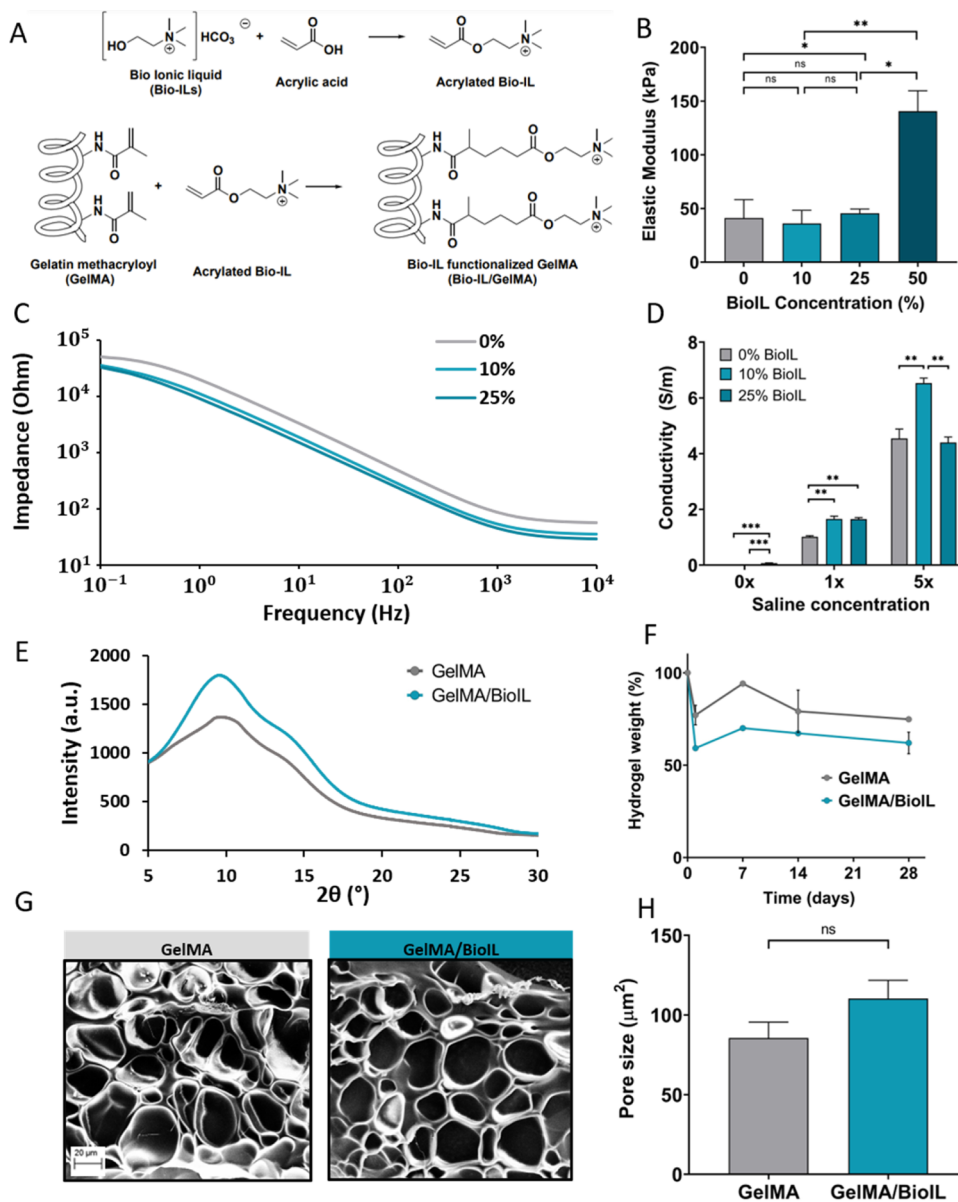


Figure 1. Synthesis and characterization of GelMA/BioIL hydrogels. (A) Schematic of BioIL functionalization and BioIL/GelMA hydrogel synthesis. Schematic of material synthesis adapted and redrawn by the authors based on Noshadi et al. (2017). (B) Elastic modulus of GelMA and GelMA/BioIL hydrogels up to 50% BioIL ($n = 3$). (C) Impedance spectrogram of hydrogels ($n = 3$) in physiological concentration of saline. (D) Ionic conductivity of hydrogels with different concentrations of conjugated BioIL, in solutions of different ion concentrations. (E) WAXS results of GelMA and GelMA/BioIL (10% BioIL) hydrogels. (F) Hydrogel stability over 28 days ($n = 3$). (G) SEM images of hydrogel pores. (H) Quantification of the hydrogel pore area. All data presented as mean + SEM. Data from B, C, and D were analyzed using one-way ANOVA with Tukey's post hoc test and from H using Student's t test. * $p < 0.05$, ** $p < 0.01$. $n = 3$ for mechanical and conductivity characterization.

2.12. Immunohistochemistry

Sectioned tissue samples were first fixed by 4% PFA for 10 min. The samples were then blocked for 1 h before overnight incubation with primary antibodies. Nerve axons were stained with antineurofilament 200 (1:200, Sigma-Aldrich, N-4142). Samples were then washed in PBS before being incubated in secondary antibodies. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1:2500).

Immunostained tissues were imaged using confocal microscopy (Leica SP8). Axonal cross sections were identified by a supervised neural-net image analysis model (Aivia, Leica), followed by manual segmentation.

2.13. Toluidine Blue Staining and Myelin Thickness Quantification

Nerve tissues for analysis of myelin thickness were fixed in cold 3% glutaraldehyde and 0.1 M sucrose in 0.1 M cacodylate buffer overnight. Fixed samples were then washed and postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer. Samples were dehydrated through a series of increasing ethanol solutions. Dehydrated samples were then embedded in Eponate 12 resin and polymerized for 48 h at 60 °C. Sections of 1 μm were stained in 0.5% toluidine blue in 1% borate buffer for visualization of myelin sheaths. Toluidine blue stains were visualized under bright-field microscopy, and the thickness of the myelin sheaths was quantified using ImageJ.

2.14. Statistical Methods

Student's *t* test or one-way ANOVA with Tukey's post hoc tests were used to analyze the data as appropriate. Quantitative data are presented as the mean $\pm$ standard error of the mean (SEM).

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Ionic Conductive Hydrogels

Ionic conductive hydrogels were synthesized by photo-cross-linking acrylated 10% GelMA/BioIL prepolymer solution (Figure 1A). To more closely simulate properties of the *in vivo* implanted hydrogels, hydrogels were submerged in phosphate-buffered saline (PBS) for 24 h prior to characterization. The mechanical properties of the hydrogels were measured by a tensile test using an Instron mechanical tester. Young's modulus of a 10% GelMA alone was measured as 41.05 ± 17.12 kPa. Young's modulus of the hydrogel did not increase with the addition of 10% BioIL (35.93 ± 12.37 kPa) or 25% BioIL (41.46 ± 4.05) (Figure 1B). However, the modulus of the hydrogel greatly increased with the addition of 50% BioIL to 140.50 ± 19.05 kPa. While a healthy sciatic nerve has Young's modulus of approximately 580 kPa,³⁶ conditions of the healthy nerve may not be ideal for stimulating axon regeneration. Rather, studies using substrates of stiffnesses from 1882 to 18 kPa found that dorsal root ganglia neurite extension is greatest at a modulus of approximately 88 kPa. However, regeneration of functional peripheral nerves also requires the proliferation of Schwann cells for axon myelination. Schwann cell proliferation and migration were observed to be greatest on substrates of lower stiffness, approximately 7.45 kPa.³⁷ GelMA/BioIL hydrogels of up to 25% BioIL have Young's moduli measuring within the range of 88 and 7.45 kPa. Elasticities of these hydrogels suggest that they may support both axon extension and Schwann cell proliferation processes of peripheral nerve regeneration. As 50% BioIL results in higher stiffness and may reduce necessary Schwann cell processes, the formulation was no longer continued in further experiments.

Conductivity of the hydrogels was measured by a frequency spectroscopy method.³⁸ Hydrogels were incubated in saline to approximate ion conditions *in vivo* and measured while hydrated; additionally, hydrogels were measured after incubation in water and 5 $\times$ physiological concentration of saline for the purpose of understanding conduction mechanisms. Ionic conductivity of the hydrogel was calculated from the intercept of the first semicircle in EIS measurements. Each hydrogel displayed frequency-dependent behavior, consistent with conduction through ionic mechanisms (Figure 1C). However, BioIL containing hydrogels demonstrated increased conductance as compared to pure GelMA hydrogel throughout the frequency spectrum (0.1 Hz to 10 kHz). Conductivity of the hydrogel was calculated based on observed impedance and gel dimensions (Figure 1D). Hydrogels swollen in water exhibited low conductivity. Conjugation of BioIL to the hydrogel backbone produced moderate improvements, consistent with a limited charge transport through chain-associated groups. Incubation in physiological saline further increased the conductivity, reflecting the higher ion concentration. Notably, BioIL-conjugated hydrogels in saline displayed a greater enhancement (150%) than in water (126%), suggesting that BioIL not only contributes through conjugated charge mobility but also promotes ion accumulation via electrostatic

interactions, consistent with the Nernst–Einstein relationship. Collectively, these results indicate that GelMA/BioIL hydrogels conduct through a dual mechanism involving both mobile conjugated charges and recruited free ions.

At physiological salinity, the conductivity of the GelMA hydrogel with 10% BioIL (1.6565 ± 0.2020 S/m) was significantly greater than that of GelMA alone (1.016 ± 0.06 S/m). Conductivity of GelMA containing 25% BioIL was similarly increased. However, no further increases in conductivity were seen by increasing the BioIL concentration from 10 to 25%. A possible explanation for this plateau in conductivity could involve charge shielding interactions, where ionic interactions might shield chain-to-chain repulsion and reduce the width of the conducting channels. Similar phenomena have been observed in polyelectrolyte hydrogels, where increased salt concentration can shield electrostatic repulsion between charged groups and alter polymer conformation.³⁹ Similar effects were also seen in hydrogels submerged in 5 $\times$ concentration saline: while 10% BioIL conjugation leads to increased conductivity, a further increase of BioIL concentration to 25% rather leads to no increase in conductivity. Due to the limited increase in conductivity from 10 to 25% BioIL, only 10% GelMA/BioIL hydrogels were used for further studies. Nevertheless, the ionic conductivity of GelMA/BioIL hydrogels in physiological conditions is similar to that of nerve tissue measured via magnetic resistance electrical properties tomography *in vivo* (1.47–3 S/m).⁴⁰ GelMA/BioIL gels also achieve a conductivity similar to that used for other conductive nerve conduits synthesized using conducting polymers.^{10,41–45,48} Increased conductivity of the GelMA/BioIL hydrogel at 10 kHz also suggests that the nerve conduit may support transmission of low-frequency electrical stimulation used to treat peripheral nerve injury.⁴⁶ Further studies may be performed to assess the effect of hydrogel conductivity on the effectiveness of stimulation treatments.

Ion-conduction mechanisms through the GelMA/BioIL hydrogel were further assessed by structural analysis. Structural analysis was conducted through WAXS (Figure S1). Both GelMA and GelMA/BioIL hydrogels exhibit a broad diffraction peak near $2\theta \approx 10^\circ$, indicating predominantly amorphous polymer chain structure (Figure 1E). However, the sharpness and intensity of the peak corresponding to BioIL-conjugated GelMA hydrogel were increased compared to that of pure GelMA hydrogel, indicating greater directional alignment of polymer chains. This suggests enhanced local ordering of the chains into structured crystalline domains, likely due to ionic interactions between BioIL and polymer chains. Such partial ordering may contribute to the formation of more stable and efficient ion-conducting pathways, thus supporting the observed increase in the ionic conductivity. These results reveal that BioIL not only increases hydrogel conductivity through the increase of charge carrier density but also modulates the polymer microstructure to create a more favorable environment for ion transport. Stability of the GelMA/BioIL hydrogel was assessed by incubation in a serum solution. Hydrogel dry weight was measured prior to incubation and then measured at several time points up to 28 days (Figure 1F). Mass of both hydrogels decreased in the initial 2 days of incubation, potentially due to leaching of unconjugated GelMA. Over 60% of the hydrogels remained for the remainder of the duration up to 28 days. No significant difference in the decrease was observed between GelMA and GelMA/BioIL hydrogels. The stability of the hydrogels implies

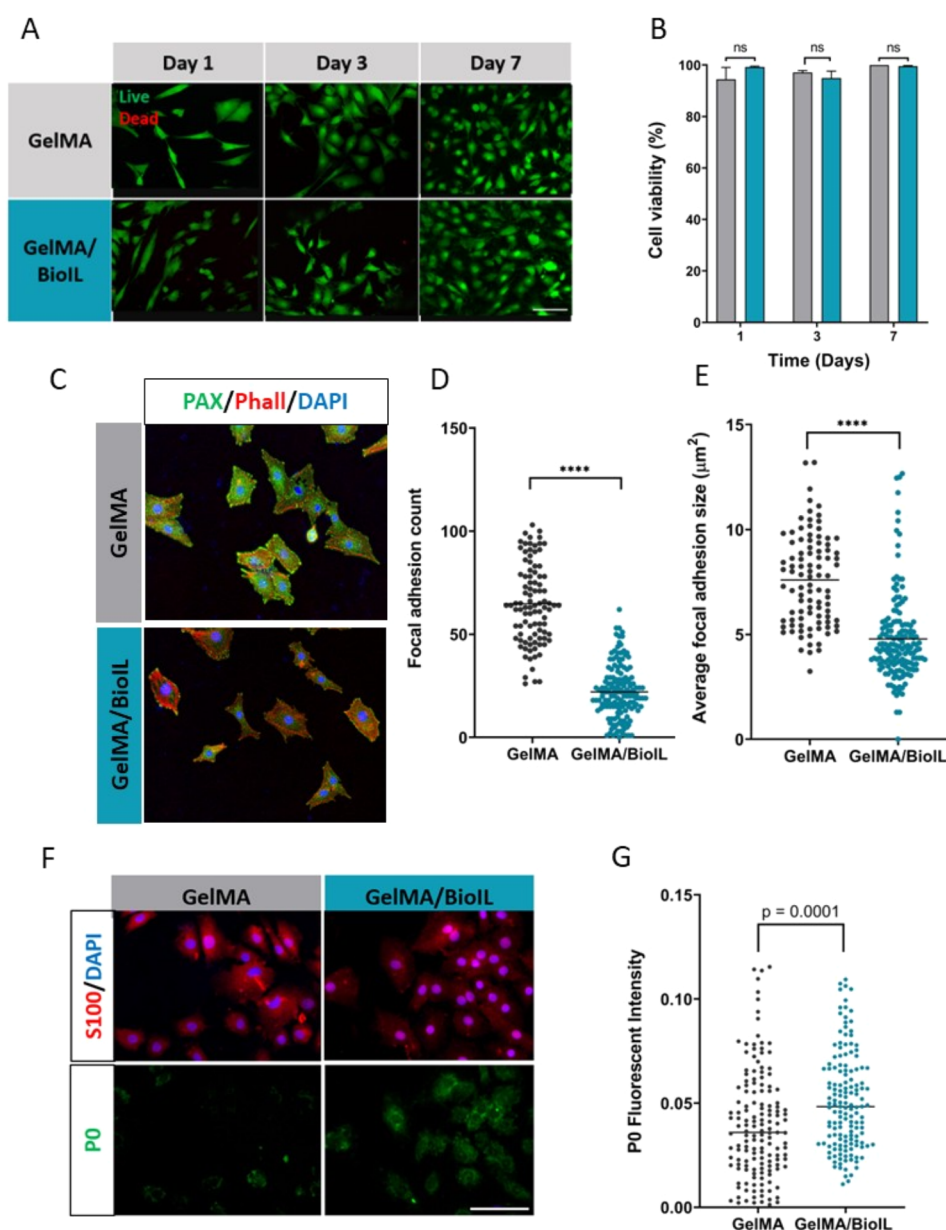


Figure 2. Biocompatibility of GelMA/BioIL hydrogels and their effects on Schwann cells. (A) Representative images of cell viability on hydrogels. (B) Quantification of cell viability of S16 on hydrogels ($n = 4$). (C) Immunofluorescence images of Schwann cell cytoskeleton and focal adhesions on hydrogels using phalloidin and PAX. (D) Quantified number of focal adhesions per cell. (E) Quantification of average focal adhesion area for each cell. (F) immunofluorescence images of P0 in S16 cells cultured on hydrogels for 3 days. (G) Quantification of P0 fluorescence intensity per S16 cell. Data from B were analyzed using one-way ANOVA with Tukey's post hoc test and from D, E, and G using Student's *t* test. Scale bars represent 100 μm .

that implanted nerve conduits may maintain structural support for the critical 4 week duration of axon outgrowth. However, the degradation of GelMA hydrogel *in vivo* may be facilitated by enzymatic degradation. Our previous studies demonstrated that implanted GelMA/BioIL hydrogels degrade steadily over 28 days, with approximately 50% of the hydrogel remaining at the end of the period. Implantation of the hydrogel elicited minimal sustained inflammation and fibrosis. The material also supported tissue ingrowth over 28 days. While degradation was not monitored past 28 days, GelMA is known to be biodegradable with a half-life of approximately 5 weeks.^{47,48}

Porosity of the GelMA/BioIL hydrogels with 10% GelMA was visualized through scanning electron microscopy (SEM) and quantified for the area of the pores (Figure 1G). Porosity

of the hydrogels did not differ significantly with the addition of BioIL. However, both hydrogels displayed a porosity sufficient for the growth of myelinated axons through the material (Figure 1H). The cross-sectional area of myelinated peripheral axons averages $27.8 \mu\text{m}^2$.⁴⁹ Pores of the GelMA hydrogel were $85.6 \pm 9.95 \mu\text{m}^2$, while those of GelMA/BioIL gels were $110.3 \pm 11.48 \mu\text{m}^2$. A greater area of hydrogel pores compared to the axon cross section suggest that the hydrogels may be used to provide structural support, without impeding axon regeneration.

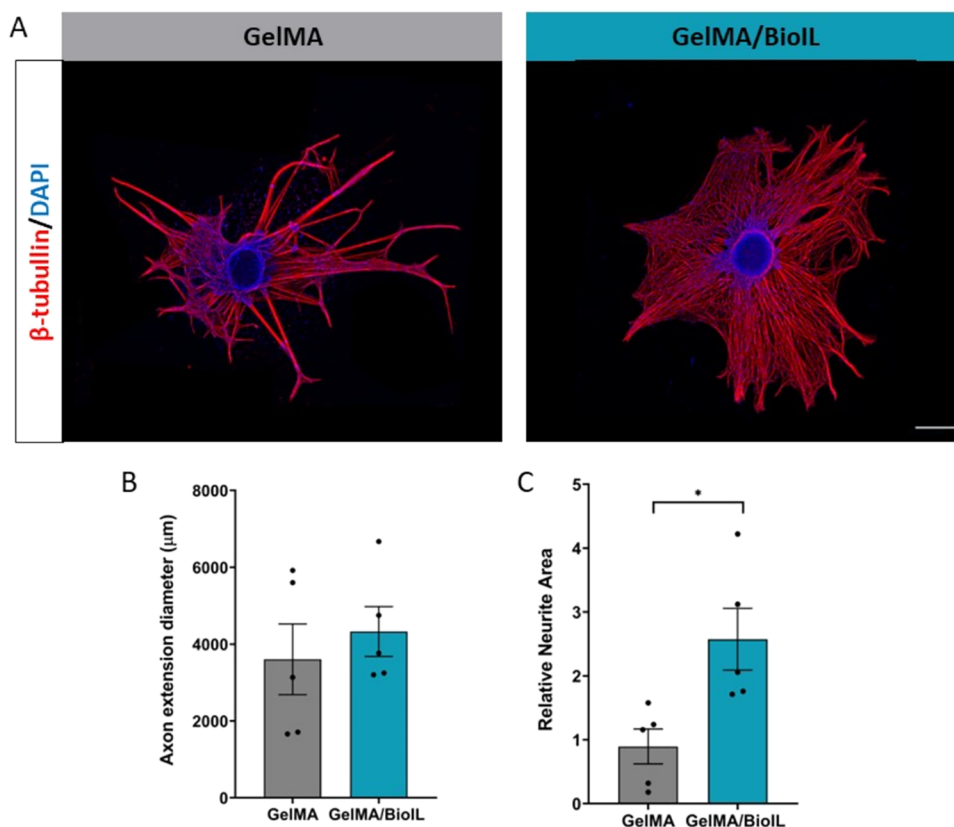


Figure 3. Axon outgrowth from dorsal root ganglia (DRG) on hydrogels. (A) Immunofluorescence images of DRG seeded on hydrogels. (B) Quantification of neurite extension diameter. (C) Quantification of neurite coverage area. Data were analyzed using Student's *t* test. Scale bars represent 500 μ m.

3.2. *In Vitro* Evaluation of Ionic Liquid Hydrogel Biocompatibility

S16 Schwann cells from rat sciatic nerves were cultured on the GelMA/BioIL hydrogels to evaluate biocompatibility. Cell viability was assessed on days one, three, and seven using live/dead fluorescence staining, and images were quantified (Figure 2A). Viability of cells on GelMA hydrogels with 10% BioIL was compared to that on GelMA hydrogels (Figure 2B). No differences in viability were seen between the GelMA/BioIL and GelMA hydrogels at any tested time point. Viability of Schwann cells suggests that GelMA/10% BioIL hydrogels may be used in peripheral nerve implantation without cytotoxicity. Moreover, survival of Schwann cells on the material indicates that the hydrogel may be used for functional regeneration of the peripheral nerve, as Schwann cell survival is critical to restoring signal transmission of regenerated axons.⁵⁰

3.3. Evaluation of Schwann Cell Adhesion on Ionic Liquid Hydrogel

To elucidate the potential mechanism by which GelMA/BioIL hydrogels affect the function of Schwann cells, Schwann cells cultured on the hydrogels were stained for the cytoskeleton and adhesion structures. Schwann cells cultured on GelMA/BioIL showed a reduced amount of PAX puncti (Figure 2E). Quantification of PAX puncti showed a decrease in both the number (Figure 2F) and size (Figure 2G) of focal adhesion complexes on the ionic conducting hydrogel. The observed effects suggest that the material's surface charge and ionic conductivity modulate Schwann cell behavior by altering integrin–matrix interactions, leading to partial inhibition of

integrin binding.^{51,52} Since persistent activation of focal adhesions is associated with the maintenance of an immature Schwann cell state,⁵³ the observed reduction in signaling suggests that ionic conductivity may bias these cells toward maturation. These findings imply that ionic conductivity affects behavior and potentially promotes Schwann cell maturation through focal adhesion-dependent signaling pathways.

3.4. *In Vitro* Evaluation of Schwann Cell Function on Ionic Liquid Hydrogels

Following observations of focal adhesion changes, GelMA/BioIL hydrogels were evaluated for effects on the Schwann cell myelination function. S16 cells were immunostained for Schwann cell marker S100, and no significant change was observed in the hydrogel materials (Figure S2). Cells were also immunostained for myelin protein 0 (P0), a crucial protein for *in vivo* myelination, to examine the potential of Schwann cells.^{54,55} Schwann cells cultured on GelMA/BioIL showed a significant increase in P0 expression (Figure 2D). P0 expression was also measured by Western blotting analysis (Figure S3), demonstrating a similarly significant increase in GelMA/BioIL hydrogels. The increased P0 expression is an indication of a myelinating phenotype of Schwann cells.⁵⁶ Increased P0 expression of S16 Schwann cells on GelMA/BioIL hydrogels suggests that nerve conduits filled with the same material may promote myelination in the recovery of PNI.

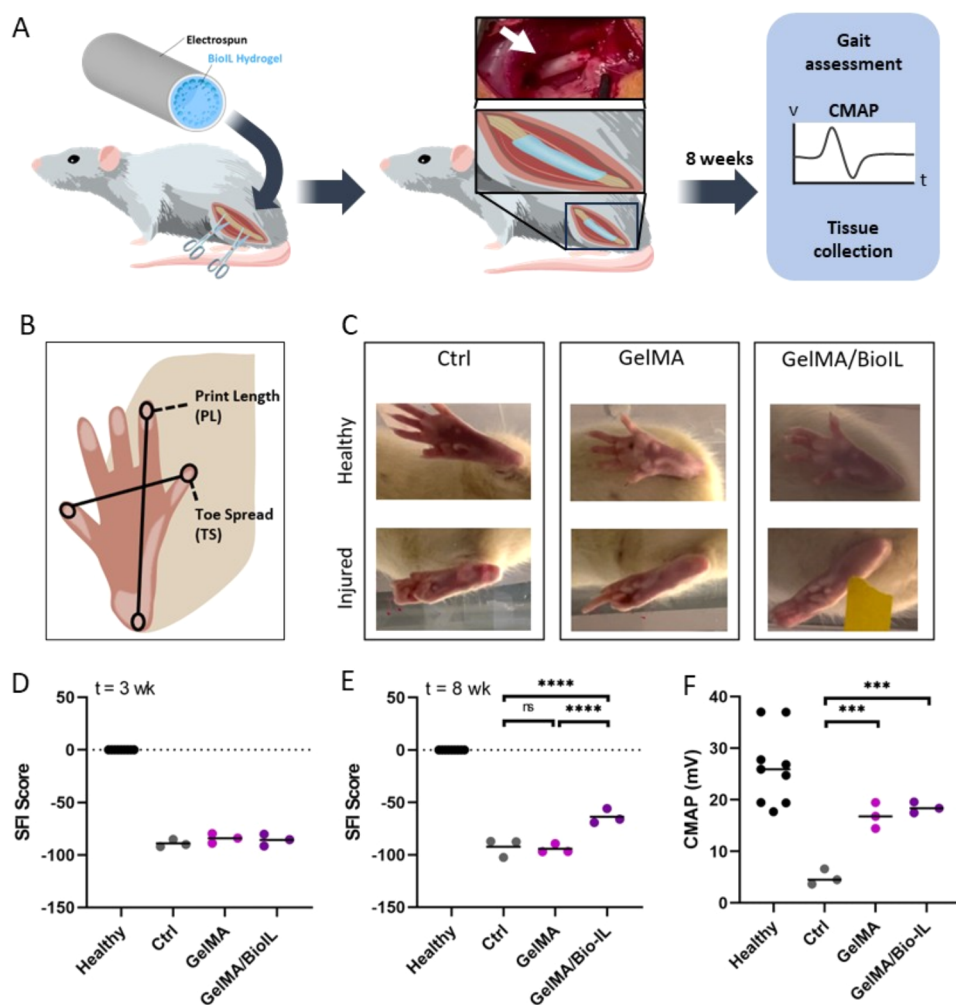


Figure 4. Functional analysis of peripheral nerve regeneration. (A) Schematic of nerve conduit and implantation into a rat sciatic nerve injury model, with image of implanted nerve conduit. White arrow indicates the implanted nerve conduit. (B) Diagram of metrics used in the sciatic functional index (SFI) measurement. (C) Representative images of rat hindlimbs used for calculation of SFI 8 weeks following injury. Injured limb (bottom) values were calculated against values of the healthy limb (top). Sciatic functional index of rats with hydrogel or nerve conduits at (D) 3 and (E) 8 weeks postsurgery, showing gait abnormalities compared to a healthy foot. (F) Compound muscle action potential (CMAP) of nerve conduit-implanted animals ($n = 9$ for healthy, $n = 3$ in experimental groups for D, E, and F). All data were analyzed using one-way ANOVA with Tukey's post hoc test. $p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

3.5. In Vitro Evaluation of Axon Outgrowth from Dorsal Root Ganglia on Hydrogel

To assess the hydrogel effects on axon extension, primary dorsal root ganglia were isolated from rats and cultivated on hydrogels for 2 days. Axon outgrowth from the neurite was then visualized through the immunostaining of β -tubulin. DRGs cultured on ion-conductive GelMA/BioIL hydrogels showed denser axon outgrowth compared to those on GelMA hydrogels (Figure 3A). Quantification of neurite extension diameter showed no significant difference between DRG cultured on each hydrogel (Figure 3B). However, DRGs cultured on GelMA/BioIL hydrogels exhibited more neurite outgrowth, forming a denser and more highly interconnected network compared with GelMA hydrogels. The amount of neurite outgrowth from the DRGs was quantified by the neurite coverage area. Axons extended from DRGs on GelMA/BioIL covered a larger area (Figure 3C), showing a greater amount of neurite outgrowth. The increased neurite outgrowth from the ion-conductive hydrogels implies that the use of these hydrogels in a nerve conduit may stimulate axon extension and

improve peripheral nerve regeneration. Taken together with the enhanced Schwann cell myelination activity, these findings suggest that incorporating GelMA/BioIL hydrogels into nerve conduits could actively promote axon extension and support the regeneration of functional, myelinated peripheral nerve tissue.

3.6. In Vivo Evaluation of Functional Recovery in a Rat Model of Peripheral Nerve Injury

The ability of conductive GelMA/BioIL hydrogels to facilitate axon regeneration was then evaluated in a rat model of PNI (Figure 4A). PNIs were made by resection of 1 cm of the sciatic nerve from the rat hindlimb. Transection of 1 cm⁶³ simulates a critical defect size at which the nerve is unable to regenerate without medical intervention;⁵⁷ this critical defect model represents clinical situations where surgical or medical repair is necessary. To create a suturable hydrogel conduit to bridge the critical gap,³⁴ nerve conduits were fabricated by filling electrospun PLCL/PLG tubes with GelMA hydrogels functionalized with 10% BioIL. The use of PLCL/PLG outer conduit provides sufficient mechanical support, with flexibility

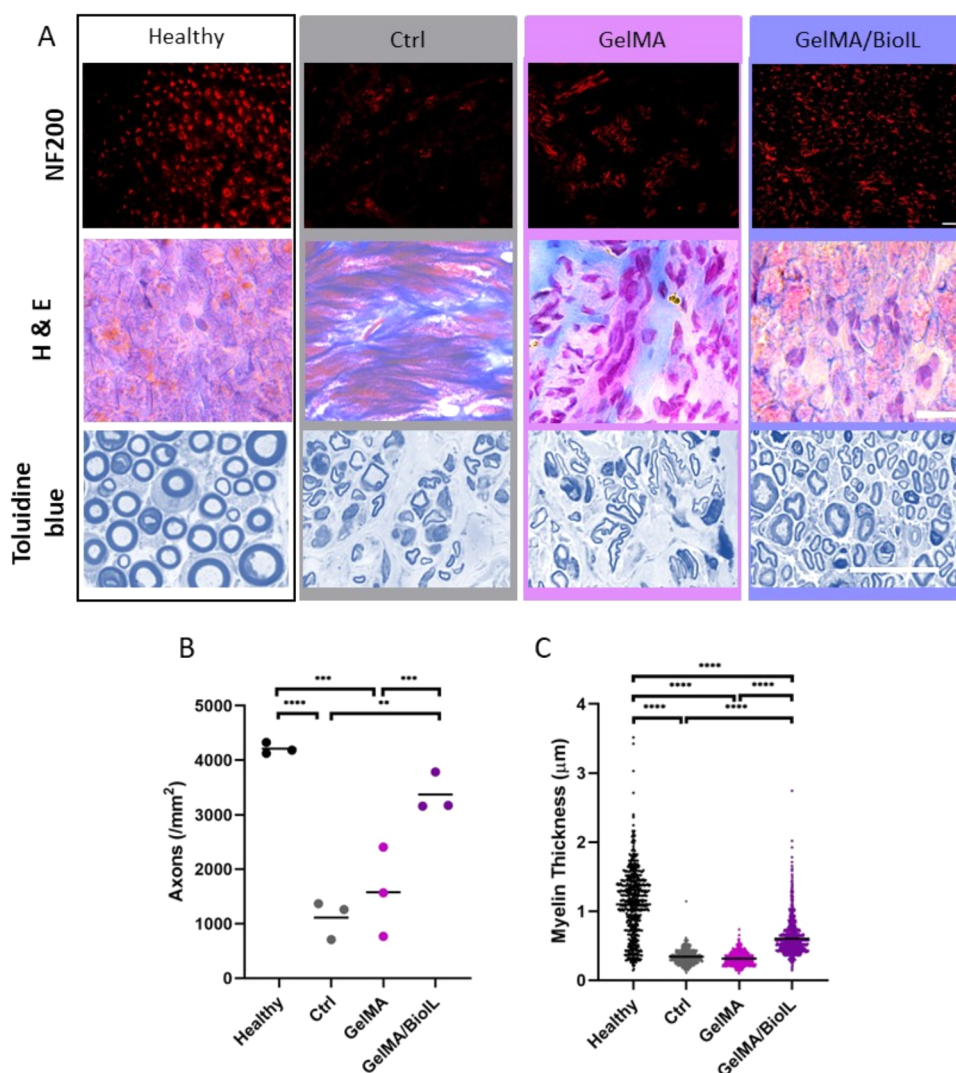


Figure 5. *In vivo* nerve regeneration through BioIL nerve conduits. (A) Cross section images from the center of nerve conduit. Stained for peripheral nerve axons (NF200) using immunofluorescence, Masson's trichrome, and for myelin sheaths using toluidine blue. Scale bars represent 20 μm . (B) Axon density in nerve cross sections, quantified from immunofluorescent images ($n = 3$). (C) Quantified thickness of myelin sheath from toluidine blue images (individual neurons quantified from three images per group). Data from B and C were analyzed using one-way ANOVA with Tukey's post hoc test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

for animal movement.³⁴ Hydrogel-filled conduits were then trimmed and sutured to connect the gap. Functional analysis and collection of histological samples were performed 8 weeks postimplantation. All experiments performed on rats were performed following the protocol ARC-2016–101 approved by UCLA's Animal Research Committee.

To evaluate potential chronic immune responses to the hydrogel, rats were sacrificed at 2 weeks postimplantation. Implanted conduits, as well as attached tissues, were collected and stained for inflammatory marker CD45 to assess the inflammatory response (Figure S4). While initial inflammation assists in clearing damaged tissue, sustained inflammation can impair regeneration.^{58,59} CD45 staining showed minimal to no presence of inflammatory cells within the hydrogel. Some CD45 staining was observed on the surface of the nerve conduits, possibly due to slow degradation of the nerve conduit. By 2 weeks postimplantation, inflammation had largely resolved, indicating that immune responses to the hydrogel are unlikely to hinder regeneration at later stages.

At 2 weeks postimplantation, a partial degradation of the hydrogel could be observed in the cross-sectioned sample (Figure S5). While quantification of total hydrogel volume is not possible from microscopy images, our prior studies demonstrated approximately 35% GelMA/BioIL degradation *in vivo*.³⁰ Future studies may be required to quantify the degradation of the nerve conduit-loaded hydrogel.

The animals were evaluated for gait recovery as well as motor functionality of the hindlimb. Gait recovery was measured at 3 and 8 weeks following injury. Sciatic nerve function was evaluated through the animal's control of the injured hind paw during locomotion. Toe spread and print length of the animal's injured hindfoot (Figure 4B) were measured from recorded images (Figure 4C) and normalized according to equivalent measurements of the animal's healthy foot to produce the sciatic functional index (SFI). No differences were observed between experimental groups at the early time point of 3 weeks (Figure 4D). However, by 8 weeks, GelMA/BioIL nerve conduit-implanted animals demonstrated a greater ability to extend the paw to achieve a

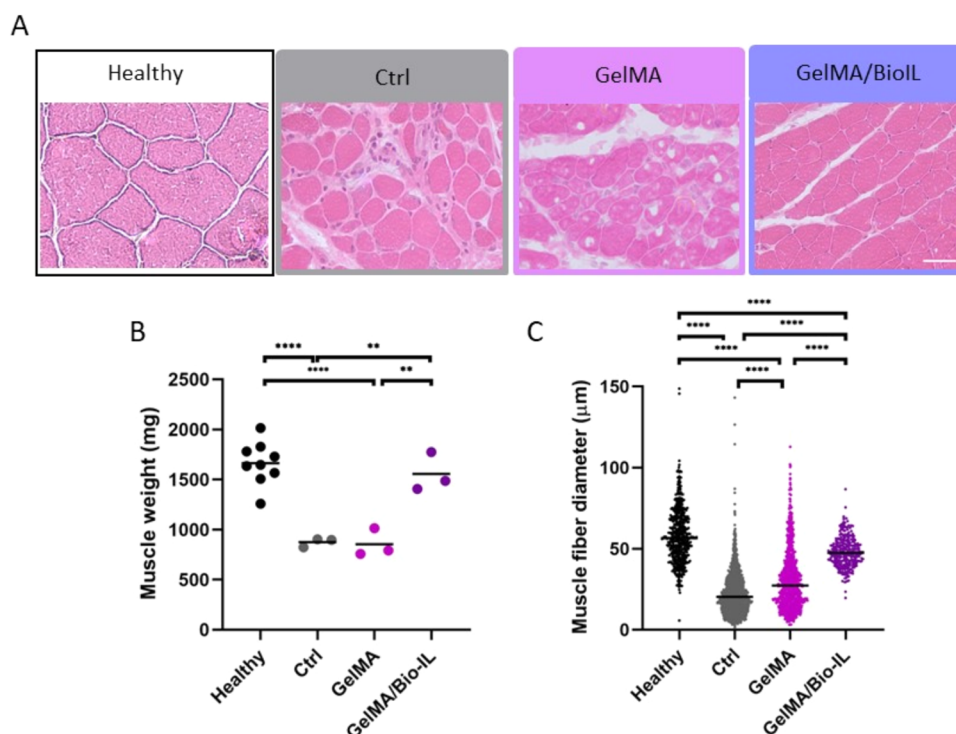


Figure 6. BioIL hydrogel nerve conduit effects on gastrocnemius muscle following sciatic nerve injury. (A) H&E stains of gastrocnemius muscle cross sections at 8 weeks postinjury. Scale bar represents 20 μm . (B) Wet weight of gastrocnemius muscles from healthy and nerve conduit-implanted animals ($n = 9$ for healthy, $n = 3$ for experimental groups). (C) Diameter of gastrocnemius muscle fibers (individual muscle fibers quantified from 9 images per group). All data were analyzed using one-way ANOVA with Tukey's post hoc analysis. $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

healthier gait (Figure 4C). In groups that received a GelMA/BioIL-filled conduit, animals had a significantly higher SFI compared to those of both GelMA-filled and empty conduit groups (Figure 4E). Meanwhile, animals that received conduits filled with GelMA alone did not show any effect on resulting SFI in comparison to the empty conduits, suggesting that conjugation of ion-conductive BioIL to the hydrogel aids in restoring sciatic nerve function. Functionality of motor neurons was measured by the resultant CMAP upon electrical stimulation of the proximal nerve (Figure 4F). Stimulation elicited a greater subsequent CMAP in hydrogel-filled conduit groups when compared with hollow control conduits. However, no significant difference was seen between the GelMA and GelMA/BioIL conduit groups. GelMA and GelMA/BioIL conduit-implanted rats reported CMAP amplitudes averaging 16.90 and 18.47 mV, amounting to approximately 70.50 and 77.04% recovery from healthy controls, respectively. Both conduits performed similarly to the reported CMAP recovery of autograft-treated animals (approximately 60%) in similar sciatic nerve injury models.⁶⁰ The empty control conduit, on the other hand, showed only 20.5% recovery of CMAP amplitude. The difference in recovery as measured by the two different methods suggests an accelerated regeneration in the GelMA/BioIL nerve conduits. The elicited CMAP measures the reinnervation of muscle.⁶¹ Meanwhile, SFI measures overall recovery, including the recovery of hindlimb control, coordination, and functionality. Improvements in CMAP in both GelMA and GelMA/BioIL groups indicate successful reinnervation of the target muscle by the 8 week time point. However, a lack of improvement in the SFI of the GelMA group suggests a delay in reinnervation, such that a part of the target muscle has

already atrophied. By contrast, GelMA/BioIL-implanted rats saw improvements in both CMAP and SFI, indicating more rapid reinnervation, preventing atrophy. Overall, these results demonstrate the potential of BioIL-conjugated, ionically conductive hydrogels to restore function in peripheral nerve injury. Ionic conductive GelMA/BioIL nerve conduits demonstrated comparable functional improvements to those made with conducting polymer or carbon materials,^{41,62,63} while offering improved biodegradability.

3.7. Evaluation of *In Vivo* Peripheral Nerve Regeneration through Histology and Immunofluorescence Staining

To evaluate axon regeneration through the conduits, histological sections of the regenerated nerves were collected. Cross sections of each nerve conduit were stained for neuron marker neurofilament protein (NF200) to visualize regenerated axons (Figure 5A). Sections from the unfilled control samples showed the regeneration of a few axons through the conduit in small bundles. GelMA-filled conduits similarly demonstrated few regenerated nerve bundles with small numbers of axons. In contrast, GelMA/BioIL-filled conduits showed the extension of a great number of axons into the conduit. The regenerated axons also formed large nerve bundles with structures similar to those of healthy nerve fascicles. Quantification of axon numbers in the nerve cross sections revealed doubled axon extension through GelMA/BioIL nerve conduits compared to empty or GelMA-filled conduits (Figure 5B). Axon regeneration in GelMA/BioIL nerve conduits resulted in densities similar to those found in healthy sciatic nerves. Histological staining of the regenerated nerves using Masson's trichrome showed collagenous fibrotic tissue formation between regenerated axons in control and

GelMA conduit samples. The excessive formation of fibrotic tissue can impede the regeneration of the nerve and potentially cause neuropathic pain.⁶⁴ Meanwhile, GelMA/BioIL samples showed minimal fibrosis, with a morphology similar to that of a healthy nerve. Functional nerve regeneration requires restoration of myelin sheaths that allow for efficient signal transmission in addition to axon extension. Myelination of regenerated axons through implanted nerve conduits was analyzed by toluidine blue staining of the myelin sheath (Figure 5A). Visualization of toluidine blue-stained nerve sections showed the formation of thicker myelin sheaths surrounding axons in the GelMA/BioIL nerve conduit compared to those in empty or GelMA nerve conduits (Figure 5C), as well as a lower density of myelinated axons. Increased thickness of the myelin sheath is correlated with increased axon conduction velocity of regenerated axons.^{65,66} Histological analysis of increased regeneration of axons as well as axon myelination indicates stronger and more rapid signal transmission through regenerated nerves, correlating with results observed in functional studies. Additionally, histological cross sections from within the conduit at 8 weeks demonstrate complete degradation of the conductive hydrogel and replacement with regenerated tissue. Increased myelination of regenerated axons in GelMA/BioIL nerve conduits correlates with *in vitro* observations of increased myelination markers in the Schwann cells on the hydrogel.

In addition to nerve regeneration and neuromuscular communications, muscle mass and strength are also important for functional recovery, as measured by SFI. At 8 weeks following implantation, the gastrocnemius muscle was isolated and wet weight was measured to further assess functional recovery. Rats implanted with GelMA/BioIL-filled, conductive nerve conduits experienced less muscular atrophy compared to both those with GelMA-filled and empty nerve conduits (Figure 6A). The GelMA/BioIL group maintained nearly double the amount of muscle mass (1555.0 ± 111.7 g) versus the control group (795.2 ± 47.55 g) or GelMA-filled group (853.7 ± 80.63 g) (Figure 6B). Muscle mass of the GelMA/BioIL group remained similar to that of healthy rats. Muscular atrophy of the gastrocnemius muscle was further assessed by the diameter of individual muscle fibers. Hematoxylin and eosin (H&E) stains of the muscle similarly reflected the increased loss of muscle mass found in nonconductive controls compared to GelMA/BioIL samples. Muscle samples from nonconducting conduits (empty control and GelMA) showed significant shrinking of individual fibers, as well as increased fibrous tissue in the muscle (Figure 6C). Empty conduit and GelMA-filled conduit groups experienced a large decrease in the size of the muscle fibers. In contrast, muscle fibers in the GelMA/BioIL group had only a slight decrease compared to those in the muscle tissue of uninjured limbs. Decreased muscle mass and fiber size in the GelMA group in comparison to the GelMA/BioIL group are consistent with lower SFI in the GelMA group despite the lack of significant difference in CMAP (neuromuscular communication) between GelMA and GelMA/BioIL groups at 8 weeks. Muscular atrophy occurs following PNI due to a lack of stimulation and use.⁶⁷ Extended denervation can lead to difficulties with reinnervation of the muscle, further impeding regeneration of limb function. Decreased atrophy of the gastrocnemius muscle with GelMA/BioIL nerve conduit implantation suggests accelerated restoration of signal transmission. The reinnervation of the gastrocnemius muscle in GelMA/BioIL-implanted animals was

further confirmed by the immunofluorescence staining of the neuromuscular junction (Figure S6). Images of the neuromuscular junction show acetylcholine receptor junctions, indicating synapse formation. While the conductive hydrogel nerve conduits may also conduct nerve impulses, histological analysis of nerve cross sections reveals that reduced muscle atrophy may be attributed to accelerated axon regeneration through GelMA/BioIL conduits. The use of GelMA/BioIL nerve conduits restores nerve function more rapidly to mitigate challenges in restoring muscle function. Together, these results demonstrate GelMA/BioIL as a strong candidate for use in nerve conduits. The ion-conducting hydrogel accelerates axonal regeneration at a comparable rate to those synthesized using carbon-based or conducting polymer materials.^{41,68,69} However, degradation products of GelMA/BioIL may be more easily cleared, while carbon materials such as graphene oxide may remain in the tissue even after dissolution of the encapsulating polymer,⁷⁰ causing long-term risks.

4. CONCLUSIONS

BioIL-conjugated hydrogels can significantly improve peripheral nerve regeneration, offering ionic conductivity with excellent biodegradability, biocompatibility, and processability. In this work, we demonstrated the superior regenerative effects of GelMA/BioIL hydrogels on sciatic nerve injury. The hydrogels were shown to improve the Schwann cell myelination capability and stimulate axon outgrowth. GelMA/BioIL hydrogels were then used in a two-step fabrication method to create a filled composite nerve conduit. GelMA/BioIL nerve conduits increased the regeneration of myelinated axons and the reinnervation of the target muscle, resulting in improved muscle action potential and reduced muscle loss. Structural improvements in nerve regeneration were further validated by improved sciatic functional outcomes. While additional work may be required to further elucidate the proregenerative mechanisms of conductive properties of the material, GelMA/BioIL hydrogels prove a promising alternative to current clinical options for the treatment of PNI.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.5c19623>.

WAXS diffraction patterns for GelMA and GelMA/BioIL hydrogels (1); S100 fluorescence-based quantification of Schwann cells on hydrogels (2); Western blot quantification of P0 expression in Schwann cells cultured on hydrogels (3); Immunofluorescence images of immune cells in implanted nerve conduits at 2 weeks (multiple locations and magnifications) (4); Immunofluorescence images of neuromuscular junctions in GelMA/BioIL-regenerated samples (5) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Song Li – Department of Bioengineering, University of California Los Angeles, Los Angeles, California 90095, United States; orcid.org/0000-0002-4760-8828; Email: songli@ucla.edu

Authors

Joyce Huang – Department of Bioengineering, University of California Los Angeles, Los Angeles, California 90095, United States; orcid.org/0000-0002-9729-0088

Jing Tian – Department of Bioengineering, University of California Los Angeles, Los Angeles, California 90095, United States

Tzu Chun Chung – Department of Bioengineering, University of California Los Angeles, Los Angeles, California 90095, United States; Division of Microsurgery, Department of Orthopedics, E-Da Hospital, I-Shou University, Kaohsiung 824005, Taiwan

Sam Denton – Department of Bioengineering, University of California Los Angeles, Los Angeles, California 90095, United States

Youchao Teng – Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Kun-wei Yeh – Department of Bioengineering, University of California Los Angeles, Los Angeles, California 90095, United States

Stephanie K. Seidlits – Department of Biomedical Engineering, University of Texas at Austin, Austin, Texas 78712, United States

Nasim Annabi – Department of Chemical and Biomolecular Engineering, University of California Los Angeles, Los Angeles, California 90095, United States; orcid.org/0000-0003-1879-1202

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acsami.5c19623>

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