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# Anisotropic three-dimensional peptide channels guide neurite outgrowth within a biodegradable hydrogel matrix

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**Abstract**

The objective of this study was to investigate the neurite guidance potential of concentration gradients of glycine–arginine–glycine–aspartic acid-serine (GRGDS) oligopeptides immobilized within three-dimensional patterned cylindrical volumes created in a biodegradable nerve guidance matrix. This was achieved using ultraviolet (UV) laser micropatterning of a hyaluronan (HA) hydrogel matrix modified with S-2-nitrobenzyl cysteine. Upon exposure to focused laser light, the 2-nitrobenzyl group was cleaved, exposing thiol groups which reacted with maleimide-terminated GRGDS exclusively within these laser-defined volumes. We show that the UV laser micropatterning technique can be used to create GRGDS peptide concentration gradients within the oligopeptide channels and that these channels guide neurite outgrowth from primary neural cells.

(Some figures in this article are in colour only in the electronic version)

## Introduction

Scaffolds are one of the pillars of tissue engineering providing a three-dimensional temporary supportive environment for cell growth and survival [1, 2]. In order to control cell behaviour, both scaffolds and substrates have been patterned with cell-adhesive cues in order to guide cell growth [3, 4]. Of the numerous synthetic and naturally-derived materials that have been investigated as scaffolds, polysaccharides are particularly interesting because they closely mimic the native cellular environment [5, 6]. Hyaluronan (HA) is a polysaccharide of specific relevance because it degrades into nontoxic products, and is both biocompatible and non-immunogenic [7, 8]. It has been used for a variety of tissue engineering applications, including as a scaffold for neural tissue engineering [9–11].

Scaffolds can be modified with peptides to elicit a specific cellular response. For example, neurite guidance can be achieved in scaffolds modified with oligopeptides such as RGD, YIGSR and IKVAV [3, 4, 12, 13]. Moreover, concentration gradients of IKVAV and RGD have been shown to influence cell growth on modified substrates [13, 14], likely through integrin-mediated mechanisms [13–15].

Scaffolds that provide cell guidance through the combination of chemical and physical cues have been pursued because they are precedents for guided tissue growth and integration. Some of the fabrication techniques currently being used to micropattern three-dimensional hydrogel matrices include photolithography [16, 17], microfluidic patterning [18], electrochemical deposition [19], reactive ion etching [20] and three-dimensional printing [21]. Although these

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techniques can modify the hydrogel properties in the $x$–$y$ plane, the depth of modification is limited to approximately 100 $\mu$m, requiring layering for the incorporation of biological components in the $z$-direction. Our laboratory has recently demonstrated cell guidance in a three-dimensionally modified, non-biodegradable agarose matrix by the combination of photochemistry and advanced laser technology [3].

Herein, we report cell guidance within three-dimensional peptide channels created in a 1.5 mm thick, biodegradable hyaluronan matrix for the first time. The laser technique used in this paper can be manipulated in order to create different concentration gradients within channels of varying diameters. Anisotropic GRGDS biochemical channels were created in an HA matrix using photochemistry and UV laser micropatterning, taking advantage of the optical transparency of HA to create the three-dimensional channels. The synthetic scheme used to create the channels is shown in figure 1. To investigate the cell guidance potential of the three-dimensional biochemical channels, cellular response of embryonic day 15 (E15) rat dorsal root ganglia (DRG) cells to GRGDS channels was compared to scrambled GRDGS channels. The cells possess integrin receptors to the RGD peptide and have been shown to extend neurites on substrates modified with the RGD peptide [22]. While we have previously shown that RGD peptide channels guide neurite outgrowth [3], we show, for the first time, neurite guidance up an RGD peptide concentration gradient.

## Methods

Hyaluronan (HA), with a molar mass of 1.03 $\times$ $10^6$ million g mol$^{-1}$, was purchased from NovaMatrix, Norway. Deuterium oxide ($D_2O$) was purchased from Cambridge Isotopes Laboratories (Andover, USA). All other chemicals and solvents were purchased from Sigma-Aldrich (Oakville, Canada) and used as received, unless otherwise noted. Water was distilled and deionized using Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, USA) at 18 M$\Omega$ resistance. Water for cell culture was sterile filtered through a 0.22 $\mu$m Millipore filter (Bedford, USA).

### *Modification of hyaluronan with SNBC*

S-2-nitrobenzyl cysteine (SNBC) was synthesized as described by Hazum *et al* [23] Briefly, L-cysteine was reacted with 2-nitrobenzyl bromide (1:1 molar ratio) in a solution containing triethylamine. The pure product was obtained by recrystallization in distilled water. To conjugate SNBC to hyaluronan (HA), the polymer was first dissolved in distilled water at a concentration of 0.5 wt%. Twofold molar excesses of both 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and sulfo N-hydroxysuccinimide (sulfo-NHS) (Pierce, Rockford, USA) were added to activate the carboxylic acid groups on HA. Finally, a twofold molar excess of SNBC was added and the reaction was allowed to proceed for 16 h at 75 °C. The resulting solution was dialyzed (molecular weight cutoff 12–14 000 g mol$^{-1}$, Spectra/Por membrane, Rancho Dominguez, USA) against 100 mM sodium chloride (48 h),

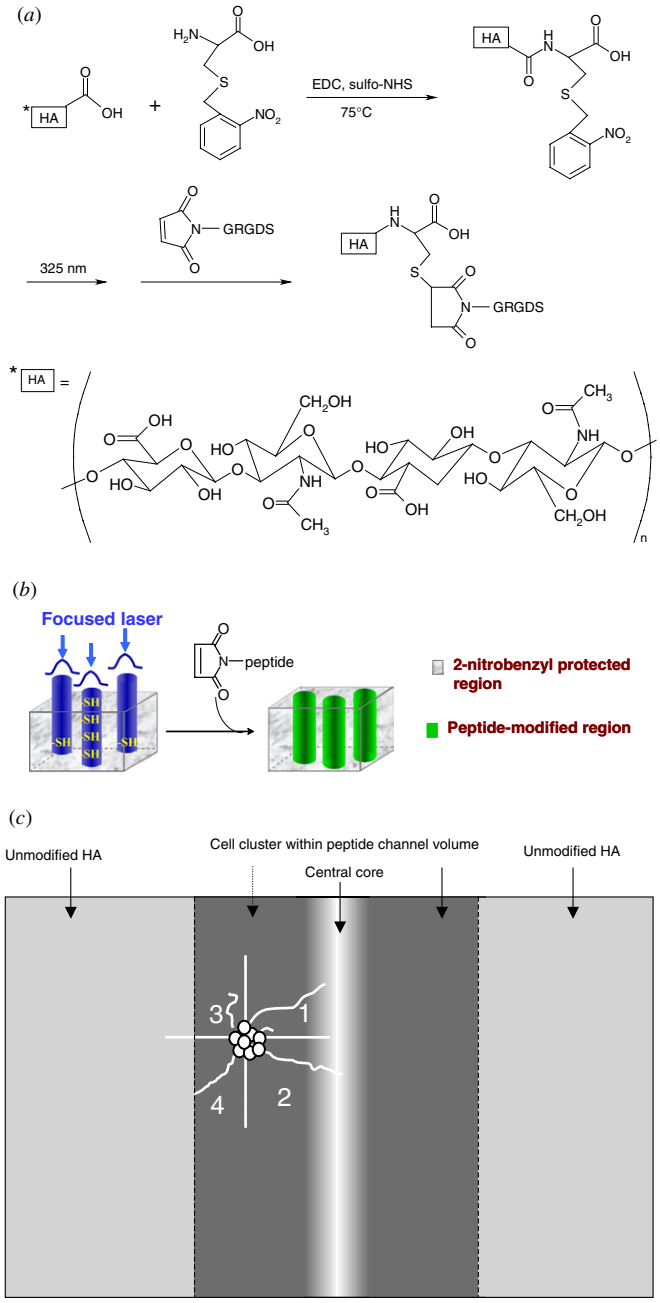


**Figure 1.** An overview of the reaction scheme used in the fabrication of three-dimensional GRGDS oligopeptide channels in hyaluronic acid. (*a*) Hyaluronan is first bulk modified with S-2-nitrobenzyl cysteine to create HA-SNBC. Irradiation with UV laser light cleaves the 2-nitrobenzyl moiety and creates free thiol groups in the irradiated cylindrical volumes. Thiol groups react with the maleimide terminal of the biomolecules to create three-dimensional biochemical channels. (*b*) Diagrammatic representation of the use of laser light with a Gaussian profile and the scheme outlined in (a) to create concentration gradients within the three-dimensional biochemical channels. (*c*) Image showing how quantitative analysis was performed to determine neurite guidance. Neurites were accounted for as extending towards the channel cores when they terminated in quadrants 1 and 2, and were accounted for as extending away from the channel cores when they terminated in quadrants 3 and 4.

followed by distilled water (48 h). The product was lyophilized and stored at 4 °C until needed. A control sample was prepared

following the same procedure except for the addition of EDC and sulfo-NHS.

The degree of modification was determined using $^1$H-NMR spectroscopy. The sample was dissolved in $D_2O$ at a concentration of 2 wt% and the $^1$H-NMR spectrum recorded on a Mercury 400 spectrometer (Varian). The degree of modification was calculated by comparing the integrated area of the signal produced by the methyl protons of the N-acetyl methyl group on HA (s, $\delta = 1.8$–$2.0$ ppm) [24, 25] with the aromatic protons of SNBC (m, $\delta = 7.4$–$8.0$ ppm). The degree of SNBC modification of HA was calculated according to:

$$\text{degree of modification} = \left(\frac{\text{total area under aromatic protons}}{4}\right) \Big/ \left(\frac{\text{total area under methyl protons}}{3}\right) \times 100\%. \quad (1)$$

*Preparation of HA-SNBC hydrogel*

The HA-SNBC hydrogel was prepared according to a modified published procedure [26, 27]. Briefly, a polymer blend containing native HA and HA-SNBC (1:1 mass ratio) was dissolved in distilled water at a concentration of 0.5 wt%. A fourfold molar excess (with respect to native HA) of EDC and a twofold molar excess of ADH were added, and the pH adjusted to 4.75. After vortexing, the reaction was allowed to proceed for 12 h at room temperature and the hydrogel was washed in pH 7.4 phosphate buffered saline (PBS) solution.

*Degradation study of HA-SNBC hydrogel*

*In vitro* degradation of HA-SNBC hydrogels was performed by incubating the hydrogels with different concentrations of testicular hyaluronidase EC 3.2.1.35 (Sigma-Aldrich, Oakville, Canada) at 37 °C with constant agitation at 160 rpm. The hydrogel samples (0.5 ml, $n = 4$) were placed in 1 ml of 0, 50, 150 or 250 U ml$^{-1}$ solutions of hyaluronidase prepared in PBS. Samples (200 $\mu$l) of each supernatant were taken at predetermined time points and replaced with fresh hyaluronidase solution (200 $\mu$l). The collected samples were diluted 50 times in distilled water saturated with benzoic acid, and analysed for uronic acid content according to the carbazole assay [28].

*Peptide synthesis*

N-$\alpha$-(3-maleimidopropionyl)-GRGDS (mi-GRGDS), and the scrambled peptide N-$\alpha$-(3-maleimidopropionyl)-GRDGS (mi-GRDGS), were prepared by solid-state peptide synthesis based on 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The oligopeptide sequences, GRGDS and GRDGS, were custom synthesized, without cleaving the protecting Fmoc groups on the side chains of the amino acids, using a peptide synthesizer (Pioneer, BioApplied Systems) in order to selectively modify the amine terminus prior to cleavage. A tenfold molar excess of 3-maleimidopropionic acid was activated using dicyclohexyl carbodiimide in dichloromethane for 1 h under nitrogen protection. The activated acid was then reacted with the amine terminal of the peptide on the resin for 2 h. The resin was washed sequentially with dichloromethane, isopropanol and methanol before being dried under vacuum. The maleimide-modified peptide (mi-GRGDS or scrambled mi-GRDGS) was de-protected and cleaved from the resin using 95% aqueous trifluoroacetic acid (2 h). The de-protected peptide was precipitated in cold ether solution and lyophilized. Fluorescein-labelled oligopeptides were synthesized in the same way, except that fluorescein-labelled lysine (N-$\varepsilon$-fluorescein-lysine) (Molecular Probes, Burlington, Canada) was added to the amine terminal of the oligopeptide sequence giving N-$\alpha$-(3-maleimidopropionyl)-N-$\varepsilon$-fluorescein-lysine-GRGDS (mi-fGRGDS) and N-$\alpha$-(3-maleimidopropionyl)-N-$\varepsilon$-fluorescein-lysine-GRDGS (mi-fGRDGS).

*Fabrication of biochemical channels in the hyaluronan matrix*

In order to create three-dimensional (3D) cylindrical channels, HA-SNBC hydrogel was prepared as described above to give a 1.5 mm thick matrix. The hydrogel was incubated in PBS buffer containing 1 mg mL$^{-1}$ peptide (mi-GRGDS: mi-fGRGDS, 3:1 mass ratio) for 3 h. The solution was then aspirated and in order to couple the peptide onto HA-SNBC, the hydrogel was irradiated with laser light from a He–Cd laser (4.0 mW, Omnichrome, Model 3074R-S-A03, Melles Griot) with a wavelength of 325 nm for approximately 1 s. The laser was focused by a convex fused-silica lens (focal length = 10 cm, theoretical focal spot size and depth of focus are 197 $\mu$m and 7 cm, respectively), and the gel was placed at the focal spot of the lens and moved using an *XY* stage. After irradiation, the gel was thoroughly washed to remove any unreacted reagents, and the channels were observed under a laser scanning confocal microscope (Olympus IX70, Japan). The amount of light that was transmitted by the hydrogel was determined, by measuring the energy of the laser beam before and after penetrating the sample, using an optical power meter (Model 480, Newport).

*Culturing neural cells on HA matrices modified with biochemical channels*

Neural cells were dissociated from embryonic day 15 (E15) Sprague-Dawley rat dorsal root ganglia (DRG) [29, 30], and plated on hydrogel matrices to study the ability of the GRGDS oligopeptide channels to guide neurite outgrowth. The hydrogel samples were prepared in polystyrene cuvettes (Fisher Scientific, Ottawa, Canada) under sterile conditions and all the solutions used were sterile filtered using 0.2 $\mu$m filters. Dissociated cells from DRGs were plated on top of hydrogel matrices ($6 \times 10^4$ cells cm$^{-2}$) containing three-dimensional channels made from either GRGDS or the scrambled peptide, GRDGS. The samples were cultured in medium containing Eagle's minimum essential medium, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamine, 50 ng ml$^{-1}$ nerve growth factor, and 10 $\mu$M uridine and 5-fluoro-2′-deoxyuridine (all supplied by

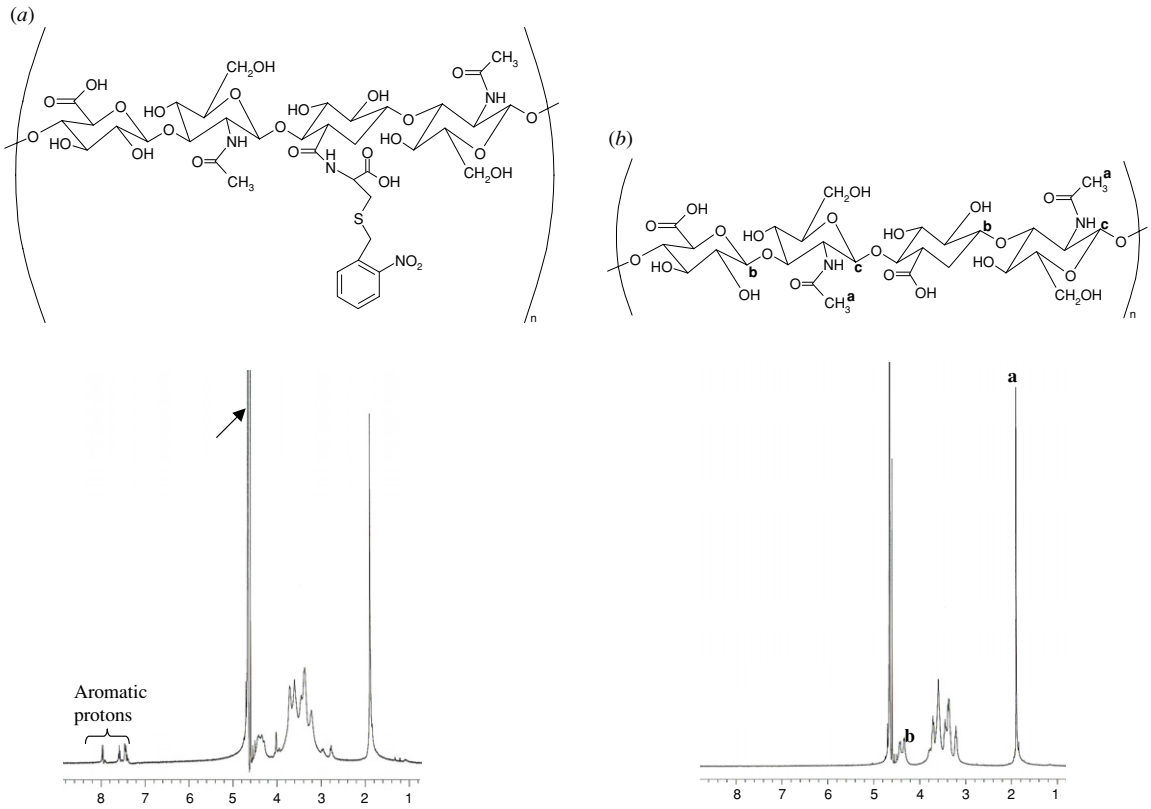


**Figure 2.** The $^1$H-NMR spectra in $D_2O$ at 400 MHz show that hyaluronan was successfully modified with S-2-nitrobenzyl cysteine: (*a*) HA-SNBC shows the characteristic methyl protons of the N-acetyl methyl group on HA (s, $\delta = 1.8$–$2.0$ ppm) and the aromatic protons of SNBC (m, $\delta = 7.4$–$8.0$ ppm); whereas (*b*) the control HA sample, where HA and SNBC were mixed in the absence of EDC and sulfo-NHS and dialyzed, shows only the characteristic HA methyl protons and no aromatic protons of SNBC.

Gibco, Burlington, Canada), and incubated (37 °C, 5% $CO_2$ and 100% humidity). The cell culture medium was replenished every 48 h. After 7 days, 5 randomly selected, separate fields containing channels in each cuvette were recorded at 10× magnification using a phase contrast/fluorescent microscope (LM410, Zeiss) to calculate the average length of neurites extending towards and away from the centre of the channels. Fifteen channels were recorded and a total of 30 neurites were analysed per channel type (GRGDS channels and scrambled GRDGS channels). Only those neurites longer than 1 cell diameter were analysed. Each experiment was conducted in triplicate. Neurites were counted as extending towards ('to') the GRGDS (or GRDGS) channels when they extended in quadrants 1 and 2, and were counted as extending 'away' from GRGDS (or GRDGS) channels when they extended in quadrants 3 and 4 (as shown in figure 1(*c*)). For all analyses, only neurites penetrating into the hydrogel matrix were analysed, those extending along the surface were not analysed because we were interested in learning about the impact of three-dimensional patterning on guidance. Variance analysis using two-way ANOVA (direction of neurites and peptide) was used for statistical analysis, followed by a posthoc Tukey test. Differences were considered significant at $p < 0.05$.

## Results and discussion

### *HA-SNBC hydrogel: synthesis and properties*

To prepare a photolabile matrix for three-dimensional photofabrication, the photosensitive S-2-nitrobenzyl cysteine (SNBC) was first coupled to the hyaluronan polymer using carbodiimide chemistry. The degree of SNBC modification of HA was calculated from the $^1$H NMR data (shown in figure 2) according to equation (1) and found to be approximately 6%, which corresponds to an SNBC concentration of 0.8 mM in a 0.5 wt% hydrogel solution. Covalent coupling of SNBC was confirmed by the absence of aromatic signals in the $^1$H-NMR spectrum of the control sample, where HA and SNBC were mixed in the absence of EDC and sulfo-NHS and then dialyzed prior to characterization by $^1$H NMR.

A polymer blend of HA-SNBC and native HA (referred to simply as HA-SNBC hydrogel) was successfully cross-linked using EDC and ADH. The hydrogel began to form 60 s after mixing, and the reaction mixture was left to react for 12 h in order to allow the gel to solidify. The resulting hydrogel was transparent and did not flow when inverted for 1 min.

The resulting hydrogel was analysed for degradation with hyaluronidase and analyzed by the standard carbozole assay. The HA-SNBC hydrogel was completely degraded by the accelerated degradation study using 250 U ml$^{-1}$ of

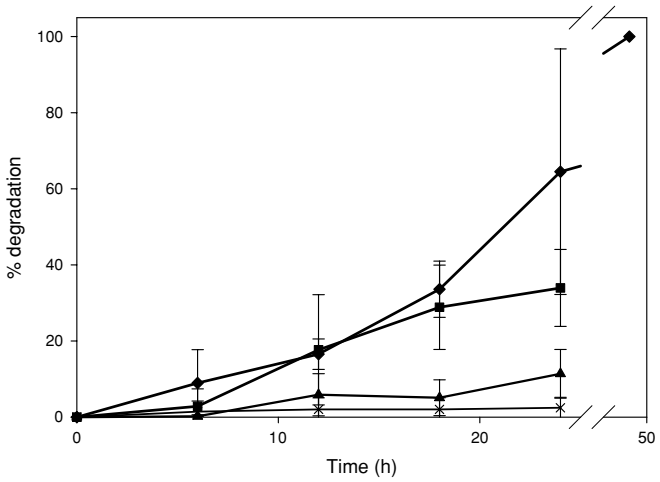


**Figure 3.** Degradation rates of HA-SNBC hydrogel in (×) 0 U ml$^{-1}$; (▲) 50 U ml$^{-1}$; (■) 150 U ml$^{-1}$; (♦) 250 U ml$^{-1}$ of hyaluronidase prepared in pH 7.4 PBS and incubated at 37 °C. (Data shown as means ± 95% confidence intervals, $n = 4$.)

hyaluronidase after 48 h, demonstrating that the crosslinked and SNBC-modified HA hydrogel remained degradable. The percent degradation was calculated by dividing the amount of uronic acid released at a given time point by the final amount of uronic acid measured when a reference hydrogel was completely degraded, as shown in figure 3. Since the DRG neurons do not secrete hyaluronidase, it is unlikely that the biochemical RGD peptide channels created in the HA hydrogel were affected during the cell guidance experiments.

### *3D patterning of HA-SNBC hydrogels*

The HA-SNBC hydrogel matrix was irradiated with a He–Cd laser in order to fabricate three-dimensional biochemical channels. The peptide channels were visualized as isolated cylindrical volumes throughout the 1.5 mm thick hydrogel matrix using fluorescently labelled f-GRGDS and scrambled f-GRDGS (figure 4). Reconstructed confocal microscopy *XY* cross-section micrographs, recorded at 20 $\mu$m intervals for a distance of 0.5 mm, were used to create the three-dimensional image of the channels (figure 4(*b*)). In order to view six channels in the same field of view at a magnification of 10×, the hydrogel matrix was irradiated at intervals of 150 $\mu$m and the contrast of the micrographs was adjusted in order to clearly view the isolated channels shown in figures 4(*a*) and (*b*).

The peptide channels had a GRGDS concentration gradient in the cross-sectional plane, as determined by the fluorescence intensity profile along a line drawn through the centre of the cross-sectional image. Figure 4(*c*) is a representative profile obtained from an image where the hydrogel was irradiated at 1 mm intervals and the contrast was not adjusted. The highest peptide concentration was in the centre of the channels and it gradually decreased in a Gaussian way towards the periphery of the *x*–*y* plane of the cylindrical channels, indicating a cylindrical channel radius of 250–300 $\mu$m. The peptide concentration gradient along the radius of the channel was estimated at 0.4 $\mu$g $\mu$l$^{-1}$ $\mu$m$^{-1}$.

The longitudinal dimensions are determined by both the depth of focus of the laser beam (defined as the distance over which the focal spot size changes by ±5%), and the transmittance of light through the hydrogel material. The UV light is absorbed by SNBC, as it travels through the HA-SNBC hydrogel. While the channels were observed to penetrate the entire hydrogel matrix, fluorescence intensity of the channels became more diffuse deeper into the hydrogel matrix. Importantly, analysis of the peptide channels over a 3 week period showed no change in either channel diameter or geometry, indicating that the peptide channels were stable during the 2 week period required to complete the cell culture experiments, especially given that DRGs do not produce hyaluronidase.

### *Cell and neurite guidance in GRGDS-peptide channels*

In order to determine neural cellular response to the defined three-dimensional GRGDS volumes within the HA matrix, E15 rat dorsal root ganglia (DRG) cells were plated on the top of the hydrogel matrix containing the biochemical channels. We observed cell migration into the hydrogel matrix to a depth of 600 $\mu$m (figure 5). The GRGDS gradient across the *x*–*y* plane of the biochemical channels affected neurite outgrowth such that neurites extended preferentially up the concentration gradient, towards the central core of the peptide channel, when the cells were within the 250 $\mu$m radius of the channels (figures 5(*a*) and (*b*)). In contrast, for the scrambled GRDGS peptide gradient channels, neurite extension was haphazard and neither preferentially up (or 'to') the concentration gradient nor away from the concentration gradient (figure 5(*c*)). DRG neurons and neurites grew only within the peptide-modified channels of the HA hydrogels and not within the volumes separating the peptide channels. Importantly, DRG neurons have integrin receptors for RGD and it is only within these channels that the peptide concentration gradient resulted in guidance up the gradient. The guidance observed was towards the high concentration gradient at the core of the peptide channels; this marks the first time that an RGD peptide concentration gradient has demonstrated neurite guidance within a three-dimensional volume. In our previous study in agarose, primary dorsal root ganglia neurons also only grew in peptide-modified channels; however, unlike the hyaluronan study where cells grew in both RGD and scrambled RDG modified channels, in agarose the cells only grew in the RGD-modified channels. Interestingly, in agarose, the concentration gradient did not inform our cell analysis, yet was important to demonstrating specificity in hyaluronan. Thus in the hyaluronan study, cell adhesion was non-specific to the peptide yet neurite outgrowth up the concentration gradient was specific to the integrin-ligand interaction between primary dorsal root ganglia neurons and RGD-modified channels.

The lengths of neurites were quantified in order to investigate the guidance potential of GRGDS channels relative to the scrambled GRDGS control channels created in hyaluronan hydrogels. Specifically, neurites were

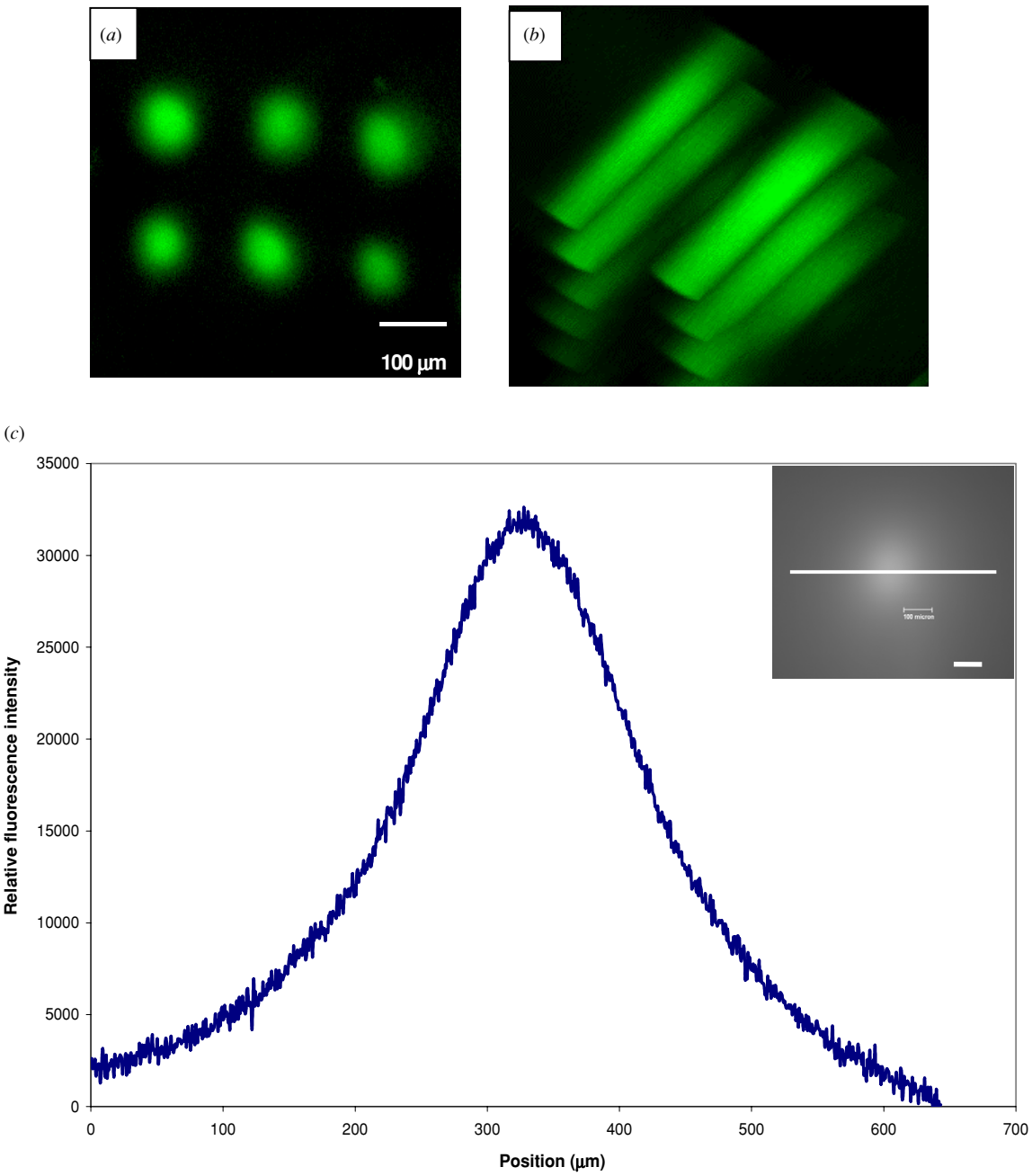


**Figure 4.** Biochemical channels synthesized in hyaluronan hydrogel were visualized and characterized using a fluorescein-tagged GRGDS peptide. (*a*) A representative *XY* cross-sectional image of green, fluorescently-labelled oligopeptide channels (contrast adjusted to clearly view the isolated regions). (*b*) A representative longitudinal image of green, fluorescently-labelled oligopeptide channels constructed from a series of *XY* cross-section micrographs taken at 20 $\mu$m intervals over a depth of 0.5 mm (contrast adjusted to clearly view the isolated regions). (*c*) The relative intensity profile of a line passing through the centre of a cross-sectional image showing a GRGDS concentration gradient in the *x*–*y* plane of a modified cylinder, as well as the contrast between the modified and unmodified regions. The gradient is greatest in the centre and least at the periphery, having a radius of 250–300 $\mu$m (scale bar: 100 $\mu$m). (*d*) Longitudinal fluorescence intensity profile along the central axis of the channel showing a decrease in fluorescence intensity with depth.

characterized as either growing 'to' the central core, i.e., up the gradient, or 'away' from the central core as was shown schematically in figure 1(*c*) and as an overlay to the data in figure 6(*a*). As shown in figure 6(*b*), cells extended significantly longer neurites towards the GRGDS channel cores than away from the channel cores ($p < 0.001$). Additionally, cells extended significantly longer neurites towards GRGDS channel cores than towards scrambled GRDGS control channel cores ($p < 0.001$). There was no statistical difference between neurite extension towards and away from scrambled GRDGS channels. The lack of guidance observed with scrambled GRDGS control channels showed that the guided neurite extension observed with GRGDS channels was due to the local biochemical environment within the channels. The comparison between GRGDS and the scrambled GRDGS control indicates that the guidance effect in

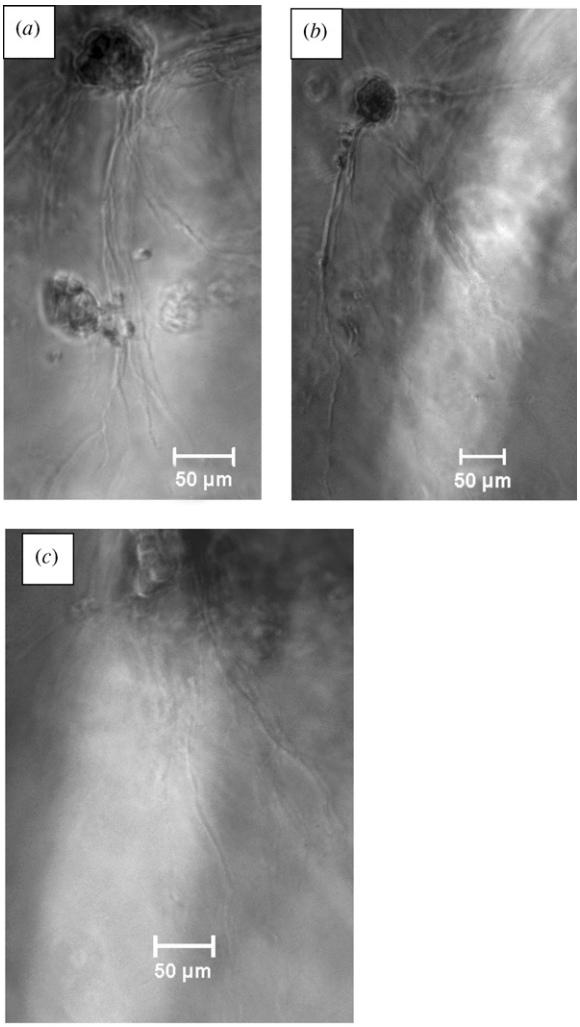


**Figure 5.** Micrograph showing DRG neurite outgrowth from cells (*a*) directly on top of, (*b*) near the GRGDS channel core, and (*c*) directly on top of the scrambled GRDGS channel created in a hyaluronan matrix. The cells migrated into the hydrogel matrix and the neurites extended predominantly to the core and up the GRGDS concentration gradient, while the cells extended neurites haphazardly, with no general preference of either to or away from the scrambled GRDGS channel core. (Light areas show the cores of the channels.)

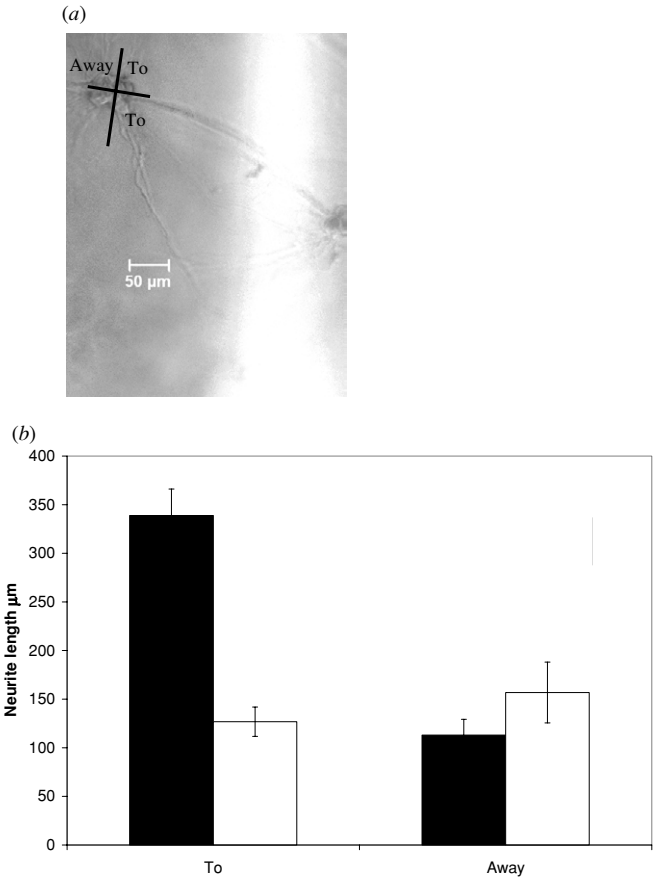


**Figure 6.** (*a*) A representative image of neurites extending to the channel core (bright white channel) and how the quadrant methodology was applied to distinguish neurites extending 'To' versus 'Away' from the core. (*b*) Quantitative analysis of rat DRG neurite outgrowth from cell clusters within 250 $\mu$m of the central core of the (■) GRGDS channels versus (□) GRDGS channels. Statistically significant differences were found between neurite length To GRGDS channels and To scrambled GRDGS channels ($p < 0.001$), as well as neurite length Away from GRGDS and scrambled GRDGS channels ($p < 0.001$). There was no statistical difference between neurite extension towards and away from GRDGS channels ($p = 0.364$). (Data shown as means ±95% confidence intervals, $n = 15$ channels.)

GRGDS biochemically-defined channels is due to an integrin-mediated response to the concentration gradients within the GRGDS-modified regions of the hydrogel. It is unlikely that protein adsorption in the GRGDS channels or local mechanical properties within the channels would lead to the difference observed between GRGDS and the scrambled GRDGS.

Concentration gradients of RGD peptide have been shown to influence fibroblast cells to align and spread parallel to the axis of the concentration gradients [14]. Gradients of neurotrophins [31, 32] and laminin-derived IKVAV [13] have also been shown to promote growth cone turning and migration up the substratum-bound gradients. However, to our knowledge, this is the first report of RGD concentration gradients guiding neurite outgrowth in the direction of increasing concentration within a three-dimensional volume. By manipulating the UV laser source, the peptide concentration gradient within the channels can be varied in order to investigate whether a minimum GRGDS concentration gradient is required to guide neural cellular outgrowth, as has been observed for neurotrophins [33].

## Conclusions

We report a micropatterning technique that can be used to create biochemical concentration gradients within three-dimensional biodegradable hydrogels. The oligopeptide concentration gradients and the cell-invasive nature of the hyaluronan hydrogels allowed for guided neurite extension in the peptide-modified regions of the hydrogel. Due to the enzymatically-degradable nature of hyaluronan, these materials may be useful as temporary scaffolds for guided axonal regeneration *in vivo*.

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## References

[1] Langer R and Vacanti J P 1993 Tissue engineering *Science* **260** 920–6
[2] Gomes M E and Reis R L 2004 Tissue engineering: key elements and some trends *Macromol. Biosci.* **4** 737–42
[3] Luo Y and Shoichet M S 2004 A photolabile hydrogel for guided three-dimensional cell growth and migration *Nat. Mater.* **3** 249–53
[4] Saneinejad S and Shoichet M S 2000 Patterned poly(chlorotrifluoroethylene) guides primary nerve cell adhesion and neurite outgrowth *J. Biomed. Mater. Res.* **50** 465–74
[5] Shieh S J and Vacanti J P 2005 State-of-the-art tissue engineering: from tissue engineering to organ building *Surgery* **137** 1–7
[6] Tabata Y 2004 Tissue regeneration based on tissue engineering technology *Congenit. Anom. (Kyoto)* **44** 111–24
[7] Hu M, Sabelman E E, Cao Y, Chang J and Hentz V R 2003 Three-dimensional hyaluronic acid grafts promote healing and reduce scar formation in skin incision wounds *J. Biomed. Mater. Res.* **67B** 586–92
[8] Duranti F, Salti G, Bovani B, Calandra M and Rosati M L 1998 Injectable hyaluronic acid gel for soft tissue augmentation. A clinical and histological study *Dermatol. Surg.* **24** 1317–25
[9] Adanali G, Verdi M, Tuncel A, Erdogan B and Kargi E 2003 Effects of hyaluronic acid-carboxymethylcellulose membrane on extraneural adhesion formation and peripheral nerve regeneration *J. Reconstr. Microsurg.* **19** 29–36
[10] Tian W M, Hou S P, Ma J, Zhang C L, Xu Q Y, Lee I S, Li H D, Spector M and Cui F Z 2005 Hyaluronic acid-poly-D-lysine-based three-dimensional hydrogel for traumatic brain injury *Tissue Eng.* **11** 513–25
[11] Leach J B, Bivens K A, Collins C N and Schmidt C E 2004 Development of photocrosslinkable hyaluronic acid-polyethylene glycol-peptide composite hydrogels for soft tissue engineering *J. Biomed. Mater. Res.* A **70** 74–82
[12] Saneinejad S and Shoichet M S 1998 Patterned glass surfaces direct cell adhesion and process outgrowth of primary neurons of the central nervous system *J. Biomed. Mater. Res.* **42** 13–9
[13] Adams D N, Kao E Y, Hypolite C L, Distefano M D, Hu W S and Letourneau P C 2005 Growth cones turn and migrate up an immobilized gradient of the laminin IKVAV peptide *J. Neurobiol.* **62** 134–47
[14] Kang C E, Gemeinhart E J and Gemeinhart R A 2004 Cellular alignment by grafted adhesion peptide surface density gradients *J. Biomed. Mater. Res.* A **71** 403–11
[15] Giancotti F G and Ruoslahti E 1999 Integrin signaling *Science* **285** 1028–32
[16] Yamato M, Konno C, Koike S, Isoi Y, Shimizu T, Kikuchi A, Makino K and Okano T 2003 Nanofabrication for micropatterned cell arrays by combining electron beam-irradiated polymer grafting and localized laser ablation *J. Biomed. Mater. Res.* A **67** 1065–71
[17] Miller C, Jeftinija S and Mallapragada S 2001 Micropatterned Schwann cell-seeded biodegradable polymer substrates significantly enhance neurite alignment and outgrowth *Tissue Eng.* **7** 705–15
[18] Sharma S, Tan W and Desai T A 2005 Improving the integrity of three-dimensional vascular patterns by poly(ethylene glycol) conjugation *Bioconjug. Chem.* **16** 18–22
[19] Khademhosseini A, Suh K Y, Yang J M, Eng G, Yeh J, Levenberg S and Langer R 2004 Layer-by-layer deposition of hyaluronic acid and poly-L-lysine for patterned cell co-cultures *Biomaterials* **25** 3583–92
[20] Miller C, Shanks H, Witt A, Rutkowski G and Mallapragada S 2001 Oriented Schwann cell growth on micropatterned biodegradable polymer substrates *Biomaterials* **22** 1263–9
[21] Amirpour M L, Ghosh P, Lackowski W M, Crooks R M and Pishko M V 2001 Mammalian cell cultures on micropatterned surfaces of weak-acid, polyelectrolyte hyperbranched thin films on gold *Anal. Chem.* **73** 1560–6
[22] Isahara K and Yamamoto M 1995 The interaction of vascular endothelial cells and dorsal root ganglion neurites is mediated by vitronectin and heparan sulfate proteoglycans *Brain Res. Dev. Brain Res.* **84** 164–78
[23] Hazum E, Gottlieb P, Amit B, Patchornik A and Fridkin M 1981 A photocleavable protecting group for the thiol function of cysteine *Proc. 16th European Peptide Symp. (Copenhagen)* p 105
[24] Sicinska W, Adams B and Lerner L 1993 A detailed 1H and 13C NMR study of a repeating disaccharide of hyaluronan: the effects of temperature and counterion type *Carbohydr. Res.* **242** 29–51
[25] Pfeiler E, Toyoda H, Williams M D and Nieman R A 2002 Identification, structural analysis and function of hyaluronan in developing fish larvae (leptocephali) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **132** 443–51
[26] Prestwich G D, Marecak D M, Marecek J F, Vercruysse K P and Ziebell M R 1998 Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hydrazide derivatives *J. Control Release* **53** 93–103
[27] Vercruysse K P and Prestwich G D 1998 Hyaluronate derivatives in drug delivery *Crit. Rev. Ther. Drug Carrier Syst.* **15** 513–55
[28] Bitter T and Muir H M 1962 A modified uronic acid carbazole reaction *Anal. Biochem.* **4** 330–334
[29] Erzurumlu R S, Jhaveri S, Takahashi H and McKay R D 1993 Target-derived influences on axon growth modes in cultures of trigeminal neurons *Proc. Natl Acad. Sci. USA* **90** 7235–9
[30] Erzurumlu R S and Jhaveri S 1995 Target influences on the morphology of trigeminal axons *Exp. Neurol.* **135** 1–16
[31] Cao X and Shoichet M S 2003 Investigating the synergistic effect of combined neurotrophic factor concentration gradients to guide axonal growth *Neuroscience* **122** 381–9
[32] Kapur T A and Shoichet M S 2004 Immobilized concentration gradients of nerve growth factor guide neurite outgrowth *J. Biomed. Mater. Res.* A **68** 235–43
[33] Cao X and Shoichet M S 2001 Defining the concentration gradient of nerve growth factor for guided neurite outgrowth *Neuroscience* **103** 831–40