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Channel density and porosity of degradable bridging scaffolds on axon growth after spinal injury

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ABSTRACT

Bridges implanted into the injured spinal cord function to stabilize the injury, while also supporting and directing axon growth. The architecture of the bridge is critical to its function, with pores to support cell infiltration that integrates the implant with the host and channels to direct axon elongation. Here, we developed a sucrose fiber template to create poly(lactide-co-glycolide) multiple channel bridges for implantation into a lateral hemisection that had a 3-fold increase in channel number relative to previous bridges and an overall porosity ranging from approximately 70%–90%. Following implantation into rat and mouse models, axons were observed within channels for all conditions. The axon density within the bridge increased nearly 7-fold relative to previous bridges with fewer channels. Furthermore, increasing the bridge porosity substantially increased the number of axons, which correlated with the extent of cell infiltration throughout the bridge. Analysis of these cell types identified an increased presence of mature oligodendrocytes within the bridge at higher porosities. These results demonstrate that channels and bridge porosity influence the re-growth of axons through the injury. These bridges provide a platform technology capable of being combined with the delivery of regenerative factors for the ultimate goal of achieving functional recovery.

1. Introduction

Spontaneous regeneration of the spinal cord is limited by numerous factors from the injury that are generally described as an insufficient supply of growth promoting stimuli and an abundance of growth inhibitors. Cells at the injury site are induced to secrete cytokines that recruit macrophages and immune cells as well as promote the migration of progenitors and myelinating cells. Macrophages function to clear debris that would otherwise inhibit the extension of regenerating axons; however, they also secrete cytokines that induce gliosis in astrocytes [1,2]. These activated astrocytes then influence the differentiation of recruited progenitor cells towards astrogenesis and gliosis rather than to mature oligodendrocytes that would re-myelinate spared axons to enhance their growth, survival, and conductance [3–6]. The accumulation of glial and fibrous scar tissue inhibits the extension of axons to and beyond the injury, as well as their myelination, which prevents the re-establishment of neural circuitry required for functional recovery [2]. Instead, the lack of functionality in regenerating neurons results in the demyelination and retraction of axons, a phenomenon known as Wallerian degeneration.

Biomaterial scaffolds implanted into the spinal cord are termed bridges, and they provide a central tool for modulating the local environment after spinal cord injury and facilitating nerve regeneration. Bridges with channels that span the length of the implant can provide and maintain a path for axon extension across an injury. *In vitro*, these aligned channels enhanced neural elongation compared to randomly-oriented interconnected pores [7]. Additionally, implantation of bridges with aligned channels orients cells

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within the channels that can direct axon extension [8] and enhances functional recovery after injury [9]. Previously, we reported on the development of porous, multiple channel poly(lactide-co-glycolide) (PLG) bridges. The combination of linear channels and interconnected pores in these bridges permitted the rapid ingrowth of cells *in vivo* to prevent the formation of cavities and stabilize the injury site. These infiltrating cells supported axon growth into and through the bridge, and a reduction in the presence of inflammatory cells was noted [8].

In this report, we investigated the role of bridge architecture, namely channels and porosity, in order to create a more permissive cellular environment for axon extension. Our gas foaming-based fabrication process was modified to include a sucrose fiber template that substantially increased the channel density allowing for the fabrication of bridges for both rat and mouse models and provided greater control over porosity in multiple channel PLG bridges. Using this fabrication method, we deconvoluted the influence of interconnected pores and channels on the cell types that occupy the bridge, as well as their impact on neurite extension into and through the bridge. Neurite density (neurofilaments per mm^2) and cellular residency (defined as percent area following staining with a cell-specific antibody) in the bridge were quantified using an image transformation method applied to sections stained with 3,3-diaminobenzedine (DAB) as the chromagen. This method accurately differentiates DAB^{pos} from DAB^{neg} and $hematoxylin^{pos}$ areas, which has posed a challenge historically [10]. These studies investigated key design features of the bridge, which can be further developed to serve as a platform for promoting regeneration after spinal cord injury.

2. Materials and methods

2.1. Multiple channel bridges

Bridges were fabricated using a combination of a gas foaming technique that was previous described [8,11] and a recently-developed sacrificial template technique [12]. PLG (75:25 ratio of D,L-lactide to L-glycolide, inherent viscosity: 0.76 dL/g; Lakeshore Biomaterials, Birmingham, AL) was dissolved in dichloromethane (6% w/w) and emulsified in 1% poly(vinyl alcohol) using a homogenizer (PolyTron 3100; Kinematica AG, Littau, Switzerland) at 3000 rpm to create PLG microspheres. D-sucrose was caramelized at approximately 220 °C, cooled to approximately 103 °C, and drawn from the solution using a Pasteur pipette to create sugar fibers. The fibers were coated with a mixture of PLG microspheres and salt particles (63–106 μm) and placed into an aluminum mold lined with salt to ease removal. The materials were pressed together by hand and placed into a pressure vessel. The constructs were equilibrated at 800 psi CO_2 for 12 h, with the pressure released at 60 psi/min. Bridges were then sectioned to the desired length, leached in distilled water for 2 h to remove the porogens, disinfected in ethanol for 2 min and dried overnight. All materials mentioned were from Fisher Scientific unless otherwise described. The final bridge dimensions were 3.8 mm in length, 2.5 mm in width, and 1.5 mm in height for the rat model, and 2.25 mm in length, 1.25 mm in width, and 0.75 mm in height for the mouse model. The channels were characterized by measuring the diameters of bridges ($n = 6$) acquired using light microscopy images (Leica Microsystems, Wetzlar, Germany) with ImageJ. The cross sectional area occupied by the channels and the porosities of these bridges were calculated using equations established previously [13,14]. Bridges were imaged using the Leo Gemini 1525 (Zentrum für Werkstoffanalytik Lauf, Pegnitz, Germany) at 10 kV after coating with osmium tetroxide.

2.2. Spinal cord injury

Animals were treated according the Animal Care and use Committee guidelines at Northwestern University. Surgery was performed as previously described ($n = 4$ per scaffold design and time-point for rats, $n = 3$ each for mice) [8,11]. Female Long-Evans rats (200 g, Charles River) and female C57Bl6 mice (20 g, Charles River) were anesthetized using isoflurane (2%). A laminectomy was performed at T9-T10 to allow for a 4 mm (rat) or a 2.25 mm (mouse) lateral hemisection for bridge implantation. The injury site was protected using Gelfoam and secured in place after suturing the muscles together and stapling the skin. Postoperative care included administration of Baytril (enrofloxacin 2.5 mg/kg, once a day for 2 weeks), buprenorphine (0.01 mg/kg for rats or 0.1 mg/kg for mice, twice a day for 3 days), and lactate ringer solution (5 mL/100 g, once a day for 5 days). Bladders were expressed twice daily until function recovered.

2.3. Immunohistochemstry

Bridges were implanted into the spinal cord for 2 or 8 weeks. Upon retrieval, bridges extracted from rats were frozen and sectioned transversally in 12 μm thick slices, and every third slice was collected serially. Bridges extracted from mice were frozen and sectioned transversally in 18 μm thick slices and collected serially. Sections from the middle of the bridge were fixed using 4% paraformaldehyde. To detect overall cell presence these sections were stained with eosin and counter-stained with Mayer's hematoxylin (Surgipath Medical Industries). To detect myelin, sections were stained with 0.1% solution of luxol fast blue and counter-stained with a 0.1% solution of cresyl violet. To detect type I and III collagen, sections were stained using a Masson's trichrome kit (Polysciences, Warrington, PA).

Bridge sections from rats were stained using the following primary antibodies to detect neurites (Neurofilament-200/NF200, 1:5000 dilution), macrophages (ED-1/CD68, 1:1000 dilution; Chemicon), astrocytes (Glial fibrillary acidic protein/GFAP, 1:5000 dilution), fibroblasts (anti-rat prolyl 4-hydroxylase/rPH, 1:500; Acros Antibodies), Schwann cells (S-100β, 1:100), endothelial cells (anti-rat endothelial cell antigen-1/RECA-1, 1:150; AbD Serotec), and oligodendrocytes (RIP, 1:2500; Chemicon). To detect these antibodies, the vectastain Elite ABC kit (Vector Laboratories) was used with diaminobenzedine (DAB; Vector Laboratories) and counter-stained with Mayer's hematoxylin. Bridges retrieved from mice were sectioned and stained using the following antibodies to detect neurites: Neurofilament 150/NF150 as the primary (MAB1621, 1:2000 dilution; Millipore) and anti-mouse IgG Alexa-Fluor 555 (1:1000, Invitrogen) as the secondary. All materials mentioned were from Sigma–Aldrich unless otherwise described. Images were captured at 40× or 20× for light or fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

2.4. Selection of DAB^{pos} areas

DAB-immunostained images ($n = 17$) were split into their color channels and were transformed into Normalized Red images using the equation *Normalized Red* = (R + G)/R, where R and G represent red and green channels respectively, and into Normalized Blue, Green/Blue, and Brown images using previously established equations [10]. DAB^{pos} areas of the transformed images were selected and compared to manual tracings. Three random fields of view from each image were traced by hand to quantify the misclassified pixels and area selections of transformed images, and their correlation to manual area analysis.

2.5. Cellular response

Immunostained samples at the middle of the implant site were imaged in 4 random fields of view. To quantify the neurite density in the bridges for a rat spinal cord injury model, a linear regression of DAB^{pos} area and neurofilament number from a training set ($n = 17$) correlated the selected area to the neurite density of regenerating neurons. For mouse, the neurite densities were assessed manually given their relatively small cross-sectional areas. The residency (percentage area that is positively stained) of S100β+, GFAP+, ED1+, and GFAP+ cells was quantified by selecting DAB^{pos} areas (black) thresholded manually. To quantify myelin content in the bridge, LFB-positive areas (blue) were selected, which become white in the transformed images.

2.6. Statistics

Multiple comparisons pairs were analyzed using a one-way ANOVA with a Bonferonni post-hoc test. Significance was defined at a level of $p < 0.05$ unless otherwise noted.

3. Results

3.1. Bridge structure

Bridges with a high density of channels were fabricated by adapting a previously established gas foaming/particulate leaching procedure to include caramelized sucrose fibers as a template for the channels [8,11,12]. Sucrose fibers were rolled in a mixture of PLG microspheres and salt crystals, and subsequently packed into a mold that is matched to the dimensions of a rat lateral hemisection. A total of 22 fibers could be packed within the mold, and the construct was subsequently foamed and immersed in water to remove the salt and sugar, and thereby, create a porous, multiple channel bridge (Fig. 1A). A similar procedure was employed to create bridges for a mouse lateral hemisection, which had 7 channels (Fig. 1B).

The gas foaming, sugar template-based fabrication process at this channel size and density yields bridges with a minimum