Fiber templating of poly(2-hydroxyethyl methacrylate) for neural tissue engineering

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Abstract

We have developed a method to create longitudinally oriented channels within poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels for neural tissue engineering applications. Incorporated into an entubulation strategy, these scaffolds have the potential to enhance nerve regeneration after transection injuries of either the spinal cord or the peripheral nerve by increasing the available surface area and providing guidance to extending axons and invading cells. The fabrication process is straightforward and the resultant scaffolds are highly reproducible. Polycaprolactone (PCL) fibers were extruded and embedded in transparent, crosslinked pHEMA gels. Sonication of the pHEMA/PCL composite in acetone resulted in the complete dissolution of the PCL, leaving longitudinally oriented, fiber-free channels in the pHEMA gel. Regulating the size and quantity of the PCL fibers allowed us to control the diameter and number of channels. Small and large channel scaffolds were fabricated and thoroughly characterized. The small channel scaffolds had 142$\pm$7 channels, with approximately 75% of the channels in the 100–200$\mu$m size range. The large channel scaffolds had 37$\pm$1 channels, with approximately 77% of the channels in the 300–400$\mu$m range. The equilibrium water content (EWC), porosity and compressive modulus were measured for each of the structures. Small and large channel scaffolds had, respectively, EWCs of 55.0$\pm$1.2% and 56.2$\pm$2.9%, porosities of 35$\pm$1% and 40$\pm$1% and compressive moduli of 191$\pm$7 and 182$\pm$4 kPa.

Keywords: pHEMA; Polycaprolactone; Fibers; Spinal cord injury; Regeneration; Nerve guidance channel; Scaffold; Orientation; Hydrogel

1. Introduction

Nerve injury is debilitating in both the peripheral nervous system (PNS), where regeneration is limited to small gaps [1], and the central nervous system (CNS), where regeneration is essentially non-existent due to both physical (i.e. glial scar) and chemical (i.e. myelin proteins) inhibitors [2,3]. The standard treatment for peripheral nerve injury across gaps greater than a few millimeters involves the use of autografts, nerve tissue taken from a donor site and grafted into the injured site [4]. However, this strategy is frequently associated with donor site morbidity and incomplete functional recovery. To improve on these results, several laboratories are investigating the use of tubes, or nerve guides, to bridge the gap between transected nerves in both the CNS and PNS. Of the numerous entubulation studies reported to date, some of the best results have been achieved in the PNS over short distances with nerve guides consisting of poly(glycolic acid) [5], poly(caprolactone-co-lactic acid) [6] and collagen [7].

We have been actively pursuing synthetic alternatives to replace the autograft for use in transection injury treatment strategies in either the PNS or CNS. To this end, we have developed a new methodology to produce hollow fiber membranes [8,9] and have begun to test these devices in vivo with some success [10–12]. In order to further enhance recovery, we hypothesize that regeneration will be augmented by the inclusion of a scaffold that increases surface area and provides both haptotactic [13] and chemotactic cues [14,15]. The focus of this paper is on the creation of longitudinally oriented
porous scaffolds that will fill the interior of a nerve guidance channel. We expect that this scaffold will provide greater surface area and directionality to regenerating nerve fibers.

To achieve our goals for an oriented scaffold, we wanted to design a structure that would support the systems’ natural pattern of growth [16] which, in the spinal cord, for example, consists of the natively oriented ascending sensory pathways and descending motor pathways. Longitudinally oriented scaffolds would meet the biological requirements of physically supporting and guiding regenerating fibers within the tube and ultimately aid in functional recovery. Others have followed a similar strategy for enhanced regeneration in the past, using, for example, filaments of: aligned collagen [17–19], carbon [20], poly(l-lactide) [21] or polyamide [22]. In each of these strategies, longitudinal orientation was achieved with fibers. While nerve regeneration over long peripheral nerve gaps improved with these fiber-filled devices versus empty tubes [23,24], possibly due to increased surface area, the arrangement of the filaments within the guidance channels was irregular and difficult to reproduce. We propose a different approach to the fiber-filled tube—a scaffold with microtubular architecture, where regenerating axons extend through open longitudinal channels, as they would normally extend through endoneurial tubes of a peripheral nerve. This latter strategy promises to be more effective and reproducible.

Of the wide diversity of materials available, we chose to work with synthetic hydrogel scaffolds because they have been used in several biomedical applications due to their versatile nature [25–27]. Hydrogels are soft and flexible, exhibiting physical characteristics similar to those of soft tissue. Poly(2-hydroxyethyl methacrylate) (pHEMA) is particularly attractive for biomedical engineering applications because its physical properties of fiber diameter on guidance in vitro [33], we chose to create channels having the majority of pores between 100 and 200 μm (small channels). To demonstrate the versatility of this technique, we also made scaffolds with channels having the majority of pores between 300 and 400 μm (large channels). Ultimately, cell-adhesive peptides and/or growth-promoting neurotrophins could be incorporated into this scaffolding structure to further enhance regeneration [14,34].

2. Materials and methods

2.1. Materials

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received. Water was distilled and deionized using a Millipore Milli-RO 10 Plus filtration system at 18 MΩ resistance.

2.2. Polymerization

HEMA was polymerized at room temperature in a glass tubular mold, with an inner diameter of 4.0 mm, which was capped at both ends with rubber septa. HEMA was polymerized by a redox-initiator in the presence of a crosslinking agent in an aqueous solution. The formulation consisted of: 60 wt% HEMA, 0.5 wt% ammonium persulfate (APS), 0.4 wt% tetramethylethylenediamine (TEMED), 0.1 wt% ethylene dimethacrylate (EDMA) crosslinker, and 40 wt% aqueous solution, of which 90 wt% was water and 10 wt% was ethylene glycol (EG). The APS, TEMED and EDMA concentrations are expressed as weight percentages of the total monomer concentration. A 10 wt% aqueous solution of APS initiator was prepared prior to every use. This formulation resulted in transparent pHEMA gels, facilitating visual analysis of the channels within the resultant structures.

2.3. Fiber extrusion

A high-pressure piston extruder with a fixed orifice of 0.3 mm (SpinLine, DACA Instruments) was used to form fibers from PCL pellets having a weight average molar mass of 80,000 g/mol. The pellets were melted in the heated 10 ml barrel at 67°C for 2 h prior to extrusion to ensure a consistent molten solution and thereby minimize air bubbles in the molten polymer. The force exerted by the piston and the speed of the winder controlled the diameter of the fibers produced. Two different sizes of fibers were extruded in order to create scaffolds with different channel diameters. To fabricate small diameter fibers, the piston advanced at a rate of 1.0 mm/min and the winder speed was set at 2.5 m/min.
For the larger diameter fibers, the piston speed was 1.6 mm/min and the winder speed was 0.8 m/min.

2.4. Scaffold fabrication

Two different types of scaffolds were created—small channel and large channel—based on the size of the extruded PCL fibers. After extrusion, the fibers were removed from the winder, grouped into bundles of like fiber diameter and then fused by melting the ends of the strands together with a Bunsen burner. The PCL bundles were inserted into 4.0 mm ID glass tubing, threaded through with the fused end first. The ends of the bundles were trimmed and the tubes sealed with rubber septa. For each sample, the relevant quantities of HEMA, water and EG were placed in an amber vial and the monomer mixture was degassed under vacuum. The appropriate quantities of initiator and accelerating agent were added to the vial and the mixture was agitated gently for 30 s. The mixture was then injected into the fiber-filled polymerization mold, displacing all of the air within the vessel. Polymerization proceeded for a minimum of 8 h, after which the pHEMA/PCL composites were removed from the glass tubing and immersed in distilled water until the next stage of processing.

The polymerized samples were cut into 1 cm long sections and placed in scintillation vials filled with acetone. The vials were sonicated for 75 min, resulting in the complete dissolution of the PCL fibers in the solvent. The samples were then removed from the vial and rinsed 3 times with fresh acetone to remove any residual PCL. The etched scaffolds were Soxhlet extracted in water for 12 h to remove residual acetone, followed by immersion in distilled water for a minimum of 24 h.

2.5. Structural characterization

Optical microscopy and scanning electron microscopy (SEM) were used to characterize the scaffolds for channel count, channel diameter and scaffold porosity. To create contrast for optical microscopy, the scaffolds were stained with 0.4% Giemsa methanol stain and then immediately washed in water. For SEM imaging, the samples were cut to the required mounting sizes, quenched in liquid nitrogen and then freeze-dried. Dried scaffolds were then attached with carbon paint to microscopy sample studs and sputter-coated with gold for 60 s. The samples were then placed on the SEM stage (Model S-570, Hitachi) for imaging. Operating conditions included a working distance of 15 mm and an accelerating voltage of 20 kV to minimize damage to the polymer structure.

2.5.1. Channel count

The number of channels present in each type of scaffold was counted from the SEM micrographs. A total of six different samples of each type, small channel and large channel, were analyzed to calculate an average and standard deviation in channel number.

2.5.2. Channel diameter

The diameters of the channels were measured from SEM micrographs using Scion Image Analysis 4.0.2 software. A total of 5 different samples of each type of scaffold were analyzed. In the small channel samples, approximately 130 channels per specimen were measured to obtain a size distribution. In the large channel samples, approximately 30 channels were measured. Averages and standard deviations are reported.

2.5.3. Scaffold porosity

The porosity of each type of scaffold was calculated using stereomicroscopic images of Giemsa-stained cross-sections, to clearly identify the channels within each structure by differentiating between the background and the gel phase. Scion Image Analysis 4.0.2 software was utilized to measure the surface area of the entire scaffold. The staining of the scaffold facilitated the use of the thresholding function in this software package to determine the surface area of only the gel portion of the scaffold, excluding the area occupied by the channels. The porosity was calculated according to the following equation:

\[
\text{Porosity} = \frac{A_{\text{scaffold}} - A_{\text{gel}}}{A_{\text{scaffold}}} \times 100\% ,
\]

where \(A_{\text{scaffold}}\) is the surface area of the entire scaffold, including channels, and \(A_{\text{gel}}\) is the surface area of the gel only. Three samples of each type of scaffold were analyzed at 3 different points along their length. By calculating the porosity at various points within the same sample, it was possible to assess scaffold uniformity and channel continuity within the structures. Hence, a total of 9 different porosity measurements were obtained for each type of scaffold. The averages and standard deviations are reported.

2.6. Equilibrium water content

For equilibrium water content (EWC) measurements of the scaffolds following acetone fiber etching, five samples of each scaffold type were placed in deionized water, exchanged daily, for 2 weeks. The residual surface water was then removed and each sample was weighed to measure the hydrated scaffold mass. The samples were then dehydrated at 50°C over a 2-week period. Each scaffold was then re-weighed to ascertain the dry mass. The EWC was calculated according to the following equation:

\[
\text{EWC} = \frac{(w_h - w_d)}{w_h} \times 100\%
\]
where \( w_h \) is the hydrated and \( w_d \) the dry mass of the scaffolds.

### 2.7. Compressive modulus

The elastic (Young’s) modulus of each type of scaffold was assessed using a micro-mechanical tester (Dynatek Delta). All tests were conducted in triplicate, with 20% compression of 2.5 mm length sections over a 180 s time interval. This ensured results within the linear range of behavior for the material. The load was applied parallel to the longitudinal architecture of the channels. The samples were placed in an aqueous chamber during the testing procedure to maintain hydration of the scaffold. The cross-sectional area of the entire scaffold was measured using a stereomicroscope. Additionally, the modulus of the gel-phase portion of each scaffold was calculated. For this procedure, the cross-sectional area was determined through structural analysis of stereomicroscope images using Scion Image Analysis 4.0.2. The measured area included only the gel phase of each scaffold, with the area occupied by the empty channels excluded from the calculation. Based on previous studies, the expected value for the modulus of transparent pHEMA gels was approximately 290 kPa [35].

### 3. Results and discussion

The primary goal of this investigation was to design and characterize a scaffold with oriented, longitudinal channels that would provide increased surface area within a tubular structure for use in neural tissue engineering applications. Such a scaffold could support and guide extending axons subsequent to nerve injury in a cell-adhesive scaffold. We chose to create an oriented scaffold using pHEMA gels because we had shown that pHEMA tubes were conducive to cell-penetration in vivo [10,12]. Furthermore, by working with high HEMA concentrations, we could create transparent gels that facilitated characterization.

To develop a safe, effective and reproducible method for scaffold formation, we used a chemical dissolution technique for the creation of fiber-free longitudinally oriented channels in transparent pHEMA. By varying the diameter of the extruded fibers, scaffolds of differing channel diameters were formed. PCL, unlike poly(lactic acid) and poly(lactic-co-glycolic acid), was particularly appropriate to use in this method because PCL was insoluble in HEMA yet soluble in acetone. The acetone, used to dissolve the PCL fibers, did not significantly swell the pHEMA gel, and the crosslinked polymer maintained good structural integrity during the acetone washing process. As summarized in Table 1, fiber diameter influenced channel count the most, varying from 37 for large diameter fibers to 142 for small diameter fibers.

The optical and electron micrographs revealed that the scaffolds were consistent along the longitudinal axis, with the channels distributed throughout the gel phase portion of the scaffolds. Fig. 1 shows representative images of the two types of scaffolds produced. There was no evidence to suggest merging of individual channels, indicating that there was good wetting of the PCL fibers by the monomer mixture during the polymerization/fabrication process. Further, the gel provided sufficient support to prevent structural collapse following fiber dissolution. Visual analysis confirmed longitudinally oriented, fiber-free channels running throughout the transparent pHEMA gel.

#### 3.1. Channel count

The number of channels within each scaffold (small and large) was consistent and reproducible between batches. The ends of the thermoplastic PCL fibers were melted together to form bundles, which could be easily introduced into the glass mold prior to the injection of the HEMA monomer formulation. For extruded fibers of similar diameter, modulating the packing density of the fiber bundles within the glass molds controlled channel count. Thus, by filling the molds to capacity with the fibers, there was little variation in the number of channels per sample. In the small diameter channel samples, an average of 142 ± 7 channels were present per sample. Due to the increase in fiber diameter in the large channel samples, the packing density of fibers was significantly lower and consequently easier to control. In the large diameter channel samples, an average of 37 ± 1 channels were present per sample.

The ability to control both the size and density of the channels may be important to the success of the scaffold. In neural applications, for example, it may be

<table>
<thead>
<tr>
<th>Channels</th>
<th>Channel count ((n = 6))</th>
<th>Equilibrium water content ((%, n = 5))</th>
<th>Porosity ((%, n = 3))</th>
<th>Compressive modulus ((kPa, n = 3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>142 ± 7</td>
<td>55.0 ± 1.2</td>
<td>35 ± 1</td>
<td>191 ± 7</td>
</tr>
<tr>
<td>Large</td>
<td>37 ± 1</td>
<td>56.2 ± 2.9</td>
<td>40 ± 1</td>
<td>182 ± 4</td>
</tr>
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</table>
beneficial to have many channels of different dimensions, thereby increasing the surface area available for regeneration, while at the same time minimizing the amount of polymer used and maximizing the space available to regenerating axons. While we have not yet investigated the optimal channel diameter, we and others have observed neurite guidance on fibers with diameters of \( \sim 150 \mu m \) and less.

3.2. Channel diameter

In both the small and large channel scaffolds, a distribution in the diameter of the individual channels was observed due to variations in the diameters of the extruded PCL fibers. During the extrusion process, diameter fluctuations occurred as the fibers produced were extremely fine and the liquefied PCL was occasionally prone to breaking or stretching. A more controlled extrusion system would reduce the PCL fiber diameter variations, thereby narrowing the channel diameter distribution in the resultant scaffolds. The diameter of the small channels ranged from 50 to 300 \( \mu m \), as shown in Fig. 2, with \( \sim 75\% \) of the channels in the 100–200 \( \mu m \) diameter range. The large channel scaffolds were investigated to demonstrate the flexibility of the fiber dissolution system to produce oriented scaffolds with varying dimensions. In the large channel samples, the channel diameters ranged from 250 to 450 \( \mu m \), as shown in Fig. 2, with \( \sim 77\% \) of the channels in the 300–400 \( \mu m \) diameter range. Based on size alone, cellular invasion in both small and large diameter channels should be facile.

Based on average fiber counts and diameters, we estimated the increase of available surface area of a scaffold-filled vs. an empty hollow fiber membrane of similar dimensions. The small channel scaffolds, with an average channel diameter of approximately 152 \( \mu m \) and an average of 142 channels, augmented the surface area by an estimated 540\% whereas the large channel scaffolds, with an average diameter of approximately 332 \( \mu m \) and an average of 32 channels, resulted in an estimated area increase of 310\%. By mixing fiber sizes, a
higher density of channels and thus a greater increase in available surface area should be possible. Augmenting the surface area could significantly improve regeneration by promoting cell attachment and directional growth.

3.3. Scaffold porosity

Scaffold porosity was calculated, according to Eq. (1), by comparing the ratio of the area of the entire scaffold to that of the gel phase. In the small channel samples, the porosity was calculated to be 35 \( \pm \) 1\%, while that of the large channel samples was 40 \( \pm \) 1\%. Hence, although there were substantially fewer channels in the large samples, the increase in channel size resulted in the creation of a more porous scaffold due to a reduction in the amount of gel between the channels. The porosity calculation was also utilized to assess channel continuity and scaffold uniformity within individual samples. We calculated porosity at 3 different points along the length of each sample and found that it varied within 1% for the small channel scaffolds and 3% for the large channel scaffolds, thereby confirming that the channels were continuous and the structures were homogeneous.

3.4. Equilibrium water content

The EWC was calculated according to Eq. (2) and was 55.0 \( \pm \) 1.2\% for the small channel scaffolds and 56.2 \( \pm \) 2.9\% for the large channel scaffolds. These values are substantially higher than the 39.6 \( \pm \) 0.3\% EWC measured for transparent, non-perforated pHEMA gels of the same formulation. The elevation in EWC is likely due to the presence of water inside the scaffold channels, which could not be easily removed prior to weighing the wet mass.

3.5. Compressive modulus

The compressive modulus was 191 \( \pm \) 7 kPa for the small channel scaffolds and 182 \( \pm \) 4 kPa for the large channel scaffolds. Additionally, the modulus of the gel phase portion of each type of scaffold was calculated and compared to the expected value of approximately 290 kPa for transparent pHEMA gels [35]. By considering only the gel phase in our calculation of area, the gel phase moduli were determined to be 291 \( \pm \) 11 and 301 \( \pm \) 6 kPa for the small and large channel scaffolds, respectively, thereby confirming the accuracy of our experimental methods and the data reported.

The scaffold structures were strong, yet compliant and flexible, and had moduli comparable to that of the spinal cord, which is between 240 and 260 kPa for the feline model [37,38]. Matching the mechanical properties of the device to the site of implantation is important for success in in vivo applications. As with many tissue-engineered constructs, the scaffold must be strong enough to resist structural collapse upon implantation, but must not be so rigid as to damage the surrounding tissues, which may lead to inflammation and subsequent device failure [26,39].
4. Conclusions

Oriented pHEMA scaffolds were successfully fabricated using a fiber templating technique involving the solubilization of embedded PCL fibers dispersed in pHEMA gels. The process was safe, effective and reproducible, resulting in the production of scaffolds with highly controlled dimensions. Each scaffold had longitudinally oriented, fiber free channels that were continuous along the entire length of the structure. The size and arrangement of the PCL fibers in the transparent pHEMA controlled the diameter and patterning of the resultant channels. The channels may act as a bridge, providing support and contact guidance for extending axons and invading cells. These scaffolds may be useful for guided regeneration and we are investigating them for inclusion in a multi-component tissue engineered device to promote regeneration after transection injury in either the peripheral nerve or spinal cord.

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References


