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Ice-Templated Scaffolds with Microridged Pores Direct DRG Neurite Growth

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Successful spinal cord repair is thought to be promoted with hierarchically structured scaffolds. These should combine aligned porosity with additional linear features on the micrometer scale to guide axons across multiple length scales. Such scaffolds are generated through the carefully controlled directional solidification of an aqueous biopolymer solution, followed by lyophilization. Under specific freezing conditions this yields a highly regular and aligned lamellar architecture. This architecture exhibits uniform ridges of controlled height and width on the lamellar surface. These ridges run parallel to the pore axis, serving as secondary guidance features. The ridges are capable of linearly aligning 62.4% of chick dorsal root ganglia neurites to within ±10° of the ridge direction. Notably, neurites sprouting perpendicular to the ridge are guided into alignment with these microridged features.

1. Introduction

A functional neuronal scaffold must simultaneously fulfill multiple structural, mechanical, and biochemical requirements. These range from controlled biodegradability to presentation of chemical cues. Freeze casting, the directional solidification of a polymer solution, offers a little explored one-step method to custom design key structural and mechanical properties of a three-dimensional (3D) construct with hierarchical architecture.[1,2] Freeze casting allows for the careful control of both the overall pore morphology of the scaffold and the microtopography of the scaffold walls, which are critical for axon guidance and the scaffold's mechanical performance. The importance of a match in mechanical properties between the scaffold and the native tissue is well established. However, the Young's modulus reported in the literature for the spinal cord varies greatly from 3 kPa to 3 MPa.[3] Values in the MPa range are for spinal cords with the meninges intact, while tests on isolated gray and white matter yield values three orders of magnitude lower. Since the axons traverse only the gray and white matter, preferred scaffolds should possess moduli in the low kPa region. The scaffold must also be highly porous and possess oriented pores capable of linearly guiding the axons. To date, microchannel axonal guidance has been achieved primarily on 2D substrates, using processes such as photolithography, electron-beam lithography, and liquid-liquid centrifugal spinning.[4–6] These channels have been shown to linearly orient axons, but because of the nature of their production, 3D constructs made from these have been limited to one level of hierarchy of aligned porosity. We were able to generate 3D cylinders consisting of highly aligned and ridged lamellae that traverse the length of the cylinder. The ridges are aligned parallel to the pores generated by the spaces between the lamellae (**Figure 1**). In vitro testing was performed on both 3D cubes of the scaffold and on isolated 2D lamellae that had been teased apart from the overall structure. The isolated lamellae were used to image and easily visualize the guidance of the ridges on the cultured neurons.

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DOI: 10.1002/adfm.201201323

2. Results and Discussion

2.1. Freeze-Cast Chitosan Scaffolds

Complex scaffolds, which combine several levels of hierarchy require at least two, frequently more processing steps that often are elaborate.[7] Freeze casting, in contrast, permits the generation of scaffolds with several levels of hierarchy in one straightforward processing step. Directionally solidifying a chitosan solution, we found that we could achieve the desired combination of a highly aligned porosity with lamellae that feature ridges of uniform height and width (Figure 1) on their surface, parallel to the long pore axis.[2] Lamellar spacings of 71 ± 22.1 μm were achieved when freeze-casting a 2.4% (w/v) chitosan in 1% (v/v) acetic acid solution with a freezing front velocity greater than 16.7 μm/s, and a local cooling rate higher than 3.5 K/min. Chitosan, the partially deacetylated form of chitin, was the biopolymer of choice in these experiments because of its relatively straightforward processing requirements and its previous successes in spinal cord repair.[8]

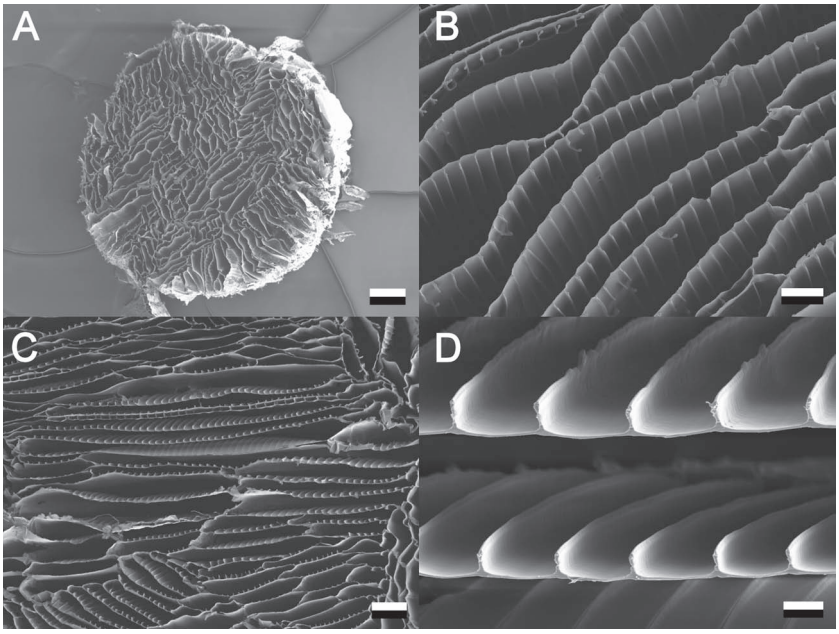

Figure 1. Cross-section SEM images of 3D lamellar scaffold with uniform ridge formation. Scale bars: A) 200 µm, B) 40 µm, C) 200 µm, and D) 10 µm.

The highly regular ridges on the cell walls are templated, we hypothesize, by the ice-dendrites, which form during freezing when the polymer solution phase separates into pure water ice crystals and an increasingly viscous supersaturated polymer solution that ultimately vitrifies. We observed that by varying the rate at which the solution is cooled, we could carefully control the thermal diffusion, the local cooling rate within the solution, and the freezing front velocity of the ice crystals. Through these parameters, it was possible to vary the size and the morphology of the ice crystals and hence to determine the final structure of the templated polymer scaffold. This is in accordance with the empirical structure-processing correlation of $\lambda \propto \frac{1}{v^n}$, where λ is the lamellae spacing, v is the freezing front velocity, and typical values for the exponent n range from 0.5 to 2.[9]

Through mechanical testing in compression at a strain rate of 0.01/s in a phosphate buffer solution to simulate in vivo conditions, the modulus of the scaffolds was determined to be 10 ± 0.18 kPa, a value close to the 3–5 kPa reported for isolated gray and white matter.[10] Correlating mechanical properties with processing conditions we found, assuming a buckling-dominated deformation, that the scaffold's plateau or crushing strength scales with the scaffold's modulus as $\sigma^*_{pl} = 0.1(E^*)^{0.91}$, with the modulus, E^*, ranging from 2 to 12 kPa for freezing rates from 1–10 °C/min. We interpret these results as a confirmation of the highly aligned, honeycomb-like pore structure in the freeze-cast chitosan scaffolds.[11]

2.2. In Vitro Testing with Embryonic Chick Dorsal Root Ganglia

Embryonic chick dorsal root ganglia (DRGs) were used for in vitro testing of the scaffolds' ability to align the growing neurites aided by the ridges. The confocal microscopy image of **Figure 2** shows the excellent neurite alignment during growth within the 3D scaffold section. Additionally, for easier visualization and quantification of neurite growth and alignment, isolated DRGs were seeded on 2D single lamellae isolated from the scaffolds (**Figure 3**). These single lamellae were physically teased apart from the overall 3D structure and placed ridge-side up on a coverslip. The DRG explants were then cultured directly on top of the lamellae, and were placed at one end of the isolated lamellae, allowing some of the neurites to propagate the length of the sample, and others to extend off the edge of the ridged sample and grow onto the flat surface that acted as a second level of control. Both the lamellae and plain glass coverslips, used as a negative control, were coated with laminin to promote DRG attachment and neurite outgrowth.

Figure 3 illustrates the strength of the structural cue of the ridges. They linearly oriented the neurites extending from the DRGs, and neurites that initially sprouted perpendicular to the ridges were guided into alignment. Neurites that extended from the ridged lamellae surface onto the flat coverslip did not maintain their linearly aligned growth, but instead grew in a disoriented manner. Neurite alignment was quantified by measuring the angle of orientation between the neurites and

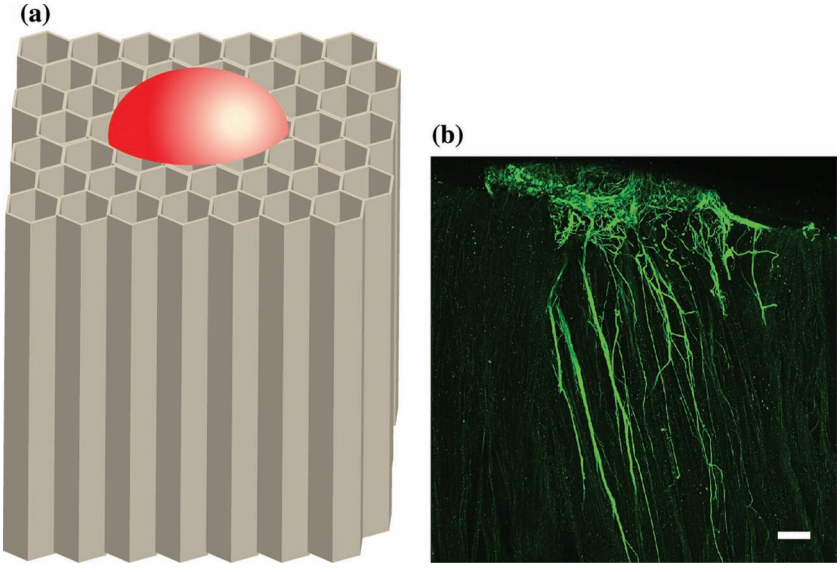

Figure 2. Schematic (left) and confocal microscopy image (right) of an immunostained DRG on a laminin-coated chitosan scaffold. Scale bar: 200 µm.

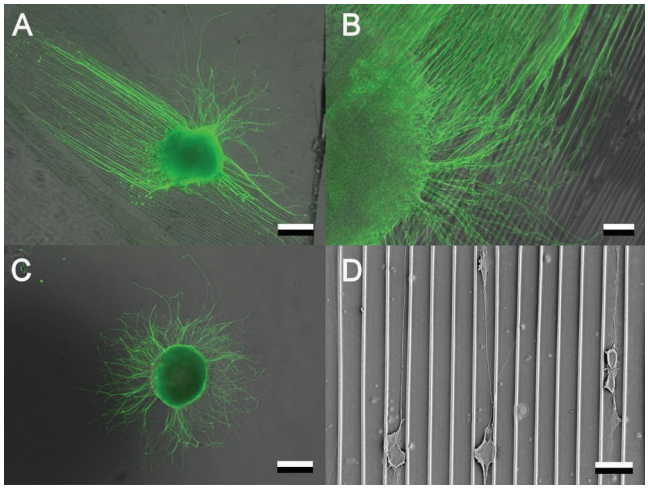

Figure 3. A,B) Fluorescent microscopy of a DRG seeded on an isolated lamella. C) Fluorescent microscopy of a DRG seeded as a control on a flat substrate. D) SEM image of dissociated DRG neurons growing within the grooves of an isolated lamella. Scale bars: A) 300 μm, B) 100 μm, C) 300 μm, and D) 30 μm.

the ridges (defined as 0°) and separating the angles into bins of 10° increments. **Figure 4** shows that 62.4% of DRG neurites growing on laminin-coated chitosan had orientation angles within ±10° relative to the ridge direction. A Gaussian distribution was fitted to the data and resulted in a mean angle of 0.99°± 8.48°. These data provide quantitative confirmation that the neurites are highly aligned with the lamellae ridges. Conversely, DRG explants seeded on control laminin-coated coverslips extended neurites in all directions, lacking any alignment (Figure 3C). No Gaussian distribution could be fit to the control data, as there is no significant difference between the bins of angles of neurite orientation (**Figure 5**).

Neurites grew predominantly within the grooves between the ridges; however, they were not confined by the groove walls. This suggests that the neurites were not physically constrained by the ridges. In the absence of chemical or electrical gradients,

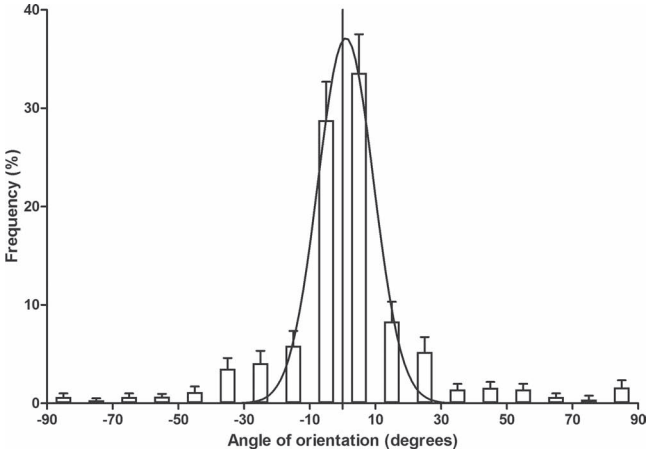

Figure 4. Histogram of neurite orientation on laminin-coated chitosan scaffold. 0° represents ridge direction.

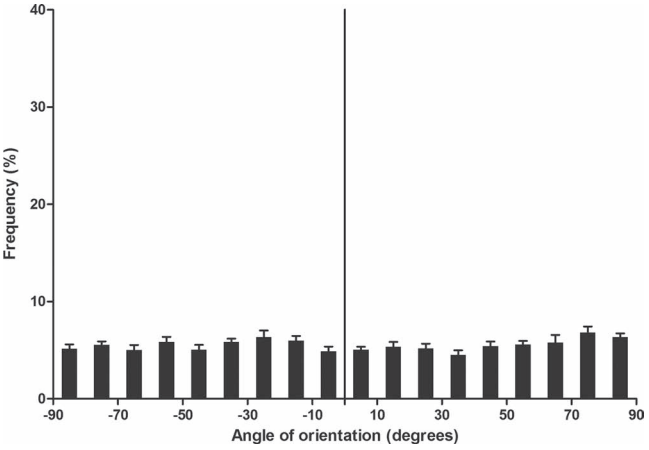

Figure 5. Histogram of neurite orientation on laminin-coated coverslip.

contact guidance from the microridged chitosan scaffold provided directional cues to the growing DRG neurites, bringing them into parallel alignment with the ridge direction. Similar contact guidance of neurites has been observed on other grooved substrates.[12–17]

3. Conclusions

Although other 3D biopolymer scaffolds have been produced for use as nerve guidance conduits,[18–21] those presented here are the first that combine an aligned porosity and ridged pore walls capable of topographical contact guidance. While freeze casting has been used to produce 3D collagen[22] matrices with longitudinal pores for neural guidance, these matrices lack a ridged microstructure and rely on the formation of columnarly aligned Schwann cells to guide the growth of DRG neurites. The present data suggest that our 3D, porous, aligned, and microgrooved chitosan scaffolds are promising candidates for the promotion of linearly oriented nerve regeneration in the central nervous system (CNS) or peripheral nervous system (PNS).

4. Experimental Section

Sample Preparation: Scaffolds were prepared as described previously.[1,2] Briefly, a 2.4% (w/v) solution of chitosan was created by dissolving 2.4 g of low molecular weight chitosan powder in 100 mL of 1% (v/v) glacial acetic acid in deionized water. This solution was then mixed on a Wheaton bench top roller machine at approximately 10 rpm for a minimum of 24 h to ensure complete dissolution of the chitosan. The solution was then freeze cast in a 50 mm tall cylindrical mold of 18 mm inner diameter, sealed at its bottom with a tightly fitting copper plate to provide the best possible thermal transfer from the cold finger on which the cylinder was placed for freezing; the top of the mold was left open to ambient conditions. The empty cylinder was secured on the cold finger with electrical tape. Once secured, the solution was pipetted into the mold and the copper cold finger cooled with liquid nitrogen. A thermocouple placed directly under the cold finger surface measured the temperature at the cold finger-mold interface. A band heater wrapped around the top portion of the copper cold finger counteracted the

thermal diffusion from the liquid nitrogen. A PID controller was used to create a defined cooling rate at the top of the cold finger. Once the freezing process was completed, the samples were removed from the molds, wrapped in tissue paper, and placed in a freeze dry system. The inner coil of the lyophilizer was held at –50 °C and the samples themselves at 0.18 mBar for a minimum of 48 h to ensure complete sublimation of the ice.

Mechanical Testing: Mechanical testing was performed in compression in the axial direction, thus the load was applied parallel to both pores and lamellae walls, with a cross-head speed of 0.05 mm/s, corresponding to a strain rate of 0.01. Samples were tested in PBS; however, prior to testing the positive charge on the chitosan scaffold was neutralized by a 15 min soak in a 0.4% glacial NaOH in 95% ethanol solution, followed by rinsing in ethanol for 30 s.

In Vitro Testing: Dorsal root ganglia were isolated from embryonic day 10 chick embryos and collected in sterile Hank's buffered saline solution. All 3D scaffold sections and isolated lamellae were submerged in 25 µg/mL laminin at 37 °C overnight and washed three times with PBS prior to seeding the surface with DRGs. DRG explants were seeded onto a cross-sectional scaffold surface in order to evaluate neurite growth within the bulk scaffold. To better visualize neurite growth and alignment, DRG explants were placed directly on top of the lamellar surface and incubated in growth medium (Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic, and 100 ng/mL nerve growth factor (NGF)) at 37 °C and 5% CO_2. DRG explants were also cultured on laminin-coated glass coverslips as a control condition. Explants were incubated for 48 h in vitro prior to immunocytochemical analysis. After removal of the medium, DRGs were fixed in 4% paraformaldehyde for 30 min at room temperature and washed three times in PBS. The fixed cells were blocked in 0.1% Triton X-100 with 10% normal goat serum in PBS for 1 hour at room temperature, followed by an overnight incubation at 4 °C in blocking solution containing mouse monoclonal antibody anti-neurofilament 200 kDa. The cells were washed three times in PBS and the secondary antibody Alexa Fluor 488 goat anti-mouse IgG was applied for 1 h at room temperature. DRGs seeded on 3D scaffold sections were visualized and photographed using an Olympus IX81 inverted confocal microscope. A z-stack over approximately 20 sections of 10 µm thickness was performed, and single layers were merged into one image using FluoView software version 1.7. DRGs on isolated lamellae were visualized and photographed using an Olympus IX71 fluorescence microscope and SPOT RT3 microscope camera. The angle of neurite orientation relative to the ridges on the chitosan lamellae was measured from the point of origin at the DRG explant to the tip of the neurite, with the direction of the ridges represented as 0°. Measured neurite angles were separated into bins consisting of 10° increments between –90° and +90°.

Acknowledgements

The authors thank Philipp M. Hunger and Amalie E. Donius for experimental assistance and Dr. A. Lowman for kindly granting access to his facilities, and gratefully acknowledge the support by the Centralized Research Facilities in the College of Engineering at Drexel University. U.G.K.W. wishes to express her gratitude to Anne L. Stevens for the generous support of her research and group while at Drexel University.

Received: May 15, 2012
Published online: July 27, 2012

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