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Aligned hydrogel tubes guide regeneration following spinal cord injury

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Abstract

Directing the organization of cells into a tissue with defined architectures is one use of biomaterials for regenerative medicine. To this end, hydrogels are widely investigated as they have mechanical properties similar to native soft tissues and can be formed in situ to conform to a defect. Herein, we describe the development of porous hydrogel tubes fabricated through a two-step polymerization process with an intermediate microsphere phase that provides macroscale porosity (66.5%) for cell infiltration. These tubes were investigated in a spinal cord injury model, with the tubes assembled to conform to the injury and to provide an orientation that guides axons through the injury. Implanted tubes had good apposition and were integrated with the host tissue due to cell infiltration, with a transient increase in immune cell infiltration at 1 week that resolved by 2 weeks post injury compared to a gelfoam control. The glial scar was significantly reduced relative to control, which enabled robust axon growth along the inner and outer surface of the tubes. Axon density within the hydrogel tubes (1744 axons/mm²) was significantly increased more than 3-fold compared to the control (456 axons/mm²), with approximately 30% of axons within the tube myelinated. Furthermore, implantation of hydrogel tubes enhanced functional recovery

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The authors have no competing interests to declare.

relative to control. This modular assembly of porous tubes to fill a defect and directionally orient tissue growth could be extended beyond spinal cord injury to other tissues, such as vascular or musculoskeletal tissue.

Graphical Abstract

Keywords

modular biomaterial; tissue repair; spinal cord injury; axon elongation

1. Introduction

Tissue engineering aims to restore tissues following damage incurred through traumatic injury or prolonged disease progression. Biomaterials can be used to fill defects, support cell infiltration, and/or deliver therapeutic factors. Delivery of a single biomaterial to fill a tissue void offers advantages in terms of the ease of implantation and juxtaposition with intact tissue, however, this method requires prior knowledge of wound geometries for a scaffold implant or may lack guidance cues in the case of hydrogels that polymerize in situ. Modular biomaterial implants comprised of multiple smaller structures can conform to the injury site without known wound metrics and can utilize the individual modules to provide guidance cues. A modular implant could be an off-the-shelf product that does not need to be fabricated on an individual patient basis, but can still conform to an injury and maintain close apposition with intact tissue while supporting regeneration. Moreover, the use of modules can lead to discrete regions that promote tissue-specific regeneration. For example, these modular biomaterials can promote *de novo* formation of the primary injured tissue (i.e. muscle, renal, liver, etc.), as well as the supporting tissues (nerves and vessels) that provide motor control and nutrients to the regenerating tissue [1, 2]. Modular biomaterials facilitate the partitioning of cell- or tissue-specific regions of an implant and can also be used to direct regeneration. These capabilities are essential in many tissue engineering applications as the function of a tissue is predicated on the tissue structure. Structure is particularly impactful within the spinal cord, in which the tissue is organized into specific neural tracts and a loss of structural organization due to injury leads to profound functional deficits in mobility and/or sensory function [3]. Trauma to the spinal cord can present as either contusive or penetrating injuries and, while there are several clinical trials underway, there are no viable clinical treatment options to repair the damaged tissue and restore function in either injury paradigm [4]. Spinal cord injuries frequently have an extensive inflammatory response, and

there are nuances in the infiltration and extent of inflammation associated with each model that hinder regeneration and functional recovery [5–8]. Biomaterials can be used to modulate this microenvironment, while providing support to guide regenerating tissue through the inflammatory environment, ultimately leading to restoration of motor and/or sensory function [9].

Regeneration of structurally organized tissues benefits from physical guidance systems to ensure proper organization of repaired tissues [10, 11]. Preformed structures created from templates or molds, as well as aligned fiber scaffolds have been used to promote directed growth following damage to blood vessels [12–15], intestine [16], muscle [17–20], bone [21], and nerve [9, 11, 22–35]. The goal of these implants is to restore the highly organized structures that lead to functional tissue restoration, whether it is fluid transport, generation of force, or signal conduction. While these scaffolds have been developed to guide repair and regeneration after injury, many of these scaffolds only address bi-directional guidance and do not account for variable defect geometries. For example, the spinal cord is a highly organized structure with predominantly rostral-caudal alignment of axons and myelin, making it an ideal tissue for evaluating aligned biomaterial bridges. Previous work using multi-channel bridges has shown robust directed axon elongation [9, 36–40]. An additional design feature of these bridges is their high degree of porosity [37], allowing for infiltration of progenitors that differentiate into myelinating oligodendrocytes resulting in not only axon regrowth, but also myelination of these axons [36, 41]. While these bridges hold great promise, they are pre-formed into a specific shape prior to implantation and cannot be easily modified. Additionally, other biophysical cues, namely modulus and viscoelastic properties, have not been designed to match the properties of the spinal cord [37]. Looking to other classes of biomaterials may help address these short-comings and expand the application to both penetrating and contusive injuries.

The alternative to a pre-formed biomaterial is a material that can be customized at the time of implantation. Some hydrogels can be delivered as a solution that undergoes gelation *in situ* and can conform to any defect (e.g., size, shape) to maintain good apposition with host tissue and provide mechanical stability. These hydrogels can also generally match the appropriate biophysical cues (modulus, viscoelasticity, porosity, etc.). This *in situ* gelation of a solution, however, does not provide directional cues, which can be critical to applications such as guiding re-growing axons following spinal cord injury. A lack of guidance cues could limit a material's usefulness for supporting regeneration, particularly in large gap defects [37, 42]. Novel hydrogels that incorporate guidance cues have been developed for muscular [43, 44], vascular [12, 13, 45], and neural [12, 22, 24, 25, 28–30, 46–50] applications. These hydrogel composites are comprised of a bulk hydrogel material with magnetically responsive additives used to align structures within the gels, such as fibers with iron oxide or carbon nanotubes [43, 44, 51, 52]. Alternatively, conduits with larger outer dimensions (1–4 mm; [13, 28, 33, 53, 54]) that are filled with hydrogels have been used, but alignment predominantly occurs along the perimeter of these systems due to the absence of cellular guidance cues within the implant core. Further, larger conduits do not readily conform to abnormal geometries due to their large diameter and poor cellularization at the material interfaces. Tubes with a lumen diameter on the order of 150–250 μm have been more successful at guiding regeneration throughout an injury site [37], as there is an

Genscript, Piscataway, NJ) [60] in HEPES was added to the PEG-oil mix and homogenized for an additional 60 seconds. The resulting solution was diluted with methanol and diH₂O and then centrifuged at 10,000 g for 5 minutes. The solution was decanted, then rinsed and centrifuged once each with 1% Tween-20 in diH₂O and diH₂O alone. Microspheres were used to generate tubes and bridges on the same day to ensure that the maleimide functional groups did not hydrolyze and could undergo a second phase of polymerization.

Remaining functional groups within the PEG microspheres were crosslinked via free radical polymerization. Irgacure 2959 photoinitiator (Sigma) dissolved in N-vinylpyrrolidinone (660 mg/mL; Sigma) was added to the microspheres at a final concentration of 1% w/v. The resulting microspheres were then packed into either a) polydimethylsiloxane (PDMS, Dow Corning, Midland, MI) molds to generate PEG tubes or b) 3D-printed acrylic molds to generate PEG bridges, and exposed to an ultraviolet lamp for 3 minutes to initiate free radical polymerization. Microspheres used to generate these structures were hydrated prior to packing in molds to eliminate swelling and facilitate the formation of desired shape dimensions and geometries, which are reported in Fig. 1. The crosslinked structures were immediately chilled to -20°C for 5 minutes, then gently removed from their respective molds. Tubes and bridges were rinsed in PBS (Gibco, Grand Island, NY) for 1 hour at room temperature to ensure removal of excess photoinitiator. Tubes were cut to the length of the bridge molds and 5 tubes were packed into each mold. Tubes were placed in molds to control for size and shape when compared directly to the PEG bridges to avoid potential bias. A solution of 2 mg/mL fibrinogen (Millipore, Billerica, MA), 5 U/mL thrombin (Sigma), and 2.5 mM CaCl₂ (Sigma) in tris buffered saline (TBS; Sigma) was added to the molds with the PEG tubes. The molds were incubated for 10 minutes at 37°C to allow the fibrin solution to gel and secure the tubes into a hydrogel composite, which will be referred to as the 5-tube composite. Bridges and 5-tube composites were cut to final lengths of 2 mm, sterilized with 70% ethanol for 60 seconds, then stored at -80 °C until implantation. Throughout the fabrication process the microspheres, tubes, bridges, and 5-tube composites were evaluated under light microscopy to evaluate dimensions and confirm the presence of open channels.

2.2 Evaluation of microspheres, tubes, and bridges

Microspheres were suspended in MilliQ water and evaluated with a Mastersizer 2000 (Malvern Instruments, UK) to evaluate size distribution. Porosity was assessed using both weight ratios and scanning electron microscopy. Tubes and bridges were dried in a lyophilizer, sputter-coated with gold (SPI Supplies), and analyzed on a scanning electron microscope (FEI, Quanta 200 3D). Acquisition conditions were 10 kV, 1.7 nA, 15 mm distance. The resulting images were converted to binary and the percent porosity was averaged across 6 samples. Porosity was confirmed by weighing hydrated materials, lyophilizing, and re-weighing to get an estimate of the volume that was attributed to the PEG relative to the void space filled by water. A microsquisher (Cell Scale, Ontario, CA) was used to measure the compressive modulus of slab PEG gels to evaluate the bulk properties without including confounding geometries from the tube and bridge structures. A 59.5 mm cantilever beam with a 0.5588 mm bearing was used to indent and measure the displacement of three PEG samples: (1) PEG-YKND microspheres crosslinked with

photoinitiator, (2) PEG crosslinked with YKND and then photoinitiator, and (3) PEG crosslinked with only photoinitiator. PEG crosslinked with YKND alone was not tested as a smooth PEG slab was not reproducibly fabricated. The PEG gels evaluated were chosen to evaluate the contribution of the crosslinking methods and the importance of an intermediary microsphere step in the bulk modulus.

2.3 Surgical implantation of PEG structures

All animal work was performed with prior approval and in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Michigan. A T9-10 lateral hemisection spinal cord injury was created in adult C57BL/6J female mice aged 6-8 weeks, as previously described [37, 38, 61]. Briefly, mice were anesthetized with 2% isoflurane and provided preemptive pain management (1 mg kg⁻¹ bupivacaine). After confirmation of sufficient anesthesia, a 2 cm incision was made in the skin between the scapula and a laminectomy was performed between T9-10. A 2.25 mm lateral hemisection was excised and then PEG bridges or PEG tube composites were implanted into the injury site. Gelfoam was used to secure the injury site in all conditions, after which the muscles were sutured and skin stapled. A subset of mice (no-treatment control group) did not receive an implant but did receive gelfoam over the injured spinal cord. Mice were immediately provided post-operative antibiotics (enrofloxacin 2.5 mg kg⁻¹ once a day for 2 weeks), analgesics (0.1 mg kg⁻¹ buprenorphine twice a day for 3 days), and supportive hydration (1 mL 20 g⁻¹ lactated ringer solution once a day for 5 days). Bladders were expressed twice daily until function recovered and staples were removed after 10 days. Surgical controls were put in place to limit lesion size variance, including the order of the incisions made to limit the effects of swelling to cut lines, measuring the distance between the rostral and caudal cuts, and verifying the absence of bruising to the contralateral tissue. Exclusion criteria include any deviations to the surgical controls, as well as any variance to the recovery timeline, including an inability to perform contralateral hindlimb ankle movement by post-operative day 3. No mice met these exclusion criteria for this study. Mice were euthanized and spinal cord segments (T8-11) were collected after 1, 2, or 8 weeks. For each condition, n = 3-5 mice at each time point, with n = 5 for flow cytometry, n = 3 for 2 week longitudinal histology, and n = 6 for 8 week transverse histology.

2.4 Immunohistochemistry

Isolated spinal cords were flash frozen, and then cryosectioned transversely (8 week tissue) or longitudinally (2 week tissue) in 18 µm sections. Samples were fixed, permeabilized as necessary, and incubated overnight at 4°C with primary antibodies. The following antibodies were used for primary detection: rat anti-F4/80 (1:200, Abcam, Cambridge, United Kingdom), goat anti-arginase (1:100, Santa Cruz, Dallas, TX, USA), rabbit anti-neurofilament-200 (1:200, Sigma), goat anti-myelin basic protein (MBP; 1:500, Santa Cruz), chicken anti-P0 (1:250, Aves Labs, Tigard, OR), chicken anti-GFAP (1:1000, Aves Labs). Species-specific fluorescent secondary antibodies were used for detection at 1:1000 (Life Technologies, Carlsbad, CA, USA). Hoechst 33342 (Life Technologies) was used as a counterstain in all tissue sections. Immunostained tissue sections were imaged using an AxioObserver inverted fluorescent microscope (Zeiss) using a 10× dry objective.

Axon Density and Myelination: Semi-automated counting software, previously described by McCreedy et. al.[62], was used to quantify axons and the co-localization of myelin with axons in transverse sections taken from the rostral, middle, and caudal regions of the implant. The bridge was divided into these three segments (0.75 mm lateral regions) to delineate between the rostral, middle, and caudal regions of the bridge, as axons will grow from the rostral and caudal ends into the bridge at 8 weeks and will therefore have higher densities at the rostral and caudal ends of the biomaterial than the middle of the implant. Three tissue sections (each spaced ~130 μm apart) were used for each region (rostral, middle, caudal) within the implant to obtain the regionally-specific average density for each animal. Briefly, the software was calibrated using manual NF-200⁺ and NF-200⁺MBP⁺ counts from a subset of transverse 10X images taken from different animals and regions of the implant. The software then used a series of Hessian filters and threshold functions within the bridge region to reduce noise for selected NF-200 and MBP images [62]. The software then output total axon counts, as well as the myelinated axon counts based on the curvilinear MBP co-localizing with axons; image acquisition and analysis was performed by investigators blinded to treatment condition. ImageJ (NIH, Bethesda, MD, USA) was used to analyze all other fluorescent images and define the bridge area.

Cell Density and Glial Scar Thickness: Cells positive for F4/80⁺ (macrophages) and F4/80⁺arginase⁺ (M2 macrophages) containing Hoechst⁺ nuclei were counted manually by two blinded researchers to quantify macrophage infiltration. Glial scar thickness was quantified via GFAP⁺ staining at the rostral margin of the injury, as astrocytes that comprise the glial scar become highly positive for GFAP following injury. The thickness, rather than area, of GFAP staining was quantified to limit variance due to histological tissue size. Additionally, the thickness of the glial scar is a more relevant parameter for a hemisection lesion as it defines the physical barrier the axons must penetrate to enter the biomaterial, which is free of scar tissue. Three manual measurements of the GFAP⁺ thickness (representative images of regions quantified as “positive” are provided in Fig. 4) were averaged within each tissue with 3 tissues (18 μm thick tissues spaced evenly over a thickness of 300 μm) used per each of the 3 slides (spaced 400 μm apart) used per animal. A total of $n = 3$ animals were used per condition. To reduce bias, all samples were stained within a single batch to ensure consistent exposure time and intensity profiles. Additionally, researchers were blinded to condition during image acquisition and quantification to eliminate bias for all histological analyses.

2.5 Flow cytometry

Spinal cords isolated 1 week after injury and implantation were collected with the bridge and contralateral, but not rostral or caudal tissue, to limit myelin debris. Tissue was digested with 1 U mL^{-1} liberase at 37°C for 6 minutes in thermomixer (Thermo Scientific) at 1400 RPM. Live cells were detected with a blue fix exclusion dye for 15 minutes at 4 °C. Cells were then incubated for an additional 30 minutes with Ly6G (PE, 1:1000, Biolegend), arginase (FITC, 1:1000, Abcam), CD4 (PECy7, 1:1000, Biolegend), F4/80 (Alexafluor 700, 1:1000, Biolegend), CD11c (PacBlue, 1:1000, Biolegend), GFAP (APC, 1:1000, BD), and CD45 (brilliant violet 510, 1:1000, Biolegend). Cells were then rinsed with saline, fixed with 4% paraformaldehyde for 10 minutes, and rinsed twice more. Samples were analyzed on a

MoFlo Astrios flow cytometer using appropriate excitation lasers and emission filters (Beckman Coulter, Brea, CA, USA). Data was analyzed with FlowJo software (FlowJo, Ashland, OR, USA) by investigators blind to the condition.

2.6 Basso Mouse Scale (BMS)

BMS was used to evaluate mice in an open-field locomotion test. Two researchers scored the mice on ankle movement, hindlimb placement, hindlimb stepping, and trunk stability and must reach a consensus to ensure accuracy. Mice were acclimated to the open field prior to testing and for the raters to become familiar with the normal gait pattern of the mice. Mice were scored for 8 weeks after surgery.

2.7 Statistics

Multiple comparison pairs were analyzed using a one-way or two-way ANOVA with Tukey post-hoc test. Significance was defined at a level of $p < 0.05$, unless otherwise noted. For all conditions, $n = 6$ mice per condition per time point for histological analysis of neurofilament within transverse tissue sections, while $n = 3$ was used for analysis of glial scar thickness within longitudinal tissue sections. For flow cytometry an $n = 5$ mice per condition was used. For BMS an $n = 6$ was used for each condition. All values are reported as mean $\pm$ standard deviation.

3. Results

3.1 Hydrogel porosity and structure are controlled through 2-step crosslinking of PEG-MAL

Using a water-oil emulsion, a subset of the thiol groups on 8-arm PEG-MAL were crosslinked via Michael-type addition with a slow-degrading plasmin sensitive YKND peptide [60] resulting in PEG microspheres (Fig. 1A). The resulting PEG microsphere diameters ranged from 15-105 μm with an average diameter of 45 μm (Fig. 1B). As several of the maleimide side chains were not used to form the microspheres, tertiary structures, such as bridges or tubes, can be generated through a second or even third crosslinking phase of these extra maleimide side chains (Fig. 1C.)

Microspheres were mixed with photoinitiator, cast into molds using pins to create channels, and exposed to ultraviolet light to anneal the microspheres. The molds were designed to assemble the spheres into tubes or bridge macrostructures with porosity controlled by the use of microsphere building blocks to generate our macrostructures, and these structures were confirmed with light microscopy (Fig. 2A-B). Similar pore size and pore distribution was observed between the two structures using SEM (Fig. 2C-D). Bridges and 5-tube composites (tubes assembled into composite bridge structures with fibrin) had similar porosity and water content, despite differences in channel size and overall composition (Fig. 2E). Compressive modulus was evaluated for PEG slabs to evaluate bulk differences between the crosslinking methods. Two-step crosslinking using an intermediate microsphere phase resulted in a compressive modulus of 12.5 kPa (Fig. 2F), which is within range of the modulus of a healthy mouse spinal cord (1-300 kPa, [63–65]). Conversely, 2-step crosslinking without an intermediate microsphere phase resulted in a 10-fold increase in

modulus and a 100-fold increase was observed when only the photoinitiator was used to crosslink the PEG-MAL (Fig. 2F). The increase in modulus is likely due to the reduction in porosity.

3.2 PEG scaffolds lead to a transient increase in immune cell infiltration

A 5-tube composite or a 5 channel PEG bridge was implanted into mice that underwent a T9-10 lateral spinal cord hemisection (Fig. 3A-B). The 5-tube composite had the same outer dimensions as the PEG bridges to control for cross-sectional area available to regrowing axons. A third cohort of mice did not receive any treatment, other than the gelfoam that was used to stabilize the surgical site in all conditions. The injury and contralateral tissue was isolated after 1 week to evaluate cell infiltration using flow cytometry. Interestingly, despite the presence of an implant, no differences were observed in the percentage of GFAP⁺ astrocyte or CD45⁺ leukocyte in the injury across the conditions (Fig. 3C-D). Evaluation of the distribution of CD45⁺ leukocyte subtypes identified significant differences across the conditions. An increase in CD11c⁺ dendritic cells was observed in both the 5-tube composites ($32 \pm 4\%$) and bridges ($27 \pm 3\%$) compared to the gelfoam control ($12 \pm 8\%$; Fig. 3E). Similarly, more F4/80⁺ macrophages were observed in the 5-tube composites ($52 \pm 7\%$) compared to the gelfoam control ($34 \pm 15\%$), however, no difference in the percentage of anti-inflammatory arginaseE macrophages were observed. By 2 weeks post-implantation, no significant difference in total, pro-inflammatory M1, or antiinflammatory M2 macrophage density was observed with histology across conditions (Fig. SI). No significant differences in the percentage of CD45⁺ cells expressing Lyg6 neutrophil or CD4 T-cell markers were observed at 1 week post-injury (Fig. 3E).

3.3 Glial scar formation is limited by PEG bridge/composite implantation

A glial scar was observed 2 weeks after spinal cord injury, and this scar can be a physical and biochemical barrier to axon regeneration. Implantation of a scaffold is thought to limit glial scar formation, which is primarily comprised of astrocytes, to the periphery of the hemisection injury. Elongating axons will need to traverse the thickness of the glial scar at the periphery to enter the growth supportive microenvironment within the biomaterial implant. Using longitudinal tissue sections, GFAP⁺ astrocyte thickness at the rostral margin was quantified to approximate the extent and thickness of the physical glial scar. 5-tube composites and bridges significantly reduced glial scar formation to $127 \pm 73 \mu\text{m}$ and $124 \pm 44 \mu\text{m}$, respectively, compared to the $337 \pm 169 \mu\text{m}$ glial scar thickness in the gelfoam control (Fig. 4).

3.4 Hydrogels with rostro-caudal aligned channels increase axon elongation

5-tube composites were next evaluated for their ability to support and guide axon elongation through the injury compared to the hydrogel bridge and gelfoam control. Axons were identified by neurofilament (NF-200) staining, and transverse tissue sections were separated into three 0.75 mm sections of the implant site: rostral (R), middle (M), and caudal (C) for tissue at 8 weeks (Fig. 5). NF-200⁺ axons were present in all sections of bridge in each condition (Fig. 5A-C, rostral sections shown). Axons grew into the injury from both the rostral and caudal regions of the bridge resulting in significant increases in axon densities within the 5-tube composites (Fig. 5D). No difference in axon density was observed between

conditions in the middle region of the injury at 8 weeks, which likely reflects this relatively early time point and the distances that axons must travel to reach the middle of the bridge. Mice that received the gelfoam control exhibited modest axon densities (456 ± 113 axons/mm²) relative to the PEG conditions across all regions of the injury site, likely due to the lack of guidance through the injury (Fig. 5D). A robust increase in axon density was observed in the 5-tube composites at the rostral (1744 ± 920 axons/mm²) and caudal (1436 ± 567 axons/mm²) ends when compared to the gelfoam control condition. The hydrogel bridge supported axon infiltration in the rostral (1048 ± 136 axons/mm²) and caudal (811 ± 227 axons/mm²) regions of the implant compared to the 5-tube composites, however, the bridges exhibited a significant increase in axon density in only the rostral end of the bridge compared to gelfoam controls. While there was not a significant difference between the tubes and bridges, there was a trending increase in axons within the tube composites compared to the bridges, and this may be due in part to the tubes guiding axon growth through the tube lumen as well as through the space between adjacent tubes, in contrast axons are only guided down channels within the bridges.

3.5 Hydrogels support axon myelination

The extent of myelinated axons was evaluated to further characterize the extent of tissue regeneration, as healthy axons are myelinated to ensure robust signal propagation. MBP⁺ myelin was used to discern total myelinated axons, while PO⁺ myelin was used to identify myelin from Schwann cells of the peripheral nervous system with PO⁺ myelin likely being derived from oligodendrocytes native to the central nervous system. Myelinated axons from both oligodendrocytes and Schwann cells were observed in each condition (Fig. 6A). No significant differences were observed in the myelinated axon density (Fig. 6B), however, the percentage of axons myelinated when normalized to axon density was significantly lower in the tube and bridge conditions (Fig. 6C). Of the myelinated axons, a higher percent of these axons were myelinated by oligodendrocytes in the 5-tube composite ($48 \pm 19\%$) and bridge ($49 \pm 18\%$) compared to the gelfoam control that had myelination by oligodendrocytes for $25 \pm 16\%$ of the axons (Fig. 6D).

3.6 Improved locomotion following implantation of PEG tubes and bridges

An open field locomotor BMS test was performed to assess coordination and hindlimb motor function. Average BMS scores were observed to increase across all conditions over time (Fig 7). A significant increase in average BMS score was observed 2 weeks after injury in mice with PEG bridges and 4 weeks in mice with PEG tubes compared to gelfoam. This significant increase in locomotor abilities continued through the duration of the study at 8 weeks with mice receiving PEG implants achieving an average BMS = 6 (frequent hindlimb stepping with some degree of coordination), while mice with gelfoam averaged BMS = 3 (occasional hindlimb placement).

4. Discussion

In this work, we developed a system of hydrogel tubes that meet a critical need for uniaxial guidance of regeneration while providing the flexibility to conform to abnormal injury geometries. The hydrogel tubes and bridges were fabricated using a method adapted from

Griffin et. al. [59], in which a two-step annealing process is used to control bulk hydrogel porosity for wound healing applications. Within our model, the multiple crosslinking phases allows for the microspheres to increase bridge/tube porosity facilitating tissue integration, while the second polymerization allows for geometric control of the final structure to support uniaxial regeneration. Other hydrogel systems that have been used to provide guidance cues run into problems with scale, modularity, porosity, or deliverability. The scale at which we were able to generate tubes is smaller (ID: 250 μ m; OD: 450 μ m) than previously reported hydrogel tubes that have been used for nerve or vascular injuries, where these materials were as large as the structures of interest (1-4 mm; [13, 28, 33, 53, 54]) and therefore not modular. Hydrogel-based implants have previously been pre-formed to include channels that guide regeneration, but these require prior knowledge of the defect size and shape [22, 30, 46, 48, 49]. In instances where defects were geometrically understood in advance, these preformed materials were successful in guiding tissue regeneration [22, 30]. However, they did not support infiltration of support cells to remodel the implant over time as more regenerating tissue grew through the injury. Similarly, a number of non-hydrogel based polymeric scaffolds comprised of small tubes or fibers have been used, but exhibit limited tissue integration or mismatched mechanical properties [25, 34, 35, 56]. The use of these pre-molded hydrogels, based on limitations with their scale, modularity, and porosity, highlight the importance of the 2-step polymerization process used in this work to generate a porous material that facilitates tissue integration and remodeling, as well as control the overall shape of the implant. Herein, tubes were developed that could be cut and stacked as needed to match the dimensions of an injury. Hydrogel composites, such as aligned microgels [51], hydrogel-nanotubes [43, 44], and hydrogel-microfibers [52], have also been developed to meet this critical need, however, these materials have not been tested for alignment within injury models and may require additional resources (e.g. electric fields) to facilitate the alignment of these structures within the hydrogel once implanted.

The hydrogel tubes offer an improvement over previous guidance systems due to their modularity, and in addition to the standardized way these tubes were implanted in this work, they have also been implanted individually. Tubes that are cut to length can be dehydrated for 60 seconds at room temperature and then implanted one-by-one to accommodate the defect size. The dehydrated tubes are more rigid and easy to handle during implantation, after which they rehydrate within the injury. Traditionally, hydrogels that are gelled in situ may swell in the aqueous in vivo environment after injection, which requires further design considerations to ensure that the swelling does not result in further injury due to compression of the material at the injury interface [55]. Additionally, many polymers utilize innocuous precursors that generate waste materials, thus the pool of candidate materials that can undergo in situ gelation becomes increasingly smaller. As the tubes are cut while hydrated and then dehydrated, stacked, and rehydrated in the injury, they will expand back to their initial size and fit the space rather than to lead to compression of the intact tissue. This modular approach allows the material to conform to any defect size or shape, simply by cutting and stacking the tubes in the defect as necessary. Future studies will evaluate this modular system for both acute and chronic treatment of contusive spinal cord injuries, which account for a larger percentage of spinal cord injuries, but can be more challenging to delineate between intact or spared nervous tissue and regenerating tissue. For the present

study, the tubes were aligned prior to implantation and bound together with fibrin, a naturally occurring factor that is present after injury. Fibrin is used to ensure the tubes are held together within the composite during implantation to ensure proper juxtaposition of the tubes during implantation with the interfacing tissue. Tubes were bound together with fibrin to form a single implant in an effort to control the overall size and shape between the 5-tube composites and bridges. The modular system of tubes was able to support and direct uniaxial regeneration following injury. The robust regeneration within the tubes was comparable to a prefabricated hydrogel bridge, both of which surpass the regenerative potential of a no treatment gelfoam control over a large (2.25 mm) spinal cord defect. No significant difference between cell infiltration, axon extension, or re-myelination was observed between the tubes and the hydrogel bridges, suggesting that the tube approach results in good apposition with the intact spinal cord. There was, however, a trend of increasing axon density within the 5-tube composites observed relative to the hydrogel bridge, which was likely due to the tubes having both the inner lumen and outer surface to guide axons compared to only the channel lumen providing guidance cues within the bridge. While these hydrogel tubes offer great regenerative promise within the spinal cord, their application could be extended to other tissue engineering problems such as repairing vascular, musculoskeletal, or peripheral nerve injuries.

Tailoring these tubes to other applications can be achieved through either biochemical or biophysical modifications. The tubes can be modified to change the biophysical properties (i.e., topography, modulus, porosity) during the initial fabrication process to match tissue properties or target specific cell responses. Within our spinal cord injury model, the improved regenerative capacity of the aligned hydrogels is due, in part, to the channels that provide guidance for axons and expedite the repair process compared to non-aligned hydrogels, which has been shown extensively with other aligned scaffolds [9, 23, 25, 28, 30, 37, 40, 42, 48, 49, 55, 56]. Increased oligodendrocyte-derived myelin was also observed with the tubes developed in this study, suggesting the presence of an implant or perhaps the composition (compared to primarily gelfoam within the control injury) may preferentially favor central nervous system derived myelinating cells over peripherally derived glia. Mice receiving the hydrogel tubes and bridges also recovered greater locomotor function compared to the controls, that corroborate the histological findings. Interestingly, the increased axon density at 8 weeks in the 5-tube composites (1744 axons/mm²) is an improvement over that observed in PLG bridges (740 axons/mm²) implanted into a mouse hemisection model [36, 37], which may be due to the more closely matched mechanical properties of the hydrogel to the nervous tissue (1-300 kPa [63–65]). Vascular tissue and muscle require high tensile strength, and there are a number of modifications to the material that can be made to properly match these tissues. These material modifications include replacing the 20 kDa 8-arm PEG backbone, changing the maleimide side chains to vinylsulfone, and modifying one or both crosslinking methods to increase the modulus. We showed that modifying even one of these components can lead to an order of magnitude difference in modulus. Additionally, the pore size could be change by modifying the microsphere size, which has a direct correlation to pore size [59]. Each modification will lead to differences in the overall mechanical properties and host response. Modifications to pore size, cell attachment peptides, and topography have been shown to modulate immune

cell infiltration and the resulting inflammation [66], whereas utilizing maleimide side chains can increase vessel formation [67]. For the tubes used in our mouse model of spinal cord injury, there are size restraints to ensure tubes were formed and had sufficient structural stability for handling purposes. This resulted in a smaller pore size and reduced porosity compared to the PLG bridges [37]. The PLG bridges are more rigid and the increased porosity helps to reduce the macroscale mechanical properties, which is less of a concern with the hydrogel tubes. Moving forward, various lithography and printing techniques can be used to make tubes with precise mechanics, porosity, and complex geometries. Furthermore, these tubes can be modified with biochemical factors, such as tissue specific growth factors, to chemically tailor the biomaterial for a tissue-specific application.

PEG tubes facilitate integration with the host tissue through microsphere-mediated porosity and did not have prolonged increases in inflammation. Previously, we and others have shown that controlling porosity supports infiltration of immune cells and support cells. Moreover, the size and density of the pores can help shift the immune response towards a more regenerative and less inflammatory phenotype [37, 45, 58]. Given the inflammatory response that occurs after spinal cord injury, we evaluated immune cell infiltration for the distinct material platforms. The spinal cord is immune privileged, but following injury it mounts a robust inflammatory response that leads to further damage and inflammation [68, 69]. Immune cell infiltration is necessary after spinal cord injury in order to clear debris, as studies in which immune cells have been depleted result in worse regenerative and functional outcomes [70, 71]. In this study, evaluation of the total immune cell infiltration one week after injury revealed that the PEG materials did not increase total immune cell invasion, but did increase the percentage of the total immune population was comprised of macrophages and dendritic cells within the tube composites. By week 2, the macrophage density was similar across all conditions, suggesting that the elevated immune response in the PEG tubes had subsided, which is similar to trends we have reported with PLG bridges [61]. This temporal response within the tubes aligns well with immune cell infiltration timelines reported within the literature [72], and resolution of the heightened immune responses over time indicates that the tubes are a viable implant option going forward.

The tubes reduced the glial scar that develops after spinal cord injury, which normally forms to limit the deleterious effects of inflammation, yet can lead to further damage and inflammation if allowed to persist [69]. Due to the high degree of inflammation following spinal cord injury and the presence of a glial scar, this model is challenging to deliver a material to limit the deleterious effects of glial scar formation without exacerbating the immune response. Using flow cytometry, no differences in astrocyte numbers were detected at one week, but at week two, clear morphological differences were observed with astrocytes in the PEG implants. Glial scar formation is more prominent starting at two weeks post injury, and is best characterized with histological analysis to account for astrocyte compaction along the injury margin compared solely to total cell number or GFAP+ area within the tissue, as both of these metrics can be biased by the sectioning plane and initial injury margins. Moreover, biomaterials limit glial scar formation to the periphery of the injury, forming a scar that axons must penetrate in an effort to enter the biomaterial that is devoid of astrocytes. In this study, PEG tubes and bridges were able to limit the formation of the glial scar as assessed based on thickness at the periphery, while the gelfoam control mice

presented with thick glial scars that are more likely to inhibit axon growth into the injury. These astrocyte data suggest that this temporally sensitive response is consistent with previously reported infiltration timelines [68, 72]. Moreover, PEG tubes did not significantly impact glial scarring compared to PEG bridges, suggesting that the tubes achieve comparable apposition to that of the bridge, as incomplete apposition would lead to increased inflammation and scarring which would limit regeneration. Cavitation was not observed at the tube-tissue interface. This process is less pronounced within the mouse hemisection injury compared to a rat injury model. For this reason, tissue-tube interface of the PEG tubes was primarily assessed relative to PEG bridges to achieve similar regenerative and behavioral thresholds, glial scar thickness, and immune infiltration. Together, the decrease in glial scarring and the resolution of the initial macrophage influx supports the robust axon growth through the 5-tube composites and bridges, with the tubes offering the added benefit of increased cross-sectional guidance area compared to the bridges.

5. Conclusion

This study reports a novel modular system of soft hydrogel tubes that can be assembled in situ to conform to an injury, while simultaneously providing uniaxial guidance of regenerating cells. Utilizing a two-step crosslinking process we were able to ensure that the tubes are porous to facilitate tissue integration and that the tubes contain a central channel that supports uniaxial tissue growth, a feature that is essential for regeneration in highly structured tissues. The tubes can be cut to the desired length and packed to fill any injury shape, offering the benefits of a hydrogel for wound repair yet also providing an aligned structure to direct regeneration. Tube implants integrated with the host tissue and increased regeneration following a spinal cord injury. This approach has other potential applications such as cardiovascular, musculoskeletal, and peripheral nerve repair. The hydrogel could be specifically tailored for numerous tissue engineering applications by tuning the material properties, pore size, and tube dimensions, as well as through the direct conjugation of relevant factors onto the material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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8. References

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Statement of Significance:

Tissue engineering approaches that mimic the native architecture of healthy tissue are needed following injury. Traditionally, pre-molded scaffolds have been implemented but require a priori knowledge of wound geometries. Conversely, hydrogels can conform to any injury, but do not guide bi-directional regeneration. In this work, we investigate the feasibility of a system of modular hydrogel tubes to promote bi-directional regeneration after spinal cord injury. This system allows for tubes to be cut to size during surgery and implanted one-by-one to fill any injury, while providing bidirectional guidance. Moreover, this system of tubes can be broadly applied to tissue engineering approaches that require a modular guidance system, such as repair to vascular or musculoskeletal tissues.

Figure 1. Two-step polymerization can be used to generate porous macrostructures. PEG-MAL microspheres are created with a water/oil emulsion method using a plasmin sensitive YKND peptide crosslinker. Scale bar 100 μm . (A). (B) Resulting microspheres had a diameter distribution ranging from 15-105 μm with an average size of 45 μm . (C) Tubes and bridges can be fabricated from the microspheres using UV-sensitive I2959 photoinitiator to crosslink remaining open MAL side arms. Tubes can be subsequently formed into a bridge composite using fibrin hydrogel to hold the tubes together during implantation.

Figure 2. Tertiary structures, such as tubes and bridges can be created using a second crosslinking phase that capitalizes on MAL sidechains that were not utilized during microsphere fabrication.

Resulting tubes (A – longitudinal, inset - transverse, scale bars 100 µm) and bridges (B, scale bar 200 µm) generated from the PEG-MAL microspheres are porous and contain aligned channels within the material. Using SEM, pores can be seen through the hydrogel tubes (C, 50 µm scale) and bridges (D, 50 µm scale), albeit the pore structure does appear to vary between the two structures. (E) No significant differences in the porosity, swelling ratio, or water retention capacity were detected between the two structures. (F) Young's

modulus for PEG-MAL crosslinked with YKND/PI using microspheres in a two-step polymerization technique (12.52 kPa), as used to generate tubes and bridges, was significantly lower ($p < 0.0001$) than the modulus for PEG-MAL crosslinked with YKND/PI without first forming microspheres (129.2 kPa) and PI only (1588 kPa).

Figure 3. Immune cell infiltration 7 days after injury.

Gelfoam alone (negative control) or with a biomaterial implant (PEG bridges or PEG tubes) was implanted into a lateral hemisection spinal cord injury (A) and apposition to intact tissue was visually verified (B). GFAP⁺ astrocytes (C) and CD45⁺ leukocytes (D) infiltrate the injury site, however, no difference in the bulk populations of these cells are evident across the blank (gelfoam) injury or PEG scaffolds. (E) Further evaluation of the leukocyte phenotypes including CD11c⁺ DCs, F4/80⁺ macrophages, F4/80⁺arginase⁺ M2 macrophages (teal overlay), Lyg6⁺ neutrophils, and CD4⁺ T-cells identified a significant increase in DCs and macrophages within the PEG materials. Data are represented as mean $\pm$ standard deviation. n = 5, * p < 0.05.

Figure 4. Glial scar thickness 2 weeks after injury.

GFAP⁺ astrocytes were observed throughout the intact tissue with robust staining at the interface of the gelfoam (A), bridge (B), and tube (C) implants with the intact tissue. The rostral margin thickness (bottom image outlined in red for panels A-C) was measured at multiple locations for each tissue. (D) PEG implants significantly reduced glial scar thickness ($p < 0.01$) compared to the gelfoam control. Data are represented as mean $\pm$ standard deviation. $n = 3$, ** $p < 0.01$. 500 μm , 100 μm (inset) scale bars.

Figure 5. PEG implants enhance axon elongation at 8 weeks after transplantation in T9-10 hemisection

Qualitatively, NF-200⁺ (red) axon expression is greater in PEG bridge (B) and tube (C) implants compared to gelfoam (A). (D) Quantification of regenerating axons was binned into three 0.75 mm lateral sections of the implant: rostral (R), middle of the injury (M), and caudal (C) as depicted in the schematic inset. Axon density is increased at both the rostral and caudal segments in mice receiving 5-tube composites, while the axon density for mice receiving the hydrogel bridges only increased at the rostral margin compared to gelfoam at 8

weeks post injury. Data are represented as mean $\pm$ standard deviation . n = 6, ** p < 0.01.
200 μ m scale bar.

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Figure 6. Axon myelination is supported in all conditions at 8 weeks post injury.

(A) NF-200⁺ axons co-localized with oligodendrocyte derived myelin (MBP⁺; green) and with Schwann cell myelin (MBP⁺P0⁺; blue) within transverse sections of the bridge across all conditions. Unmyelinated axons (denoted by *), oligodendrocyte myelinated axons (denoted by ^), and Schwann cell myelinated axons (denoted by ▲) are observed in each condition. Nodes between myelinated areas of axons can also be observed (denoted by arrows). No significant difference was observed in the density of myelinated axons (B), however, a significant decrease in percentage of myelinated axons was observed in the bridge and tube conditions compared to gelfoam (C). (D) While the overall percent of myelination was lower, both PEG tubes and bridges did result in a significant increase in the percentage of oligodendrocyte-derived myelin (NF-200⁺MBP⁺P0⁻; $p < 0.05$) compared to gelfoam. Data are represented as mean $\pm$ standard deviation. $n = 6$, * $p < 0.05$. 10 μ m scale bar.

Figure 7. Improved hindlimb locomotor abilities achieved with PEG implants.

An open field locomotor test known as the BMS was used to assess mouse hindlimb mobility for 8 weeks after injury and biomaterial implantation. By post-operative week 2, mice with PEG bridges achieved a significantly higher average BMS score compared to mice with gelfoam (* significance of bridges compared to gelfoam; $p < 0.05$, $n = 6$). Mice with PEG tubes exhibited a significant increase in BMS score by week 4 compared to gelfoam (^ significance of bridges compared to gelfoam; $p < 0.05$, $n = 6$).