WILEY InterScience® DISCOVER SOMETHING GREAT

Peptide surface modification of methacrylamide chitosan for neural tissue engineering applications

Laura M. Y. Yu,[1,2] Karineh Kazazian,[1] Molly S. Shoichet[1–3]
[1]*Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, Canada M5S 3E5*
[2]*Institute of Biomaterials and Biomedical Engineering, Terrence Donnelly Center for Cellular and Biomolecular Research, University of Toronto, 160 College Street, RM 514 Toronto, Ontario, Canada M5S 3E1*
[3]*Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario, Canada M5S 3H6*

Received 20 April 2006; revised 8 August 2006; accepted 28 August 2006
Published online 12 February 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31069

Abstract: Nerve fibres are guided to their targets by the combined actions of chemotactic and haptotactic stimuli; however, translating these stimuli to a scaffold that will promote nerve regeneration is nontrivial. In pursuit of this goal, we synthesized and characterized cell-adhesive, biodegradable chitosan scaffolds. Chitosan amine groups were reacted with methacrylic anhydride resulting in a water soluble methacrylamide chitosan (MC) that was then crosslinked by radical polymerization resulting in a scaffold. Biodegradability by lysozyme and penetrability of the scaffold by rat superior cervical ganglion (SCG) neurons were studied. Maleimide-terminated cell adhesive peptides, mi-GDPG**YIGSR** and mi-GQASS**IKVAV**, were coupled to a thiolated form of MC to promote cell adhesion. The MC scaffold was found to be porous, biodegradable, and to allow neurite penetration. Interestingly, all of these properties were found to depend upon the amount of initiator used in crosslinking. Covalent modification of the MC scaffold with cell adhesive peptides significantly improved neuronal adhesion and neurite outgrowth. The MC can be crosslinked to form a novel scaffold, where our results demonstrate its suitability in neural tissue engineering and its potential for other engineered tissues, such as cartilage repair, where chitosan has already demonstrated some utility. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 82A: 243–255, 2007

Key words: methacrylamide chitosan; cell adhesion; biodegradable hydrogel scaffold; neurite penetration

INTRODUCTION

Traumatic injury of the spinal cord results in permanent functional impairment below the lesion site.[1,2] Unlike injury to the peripheral nervous system, the physiological response to spinal cord injury results in an environment that is inhibitory to regeneration.[3,4] To promote nerve regeneration, chemical guidance cues, therapeutic factors, and cell-based strategies have been investigated.[2] Moreover, combination strategies have gained momentum as regeneration is enhanced by multiple stimuli.[5] A permissive environment that mimics the extracellular matrix (ECM) to support cellular growth and survival is of great importance. Laminin, a major component of the ECM, plays an important role for neuronal adhesion and neurite outgrowth. YIGSR and IKVAV are two peptide sequences derived from laminin that have been shown to promote cell adhesion and neurite outgrowth, respectively.[6–8] Given the importance of combined haptotactic and chemotactic cues for growth cone guidance in development,[9] we have investigated the guidance potential of neurotrophin concentration gradients and cell-adhesive peptide channels *in vitro*; however, in those studies, nondegradable hydrogels were used,[10,11] thereby limiting clinical transferability.

Of the numerous biodegradable polymers that have been studied for tissue engineering (see review in Ref. 2), chitin and chitosan are particularly attractive because they are enzyme-degradable, cell-adhesive, and biocompatible.[12–15] Chitin, which is extracted from shells of crustaceans and exoskeletons of arthropods,[15] can be deacetylated to chitosan under alkaline hydrolysis conditions[16] with the extent of acetylation/deacetylation governing the rate of degradation.[17,18] Nevertheless, one drawback of chitosan is that it is only soluble in dilute acid.[19]

Correspondence to: M. S. Shoichet; e-mail: molly@chem-eng.utoronto.ca
Contract grant sponsor: Natural Sciences and Engineering Research Council of Canada
Contract grant sponsor: Canada Foundation for Innovation
Contract grant sponsor: Ontario Innovation Trust

© 2007 Wiley Periodicals, Inc.

Thus, various derivatives of chitosan have been reported in the literature for a variety of tissue engineering applications, including drug delivery vehicles, micelles, and scaffolds.[20–24] Moreover, porous scaffolds are interesting for tissue engineering applications as they allow cells to penetrate the matrix and promote proliferation of certain cell types, as has been shown with chrondrocytes.[25] We have previously reported that poly(2-hydroxyethyl methacrylate) (PHEMA) crosslinked using ethylene dimethacrylate as the crosslinker creates a macroporous scaffold that allowed immobilization of neurotrophin concentration gradients and cell penetration.[10] In order to extend this chemistry to biodegradable polymers, we investigated the possibility of creating a porous chitosan scaffold that required the synthesis of a water soluble chitosan derivative.

In the present study, we report a novel water soluble chitosan that was synthesized by reaction of some of the chitosan amine groups with methacrylic anhydride, resulting in methacrylamide chitosan (MC), as shown in Scheme 1. The carbon–carbon double bonds of the methacryl groups provide the reactive sites for crosslinking chitosan resulting in a macroporous scaffold that was tested in three-dimensional (3-D) cell culture for cell penetration. We investigated the biodegradability and cell adhesion of the MC hydrogel to determine whether methacryl modification altered some of the desirable properties of the natural chitosan. We propose that the MC can be modified with cell adhesive peptides to promote greater cell adhesion and neurite outgrowth. The transparency of the gel was an advantage that facilitated 3-D analysis.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (Oakville, Canada) and used as received unless otherwise stated. Methacrylic anhydride was purchased from ACP Chemicals (Montreal, Canada) and used as received. Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, USA) and deuterium chloride (DCl, 35% in D_2O) from CDN Isotopes (Pointe-Claire, Canada). Amino acids were purchased from Novabiochem (EMD Biosciences, La Jolla, CA), activators and resins were from Applied Biosystems (Foster City, CA). Dichloromethane (DCM) and trifluoroacetic acid (TFA) were purchased from Caledon Laboratories (Georgetown, ON, Canada). Water was distilled and deionized using Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, USA) at 18 mΩ resistance.

Preparation of methacrylamide chitosan and analysis of degree of methacrylation

A 3 wt % solution of chitosan was prepared by dissolving chitosan flakes (M_W 190,000–230,000) in 2 wt % acetic acid overnight at room temperature (RT) with constant shaking. Methacrylic anhydride was added at 0.4 molar equivalents per chitosan repeat unit. The mixture was allowed to stir at RT for 3 h before being dialyzed (molecular weight cut off range 12,000–14,000, Spectrum Laboratories, USA) against distilled water for 2d with the water changed twice each day. The mixture was then lyophilized and stored at −20°C until use.

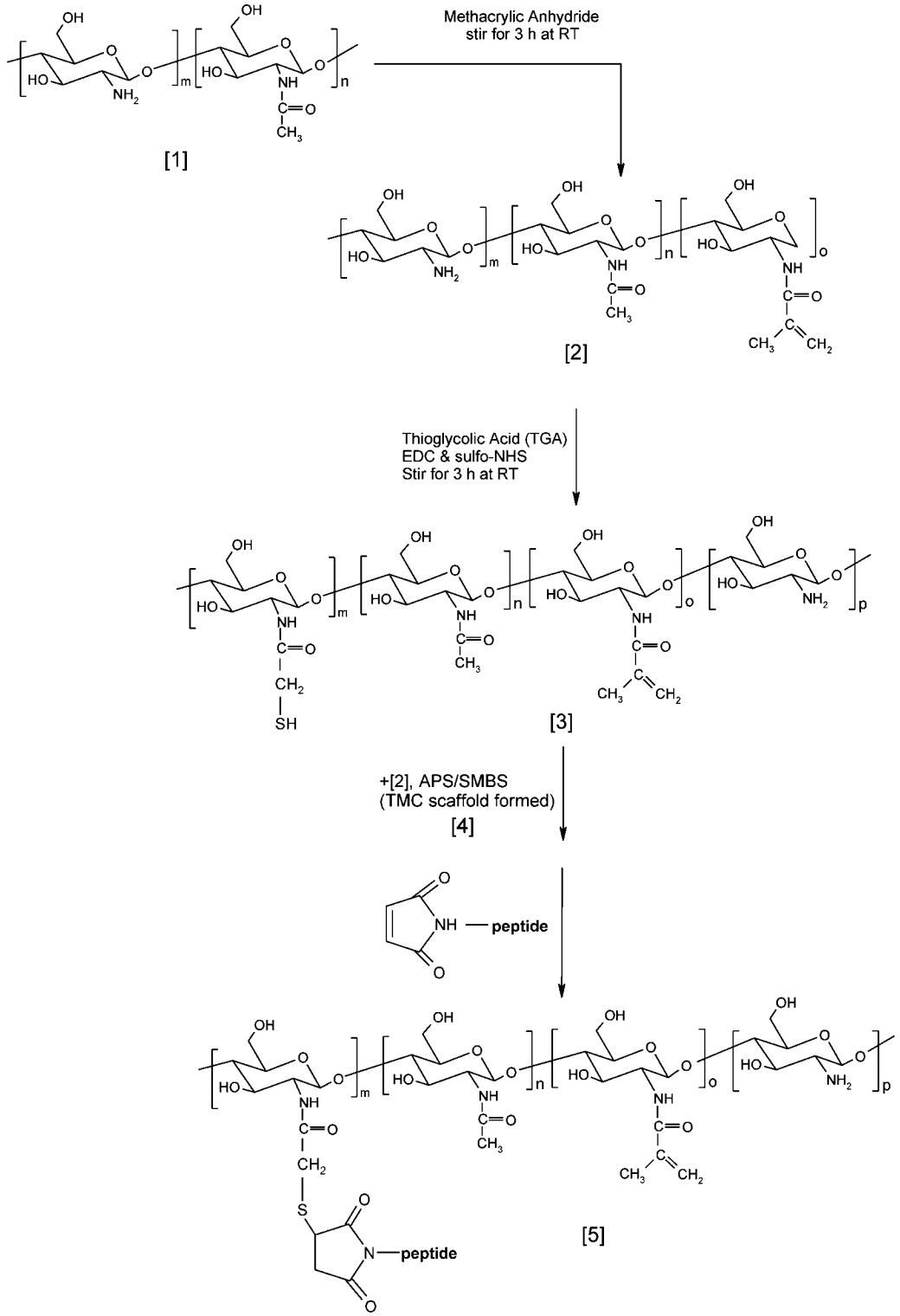
The degree of methacryl modification of chitosan was determined using ^{1}H NMR spectroscopy according to a modified published procedure.[26] Briefly, methacrylamide chitosan (MC) was dissolved in 0.25% DCl in D_2O at ~0.5% (w/v) and the ^{1}H NMR spectrum recorded (Mercury 400 spectrometer, Varian) is shown in Figure 1. The degree of methacrylation was calculated by comparing the integrated area of the H2–H6 peaks (labelled according to Ref. 26) at 2.8 to 4.0 ppm to that of the methylene peaks at 5.6 and 6.0 ppm. The degree of acetylation of chitosan before modification was found to be 12% by ^{1}H NMR using the above method.

Preparation of methacrylamide chitosan scaffold

Freeze-dried MC was reconstituted as a 2 wt % solution in distilled water and sterilized by autoclave for 20 min at 120°C. Ammonium persulfate (APS) and sodium metabisulfite (SMBS) were used to initiate and accelerate the crosslinking of MC to a scaffold. APS and SMBS were dissolved at 100 mg/mL in distilled water and sterile-filtered (0.22 μm nitrocellulose membrane). Both were added to MC at 3, 4, 5, and 7 wt % of the initial amount of MC present (referred to as wt % APS/SMBS hereafter). The scaffold was allowed to sit for at least 2 h at RT to ensure the crosslinking reaction was complete before washing in phosphate buffered saline (PBS, pH 7.4) for 3 days with at least 4–5 changes of buffer each day to remove unreacted crosslinking agents, prior to cell culture or characterization for degradation.

Preparation of thiolated methacrylamide chitosan scaffold

Thiolated methacrylamide chitosan (TMC) was prepared by covalently coupling thioglycolic acid (TGA) to MC according to a modified published procedure.[27] To 2 wt % solution of MC, *N*-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccnimide (sulfo-NHS) were added to achieve a final concentration of 50 m*M* each. Then, TGA was added to the MC solution in a weight ratio of 1:2.5. The reaction mixture was allowed to stir for 3 h at RT then dialyzed in distilled water and freeze-dried as above ([**3**] in Scheme 1). A negative control was prepared in the same manner but without EDC and sulfo-NHS added. The TMC scaffold was synthesized by blending MC (2 wt %) and TMC (2 wt %) in a 1:1 w/w ratio and crosslinked with 4 wt % APS/SMBS as before. The mixture was dispensed into 96-well plate before gelation and allowed to stand for 24 h to ensure that crosslinking reaction is complete before washing extensively for 3 days in PBS as described above. Ellman's



Scheme 1. Chitosan [**1**] primary amine groups react with methacrylic anhydride to produce methacrylamide chitosan (MC) [**2**]. Free radical polymerization of MC across the C=C on the methacrylamide group results in a MC hydrogel. The primary amine groups of MC react with thioglycolic acid (TGA) using *N*-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC) as crosslinker and *N*-hydroxysulfosuccinimide (sulfo-NHS) to enhance the efficiency of EDC coupling. The resulting thiolated methacrylamide chitosan (TMC) [**3**] is crosslinked in a 1:1 (w/w) ratio with [**2**] using ammonium persulfate (APS) and sodium metabisulfite (SMBS) to produce the TMC scaffold [**4**]. The TMC scaffold is then modified by reacting it with a maleimide-terminated cell adhesive peptide [**5**].

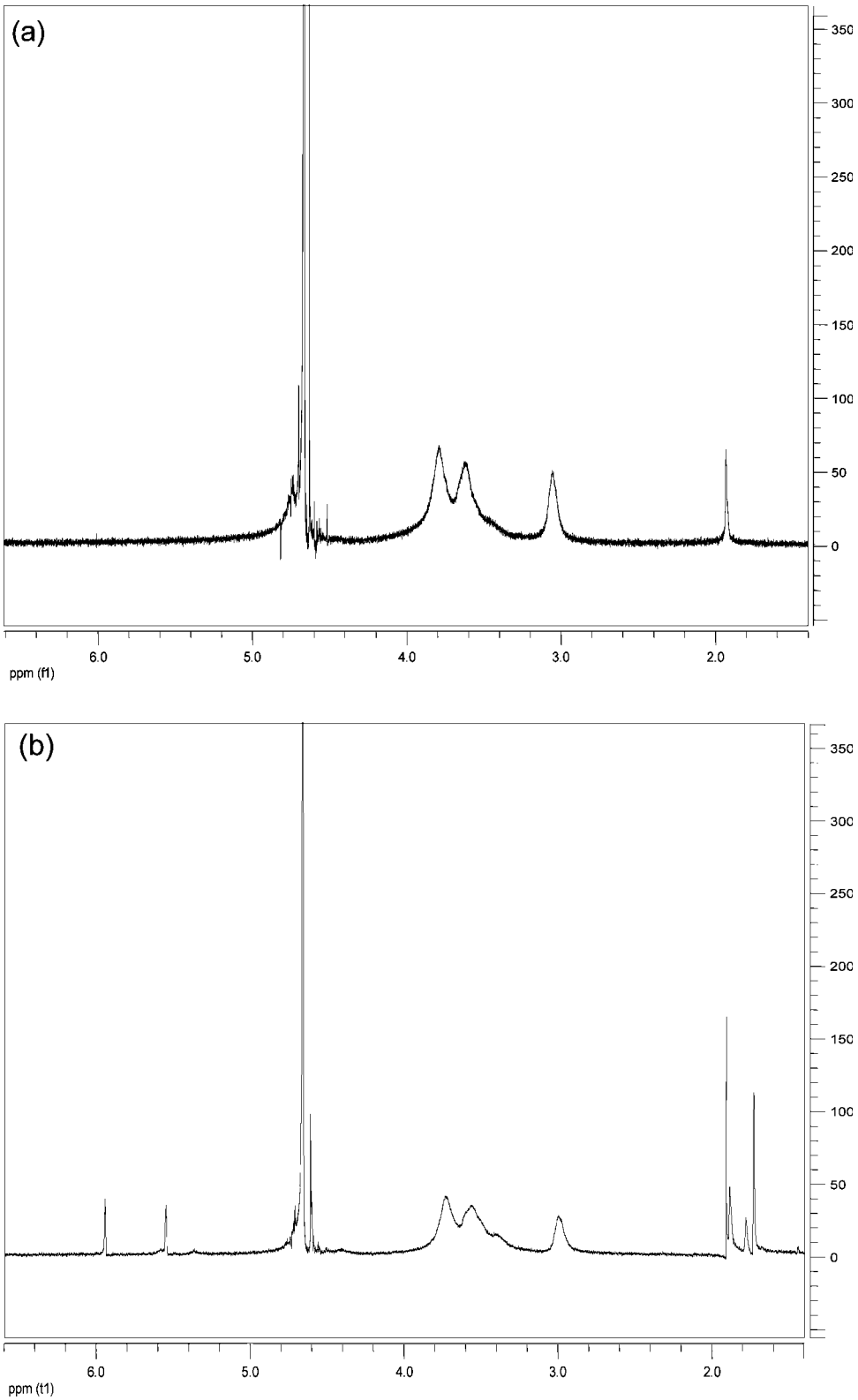


Figure 1. ^{1}H NMR of (a) chitosan before modification and (b) methacrylamide chitosan. The peaks at 2.8–4.0 ppm are the protons in the ring of the gluocosamine, and the methylene peaks on the methacrylamide chitosan are located at 5.6 and 6.0 ppm. The peak at 1.9 ppm in (a) corresponds to the acetyl group of chitosan ([**1**]) in Scheme 1). The peaks from 1.7 to 1.9 ppm correspond to the methylene carbons from the acetyl and methacrylamide pendants of methacrylamide chitosan, and acetyl pendant on unmodified regions of chitosan.

reagent [5,5′-Dithio-bis-(2-nitrobenzoic acid)] was used to quantify the amount of thiol groups present on TMC.[27] Briefly, 0.1*M* sodium phosphate buffer with 1 m*M* EDTA (pH 8) was prepared. To 2.5 mL of the buffer, 250 μL of a 0.02 wt % solution of TMC in distilled water and 50 μL of the Ellman's reagent (4 mg/mL) were added. The mixture was allowed to react for 1 h at RT, and the absorbance of the liquid was measured at 412 nm using a microplate reader (VersaMax, Molecular Devices, USA). The number of thiol groups present on TMC was determined by comparing its absorbance to a calibration curve prepared as above using cysteine hydrochloride monohydrate.

Scanning electron microscopy

The morphology of sterilized MC scaffolds, crosslinked with 3 or 7 wt % APS/SMBS, was examined using scanning electron microscopy (SEM). The scaffolds were freeze-dried under liquid N_2 for 24 h to prevent structural collapse, cut in cross-section, and mounted on SEM imaging studs with double-sided tape. The samples were coated with gold for 40 s and imaged using Hitachi S-2500 SEM.

Peptide synthesis and quantification on TMC scaffold

Maleimide-funcionalized (mi-) peptide sequences, mi-GQAAS**IKVAV** and mi-GDPG**YIGSR**, were custom synthesized by solid-state peptide synthesis on PEG-PS resin, based on Fmoc chemistry using a peptide synthesizer (Pioneer Peptide Synthesis System, Applied Biosystems). A 10-fold molar excess of 3-maleimidopropionic acid was activated by dicyclohexyl carbodiimide in DCM under N_2, which then reacts selectively with the amine terminus of the Fmoc protected amino acid on the resin. The maleimide functionalized peptide was de-protected and cleaved from the resin using 95% TFA and lyophilized. The peptide (dissolved in 99% acetic acid) was purified by reverse phase high performance liquid chromatography (RP-HPLC, VP Series HPLC Workstation, Shimadzu Scientific Instruments, Columbia, MD). Fluorescein-tagged mi-GQAAS**IKVAV** was synthesized using the same method with *N*-ε-fluorescein-lysine added to form *f*-mi-GQ*K*AAS**IKVAV** for quantifying the amount of peptide covalently coupled to TMC scaffolds. The molecular weights of the peptides were confirmed by mass spectrometry (Sciex Qstar, Applied Biosystems) (see Fig. 2).

The *f*-mi-GQ*K*AAS**IKVAV** peptide was dissolved in PBS and 200 μL (1 mg/mL) was added to the scaffold ([**5**] in Scheme 1). After 2 h at RT with shaking, the scaffolds were washed extensively with PBS, while monitoring the fluorescent intensity in the washes using a fluorescent plate reader (SpectraMax GeminiEM, Molecular Device, USA) at excitation and emission wavelengths of 490 and 515 nm, respectively. The scaffold was washed in PBS until only background signals were detected to ensure all unreacted peptides were removed. As the scaffold was transparent, the fluorescent intensity from the peptide immobilized on the surface of TMC was read directly from the scaffold using the fluorescent plate reader. A blank TMC scaffold (without *f*-mi-GQ*K*AAS**IKVAV**) was used for background signal correction. A negative control was prepared by reacting *N*-ethyl maleimide with TMC prior to adding the *f*-mi-GQ*K*AAS**IKVAV** peptide sequence. *N*-ethyl maleimide was dissolved in DMSO, diluted to 0.01 mg/mL in PBS, and then added to the surface of TMC scaffolds. The reaction was allowed to proceed for 2 h at RT with shaking and then washed thoroughly in PBS before applying *f*-mi-GQ*K*AAS**IKVAV** as before. After 2 h, the scaffolds were washed extensively in PBS, and the washes were monitored until background signal was detected as above, before the amount of *f*-mi-GQ*K*AAS**IKVAV** coupled to the negative control scaffold was quantified.

Degradation of methacrylamide chitosan scaffold

Sterile MC scaffolds were cut into circular discs with diameter of 1.5 cm and thickness of about 0.8 cm. Excess PBS was removed from the surface of the wet scaffolds using Kimwipes before weighing. The degradation of MC scaffold crosslinked with 3, 4, 5, or 7 wt % APS/SMBS was followed over a period of 28 days in the presence and absence of lysozyme (1.5 μg/mL) dissolved in PBS, with 1.5 μg/mL sodium azide, and incubated at 37°C with gentle shaking. The activity of the enzyme was 50,000 units/mg of lysozyme as indicated by the manufacturer. Lysozyme was assumed to have 100% activity throughout the duration of the study. The buffer was also refreshed each day to ensure the activity of the enzyme. The wet weight of the scaffold at each time points, 0 (M_0), 7, 14, 21, and 28 days (M_t) was weighed. The percent mass loss of the scaffolds over time was calculated using Eq. (1).

$$\% \text{ Mass Loss} = \frac{M_0 - M_t}{M_0} \times 100 \quad (1)$$

An accelerated degradation study was also performed as above with lysozyme at 0.5 mg/mL and the buffer was also refreshed each day to ensure the activity of lysozyme.

Cell penetration assay

Sterile MC scaffold was prepared as above and crosslinked with 3 and 7 wt % APS/SMBS in 1 cm by 1 cm square cuvettes which were sterilized by soaking in 70% ethanol overnight. After gelation, the scaffolds were washed extensively as before in PBS to remove excess crosslinking agents. The scaffolds were then soaked in Ultraculture General Purpose Serum-Free Medium (Cambrex Bio Science, Walkersville, MD) for at least 7 h at RT before cell culture. Superior Cervical Ganglion (SCG) was explanted from postnatal day 2 Sprague-Dawley rats according to an established procedure.[28] The explanted ganglia were rinsed in HANKS and cut into 2–3 smaller pieces using No. 5 dissection forceps. The ganglion pieces were placed on the surface of the gel and cultured in 3 mL of media supplemented with 1% penicillin/streptomyosin, 1% glutamine, 3% rat serum, 0.7% cytosine-B-D

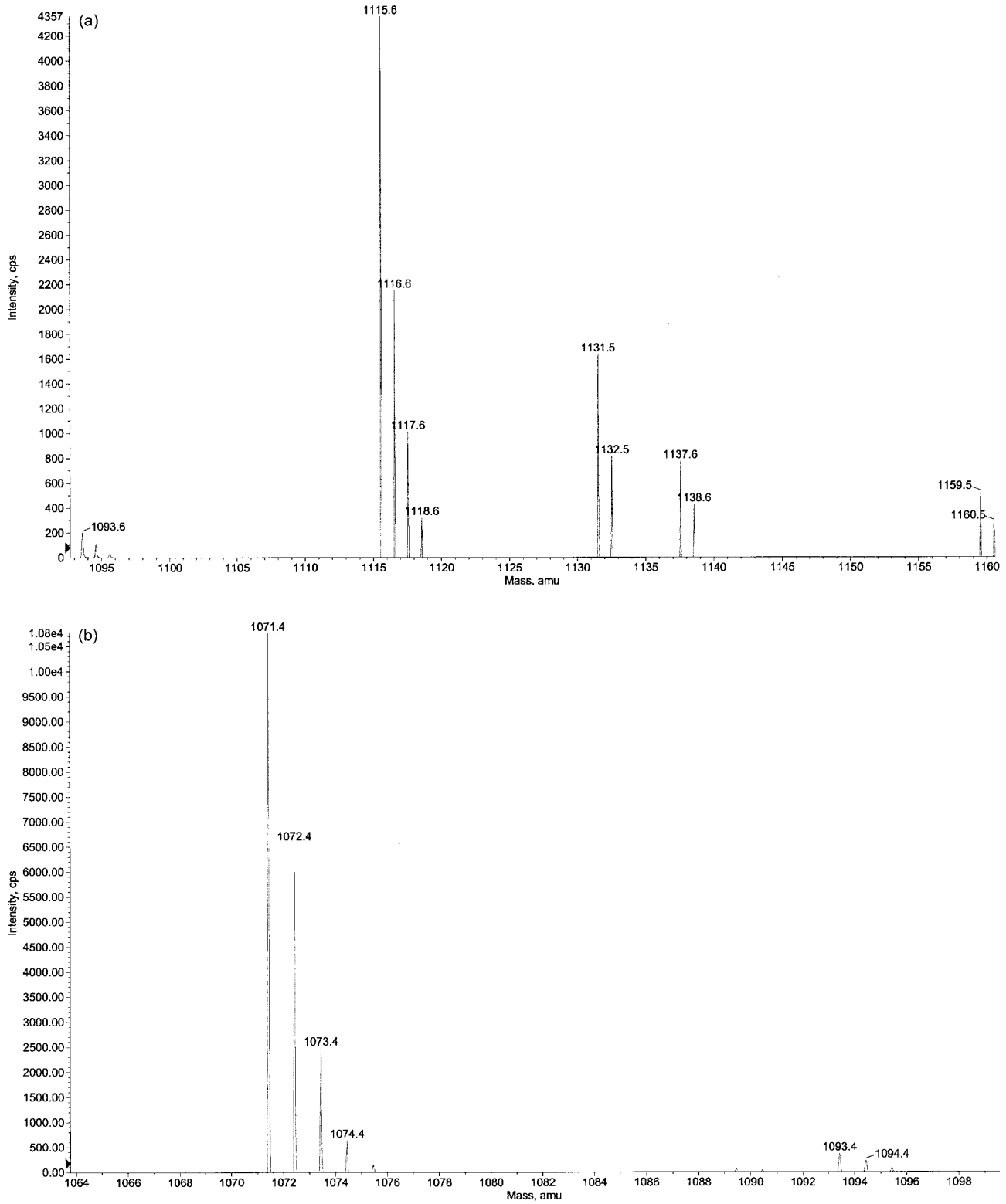


Figure 2. Mass spectra of maleimide-peptides (a) mi-GQAAS**IKVAV** and (b) mi-GDPG**YIGSR**. Mass peaks at 1093.6 and 1071.4 amu confirm the molecular weights of mi-GQAAS**IKVAV** and mi-GDPG**YIGSR**, respectively.

arabino-furanoside (CA), and 50 ng/mL nerve growth factor (NGF). The cuvette was covered by a cap with holes punched using a 20 G needle and incubated at 37°C, 5% CO_2, and 100% humidity.

On day 5, the ganglia were visualized live at 10× magnification on an inverted microscope (LM410 Zeiss, Toronto, ON, Canada) with a CCD camera (Diagnostic Instrument, Michigan, USA) using the SPOT software

(Version 3.1, Diagnostic Instrument). The cuvette was laid down on the side which is closest to the ganglion. As the MC hydrogel is transparent, the ganglion could be visualized by imaging through the hydrogel. The image was then rotated so that the ganglion appears resting on the surface of the MC hydrogel.

Cell culture on peptide-modified chitosan scaffolds

SCG ganglia were dissected as described above from postnatal day 2 Sprague-Dawley rats. The explanted ganglia were rinsed in HANKS and then trypsinized (400 U) for 30 min at 37°C followed by DNAase (400 U) treatment for 1 min at RT. The ganglia were then rinsed with cell culture medium supplemented with 1% penicillin/streptomyosin and 1% glutamine, and triturated to dissociate the ganglia into single neurons.

MC and TMC scaffolds were prepared and washed as described above, cut into discs to fit into a 96-well plate, and sterilized by autoclaving (120°C, 20 min). Differences in structural integrity or initiator concentration required to form the scaffolds were not observed whether the material was sterilized before or after gelation. Peptide solutions of mi-GDPG**YIGSR**, mi-GQAAS**IKVAV**, or a 1:1 (w/w) combination of mi-GDPG**YIGSR** and mi-GQAAS**IKVAV** were dissolved in PBS at a total peptide concentration of 1 mg/mL and sterilized by filtering through 0.22 μm filter. Peptide solution (200 μL) was added to each TMC scaffold and incubated for 2 h at RT with shaking ([**5**] in Scheme 1). The scaffolds were washed extensively in PBS to remove any unreacted peptides, following the protocol developed with fluorescently-labeled peptides (described in previous section). Prior to cell culture, all scaffolds were soaked in cell culture medium for at least 7 h at RT as before. Neurons were plated at 2×10^4 cells/cm^2, with 3% rat serum, 0.7% CA, and 50 ng/mL NGF added to the cell culture medium. After plating, the neurons were incubated at 37°C, 5% CO_2, and 100% humidity.

To determine the specificity of the peptide-cell surface receptor interaction, SCG cells were incubated with a combination of mi-GQAAS**IKVAV** and mi-GDPG**YIGSR** (0.5 mg/mL solution containing both peptides) in culture medium for 30 min at 37°C, 5% CO_2, and 100% humidity prior to plating on the mi-GQAAS**IKVAV** and mi-GDPG**YIGSR** bound scaffold surfaces as a control. The neurons were then collected by centrifugation (1500 rpm, 3 min) and plated on the peptide bound scaffold at a density of 2×10^4 cells/cm^2.

Cell adhesion and axonal growth quantification

Cells were cultured for 2d because at longer time points the neurites became entangled, making it difficult to quantify neurite length and number from individual cell bodies. Following 2d in culture, 10 random, non-overlapping fields were imaged at 20× magnification under an inverted microscope (LM410, Zeiss) with a CCD camera (Diagnostic instrument) using the SPOT software (Version 3.1, Diagnostic Instrument). The number of adherent cells, number of cells with neurites longer than the cell body, and the average length of neurites per mm^2 were measured and analyzed using the SPOT software ($n = 3$ scaffolds, $n = 60$ cells/field analyzed, 10 fields/scaffold).

Statistical analysis

Each experiment was performed in triplicate and results are presented as mean ± standard deviation. Analysis of the variance was performed using single factor ANOVA with an α-value of 0.05. Pairwise multiple comparison procedures were performed using the Bonferroni *t*-test.

RESULTS AND DISCUSSION

Synthesis and characterization of methacrylamide chitosan

Chitosan and chitin are insoluble in water because of intra- and intermolecular hydrogen bonding in the α- and β-conformations.[29,30] However, by disrupting these hydrogen bonds through the modification of the chitosan amine groups, water-soluble chitosan derivatives can be synthesized, as have been reported in the literature.[29,31] Herein, the primary amine group of chitosan repeat units was modified with methacrylic anhydride, yielding methacrylamide chitosan (MC), with a degree of modification of 23.0 ± 1.4% ($n = 3$) as determined by ^{1}H NMR. The methacryl bonds themselves are likely randomly dispersed along the chitosan backbone given that previous studies have shown the distribution of acetyl groups on chitin is random when chitosan is modified with acetic anhydride.[32] The water solubility of the MC is important for tissue engineering applications because it facilitates mixing with pH-sensitive proteins, biologics and cells, and can be easily processed into a crosslinked gel via radical polymerization across methacryl carbon–carbon double bonds using APS/SMBS initiation as shown in Scheme 1. The novel methacrylamide derivative of chitosan reported herein is advantageous over previously reported water soluble chitosan derivatives because this methodology can be used to create macroporous scaffolds in which proteins are immobilized as concentration gradients for axonal guidance during processing using, for example, a gradient maker,[10,33] which is our ultimate goal. Other photocrosslinkable chitosan derivatives[34] allow protein immobilization but not patterned immobilization, which is critical for guidance. The effect of initiator concentration on the morphology, cell penetration, and biodegradation of this novel MC scaffold were investigated.

The morphology of the MC scaffold was highly porous as shown by SEM in Figure 3, with the density of the porous network and pore size affected by the amount of initiator used in crosslinking. At

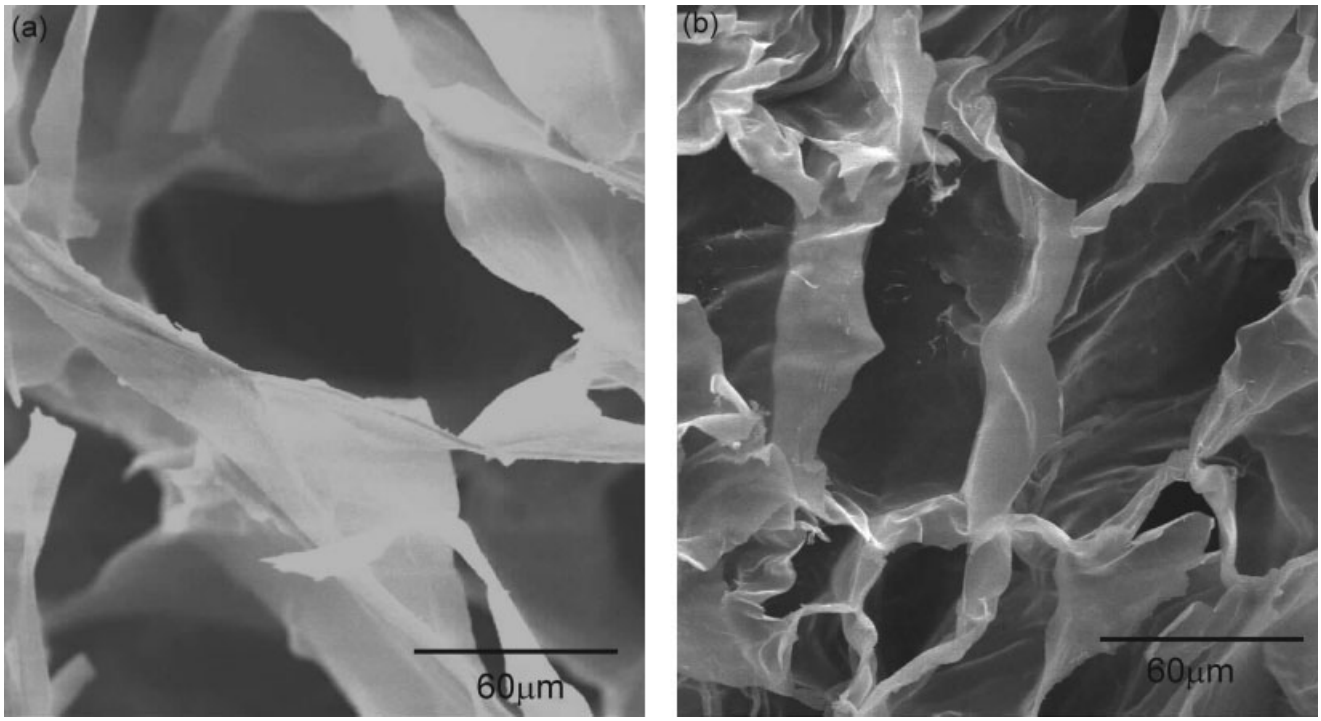


Figure 3. SEM micrograph of the methacrylamide chitosan scaffold show larger pores when crosslinked with (a) 3 wt % APS/SMBS and reduced pore size when crosslinked with (b) 7 wt % APS/SMBS.

lower initiator concentration (3 wt % APS/SMBS), the MC scaffold had larger pore sizes and formed a less dense porous network than those scaffolds formed using 7 wt % APS/SMBS. It is likely that higher initiator concentration produces a more crosslinked network resulting in smaller pores in these MC scaffolds. Interestingly, the MC maintained a porous morphology after crosslinking that resembles the honeycomb morphology previously described for chitin/chitosan gels[13] and is important for solute diffusion.

To determine the suitability of the MC scaffold for tissue engineering applications, primary SCG ganglia were plated on the scaffold and tested for the ease of neuron/neurite penetration into the gel; and single neurons were similarly plated to test cell adhesion, and neurite outgrowth. The transparency of the MC scaffold facilitated visualization of neurite penetration into the hydrogel. Interestingly, the initiator concentration, which was shown to impact scaffold morphology (cf. Fig. 3), also affected cell penetrability. As shown in Figure 4, viewed from a side perspective with the surface of the hydrogel indicated, an SCG ganglion plated on the scaffold crosslinked with 3 wt % APS/SMBS allowed neurite penetration whereas the scaffold crosslinked with 7 wt % APS/SMBS limited neurite extension to the surface of the transparent gel. The outline of the ganglion shown in Figure 4(a) appears to be less clear than the one shown in Figure 4(b) was due to its position from the wall of the cuvette where it was imaged. At lower initiator concentrations, the scaffold structure was more porous and had larger pores, thereby facilitating neurite penetration. The ability of neurites to penetrate into the matrix is important for neural tissue engineering applications, where the scaffold is designed to be a temporary scaffold through which regenerating axons are guided. For example, severed axons must be able to penetrate the polymer scaffold because extracellular cues (chemotatic or haptotactic) patterned within the scaffold will guide them to their targets in nerve repair strategies.[10] The 7 wt % APS/SMBS initiated MC scaffold did not allow neurite penetration [shown in Fig. 4(b)] because the higher crosslink density likely resulted in a scaffold with smaller pores, increased tortuosity and more numerous "dead ends". Qualitatively, the MC scaffolds were stiffer with increased concentrations of initiator and this too may have impacted neurite penetration. Importantly, neurite extension was observed on the surface of the 7 wt %-crosslinked MC scaffold, demonstrating that the excess crosslinker was successfully removed from the scaffold during the washes and that the lack of neurite penetration was independent of adhesion and dependent on penetrability and initiator concentration.

While the inherent cell-adhesiveness of chitosan was important to its initial choice for a scaffold, the methacrylamide derivative reduced the cell adhesiveness

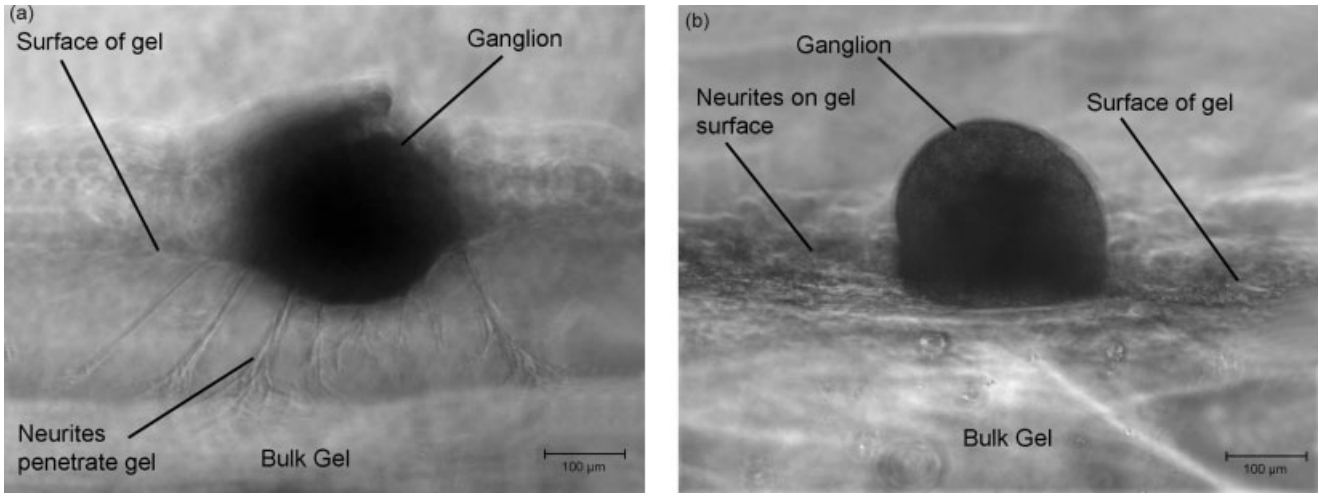


Figure 4. SCG ganglion plated on transparent methacrylamide chitosan scaffold and viewed from the side. Neurites extending from the ganglion were able to penetrate the scaffold crosslinked with (a) 3 wt % APS/SMBS, but they were unable to penetrate the network when crosslinked with (b) 7 wt % APS/SMBS. (The images were obtained by laying the cuvette on its side. The image was then rotated to the proper orientation of the ganglion on the surface of the hydrogel.)

of the MC scaffold, thereby requiring peptide modification. Laminin derived, maleimide-functionalized cell adhesive peptides (mi-GQAAS**IKVAV** and mi-GDPG**YIGSR**) were synthesized to promote cell adhesion on the MC scaffolds. The receptors present on the cell surface, which respond to laminin, are important for cell adhesion and neurite outgrowth, and have been shown to be present on the surfaces of SCG neurons. Therefore, a thiolated MC (TMC) was synthesized by coupling TGA to MC using EDC chemistry to covalently couple the peptides to the scaffold. The thiol content in TMC was measured by the Ellman's test and compared to a control where TGA was reacted with MC in the absence of EDC. In the presence and absence of EDC, the number of thiol groups present was found to be 99.6 ± 1.7 μ mol/g of TMC and 15.9 ± 0.9 μ mol/g of MC, respectively, which are significantly different ($p < 0.001$) and demonstrate covalent addition of free thiol groups to MC. Interestingly, TMC alone did not form a stable scaffold when APS/SMBS was added to initiate crosslinking. It is likely that TGA also reacted with the methacryl group through a Michael-type addition reaction. This was confirmed by ^{1}H NMR (data not shown) in which the peak areas corresponding to the protons on the methylene carbon of the methacrylamide group were greatly reduced on TMC relative to MC. Consequently, a 1:1 w/w blend of MC and TMC was used to form a stable scaffold crosslinked with 4 wt % APS/SMBS. The MC/TMC blend scaffold had 45.7 ± 3.7 μmol thiol groups/g of TMC and was used for peptide modification.

A fluorescein-labelled *f*-mi-GQ*K*AAS**IKVAV** peptide was reacted with the TMC scaffold and the extent of modification was quantified by measuring the fluorescence intensity in the transparent scaffold. To examine the maleimide-thiol addition reaction between the peptide and scaffold, a control scaffold was synthesized in which the thiol groups were quenched by reaction with excess *N*-ethyl maleimide prior to peptide addition to eliminate the reactive functional groups required for modification. The amount of *f*-mi-GQ*K*AAS**IKVAV** peptide bound to the TMC scaffold was 0.78 ± 0.12 μmol/g of TMC whereas the amount bound to the control was 0.07 ± 0.02 μmol/g of TMC, indicating that the peptide was coupled to the TMC scaffold by thiol addition across the peptide-maleimide bond. We assumed that equivalent amounts of mi-GQAAS**IKVAV** and mi-GDPG**YIGSR** were immobilized based on previously published research.[35]

The effect of the two laminin-derived peptides was compared alone and in combination relative to MC (unmodified scaffold) after 2d in culture. Peptide modification of TMC scaffolds significantly improved cell adhesion and neurite outgrowth relative to MC controls, as shown in Figures 5 and 6(a). Optimal adhesion and neurite outgrowth were observed when the surfaces were modified with both peptides, GDPG**YIGSR** and GQAAS**IKVAV**. Interestingly, when SCGs were incubated with mi-GQAAS**IKVAV** and mi-GDPG**YIGSR** in solution prior to plating, cell adhesion to the mixed mi-GQAAS**IKVAV**/mi-GDPG**YIGSR** MC surface decreased to the level of MC alone, indicating that the receptors responsible for cell adhesion were saturated with the soluble peptides, resulting in fewer adherent cells, and consequently reduced neurite outgrowth.

Figure 6(b) compares the average number of cells bearing neurites on each scaffold. All peptide-modified MC scaffolds had significantly greater

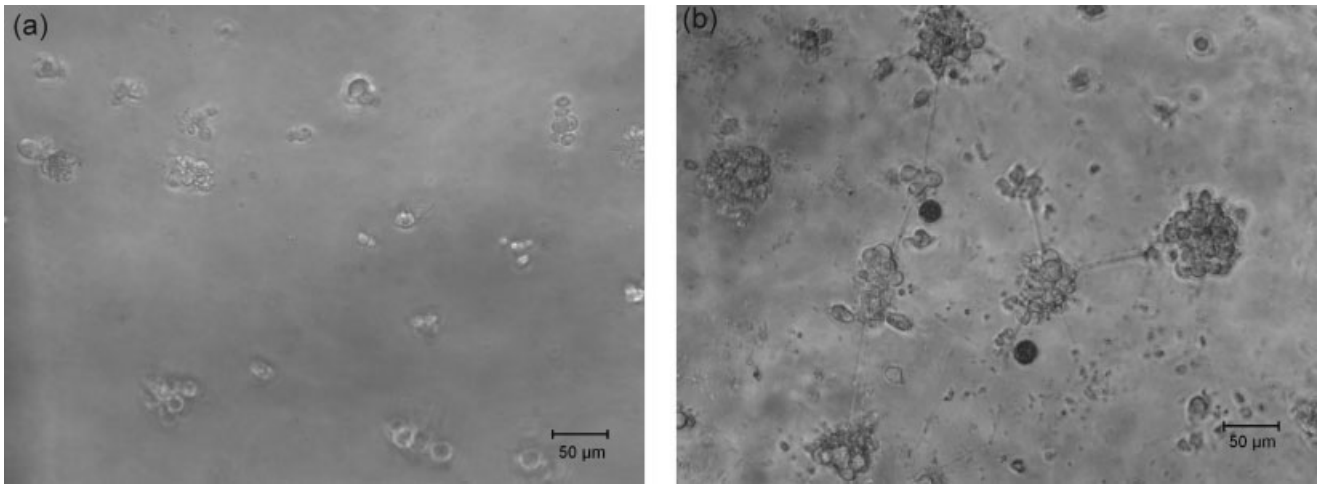


Figure 5. Response of SCG neurons to (a) unmodified MC scaffold and (b) TMC scaffold modified with the combination of **GDPGYIGSR** and GQAAS**IKVAV**. SCG neurons adhered and extended neurites on the peptide-modified scaffold.

numbers of cells bearing neurites than MC alone, with the greatest number observed on the combined mi-GQAAS**IKVAV**/mi-GDPG**YIGSR** MC surface. The combined mi-GQAAS**IKVAV**/mi-GDPG**YIGSR** MC surface had significantly ($p < 0.05$) more cells bearing neurites than either the mi-GQAAS**IKVAV** or mi-GDPG**YIGSR** modified surfaces alone, reflecting the synergism ascribed to these laminin derived

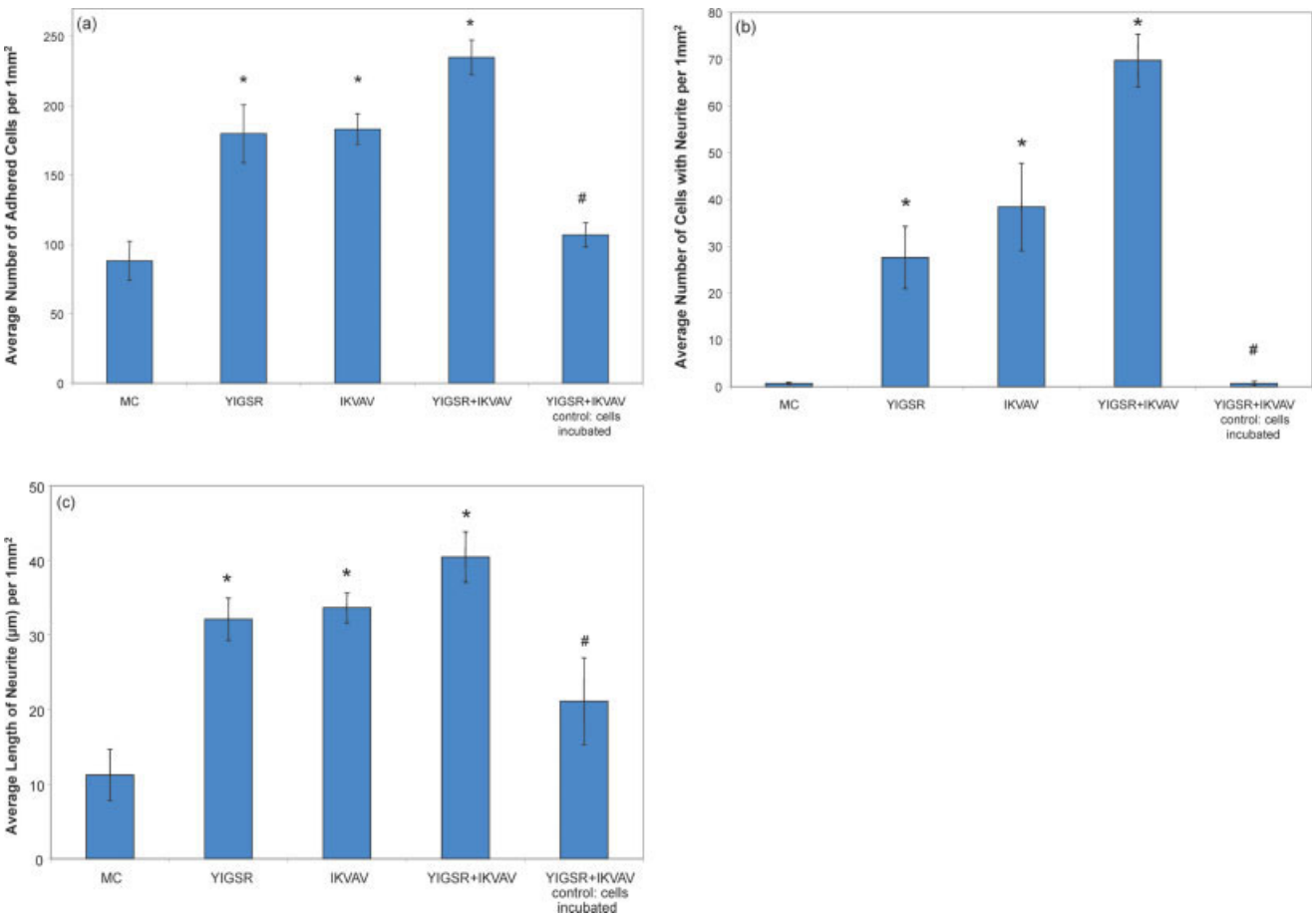


Figure 6. Modification of MC with cell adhesive peptides significantly improve the (a) adhesion of SCG neurons on the surface of the scaffold, as well as increasing (b) the number neurites per cell, and (c) average neurite length ($n = 3$ scaffolds, 60 cells/field and 10 fields/scaffold *$p < 0.05$ versus MC Control, #$p < 0.05$ versus GDPG**YIGSR**+GQAAS**IKVAV** modified scaffold). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sequences for neurite outgrowth and adhesion.[8,36] As was observed for cell adhesion, incubating the SCG cells with soluble mi-GQAAS**IKVAV** and mi-GDPG**YIGSR** prior to plating on the combined peptide surface reduced the number of adherent cells with neurites to MC levels, further confirming the specificity of the receptor-peptide surface interaction.

Peptide modified surfaces result in greater average neurite length compared to MC alone as shown in Figure 6(c), and the combined GQAAS**IKVAV**/GDPG**YIGSR** peptide-modified MC had the longest neurites of the materials compared. As was observed for other metrics of cell-material interaction, when SCG cells were incubated in soluble GQAAS**IKVAV** and GDPG**YIGSR** peptides prior to plating, the average neurite length was similar to that observed on MC alone, demonstrating specificity of the receptor-ligand interaction again. Overall, the combination of both mi-GQAAS**IKVAV**/mi-GDPG**YIGSR** on one surface consistently had the best cellular response in terms of cell adhesion and number of cells with neurites, which is consistent with previous data on different surfaces.[35] Given that YIGSR has been shown to promote cell adhesion and IKVAV to promote neurite outgrowth in sympathetic neurons,[8] the combination of both peptides is likely the most biomimetic of the surfaces presented to the cells. Furthermore, the extended peptide sequences used presented a 3-D structure similar to that found in native laminin and likely enhanced the integrin-peptide interaction.[37]

Biodegradation of methacrylamide chitosan scaffold

Lysozyme is a natural enzyme that degrades chitin and chitosan by hydrolyzing the glycosidic bonds. It contains a hexameric binding domain, designated as A-F. Subsites D and E have been shown to be the cleavage sites for sugar residues, which have high specificity for the acetyl unit, while the unit following can either be the deacetylated or acetylated residue.[38,39] The degradation characteristics of the MC scaffold was studied over a period of 28 days at 37°C using the physiological concentration of lysozyme found in serum of 1.5 μg/ml in PBS.[40] The MC scaffold crosslinked with 3 wt % APS/SMBS degraded by approximately 37% by mass within the first 7 days and by 50% at the end of the 28 days. Biodegradation rates of the 4, 5, and 7 wt % crosslinked scaffolds by lysozyme were slower compared to 3 wt % crosslinked scaffolds, with 31%, 24%, and 16% mass loss by 28 days, respectively. In an accelerated degradation study using 0.5 mg/ml lysozyme shown in Figure 7(b), the 3 wt % crosslinked scaffold was fully degraded in 21 days whereas the 4, 5, and 7 wt % crosslinked scaffolds lost 82%, 75%, and 64% of their masses, respectively. The initial biodegradation rate was higher in the first 7 days than in subsequent days; a similar degradation rate was previously reported for chitosan films and gels.[17,18] Nordtveit et al.[18] reported that the initial fast degradation is enhanced by the presence of 3 or more adjacent acetyl units for lysozyme binding. Reduction in degradation rates after 7 days can be explained by the decreasing number of binding sites for lysozyme.

Reduced biodegradation rates of crosslinked chitosan scaffolds or films have also been reported[41] and mirror our own observations; chitosan crosslinked with glutaraldehyde and dimethyl-3,3-dithiobis-proprionimidate showed markedly reduced biodegradation compared to uncrosslinked chitosan. Increased concentrations of the crosslinking agent

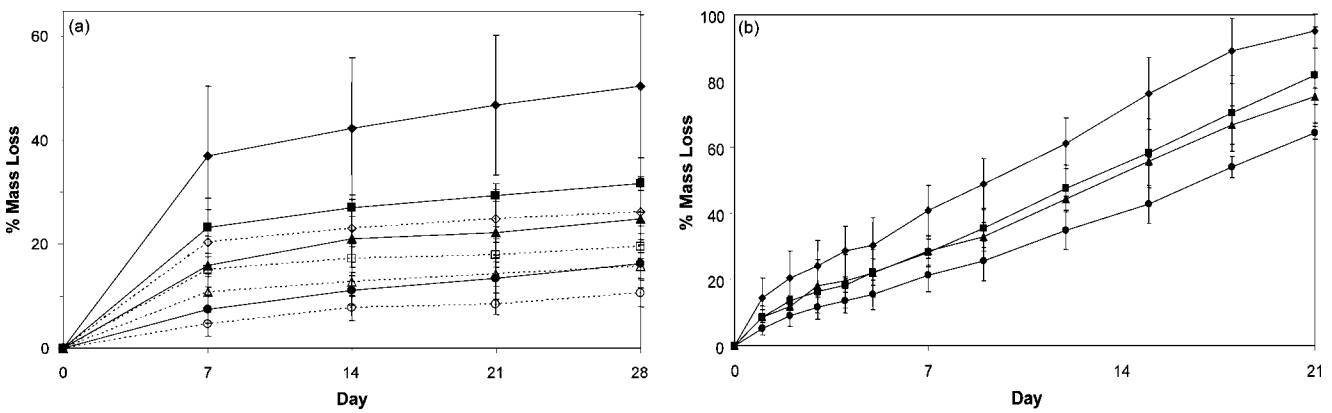


Figure 7. (a) Degradation of MC scaffold in the presence of lysozyme (1.5 μg/ml): solid line, (◆) 3 wt % APS/SMBS, (■) 4 wt % APS/SMBS, (▲) 5 wt % APS/SMBS, and (●) 7 wt % APS/SMBS, and in the absence of lysozyme: dotted line, (◇) 3 wt % APS/SMBS, (□) 4 wt % APS/SMBS, (△) 5 wt % APS/SMBS, and (○) 7 wt % APS/SMBS. Biodegradation rate of the MC scaffold decreased as the concentration of crosslinker used to form the scaffold increased. (b) Accelerated degradation of MC scaffold with 0.5 mg/ml lysozyme demonstrates the scaffold could be fully degraded especially when it is crosslinked with 3 wt % APS/SMBS (◆) [(▲) 4 wt % APS/SMBS, (●) 5 wt % APS/SMBS, (■) 7 wt % APS/SMBS].

(in this case glutaraldehyde) decreased biodegration rates of chitosan, which is similar to what we observed here with increased initiator concentrations. As the amount of initiator increased (from 3 to 7 wt % APS/SMBS), the rate of mass loss decreased (see Fig. 7) because more chains had to be cleaved before they would be solubilized. This phenomenon was evident both in the presence and absence of lysozyme.

In the absence of lysozyme, the crosslinked MC scaffolds degraded as observed by mass loss, albeit slower than what was observed for the mass loss due to lysozyme. Chitin hydrogels have been previously observed to lose mass when formed by acetylating chitosan and this has been attributed to syneresis, which is the thermodynamic imbalance that results from shrinkage accompanied by solvent expulsion from the gel.[42,43] Syneresis was found to be affected by crosslink density which is also observed here. As the initiator concentration used in crosslinking the MC scaffold increases, the chain length between crosslinks decreases and leads to stiffer scaffolds. Consequently, the mobility of the MC polymer chain is reduced, resulting in a slower rate of solvent expulsion, and thus a slower rate of mass loss.

At high initiator concentrations (i.e., 7 wt % APS/SMBS), the rate of mass loss from the MC scaffold was reduced and becomes relatively close to the rate of mass loss in the enzyme-free buffer, as crosslink density affects degradation in the presence and absence of lysozyme (see Fig. 7). Thus, more fragments must be degraded by lysozyme before significant mass loss can be observed. In the end, complete biodegradation of the MC scaffold by lysozyme was observed, demonstrating that methacrylamide modification did not inhibit lysozyme activity. The rate of biodegradation of the MC scaffold can be controlled by varying crosslink density which in turn was shown to influence neurite penetrability.

CONCLUSIONS

We have shown in the present study that a novel derivative of chitosan can be transformed into a scaffold that is biodegradable, allows neurite penetration into its matrix, and can be modified chemically to improve cell adhesion. The physical characteristics of the resulting MC scaffold can be controlled to obtain the desired morphology and biodegradation pattern by adjusting the crosslink density. Taking advantage of the thiol-maleimide coupling reaction, peptides were covalently bound to the MC scaffold. Although peptide modification on the surface does not necessitate a water soluble form of chitosan, the water soluble MC is imperative for 3-D modification of the scaffold with peptides or growth factors using similar chemistry for promoting extensive neurite penetration and axon guidance in regeneration. While we have demonstrated the use of the MC scaffold to support neuronal cell adhesion and differentiation, the results presented here may also benefit other fields of tissue engineering such as cartilage and bone engineering, where this water soluble chitosan may also find utility.

The authors greatly appreciate technical assistance from Dr. Yingfang Chen. The authors would like to thank the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, and Ontario Innovation Trust for financial assistance.

References

1. McDonald JW. Repairing the damaged spinal cord. Sci Am 1999;281:65–73.
2. Schmidt CE, Leach JB. Neural tissue engineering: Strategies for repair and regeneration. Annu Rev Biomed Eng 2003;5: 293–347.
3. Morgenstern DA, Asher RA, Fawcett JW. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res 2002;137:313–332.
4. Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 1994;13:757–767.
5. Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 2004;10:610–616.
6. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984;309:30–33.
7. Graf J, Iwamoto Y, Sasaki M, Martin GR, Kleinman RK, Robey FA, Yamada Y. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. Cell 1987;48:989–996.
8. Tashiro K, Sephel GC, Weeks B, Sasaki M, Martin GR, Kleinman HK, Yamada Y. A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration and neurite outgrowth. J Biol Chem 1989;264:16174–16182.
9. Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. Science 1996;274:1123–1133.
10. Moore K, MacSween M, Shoichet MS. Combined concentration gradients of neurotrophic factors work in synergy to guide neurite outgrowth. Tissue Eng 2005;12:267–278.
11. Luo Y, Shoichet MS. A photolabile hydrogel for guided three-dimensional cell growth and migration. Nat Mater 2004;3:249–253.
12. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials 1999;20:1133–1142.
13. Freier T, Montenegro R, Koh HS, Shoichet MS. Chitin-based tubes for tissue engineering in the nervous system. Biomaterials 2005;26:4624–4632.
14. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: A review. Biomaterials 2000;21:2589–2598.
15. Hirano S. Chitin and chitosan as novel biotechnological materials. Polym Int 1999;48:732–734.
16. Domard A, Rinaudo M. Preparation and characterization of fully deacetylated chitosan. Int J Biol Macromol 1983;5:49–52.
17. Freier T, Koh HS, Kazazian K, Shoichet MS. Controlling cell adhesion and degradation of chitosan films by N-acetylation. Biomaterials 2005;26:5872–5878.

18. Nordtveit RJ, Varum KM, Smidsrod V, Smidsrod O. Degradation of fully water-soluble, partially N-acetylated chitosans with lysozyme. Carbohydr Res 1994;23:253–260.
19. Montembault A, Viton C, Domard A. Physico-chemical studies of the gelation of chitosan in a hydroalcoholic medium. Biomaterials 2005;26:933–943.
20. Ho MH, Wang DM, Hsieh HJ, Liu HC, Hsien TY, Lai JY, Hou LT. Preparation and characterization of RGD-immobilized chitosan scaffolds. Biomaterials 2005;26:3197–3206.
21. Holappa J, Nevalainen T, Soininen P, Masson M, Jarvinen T. Synthesis of novel quaternary chitosan derivatives via *N*-chloroacyl-6-*O*-triphenylmethylchitosans. Biomacromolecules 2006;7:407–410.
22. Kubota N, Tatsumoto N, Sano T, Toya K. A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents. Carbohydr Res 2000;324:268–274.
23. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC. Thiolation of chitosan. Attachment of proteins via thioether formation. Biomacromolecules 2005;6:880–884.
24. Weiping S, Changqing Y, Yanjing C, Zhiguo Z, Xiangzheng K. Self-assembly of an amphiphilic derivative of chitosan and micellar solubilization of puerarin. Colloids Surf B Biointerfaces 2006;48:13–16.
25. Kim SE, Park JH, Cho YW, Chung H, Jeong SY, Lee EB, Kwon IC. Porous chitosan scaffold containing microspheres loaded with transforming growth factor-β1: Implications for cartilage tissue engineering. J Control Release 2003;91:365–374.
26. Lavertu M, Xiz Z, Serreqi AN, Berrada M, Rodrigues A, Wang D, Buschmann MD, Gupta A. A validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan. J Pharm Biomed Anal 2003;32:1149–1158.
27. Kast CE, Bernkop-Schnurch A. Thiolated polymers—Thiomers: Development and in vitro evaluation of chitosan-thioglycolic acid conjugates. Biomaterials 2001;22:2345–2352.
28. Mahanthappa NK, Patterson PH. Culturing mammalian sympathoadrenal derivatives. In: Banker G, Goslin K, editors. Culturing Nerve Cells, 2nd ed. Cambridge: MIT Press; 1998, p 295–299.
29. Hirano S, Yamaguchi Y, Kamiya M. Water-soluble *N*-(*n*-Fatty acyl) chitosans. Macromol Biosci 2003;3:629–631.
30. Roberts GAF. Chitin Chemistry, 1st ed. London: Macmillan; 1992.
31. Bernkop-Schnurch A, Hornof M, Zoidl T. Thiolated polymers—Thiomers: Synthesis and in vitro evaluation of chitosan-2-iminothiolane conjugates. Int J Pharm 2003;260:229–237.
32. Vachoud L, Zydowicz N, Domard A. Formation and characterization of a physical chitin gel. Carbohydr Res 1997;302:169–177.
33. Kapur TA, Shoichet MS. Immobilized concentration gradients of nerve growth factor guide neurite outgrowth. J Biomed Mater Res A 2004;68:235–243.
34. Obara K, Ishihara M, Ishizuka T, Fujita M, Ozeki Y, Maehara T, Saito Y, Yura H, Matsui T, Hattori H, Kikuchi M, Kurita A. Photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing in healing-impaired db/db mice. Biomaterials 2003;24:3437–3444.
35. Tong YW, Shoichet MS. Enhancing the neuronal interaction on fluoropolymer surfaces with mixed peptides or spacer group linkers. Biomaterials 2001;22:1029–1034.
36. Suzuki M, Itoh S, Yamaguchi I, Takakuda K, Kobayashi H, Shinomiya K, Tanaka J. Tendon chitosan tubes covalently coupled with synthesized laminin peptides facilitate nerve regeneration in vivo. J Neurosci Res 2003;72:646–659.
37. Shaw D, Shoichet MS. Toward spinal cord injury repair strategies: Peptide surface modification of expanded poly (tetrafluoroethylene) fibers for guided neurite outgrowth in vitro. J Craniofac Surg 2003;14:308–316.
38. Varum KM, Kristiansen Holme H, Izume M, Stokke BT, Smidsrod O. Determination of enzymatic hydrolysis specificity of partially N-acetylated chitosans. Biochim Biophys Acta 1996;1291:5–15.
39. Pangburn SH, Trescony PV, Heller J. Lysozyme degradation of partially deacetylated chitin, its film, and hydrogels. Biomaterials 1982;3:105–108.
40. Porstmann B, Jung K, Schmechta H, Evers U, Pergande M, Porstmann T, Kramm HJ, Krause H. Measurement of lysozyme in human body fluids: Comparison of various enzyme immunoassay techniques and their diagnostic application. Clin Biochem 1989;22:349–355.
41. Adekogbe I, Ghanem A. Fabrication and characterization of DTBP-crosslinked chitosan scaffolds for skin tissue engineering. Biomaterials 2005;26:7241–7250.
42. Vachoud L, Zydowicz N, Domard A. Physiochemical behaviour of chitin gels. Carbohydr Res 2000;326:295–304.
43. Vachoud L, Zydowicz N, Domard A. Physiochemical properties of physical chitin hydrogels: Modeling and relation with the mechanical properties. Biomacromolecules 2001;2:1294–1300.