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# Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering

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**Abstract**

A hydrogel scaffold of well-defined geometry was created and modified with laminin-derived peptides in an aqueous solution, thereby maintaining the geometry of the scaffold while introducing bioactive peptides that enhance cell adhesion and neurite outgrowth. By combining a fiber templating technique to create longitudinal channels with peptide modification, we were able to synthesize a scaffold that guided cell adhesion and neurite outgrowth of primary neurons. Scaffolds were designed to have numerous longitudinally oriented channels with an average channel diameter of $196 \pm 6\,\mu m$ to ultimately promote fasciculation of regenerating cables and a compressive modulus of $192 \pm 8\,kPa$ to match the modulus of the soft nerve tissue. Copolymerization of 2-hydroxylethyl methacrylate (HEMA) with 2-aminoethyl methacrylate (AEMA) scaffolds, provided primary amine groups to which two sulfhydryl terminated, laminin-derived oligopeptides, CDPGYIGSR and CQAASIKVAV, were covalently bound using the sulfo-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) crosslinking agent. The concentration of peptides on the scaffolds was measured at $106 \pm 4\,\mu mol/cm^2$ using the ninhydrin method and $92 \pm 9\,\mu mol/cm^2$ using the BCA protein assay. The peptide modified P(HEMA-*co*-AEMA) scaffolds were easily fabricated in aqueous conditions, highly reproducible, well-defined, and enhanced neural cell adhesion and guided neurite outgrowth of primary chick dorsal root ganglia neurons relative to non-peptide-modified controls. The copolymerization of AEMA with HEMA can be extended to other radically polymerized monomers and is advantageous as it facilitates scaffold modification in aqueous solutions thereby obviating the use of organic solvents which can be cytotoxic and often disrupt scaffold geometry. The combination of well-defined chemical and physical stimuli described herein provides a means for guided regeneration both in vitro and in vivo.


*Keywords:* Scaffold; PolyHEMA; Surface modification; Peptide; Cell adhesion; Nerve guidance; Tissue engineering

## 1. Introduction

Spinal cord injury is a devastating disorder of the central nervous system (CNS) and often results in the loss of function below the site of injury. The lack of spontaneous regeneration of CNS axons after injury likely results from both chemical (myelin inhibitors) and physical (e.g. glial scar) barriers to regeneration [1,2]. There have been several methods investigated to overcome this hostile environment for regeneration, including delivery of Rho kinase inhibitors [3] or neutralizing molecules to Nogo [4]. An alternative strategy involves the creation of a permissive pathway to regeneration, such as peripheral nerves grafted into the spinal cord [5] or synthetic grafts lined with Schwann cells [6]. In an attempt to mimic the regenerative capacity of the peripheral nerve graft, we have been investigating chemotactic [7] and haptotatic cues [8,9] for regeneration within polymeric tubes [10] that provide the physical pathway for axonal guidance in vivo [11]. To promote guided regeneration, a scaffold with longitudinally oriented channels within poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogel was synthesized where the channels were designed to enhance cell adhesion and outgrowth while at the same time increase

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the surface area available for regeneration [12]. We chose to work with poly(2-hydroxylethyl methacrylate) (PHEMA) because it is inherently non-adhesive to cells yet it can be modified with peptides to elicit specific cellular responses [13,14]. While the strategy of this fiber templating technique is promising, the methodology was limited by the non-cell-adhesive PHEMA. To overcome this limitation while at the same time taking advantage of the guided regeneration strategy of fiber templating, we developed a new co-polymer scaffold, based on PHEMA to guide cell adhesion and outgrowth. PHEMA-based scaffolds are compelling to study because they are cell-invasive [15], soft materials and PHEMA hydrogels have been used extensively in medical applications [16], particularly for artificial cornea and contact lenses [17–19]. Moreover, PHEMA has been previously modified with peptides to elicit specific cellular responses [13,14], thereby indicating the potential of peptide modification to influence neural cells and tissues. In nerve regeneration research, cell adhesion and neurite outgrowth have been enhanced by incorporating extracellular matrix proteins or peptides into biomaterials by several laboratories [20,21] including our own [8,9,22]. Laminin, an extracellular matrix protein in the basal lamina, has been shown to promote cell adhesion, migration, differentiation and gene expression by reacting with the integrin receptors on cell membranes [23]. Specifically, the YIGSR sequence (on the $\beta$1 chain) promotes neural cell adhesion [24] while the IKVAV sequence (on the A chain) promotes neurite outgrowth [25]. Surfaces modified with the extended amino acid sequences, CDPGYIGSR and CQAASIKVAV, show an improved cellular response, relative to shorter sequences of YIGSR and IKVAV, likely because they better mimic the conformations found in laminin and can thus interact more effectively with the integrin receptors on the cell surface [26]. We took advantage of this knowledge and incorporated both extended peptide sequences into our templated hydrogel scaffold, thereby extending 2D surface modification to 3D structures.

We wanted to limit our scaffold modification to aqueous solutions to obviate the use of organic solvents which would likely also disrupt the longitudinal channels that comprise the scaffold morphology; however, this objective was difficult to attain with the hydroxyl groups of PHEMA. To achieve peptide modification in aqueous solutions, we introduced an amine functional group to PHEMA by copolymerization of 2-aminoethyl methacrylate (AEMA) with HEMA. While cationic groups of primary amines present in HEMA-based copolymer hydrogels have been shown to support cell growth on two-dimensional surfaces [27], we investigated peptide-modified materials to stimulate more specific integrin interactions. As will be described herein, these peptide modified, P(HEMA-*co*-AEMA) scaffolds, carrying a combination of physical (channels) and chemical (peptides) stimuli, were shown to promote better neural cell adhesion and guide neurite outgrowth of embryonic chick dorsal root ganglia (DRG) neurons in vitro, demonstrating the importance of using this methodology for in vitro cell culture and indicating its potential to increase cell-material interaction for in vivo neural tissue engineering applications.

## 2. Materials and methods

### 2.1. Materials

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received unless otherwise specified. Distilled and deionized water was obtained using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) system at 18 MΩ resistance. All reactions were conducted at room temperature unless otherwise indicated.

### 2.2. Polymerization

HEMA was copolymerized with AEMA (Pierce, Rockford, IL) in an aqueous solution using a redox initiator-accelerator system for 24 h. A 10 wt% aqueous solution of ammonium persulfate (APS) was used as the initiator and was prepared prior to every use. Tetramethylethylene diamine (TEMED) was used as the accelerator. 0.5 wt% of APS and 0.4 wt% of TEMED, expressed as weight percentages of the total monomer concentration, were added to the monomer solutions. Ethylene dimethacrylate (EDMA) was used as the crosslinker at a concentration of 0.1 wt% relative to that of the total monomer. HEMA and AEMA monomers were co-polymerized in a glass tubular mold with a total monomer concentration of 60 wt% and a solvent concentration of 40 wt%. Of the 60wt% total monomer concentration, the following AEMA percentages were investigated: 1%, 2%, 3% and 6%. The solvent consisted of 90 wt% water and 10 wt% ethylene glycol (EG). Successful copolymerization was determined by a ninhydrin test where ninhydrin reacted with primary amines from AEMA to yield a Ruhemann's purple complex [28]. Copolymer gel samples were sprayed with a 0.2% w/v ninhydrin in ethanol solution and heated at 130°C for 5 min. Scaffolds were stained with 0.4% Giemsa methanol stain to create contrast and visualized using optical microscopy. The intensity of purple colour reflected the concentration of AEMA in P(HEMA-*co*-AEMA) samples.

### 2.3. Scaffold fabrication

P(HEMA-*co*-AEMA) scaffolds were fabricated using a fiber templating technique previously described for

PHEMA [12]. Briefly, a transparent P(HEMA-*co*-AMEA) gel was formed around polycaprolactone (PCL) fibers, which were then removed by dissolution in acetone (with sonication) for 75 min. This resulted in longitudinally oriented, fiber-free channels in the P(HEMA-*co*-AEMA) gel. Residual monomer or acetone was removed by Soxhlet extraction in water for 24 h, followed by immersion in water for an additional minimum of 24 h.

### 2.4. SEM analysis

Scaffolds were analyzed using scanning electron microscopy (SEM) for channel diameter distribution and porosity. To prepare SEM samples, scaffolds were freeze-dried for 24 h, mounted on carbon-painted stubs and gold-coated with a sputter-coater in a Polaron (Watford, Hertfordshire, UK) vapor deposition unit, at 15 mA for 60 s. SEM (Model S-2500, Hitachi) was operated at a working distance of 15 mm and an accelerating voltage of 20 kV. Three samples of each type of scaffold were analyzed by SEM at three different positions along their length for surface area of the entire scaffold ($A_{\text{scaffold}}$) and surface area of the gel ($A_{\text{gel}}$) in order to calculate scaffold porosity according to Eq. (1):

$$\text{Porosity} = \frac{A_{\text{scaffold}} - A_{\text{gel}}}{A_{\text{scaffold}}} \times 100\% \quad (1)$$

The number of channels present in the scaffold was counted and measured from SEM micrographs. A total of ten different samples were analyzed to calculate an average and standard deviation in channel number. The diameter of the channels was measured from SEM micrographs using SPOT 3.1 software and the average and standard deviation are reported ($n = 10$).

### 2.5. Equilibrium water content

The equilibrium water content (EWC) of scaffolds was calculated according to Eq. (2):

$$\text{EWC} = \frac{(W_{\text{h}} - W_{\text{d}})}{W_{\text{h}}} \times 100\%. \quad (2)$$

The hydrated mass ($W_{\text{h}}$) was measured after immersing the scaffolds in water for 2 weeks ($n = 6$) with residual surface water removed by blotting. The dehydrated mass ($W_{\text{d}}$) was measured after storing samples at 50°C for 14 days.

### 2.6. Mechanical properties

Six, 2.5 mm long scaffolds were tested in an aqueous chamber, to ensure the complete hydration, with a 20% compression of the sample over a 180 s time interval using an Instron mechanical tester (model 8501, Canton, MA). The load was applied parallel to the longitudinal axis of the channels inside the scaffolds. The elastic modulus was calculated from the linear portion of the stress–strain curve, according to Eq. (3):

$$E = \left(\frac{\sigma}{\varepsilon}\right) = \frac{(\Delta mgL)}{(A\Delta L)} \quad (3)$$

where $\sigma$ is the stress applied, $\varepsilon$ is the corresponding strain, $\Delta m$ is the change in load applied, $g$ is the force of gravity, $L$ is the length of the scaffold sample, $A$ is the cross-sectional area of the scaffold, and $\Delta L$ is the change in length during compression.

### 2.7. Peptide modification

Oligopeptides were custom-synthesized using a solid-state peptide synthesizer (Pioneer, BioApplied Systems, Foster City, CA) on the 0.1 mmol scale and then reacted with P(HEMA-*co*-AEMA) hydrogel samples using sulfo-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Pierce, Rockford, IL) coupling [29]. Specifically, P(HEMA-*co*-AEMA) samples were activated by immersion in a 1 mg/ml sulfo-SMCC solution in phosphate buffered saline (PBS) for 2 h, under gentle agitation, and then washed 3 times with PBS. These samples were then immersed in a 1.5 mg/ml peptide solution, which consisted of a 1:1 w/w ratio of two laminin-derived oligopeptides, CDPGYIGSR and CQAASIKVAV, in PBS for 12 h under gentle agitation. Peptide-modified samples were then washed 3 times with PBS to remove any unreacted reagents.

To quantify peptide concentration on modified scaffold surfaces, disc-shaped samples with a diameter of 4.25 mm and a thickness of approximately 2 mm were prepared. Eight samples were used in each peptide modification experiment and the exact dimension of each sample was measured. Peptide concentration on the scaffolds was estimated using the BCA protein assay kit (Pierce, Rockford, IL) [30] and ninhydrin method [31,32]. The BCA protein assay is an indirect method of measuring adsorbed protein which was calculated by difference between the concentration of peptide originally added and the concentration of un-reacted peptide in the PBS buffer and brief methods for BCA. The ninhydrin method is a direct measure of peptide concentration on scaffolds. Standard curves for both of these colorimetric methods were prepared using a series of concentrations of CDPGYIGSR and CQAASIKVAV peptide solutions (1:1; w/w) reacted with each of BCA or ninhydrin.

### 2.8. Cell culture experiments

Distilled deionized water was sterile-filtered (0.22 μm filter) prior to use. All hydrogel samples were disinfected in sterile-filtered 70% ethanol for 5 min, rinsed in water for 5 min and then immersed in the Eagle's minimum

essential medium (Milwaukee, WI) for 2 h prior to plating primary chick dorsal root ganglia cells (DRGs). Chick embryos were removed from fertilized eggs at E9-11 days at 37.8°C and dissected to isolate the DRG cells as previously described [33]. Briefly explanted DRGs were incubated with trypsin solution (0.0375% in HANKS buffer) for 20 min and then collected by centrifuge (1000 rpm, 5 min), immersed in cell culture medium, and dissociated by gentle trituration. Dissociated cells were then added to a 15% BSA solution and centrifuged at 750 rpm for 5 min to further separate neurons from glia. Purified neurons collected from the bottom of the centrifuge tube were then diluted to the desired concentrations with cell culture medium [α-MEM medium containing 10% horse serum, 1% glutamine, 1% penicillin/streptomycin and 50 ng/ml NGF (all supplied by Gibco, NY)]. The disinfected P(HEMA-*co*-AEMA) and peptide modified samples were placed in 96-well plates (one sample per well) and the cell solutions were plated on these substrates inside the well at $5 \times 10^4$ cells/ml. The cells were then cultured in a standard cell culture incubator (37°C, 5% $CO_2$ and 100% humidity) for 2 d. The positive control was prepared by adsorbing 50 μg/ml laminin and 0.01% poly(L-lysine) from an aqueous solution to the well surface for 2 h. The wells were then rinsed with water and allowed to air-dry prior to cell plating.

In the competitive cell adhesion assay experiment, cells were pre-incubated, prior to cell plating, for 30 min with serum free media (SFM) containing 0.5 mg/ml of a soluble peptide solution containing of a 1:1 w/w ratio of CDPGYIGSR and CQAASIKVAV.

Cells were fixed and stained with phalloidin anti-F-actin in Hank's buffer following a previously described procedure [7]. Cells were visualized with a Zeiss Axiovert 100 inverted microscope (equipped with optical and fluorescence detection light). Cell images were captured using a digital camera and analysed using SPOT software from Diagnostic Instruments (Eagan, MN).

Statistical data were evaluated with a *t* test (95% confidence interval) and the *P* values are reported. If the equal variance test failed, a post hoc analysis with a Mann–Whitney rank sum test was performed.

## 3. Results and discussion

### 3.1. Polymerization

The P(HEMA-*co*-AEMA) gels synthesized herein swelled with increased AEMA concentration, which resulted in weaker gels. The EWC of copolymers having 1%, 2%, 3%, and 6% AEMA within the 60% monomer formulation were calculated at 46±0.3%, 53±1.2%, 62±1.9%, and 86±0.7% (mean±standard deviation), respectively. P(HEMA-*co*-AEMA) gels having 1% AEMA present in the monomer mixture (referred to as P(HEMA-*co*-AEMA-1%)) were chosen for further modification because they were the strongest of the gels tested, had an EWC most similar to that of PHEMA (of 39.6±0.3%) [12], and had a sufficiently high concentration of amine groups for peptide modification to be efficacious for cell adhesion and growth.

### 3.2. P(HEMA-co-AEMA-1%) scaffold characterization

Transparent P(HEMA-*co*-AEMA-1%) scaffolds (stained with 0.4% Giemsa methanol for visualization) are shown in Fig. 1a. The number and diameter of channels in the scaffolds were controlled by those of the fibers. We prepared scaffolds with 132±5 (Fig. 1b) and 82±3 channels (mean±standard deviation, $n=9$). The fiber diameter ranged between 50 and 350 μm and approximately 85% of these were between 100 and 250 μm. The average fiber diameter was calculated at 196±6 μm. Compared to tubular structures, scaffolds

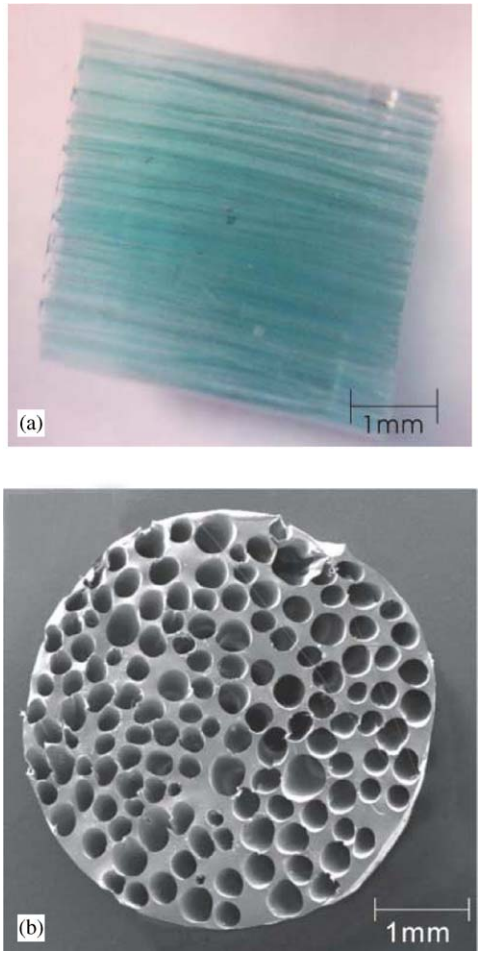


Fig. 1. A transparent P(HEMA-*co*-AEMA-1%) scaffold: (a) stained with 0.4 % Giemsa methanol and viewed by a longitudinal cross-section by light microscopy and (b) a horizontal cross-section of a scaffold of the same scaffold viewed by SEM had a mean and standard deviation of 132±5 channels.

with 82–132 channels, provide approximately 6–9 fold greater surface area, respectively, which may be advantageous for regeneration studies that depend on contact mediated cues.

The porosity was calculated at three different points along the length of each sample and found to be approximately the same, indicating channel continuity and scaffold uniformity. The porosity was controlled by the number of channels, where scaffolds having 132 channels were $47 \pm 5\%$ porous and those having 82 channels were $33 \pm 3\%$ porous (mean $\pm$ standard deviation, $n = 9$).

The EWC of the P(HEMA-co-AEMA-1%) scaffold was calculated at $61 \pm 4\%$, which is greater than that for PHEMA at $56 \pm 2\%$ [12], likely due to the greater hydrophilicity of amine functional groups in the P(HEMA-*co*-AEMA) scaffolds. The EWC of the scaffolds was also higher than the non-perforated gels of the same formulation ($46 \pm 0.3\%$) likely due to water trapped in the channels.

The mechanical properties of the scaffolds were also studied. For in vivo applications, scaffolds need to be strong enough to resist structural collapse upon implantation yet sufficiently compliant so as not to damage the surrounding tissue, which can cause necrosis and inflammation [34,35]. The compressive modulus of the P(HEMA-*co*-AEMA) scaffolds was calculated to be $192 \pm 8$ kPa, which is similar to that of PHEMA scaffolds of $191 \pm 7$ kPa [12]. This elastic modulus is on the same order of magnitude as that of the feline spinal cord, which is between 200-600 kPa [36].

### 3.3. Peptide modification

Oligopeptides were coupled to the hydrogel by the sulfo-SMCC coupling agent. The NHS end of sulfo-SMCC reacted with the primary amine groups on the scaffolds, forming a stable amide bond. The maleimide end on sulfo-SMCC reacted with the sulfhydryl groups, and was used to couple thiol-terminated oligopeptides, CDPGYIGSR and CQAASIKVAV, to the scaffolds resulting in a thiolester bond. This method ensured that the bioactive motifs, YIGSR and IKVAV, were oriented away from the surface and free to interact with the integrin receptors on the cell membranes. The spacer groups between the surface and the bioactive ligands, CDPG for YIGSR and CQAAS for IKVAV, are important for integrin receptor recognition, likely because the conformation of the peptides more closely resembles those in laminin [22].

The concentration of peptides coupled to the scaffolds was calculated as $92 \pm 9\,\mu mol/cm^2$ using the BCA protein assay and $106 \pm 4\,\mu mol/cm^2$ using the modified ninhydrin method. We assumed that the majority of the peptide coupling occurred on the scaffold surface; however, some sulfo-SMCC may have diffused into the polymer matrix and reacted with free amine groups within the bulk polymer. Thus the calculated peptide concentration on the scaffold surface may be exaggerated. It is important to note that the BCA protein assay is an indirect method of measuring the amount of coupled peptide, which was calculated by difference between the concentration of peptide originally added and the concentration of un-reacted peptide left in the PBS buffer after the reaction [30]. The ninhydrin method is a direct measure of peptide concentration on the scaffolds where the number of amine groups that react with the ninhydrin molecules is measured. There is the possibility that ninhydrin may have diffused into the bulk polymer, which may account, in part, for the slightly greater peptide concentration calculated by ninhydrin vs. BCA assays.

### 3.4. Cell culture

Cell adhesion and axon guidance were tested using primary DRG neurons. As shown in Fig. 2,

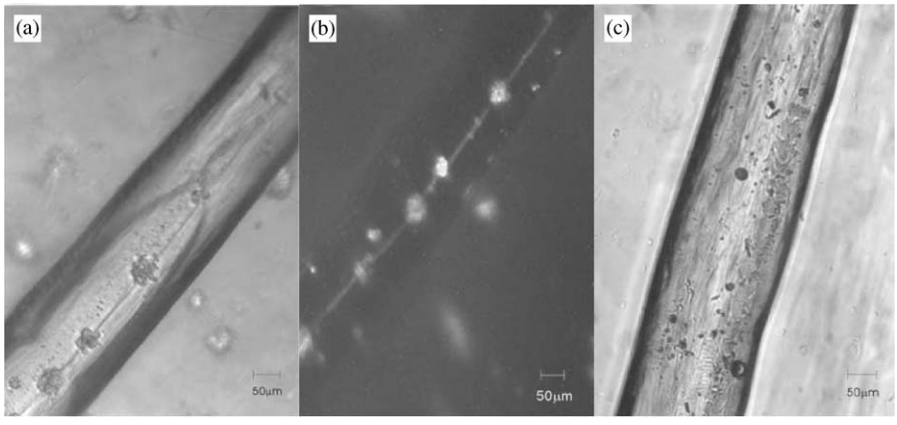


Fig. 2. Comparison of cell response on different scaffolds: (a) optical microscopy image shows that DRG cells adhere to peptide modified surfaces. (b) Fluorescent microscopy image shows that DRG cells extended neurites within the channel of peptide modified scaffolds. (c) Optical microscopy image shows that few cells adhere and no cells extend neurites in P(HEMA-*co*-AEMA-1%) control scaffolds. Images were taken one day after plating cells with a concentration of $1 \times 10^5$ cells/cm$^2$.

peptide-modified channels promoted cell-adhesion (2a) and neurite outgrowth (2b) whereas P(HEMA-*co*-AEMA) controls supported only limited adhesion of large cell clusters and limited neurite ourgrowth (2c). Peptide-modification coupled with physical channels provided pathways for guided neurite extension. As shown in Fig. 3a, there was significantly ($P \leqslant 0.001$) better cell adhesion to peptide-modified surfaces than to unmodified P(HEMA-*co*-AEMA-1%) scaffolds and there was no statistical difference between positive PLL/laminin controls and peptide-modified scaffolds ($P \leqslant 0.083$). On the P(HEMA-*co*-AEMA-1%) control surfaces, the majority of cells were washed away during the staining process, reflecting poor cell adhesion. Of the adherent cells on P(HEMA-*co*-AEMA-1%), most comprised large cell clusters, suggesting that cell–cell interaction was greater than cell–surface interaction.

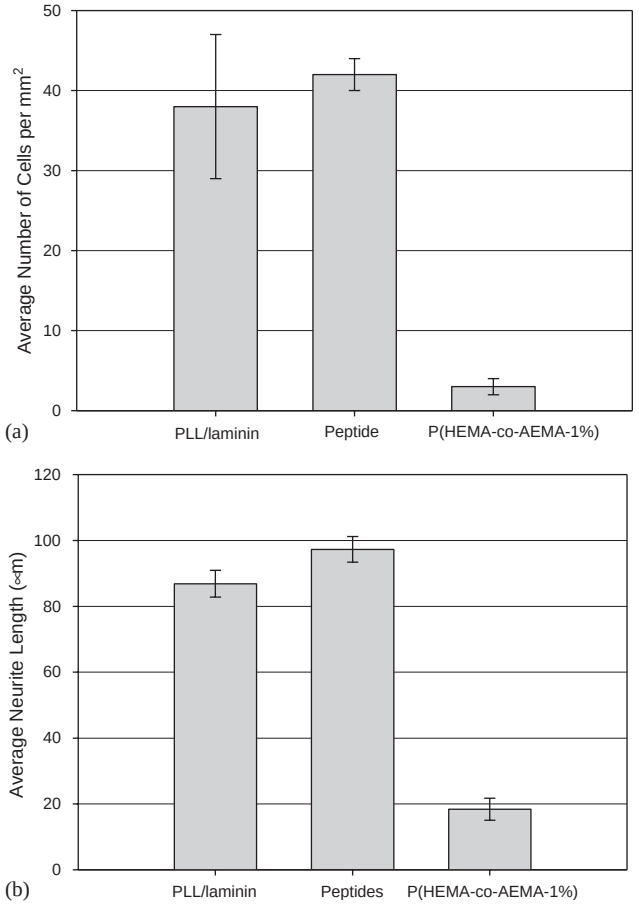


Fig. 3. Peptide-modified P(HEMA-*co*-AEMA-1%) scaffolds were compared to positive controls of laminin-modified surfaces and controls of unmodified P(HEMA-*co*-AEMA) for (a) cell adhesion and (b) neurite outgrowth. There was no statistical difference between positive control and peptide-modified P(HEMA-*co*-AEMA-1%) data for cell adhesion and neurite outgrowth. Cell adhesion and neurite outgrowth data are averages from 55 randomly picked fields per surface ($\pm$SD) on day 2 after plating cells with a concentration of $4 \times 10^4$ cells/cm$^2$.

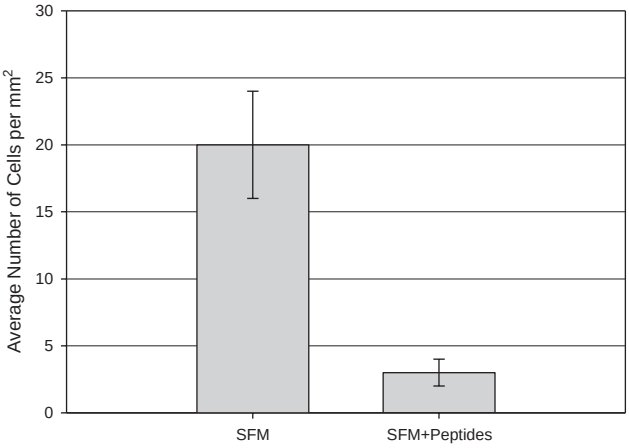


Fig. 4. The competitive cell adhesion assay demonstrated a significant reduction in the number of cells per field when the cells were pre-incubated with peptides (SFM + peptides) prior to plating relative to plating cells in medium (SFM) alone. A total 55 randomly picked fields (1 field = 1 cells/mm$^2$) per sample were analyzed ($\pm$SD) on day 2 after plating cells with an initial concentration of $4 \times 10^4$ cells/cm$^2$.

As shown in Fig. 3b, peptide modified surfaces had significantly longer neurites than P(HEMA-*co*-AEMA-1%) controls ($P \leqslant 0.001$). There was no statistical difference found between the peptide modified surface and the PLL/laminin-positive control surface ($P = 0.083$) in terms of neurite length.

To test the specificity of the neuron–peptide interaction, a competitive adhesion assay was done where cells were pre-incubated with cell adhesion peptides prior to plating. As shown in Fig. 4, cell adhesion on peptide-modified surfaces decreased significantly when cells were pre-incubated in medium containing soluble peptides ($P \leqslant 0.001$). This suggests that the cell adhesion and neurite outgrowth observed on peptide-modified channels is predominantly receptor driven, which is consistent with previous studies of other surfaces [37].

## 4. Conclusions

A new scaffold, that combines haptotactic and topological guidance cues, was synthesized by a combination of fiber templating and peptide modification, and shown to have a well defined geometry for cell invasion and adhesion. The copolymerization of AEMA facilitated peptide modification in aqueous solutions, which can be extended to other biomaterials, and obviates the use of organic solvents, thereby preserving scaffold geometry during peptide modification. The haptotactic, peptide cues stimulated specific integrin interactions, promoting cell adhesion and neurite out-

growth, while the topological, channel cues provided pathways for axonal guidance. This new copolymer scaffold holds promise for both in vitro (as shown here) and in vivo tissue engineering.

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