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Development of affinity-based delivery of NGF from a chondroitin sulfate biomaterial

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Key words: chondroitin sulfate, nerve growth factor, spinal cord injury, hydrogel, nerve regeneration, controlled release

Abbreviations: ANOVA, analysis of variance; BP, binding peptide; BSA, bovine serum albumin; C6S, chondroitin-6-sulfate; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; GAG, glycosaminoglycan; NGF, nerve growth factor; PEG, poly(ethylene glycol); Mal, maleimide; PBS, phosphate buffered saline; SE, standard error

Chondroitin sulfate is a major component of the extracellular matrix in both the central and peripheral nervous systems. Chondroitin sulfate is upregulated at injury, thus methods to promote neurite extension through chondroitin sulfate-rich matrices and synthetic scaffolds are needed. We describe the use of both chondroitin sulfate and a novel chondroitin sulfate-binding peptide to control the release of nerve growth factor. Interestingly, the novel chondroitin sulfate-binding peptide enhances the controlled release properties of the chondroitin sulfate gels. While introduction of chondroitin sulfate into a scaffold inhibits primary cortical outgrowth, the combination of chondroitin sulfate, chondroitin sulfate-binding peptide and nerve growth factor promotes primary cortical neurite outgrowth in chondroitin sulfate gels.

Introduction

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) found attached to a protein core to form a proteoglycan. Chondroitin sulfate proteoglycans (CSPGs) play an important role in the extracellular matrix in the central nervous system.¹ In neuronal development and regeneration, CSPGs modulate a wide range of activities from cell adhesion and division to synaptic plasticity and regeneration,^{2,3} and several studies have shown that the activity of CSPGs can be attributed to the sulfation pattern of the CS chains.⁴⁻⁶ Previous work has investigated the effects of the CS GAG in vitro both in solution or adsorbed onto a flat surface,⁷⁻¹⁰ but few have examined its effects on neurite outgrowth in three-dimensional gels.^{6,11,12}

CS-based biomaterials have been developed for a variety of applications, including cartilage tissue engineering^{13,14} and wound healing.¹⁵ In many of these studies, CS is modified either for covalent cross-linking for gel synthesis or for incorporation into the scaffold; however, the chemical modification of CS can interfere with potential binding sites and reduce CS bioactivity. Other systems physically entrap CS within the matrix, and diffusion of CS is controlled by the physical properties of the biomaterial.¹⁶ Other GAGs have been incorporated into hydrogels as well, and in some cases, these have been added by integrating GAG-binding domains into the biomaterial. Sakiyama-Elbert et al. have developed an affinity-based system where heparin was incorporated in fibrin matrices through heparin-binding

peptides.^{17,18} This group has also shown that growth factor activity can be regulated by GAG-binding through sequestration and localization of growth factor activity.¹⁹ Sakiyama-Elbert et al. have improved neurite outgrowth both in vitro²⁰⁻²² and in vivo²³ through affinity-based delivery of neurotrophins.

Our lab has developed a poly(ethylene glycol)(PEG)-co-peptide polysaccharide system that has tunable viscoelastic and biological properties, as seen in **Figure 1**.²⁴⁻²⁷ In earlier studies, we incorporated heparin in the system in order to bind cell-penetrating peptides. In the current study, we have modified the material specifically to incorporate CS and take advantage of native interactions between CS and nerve growth factor (NGF) for controlled release. The mechanical properties of this material are controlled both through physical interactions of GAG-binding peptides, covalently bound to eight-arm PEG, with GAGs and through the cross-linking of eight-arm PEG (black lines) with bi-functional enzymatically degradable cross-linking peptides (dark gray dumbbells) that include an integrin-binding sequence (RGD). Unmodified CS (striped stars) is entrapped within the biomaterial through interactions with CS-binding peptides (light gray hexagons) conjugated to eight-arm PEG. Finally, CS provides binding sites for the incorporation of NGF (spotted triangles).

In earlier work, we demonstrated the viability of chondroitin-6-sulfate (C6S)-based scaffolds to support outgrowth of dorsal root ganglia (DRGs) in vitro.²⁸ Thus, this system has potential for use as a therapeutic implantable hydrogel to promote regeneration of neurons in traumatic root avulsion brachial plexus

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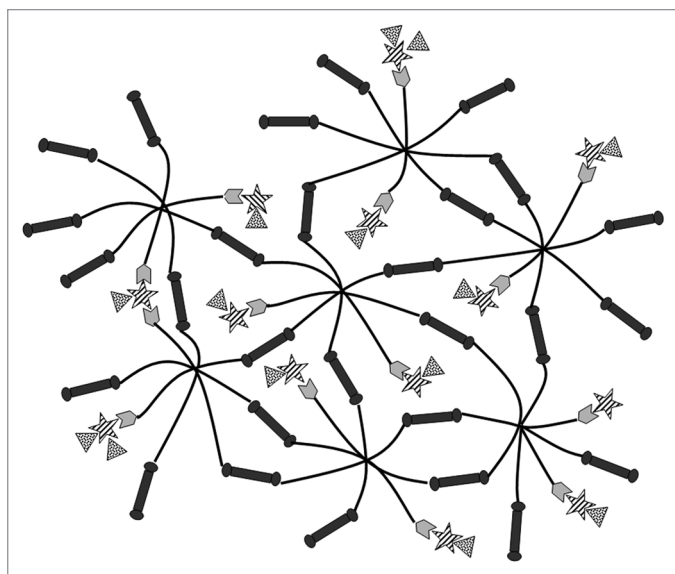


Figure 1. Affinity-based NGF delivery from PEG-co-peptide CS system. Eight-arm PEG (black lines) are modified with bi-functional cross-linking peptides (dark gray dumbbells) on 6 arms and CS-binding peptides (light gray hexagons) on 2 arms. CS (striped stars) interacts with CS-binding peptides and NGF (spotted triangles).

injuries. However, regeneration in these injuries will require both peripheral and central nerve growth, and previous studies have revealed that C6S inhibits the regeneration of central neurons.²⁹ This lack of central nervous system neuronal growth will likely prevent successful reintegration of the central and peripheral nervous systems if a C6S-based material were implanted in an *in vivo* model. Incorporation of the C6S-binding peptide described in previous work and investigated in the current work may help block these inhibitory signals and promote recovery after traumatic root avulsion brachial plexus injuries.^{29,30}

To validate, *in vitro*, the potential use of this system as a therapy, we investigated the controlled release of NGF from this C6S-based biomaterial. In addition, we investigated the effects of NGF release on primary cortical neurite outgrowth. Controlled release of NGF is achieved via non-covalent interactions between NGF, CS and CS-binding peptide. Neurite outgrowth was inhibited on gels that only included C6S, but this inhibition was overcome when NGF was incorporated into the gel.

Results

To investigate the effect peptide and CS incorporation into PEG gels had on the viscoelastic properties of gels, the compositions shown in Table 1 were investigated using rheology. As negative controls, gels without C6S and/or BP (binding peptide) were tested. Figure 2 shows the complex modulus (G^*) for the different gel compositions at 0.5–50 rad/s frequency and 0.5% strain. The PEG gel without C6S and BP (PEG) was the strongest, while the PEG gel with C6S and BP (PEG-BP-C6S) was the weakest. The two-way repeated measures ANOVA showed that

Table 1. Gel compositions

Gel name	C6S-binding	C6S	NGF
Rheology			
PEG	○	○	○
PEG-C6S	○	●	○
PEG-BP	●	○	○
PEG-BP-C6S	●	●	○
NGF Release			
PEG-NGF	○	○	●
PEG-C6S-NGF	○	●	●
PEG-BP-NGF	●	○	●
PEG-BP-C6S-NGF	●	●	●
Cortical Cell Culture			
PEG-BP	●	○	○
PEG-BP-C6S	●	●	○
PEG-BP-NGF	●	○	●
PEG-BP-C6S-NGF	●	●	●

the addition of C6S and BP significantly affected the viscoelastic properties of the gels.

From the frequency sweep, 10 rad/s was chosen from the linear viscoelastic range, and a time sweep was performed at 0.5% strain for 6 min. Figure 3 shows the averaged complex modulus for the different gel compositions. At 10 rad/s and 0.5% strain, the PEG gel (100 Pa) was significantly ($p < 0.05$) stronger than all other gels with C6S and/or BP. The weakest gel (~38 Pa) contained both BP and C6S (PEG-BP-C6S) and was not statistically different ($p > 0.05$) from gels that contained either C6S (PEG-C6S) or BP (PEG-BP). These results demonstrate that PEG gels that contain either BP or C6S are significantly weaker than gels without BP or C6S.

To demonstrate that inclusion of C6S would provide a controlled release mechanism, studies were done to investigate NGF release from the various gel compositions shown in Table 1. The amount of NGF released over 48 h was quantified with an ELISA kit. As negative controls, NGF release from gels without C6S and/or BP was monitored to determine if C6S and BP affected NGF release from PEG gels. The release profiles of the gels are shown in Figure 4. PEG gels that contained only C6S (PEG-C6S) had the fastest NGF release, while gels that contained both BP and C6S had the slowest release profile (PEG-BP-C6S). The two-way ANOVA analysis showed that the addition of BP to PEG gels was a significant factor affecting NGF release, whereas C6S was not. Table 2 shows the results from the post-hoc test to determine significance of NGF release between gels at different time points (0–48 h). At earlier time points (0–7 h), the amount of NGF released from the PEG-C6S gel is significantly greater than NGF released from gels with the BP (Table 2, light shading). At later time points (4–48 h), NGF release is significantly slower in PEG-BP-C6S than in all other gels (Table 2, dark shading). These results show that PEG gels that contain only BP have a slower NGF release profile when compared with PEG controls, while gels that contain both BP and C6S demonstrate the slowest NGF release profile.

To examine the effects of C6S, BP and controlled NGF release from the gels on neurite outgrowth, cortical neurons were cultured on PEG gels of different compositions (Table 1) for 48 h. The average and maximum length and number of neurites was quantified for each neuron. Approximately 80–200 neurons were analyzed for each gel composition. Figure 5 shows (1) the average neurite length, (2) maximum neurite length (3) and number of neurites for each gel composition. Controls consisted of neurons cultured on PEG gels that did not include C6S and/or NGF. PEG gels that included only C6S (PEG-C6S) had lower average/maximum neurite length and number of neurites compared with PEG gels without C6S (PEG-BP). Therefore, C6S in PEG gels inhibits neurite outgrowth.

When NGF was incorporated into PEG gels (PEG-BP-NGF), the average and maximum neurite lengths were significantly higher than gels without NGF (PEG-BP and PEG-BP-C6S). In gels with C6S, the neurite length with NGF (PEG-BP-C6S-NGF) was significantly higher than gels without NGF (PEG-BP and PEG-BP-C6S). These results (Fig. 5A and B) show that NGF incorporation into PEG gels not only stimulates neurite growth, but also overcomes the inhibitory effects of C6S.

Neurons cultured on PEG-BP-NGF had the same number of neurites as neurons cultured on PEG-BP. Furthermore, neurons cultured on PEG-BP-C6S-NGF had the same number of neurites as neurons cultured on PEG-C6S (Fig. 5C). Therefore, NGF had no effect on the number of neurites. The results from cortical neuron culture on PEG gels (Fig. 5) showed that C6S decreased neurite outgrowth, while NGF increased neurite length.

Discussion

The PEG-co-peptide polysaccharide biomaterial developed by Seal and Panitch²⁴⁻²⁶ is a model system to investigate the effects of affinity-based delivery of biological molecules on cortical neuron behavior. In this study, we modified the PEG-co-peptide polysaccharide system by incorporating C6S through C6S-binding peptides conjugated to eight-arm PEG rather than using heparin and heparin-binding peptides as done in the original materials. Rheological results shown in Figure 1 demonstrate that PEG gels

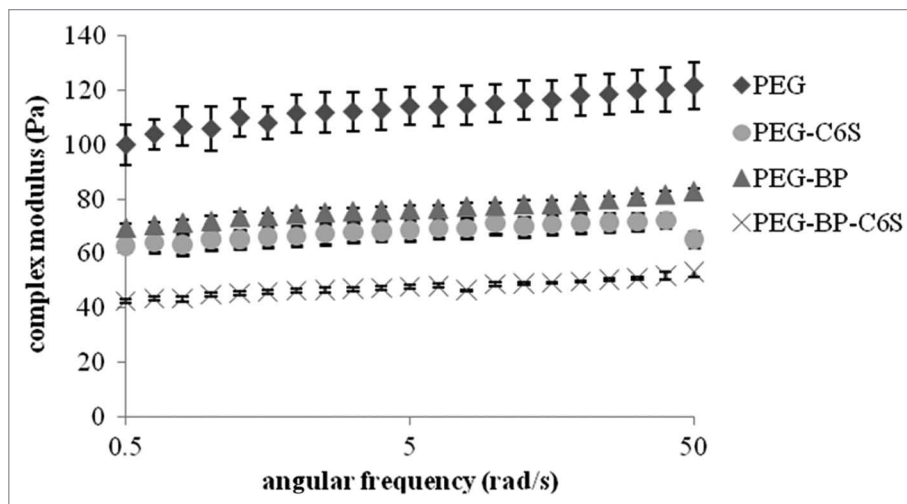


Figure 2. Frequency sweep (0.5–500 rad/s) of gels at 0.5% strain. PEG gels without C6S or BP had the highest viscoelastic properties while gels with C6S and/or BP had significantly lower complex moduli. Mean \pm SE.

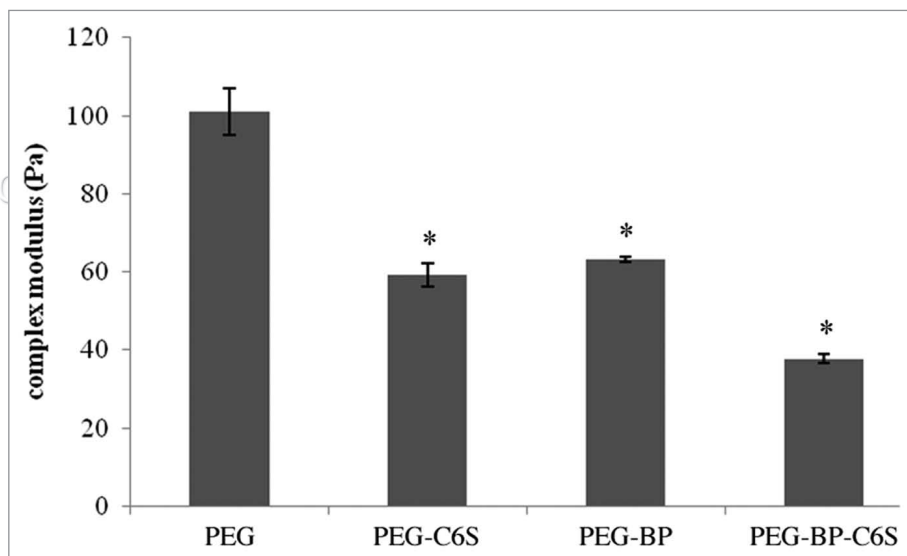


Figure 3. Time sweep of PEG gels at 10 rad/s and 0.5% strain. PEG gels that contained C6S and/or BP had significantly lower complex moduli than gels without C6S or BP. Mean \pm SE, * p < 0.05 different relative to PEG.

that included C6S-binding peptide and/or C6S were weaker than PEG gels without C6S and C6S-binding peptide. The cross-linking reaction of the bi-functional peptides with PEG-maleimide (Mal) is extremely fast. It is possible that C6S and C6S-binding peptides, which were added to the PEG-Mal solution before cross-linking, act as physical barriers between cross-linking sites on different eight-arm PEG molecules, leading to decreased intermolecular cross-linking and weaker gels. It is also possible that after conjugation of two C6S-binding peptides to the star polymer, the efficiency of the cross-linking of the remaining six arms is reduced. Indeed, when C6S and C6S-binding peptide are both incorporated into gels, the PEG gels are the weakest. Further studies are needed to elucidate why cross-linking is suppressed.

Table 2. Summary of post-hoc test of NGF release from different gels

Gels with NGF ^a	Time (hrs) ^b						
	0	2	4	7	18	32	48
PEG		BP-C6S	BP-C6S	BP-C6S	BP-C6S	BP-C6S	BP-C6S
PEG-C6S (C6S)	BP BP-C6S	BP BP-C6S	BP BP-C6S	BP BP-C6S	BP-C6S	BP-C6S	BP-C6S
PEG-BP (BP)	C6S	C6S	C6S BP-C6S	C6S BP-C6S	BP-C6S	BP-C6S	BP-C6S
PEG-BP-C6S (BP-C6S)	C6S	C6S PEG	all other gels	all other gels	all other gels	all other gels	all other gels

^aGel compositions with NGF. ^bAt each time point, the gel that is statistically significant ($p < 0.05$) from the gel composition in the left column is listed.

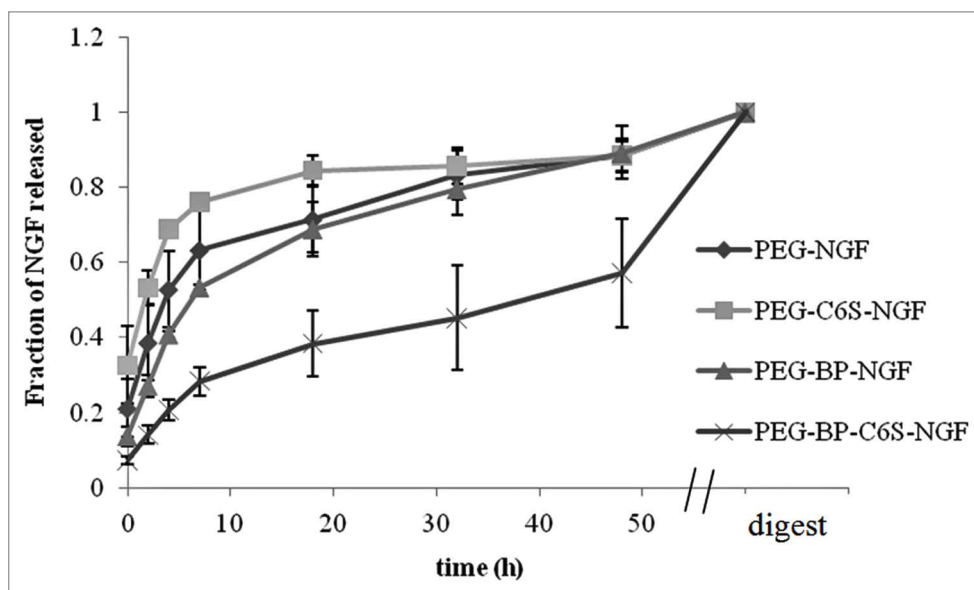


Figure 4. NGF release profile of gels with and without BP and C6S. NGF release was monitored over 48 h. After 2 d, the gels were digested and the amount of NGF quantified. PEG gels that include BP and C6S had the slowest release while PEG gels that only had C6S had the fastest release. Mean \pm SE.

The mechanical strength of the gels may affect NGF release from the PEG gels. In the NGF release assay, PEG gels with only C6S and no C6S-binding peptide had the fastest NGF release. Since these gels are weaker than PEG gels without C6S, it is likely that the cross-link density is decreased, thus increasing the molecular weight between cross-links and the average pore size. NGF could have quickly diffused out of the larger pores that are characteristic of lower cross-link density gels. The NGF assay also showed that PEG gels with C6S and C6S-binding peptide released NGF the slowest compared with all other gels. Although the PEG-BP-C6S gel was the most compliant, the binding between NGF, C6S and C6S-binding peptide was strong enough to prevent rapid diffusion from the weaker gel. These results show that C6S and the C6S-binding peptide are both important for slow diffusion of NGF from PEG gels and, possibly, point to a synergistic NGF-binding activity between C6S and the C6S-binding peptide, since NGF release was slowest when gels contained both of these molecules. This hypothesis is supported by other work with C6S gels, where fluorescence recovery

after photobleaching studies demonstrated that C6S gels containing this C6S-binding peptide exhibited lower NGF diffusivity when compared with gels with C6S only.³⁰

One of the goals of the current study was to investigate the effects of C6S immobilized in gels on cortical neuron behavior. Previous studies showed that DRG neurite extension is inhibited by chondroitin-4,6-sulfate⁶ and dermatan sulfate¹² immobilized in agarose gels, however, the CS used in these studies was modified for covalent attachment, which may interfere with CS-cell interactions. Therefore, the PEG-peptide polysaccharide system described in the current study is an ideal biomaterial for investigating the effects of unmodified CS in gels. Primary cortical neurons were used in this

study to model cellular behavior following injury to the central nervous system.³¹ Results showed that neurite length and number of neurites were significantly lower in gels with C6S compared with gels without C6S and demonstrate that C6S in PEG gels inhibits cortical neurite outgrowth.

CS can influence cell behavior not only through direct interaction, but also indirectly, through growth factor modulation.^{19,32} Thus, another goal of this study was to investigate whether the addition of NGF to the system could overcome the inhibitory effects of C6S; this was motivated by studies by Zhou et al. that showed that NGF promotes DRG growth over CSPG-coated slides.³³⁻³⁵ In the current study, the effects of C6S and NGF in gels on cortical neurons were investigated. In PEG gels with only NGF, the average and maximum neurite length was significantly greater than in PEG gels without NGF. This result agrees with previous studies that demonstrate the growth-promoting effects of NGF both in vitro and in vivo.^{34,35} In PEG gels with C6S and NGF, the neurite length was significantly greater than PEG gels without C6S and NGF. However, the number of neurites in

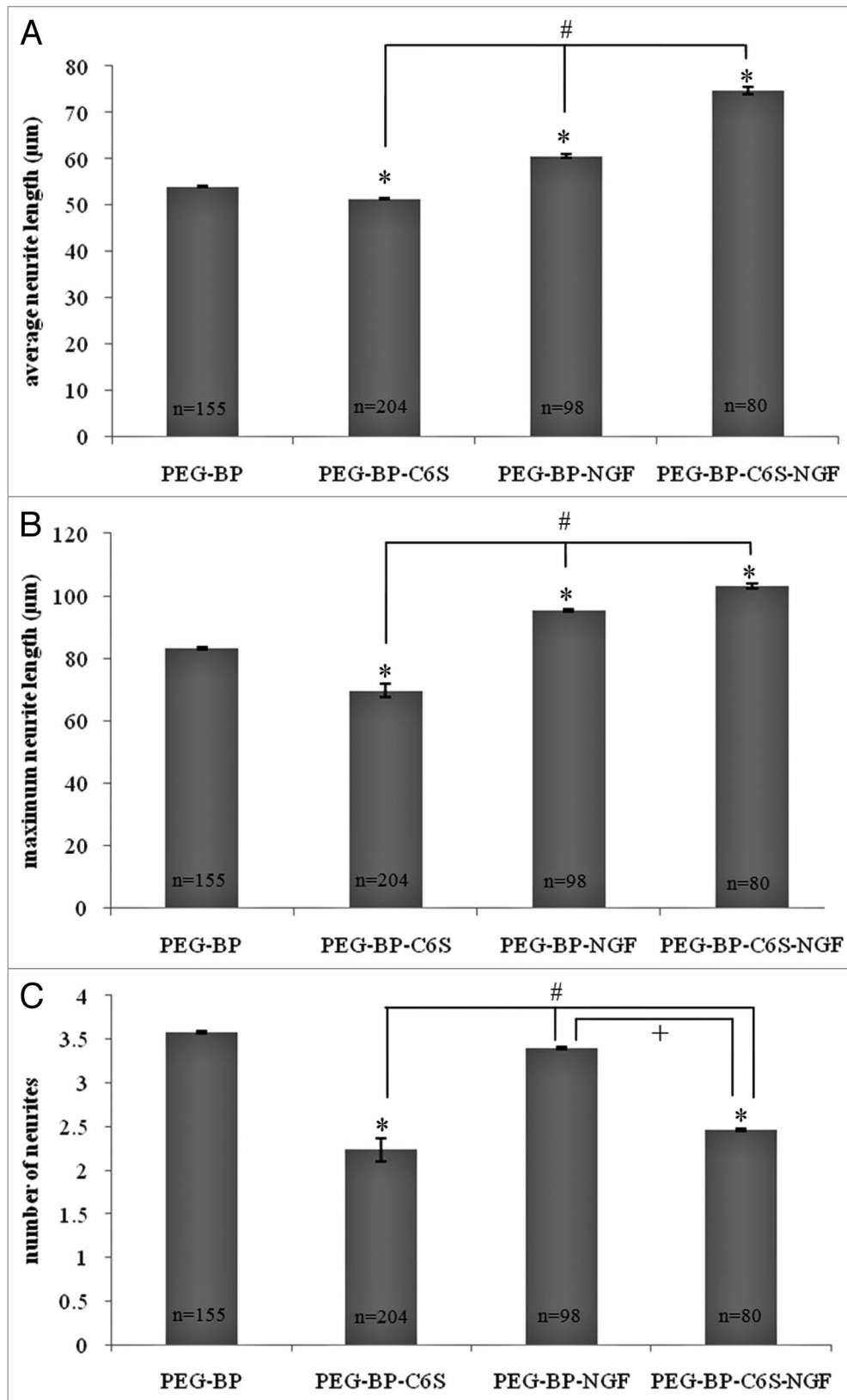


Figure 5. Effect of C6S and NGF on cortical neuron outgrowth. Neurons were cultured on gels with and without C6S and/or NGF for 48 h. The average neurite length (A), maximum neurite length (B), and number of neurites (C) were determined for each gel composition. Mean \pm SE, * $p < 0.05$ relative to PEG-BP, # $p < 0.05$ relative to PEG-BP-C6S, + $p < 0.05$ relative to PEG-BP-NGF.

Table 3. Peptide summary

Peptide name	Peptide function	Peptide sequence
BP	C6S-binding peptide	EKRIWFPYRRFGC
xlinker	bi-functional enzymatically degradable cross-linking peptide	GCR GDS GPQ GIA GQG C

PEG gels with C6S and NGF was significantly less than PEG gels without C6S and NGF. Therefore, NGF can overcome the inhibitory effects of C6S for neurite extension but not for neurite branching and outgrowth, resulting in neurons with fewer and longer neurites. Furthermore, it has been shown that addition of the C6S-binding peptide to matrices of C6S boosts their ability to support DRG growth, possibly through enhanced sequestering of NGF inside the hydrogel and blocking of any potential C6S inhibition.³⁰

In general, C6S inhibits neurite outgrowth and acts as a barrier for axon connections during development and after spinal cord injury. It is hypothesized that C6S provides an inhibitory substrate with which growth-promoting molecules, such as growth factors, can adhere, and provide guidance cues for neurite extension and connection.³² Therefore, the PEG-peptide polysaccharide system that incorporates C6S and NGF through non-covalent interactions is an ideal model for the developing central nervous system.

One of the goals of neural tissue engineering is to promote axonal regeneration for functional recovery. Often, the use of NGF and other growth-promoting molecules causes an uncontrolled increase in neurite outgrowth, which can lead to superfluous connections that cause side effects, such as neuropathic pain.³⁶ Therefore, it is important that for neural tissue engineering, the biomaterial must not only encourage neurite outgrowth but also provide guidance cues for meaningful connections.³⁷ In this study, C6S provides an inhibitory background while NGF promotes neurite outgrowth leading to fewer longer neurites. Future studies will pattern NGF on C6S substrates to guide neurite growth for functional connections.

Materials and Methods

The C6S-binding peptide was identified through peptide array screening in a previous study in reference 38. The peptides listed in Table 3 were synthesized through standard Fmoc-solid phase chemistry on a Symphony peptide synthesizer (Protein Technologies, Inc.) at 200 μ M with rink amide resin (Anaspec, 20084). Fmoc-protected amino acids were activated by O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (Synbiosci, REAG2) and added to the growing peptides in 5 M excess. After peptide synthesis, the peptides were cleaved from the resin using a cleave cocktail containing 92.5% trifluoroacetic acid (Acros Organics, 139725000), 2.5% water, 2.5% triisopropylsilane (TCI America, T1533), and 2.5% ethanedithiol (Alfa Aesar, L12865). Peptides were precipitated in 10x excess ice-cold diethyl ether (Mallinckrodt Chemicals,

0848-10) and centrifuged at 5,000 rpm for 30 min. The ether was decanted off and the peptide was resolubilized in water before lyophilization.

Peptide purification was performed using reverse phase chromatography with an ÄKTA Explorer system (GE Healthcare) equipped with a C18 column (Grace Vydac; 22 mm internal diameter, 250 mm length, 10–15 μ m particle size). After the column was equilibrated with 5 column volumes of water containing 0.1% trifluoroacetic acid, the peptide was loaded onto the column and subjected to an increasing linear gradient from 0 to 60% of acetonitrile (Sigma-Aldrich, 34998) containing 0.1% trifluoroacetic acid over 12 column volumes. The collected peptides were lyophilized, and the mass of each peptide was confirmed with matrix-assisted laser desorption/ionization-time of flight mass spectrometry on a Voyager-DE STR spectrometer (Applied Biosystems).

C6S-binding peptides were conjugated to maleimide-functionalized eight-arm PEG-Mal (MW ~40,000 g/mol, Nektar Therapeutics) following a modified version of previous studies in references 24–27. Peptides were conjugated to PEG-Mal through a Michael-type addition in 1x PBS (pH 7.4, Invitrogen, 14040182) containing 2 mM EDTA (Sigma-Aldrich, E6758). Two arms were conjugated with C6S-binding peptide, while the other six arms were cross-linked with enzymatically degradable bi-functional cross-linking peptide. First, the C6S-binding peptide was conjugated to PEG-Mal by preparing a 2% (w/v) solution of PEG-Mal and C6S-BP at 2-molar excess of eight-arm PEG. The solution was incubated in the dark at room temperature for 1 h. After conjugation, the PEG-co-C6S-BP solution was kept in the dark on ice.

A 2% (w/v) solution of C6S (Sigma-Aldrich, C4384) was added in a 1:4 molar ratio of C6S to PEG-co-peptide. Finally, the bi-functional cross-linking peptide (xlinker) in a 2% (w/v) solution was added in a 3:1 molar ratio of PEG or PEG-BP. The gels were allowed to cross-link for 1 h at room temperature. Table 1 shows the gel compositions used in this study.

The viscoelastic properties of the hydrogels were measured with a Physica MCR 101 rheometer (Anton Paar) using a parallel-plate geometry with a 20 mm diameter and 100- μ m gap. The temperature of the rheometer surface was controlled at 20°C with a built-in Peltier system. To prevent evaporation of the sample, an evaporation-blocking chamber was lowered over the sample. Gels (100 μ l) were prepared in triplicate and tested with a frequency and time sweep. The linear range of the viscoelastic response was first measured with a frequency sweep from 0.5–50 rad/s at 0.5% strain. The time sweep was performed at an angular frequency of 10 rad/s and 0.5% strain for 6 min. The complex modulus (G^*) of each gel was calculated by averaging the G^* over time. To determine the effects of the C6S-binding peptide and C6S on the viscoelastic properties of the material, gels were tested with and without BP and C6S.

The amount of NGF released from gels with and without the C6S-binding peptide and C6S was quantified. Gels (50 μ l) were prepared in triplicate in 2 ml siliconized tubes, as previously described, that contained 2 μ g/ml NGF (Invitrogen, 13257019).

After the gels were cross-linked, 2 ml of 1x PBS was added to each gel. At each time point, 500 μ l of buffer was collected from each tube and immediately replaced with 500 μ l of buffer using siliconized pipets and tubes. NGF release was monitored over 48 h, and samples were stored at -20°C . After 2 d, the gels were broken up with a spatula and digested in 10 units/ml collagenase and 0.4 units/ml chondroitinase ABC (Sigma-Aldrich, C3667) for 48 h at 37°C with gentle shaking. The amount of NGF in the collected samples was quantified with a human β -NGF ELISA development kit (Peprotech, 900-K60). The absorbance of each well was measured at 405 nm and 650 nm after 10 min of incubation with ABTS liquid substrate (Sigma-Aldrich, A3219) on a multi-well plate reader (FLUOstar Omega, BMG Labtech). The absorbance at 650 nm was subtracted from the absorbance at 405 nm to determine the relative absorbance of each well, and the amount of NGF was calculated from a standard curve.

To determine the biological activity of C6S and NGF, cortical neurons were cultured on gels with and without C6S and NGF. Gels (20 μ l) were prepared according to the previously described protocol³⁹ in silicone inserts (Sigma-Aldrich) placed in chambered glass slides (Nalgene) with two gels per chamber. Silicone inserts were sterilized by sonication with 90% ethanol (VWR Scientific, EM-EX0276) for 20 min. All materials and solutions were filtered (0.2 μ m, Millipore) for cell culture.

Cortex tissue (embryonic rat day 18) was purchased from BrainBits. Primary cortical neurons were isolated from E18 cortex tissue following a protocol from BrainBits.⁴⁰ The cortical tissue was digested in a Hibernate E media solution (BrainBits, HE) containing 2 mg/ml papain (Worthington Biochemical Corporation, LS003126) at 37°C for 30 min. The tissue was then transferred into a 2% (v/v) solution of B27 supplement (Invitrogen, 17504044) in Hibernate media and triturated. The cell suspension was filtered through a 40- μ m nylon cell strainer (BD Falcon) and collected. The filtered suspension was centrifuged at 1,100 rpm for 1 min. The supernatant was removed and the cell pellet resuspended in 3 ml B27/Neurobasal media (Invitrogen, 21103049) with 0.5 mM glutamine (Invitrogen, 25030149). The viability and density of the cell suspension was determined by mixing 20 μ l of Trypan Blue (0.4%, Sigma-Aldrich, T6146) with 20 μ l of the cell suspension. Cell density was counted using a hemocytometer. The cell suspension was diluted to a final concentration of 2×10^5 cells/ml.

Supplemented neurobasal media (100 μ l) was added onto each gel, then 6,375 μ l of the cell suspension was placed on each gel. The cells were incubated for 1 h at 37°C and 5% CO_2 before an additional 1 ml of media was added to each chamber (2 gels). Cells were incubated for 48 h before fixation.

After 2 d of culture, the cells were fixed with warm 4% (v/v) paraformaldehyde (Electron Microscopy Sciences, 19200) in 1x PBS for 1 h at room temperature. The cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, T8787) solution in PBS for 2 h. After washing 3x with PBS (20 min incubations), Image-iTTM FX signal enhancer (Invitrogen, I36933) was added to the cells and incubated at room temperature for 2 h. The cells were

again washed 3x and blocked overnight with 1% bovine serum albumin (BSA, Sigma-Aldrich, A7906) and 10% goat serum (Invitrogen, 50062Z). After blocking, the cells were washed 6x with 0.1% BSA in PBS then incubated in 5 μ g/ml mouse anti- β III-tubulin (R&D Systems, MAB1195) at room temperature for 2 h and overnight at 4°C . The cells were again washed 3x with 0.1% BSA in PBS and incubated in 2 μ g/ml Alexa-488-coupled goat anti-mouse F(ab)₂-fragment secondary antibody (Invitrogen, A11029) for 2 h at room temperature in the dark. Finally, the cells were washed 3x with 0.1% BSA in PBS before visualization.

Images were captured using a Leica DMIRB (Leica Microsystems) epifluorescence microscope. A USH-102DH-100W ultra-high-pressure mercury lamp (USHIO America, Inc.) was used as the excitatory light source. Images were viewed in the blue excitation range (filter set I3-excitation filter BP450–490 nm and emission filter BP515 nm) with a 20x objective. The images were analyzed with a custom Matlab program that measured the distance of each neurite from the center of the cell body. Approximately 100–200 neurons were analyzed for each treatment.

Statistical analysis was performed with Minitab 15 (Minitab) and SPSS 16 (SPSS). A one-way ANOVA and Tukey's post-hoc test ($\alpha = 0.05$) was performed to determine statistical significance ($p < 0.05$) of the G^* of the different gels for the time sweep. Repeated measures of two-factor ANOVA ($\alpha = 0.05$) were performed to determine statistical significance ($p < 0.05$) of the G^* of the different gels for the frequency sweep and of the NGF release of the different gels. A Tukey's post-hoc test ($\alpha = 0.05$) was performed to determine significance of different gels at 10 rad/s for rheology and at 0 to 48 h for the NGF release assay. To determine differences in cortical neuron outgrowth, a Kruskal-Wallis with a Mann-Whitney post-hoc test ($\alpha = 0.05$) was performed.

Conclusions

In this study, the effects of affinity-based delivery of C6S and NGF from gels on cortical neurite behavior were investigated. C6S and NGF were incorporated into PEG gels through C6S-binding peptides. Gels with C6S inhibited neurite outgrowth, while gels with NGF promoted neurite extension. The inhibitory activity of C6S was overcome by NGF, which was slowly released from PEG gels through non-covalent interactions between C6S-binding peptide, C6S and NGF. The affinity-based system developed in this study that incorporates C6S and NGF is an ideal biomaterial for studying neural development and for neural tissue engineering.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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