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Neural cell type-specific responses to glycomimetic functionalized collagen

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ABSTRACT

Despite their noted functional role, glycans have had limited therapeutic use due to difficulties in synthesis and quick degradation in vivo. The recent discovery of glycomimetics has provided new opportunities for their application. In this study, we have functionalized type I collagen with peptide mimics of two glycans: (1) polysialic acid (PSA) and (2) an epitope first discovered on human natural killer cells (HNK-1). These glycans and their glycomimetic counterparts have been shown to be important regulators of repair following injury through their unique and phenotypically specific effects on neural behavior. We show that these molecules retain their bioactivity following functionalization to the collagen backbone. Grafted HNK-1 encouraged motor neuron outgrowth, while grafted PSA encouraged sensory and motor neuron outgrowth and enhanced Schwann cell proliferation and process extension. These data support the potential of glycomimetic-functionalized collagen as a biomaterial strategy to increase the efficiency of synaptic reconnection following nervous system injury.

1. Introduction

Glycans are important regulators of cell and tissue fate in the nervous system [1]. Through their interactions with various neural recognition molecules, such as neural cell adhesion molecule (NCAM) and L1, glycans have been implicated in a diverse range of neurophysiologic processes including myelinogenesis, neurite outgrowth, and synaptogenesis. These molecules aid in the highly regulated spatiotemporal control of cell–cell and cell–substrate interactions during neural development, plasticity, and repair following injury [2].

Two naturally occurring glycans, an epitope first discovered on human natural killer cells (HNK-1) and polysialic acid (PSA), have been shown to be involved in axonal targeting, neuronal regeneration, and glial cell proliferation and migration [3,4]. Interestingly, HNK-1 has been selectively associated with motoneurons in the peripheral nervous system (PNS), which suggests an explicit role as part of the trophic system that regulates modality specific regeneration [5]. Both of these molecules are upregulated following neural injury, and these lesion-induced changes are understood to be prerequisites for successful regeneration [6]. Importantly, when the expression of PSA and HNK-1 is experimentally interrupted following PNS injury, regeneration and axonal targeting is significantly inhibited [3,7,8].

Despite their noted functional roles, carbohydrates in general have had limited use as therapeutics because of difficulties in their synthesis and their limited stability in vivo. For example, colominic acid – a carbohydrate derivative of PSA – has been used to functionalize electrospun scaffolds and silanized glass for studies of peripheral nerve regeneration, but the results have been mostly disappointing [9,10]. Additionally, the heterogeneity of length, high metabolic clearance, and potential for immunogenicity of colominic acid remain to be resolved [11]. Alternative strategies have been studied to upregulate glycans following injury, including electrical stimulation of damaged tissue and the implantation of exogenous, genetically modified cells [3,8,12,13]. For example, El Maarouf showed that implantation of astrocytes transfected with a viral vector that aids in the upregulation of PSA leads to increased corticospinal tract axon regeneration following spinal cord injury (SCI) [13]. Further, Eberhardt et al. show that electrical stimulation of damaged femoral nerves in the PNS increases HNK-1 expression, and thereby leads to an increase in muscle reinnervation [8]. While these approaches have produced favorable results in animal models, the likelihood of their clinical translation is limited.

Recent advances in the understanding of carbohydrate–protein interactions and the accessibility of various screening techniques have allowed for the discovery of glycomimetic peptides. These molecular mimics generally retain the functionality of their glycan

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counterparts, with the added potential benefits of ease of production, increased stability, and reduced cost [14,15]. Peptide mimics of HNK-1 and PSA have been developed using phage display screening [16,17]. Previous studies have confirmed the bioactivity of these glycomimetics in soluble form both in vitro and in vivo [11,18–21]. In a recent study using the PSA glycomimetic for repair following dorsal hemisection of the T9 mouse spinal cord, Marino et al. show that the peptide is only detectable for 48 h after single-dose delivery in solution [11]. Mehanna et al. saw improved functional recovery following spinal cord compression in the mouse when the PSA and HNK-1 glycomimetics were delivered locally for two weeks using an osmotic pump [18]. Thus, a more stable, controllable method for presentation of these cues may be required for eliciting optimal biological effects, particularly for clinical applications that require extended exposure to the molecules.

Incorporating these molecules into a biomaterial strategy may allow for the necessary improvements in stability and presentation for clinical translation to regenerative therapies, if the molecules retain their bioactivity. Many other bioactive functionalized biomaterials have been developed, including those with laminin or immobilized growth factors, but these ligands generally have broad effects on neural cell behavior. Conversely, PSA and HNK-1 have unique and phenotypically specific responses that provide interesting opportunities for its use in vivo. Instead of simply accelerating or encouraging regeneration, biomaterials for nervous system injury may be improved by including cues that increase the efficiency of synaptic reconnection.

To this end, we have functionalized type I collagen scaffolds with the PSA and HNK-1 peptide mimics. Type I collagen is non-cytotoxic, has suitable chemical groups for modification, and has been shown to promote nervous system regeneration [22,23]. Suspensions of the functionalized oligomeric type I collagen form stable hydrogels at physiologic temperature and pH. Thus, the material retains all the functional benefits of a hydrogel including high surface area to volume ratio, pore interconnectivity, complete void filling, and suitable mechanical strength for neural tissue engineering applications. Additionally, since the carbohydrates are found tethered to the extracellular matrix or presented on the cell surface [2], grafting the glycomimetics to a scaffold may provide for more physiologically relevant presentation.

In this study, we assess the bioactivity of the glycomimetics after covalent conjugation to a collagen hydrogel. We assay the response of several neural cell types that have been previously shown to be differentially affected by the presence of the glycans and/or glycomimetics in solution. Collectively, these studies provide insight into the potential use of glycomimetic functionalized biomaterials as a novel approach for neural tissue engineering.

2. Methods

2.1. Peptide mimics

From sequences identified previously as functional glycomimetics [16,17], one linear glycomimetic peptide was selected for HNK-1 (FLHTRLFV, MW: 1032.24) and for PSA (SSVTAWTTG, MW: 908.97). Additionally, a cyclic version of the PSA mimetic (cyc PSA: CSSVTAWTTGC) was tested; the peptide was made cyclical by bridging two flanking cysteine residues with a disulfide bridge. A scrambled version of the HNK-1 peptide (TVFHFRLL) and a reverse version of the PSA peptide (rev PSA: GTTWATVSS) served as controls. All peptides were acquired from a commercial vendor (Genscript, Piscataway, NJ). The scrambled and reverse sequences were queried in PepBank, an online database of peptides, to ensure their lack of known biological activity.

2.2. Functionalization of collagen

Peptide sequences were covalently conjugated onto oligomeric type I collagen using the heterobifunctional crosslinker 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as previously described [24]. Briefly, fetal calf type I collagen (EPC, Owensville, MO) was reconstituted to 3 mg/mL in 0.02N acetic acid to make an oligomeric collagen solution. To activate carboxyl groups, 2 mg of peptide were dissolved in 2 mL of MES buffer (Fluka) containing 1 mM EDC (Pierce) and reacted for 15 min at 37 °C. Following this incubation, the activated peptide was added to 5 mL oligomeric collagen solution and rotated overnight at 4 °C. The peptide/collagen solution was dialyzed overnight to remove unattached peptide with two dialysate changes. Finally, the purified solution was lyophilized and reconstituted to 3 mg/mL in 0.02N acetic acid. In a previous study using this conjugation technique, we confirmed that the resulting grafting efficiency is between 50 and 60%. Additionally, the grafting process does not significantly change mechanical properties, fiber diameter, or fiber density of the hydrogel [24].

2.3. Preparation of hydrogels

Collagen hydrogels at 2.0 mg/mL were prepared as previously described [25]. Briefly, native or functionalized oligomeric collagen solutions at 3 mg/mL were neutralized using solutions in the following ratios: 2% 1M Hepes (Fluka), 14% 0.1N NaOH, 10% 10X Minimum Essential Medium (Sigma), 5.2% M199 (Sigma), 0.1% Penicillin/Streptomycin (P/S; Sigma), 1% L-glutamine (L-glut; Sigma), 67.7% native or peptide-functionalized collagen. For simplicity, hydrogels grafted with the HNK-1 glycomimetic are referred to as HNK-grafted hydrogels. For composite hydrogels containing both of the grafted glycomimetics, 33.85% of each of the HNK- and PSA-functionalized collagen solutions were neutralized using the formulation above, and these composite hydrogels are abbreviated as PSA/HNK-grafted. The hydrogel solution was plated into a microtiter plate and incubated at 37 °C to enable self-assembly.

2.4. Collagenase assay

A collagenase assay was used to test the enzymatic stability of the modified hydrogel. Native or peptide-grafted oligomeric collagen solutions were spiked with 10% FITC-collagen solution (EPC). The fluorescently tagged collagen fibrils have an equivalent periodic pattern to native collagen when assessed using electron microscopy. Additionally, radioactively labeled collagen and fluorescently labeled collagen do not differ significantly in their degradation [26]. Thus, the activity of collagenase is not affected by the addition of the fluorophore. Lyophilized collagenase was dissolved in PBS at a concentration of 15 Units/mL. The spiked oligomeric collagen was neutralized using appropriate buffers, and then 400 μL of the 2.0 mg/mL spiked solution was added to individual wells of a 12-well microtiter plate and placed in the incubator at 37 °C. The collagenase solution was added after self-assembly and incubated at 37 °C with the hydrogel. A 50 μL sample of the supernatant was collected at various time points and added to a 96 well microtiter plate, and the fluorescence intensity was measured on a fluorescence spectrometer (Tecan Instruments). An increase in fluorescence correlated to the amount of collagen liberated from the hydrogel.

2.5. Cell culture

2.5.1. Dorsal root ganglia

Dorsal root ganglia (DRGs) were isolated from specific pathogen-free chick eggs at embryonic day 8 (Charles River Laboratories). For dissociated cultures, ganglia were added to 0.25% trypsin for 20 min. Cells were then pelleted, washed in media, mechanically dissociated and counted using trypan blue exclusion. Whole and dissociated cells were cultured in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glut, and 1% P/S, supplemented with 100 ng/ml nerve growth factor (NGF; R&D Systems, Minneapolis, MN).

2.5.2. NSC-34

NSC-34 is hybrid cell line that was produced via the fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells [27]. Notably, consistent with a motoneuron phenotype, these cells extend neurites, generate action potentials, and express neurofilament and choline acetyltransferase. Generally, cultures will contain two populations—small, undifferentiated cells and larger, non-dividing cells with processes. To maintain their active proliferation, NSC-34 cells were incubated in high-glucose DMEM supplemented with 10% FBS, 0.5% P/S, and 0.5% L-glut. Cells were passaged every 2–3 days. To induce differentiation towards the motoneuron-like phenotype, media was changed to 1:1 DMEM plus Ham's F12, 1% FBS, 1% P/S, and 1% modified Eagle's medium with non-essential amino acids for 5–7 days prior to plating on hydrogels [28].

2.5.3. Spinal cord neurons

Dissociated spinal cord neurons (SCNs) were isolated from E15 Sprague Dawley rats as previously described [29]. Spinal cords were excised and meninges removed. Cords were transferred to a 15 mL centrifuge containing 0.05% trypsin in HBSS and incubated at 37 °C for 20 min. Following this incubation, the sample was centrifuged and supernatant removed and replaced with a 10% serum-containing media. The tissue pieces were mechanically dissociated via trituration and large pieces of debris were allowed to settle. The supernatant, which contained the single cell suspension, was transferred to a 50 mL centrifuge tube and placed in a 37 °C humidified incubator. The remaining tissue pieces were subjected to a second dissociation and were

then pooled with dissociated cells from the first. The combined supernatants were filtered through a 40-μm pore-sized nylon mesh. Cells were centrifuged for 5 min at 1000 rpm at 4 °C, resuspended in 10% serum containing media, and triturated with a fire-polished Pasteur pipette. The cell suspension was transferred to a T-75 tissue culture flask and incubated for 30 min at 37 °C to enhance the purity of the culture via differential adhesion of the contaminating glia and fibroblasts vs. neurons. The neuron-enriched supernatant was removed with a serological pipette and transferred to a centrifuge tube. Following centrifugation, the cells were resuspended in neurobasal media supplemented with B-27, penicillin/streptomyocin, L-glutamine, and beta-mercapoethanol prior to plating.

2.5.4. RSC-96

RSC-96, a spontaneously transformed rat Schwann cell line, were grown in DMEM containing 10% FBS, 1% L-glut, and 1% P/S. Cells were passaged using standard cell culture techniques at 80% confluency.

2.6. Cellular assays

2.6.1. Dorsal root ganglia explant outgrowth

Explant outgrowth has been shown to have high variability from egg to egg. Accordingly, native and either PSA- or reverse PSA-grafted conditions were included as baseline conditions for all experimental days. DRG explants were plated individually in a 24 well microtiter plate on native or functionalized collagen hydrogels and incubated for seven days in the presence of NGF. For studies investigating the bioactivity of the soluble peptide, the media was supplemented with 100 μM of the glycomimetic once at the beginning of the culture period. Following this incubation period, ganglia were fixed and stained with mouse anti-neurofilament 200 (Sigma) followed by goat-anti-mouse Alexa Fluor488 secondary antibody (Invitrogen). Epifluorescent images were taken on an Olympus IX81 using a 10× objective and analyzed using ImageJ (NIH). Explant outgrowth was measured by taking the length of individual neurites extending from the perimeter of the ganglion to growth cone end.

2.6.2. Dissociated dorsal root ganglion neuron extension

Dissociated cell cultures were plated on collagen hydrogels in a 24 well microtiter plate at a seeding density of 25,000 cells/well. Cells were grown for 3 days in the presence of NGF and stained with mouse anti-NF200, followed by goat-anti-mouse Alexa Fluor 488. Approximately 10 images were taken per well using a 10× objective. Images were analyzed using ImageJ, and neurite extension was considered to be the length of the presumptive axon, or the longest neurite. Measurements were only included if the recorded length was longer than the cell body diameter [16].

2.6.3. NSC-34 extension

Differentiated cells were plated on native or functionalized hydrogels at a density of 10,000 cells/well in a 24 well microtiter plate. Cells were incubated for 7 days with one complete media change on day 3 or 4. Cultures were then fixed, and stained immunohistochemically with mouse anti-neurofilament 200, followed by goat-anti-mouse Alexa Fluor488 secondary antibody, TRITC-conjugated phalloidin (Sigma), and DAPI (Invitrogen). Approximately ten images per well were taken using a 10× objective and analyzed using ImageJ. As this culture contained both mature, non-proliferating motoneuron like cells and proliferating cells, only neurons with a large soma and neurites at least the length of their soma were measured [30].

2.6.4. Spinal cord neuron extension

Dissociated spinal cord neurons were immediately plated on native or functionalized collagen hydrogels at 75,000 cells per/well in a 24 well microtiter plate. Following a 4-day incubation period, cells were fixed and stained using rabbit anti-MAP-2 (Millipore) followed by goat anti-rabbit Alexa Fluor 488. Cultures were co-stained with TRITC-conjugated phalloidin and DAPI to identify contaminating cells and qualitatively assess the purity of the cell population. Approximately fifteen images per well were taken using a 20× objective and analyzed using ImageJ. Neurite extension was considered to be the length of the presumptive axon, or the longest neurite. Measurements were only included if the recorded length was longer than the cell body diameter [16].

2.6.5. RSC-96 proliferation

RSC-96 cells were grown in low (0.5%) serum containing media for 48 h to synchronize the cell cycle prior to plating for proliferation studies. Cells were then resuspended in 10% serum containing media and plated on collagen hydrogels at a seeding density of 10,000 cells/well in a 24 well microtiter plate. After 23 h, 10 μM bromodeoxyuridine (BrdU) was added to the cells for 1 h. Cells were then fixed and stained with an anti-BrdU goat-anti-mouse Alexa Fluor568 and counterstained with DAPI. Five images per well were captured using a 10× objective. ImageJ was used to quantify the number of $BrdU^+$ and $DAPI^+$ cells.

2.6.6. RSC-96 process extension

In an effort to slow proliferation prior to plating, cell cultures were grown in low serum containing media for 24 h. Cells were seeded on collagen hydrogels at 5000 cells/well in a 48 well microtiter plate and maintained in low serum media for 4 days. For histological staining, cells were fixed followed by incubation with TRITC-conjugated phalloidin for 1 h at room temperature. Only cells with limited contact with neighboring cells and extensions at least the length of the longest axis of the cell body were measured. Extension measurements were normalized to the average extension length on native collagen.

2.7. Statistical analysis

The number of samples per experimental condition is summarized in Table 1. Variance analysis using a two-way ANOVA (length of processes/percent proliferation and type of hydrogel) was used followed by posthoc pairwise comparisons with Tukey's test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Hydrogel degradation

Type I collagen has been used in a number of regenerative strategies for nervous system repair. It is biocompatible, provides a biologically active scaffold, and can be formed into a hydrogel, sponge, or powder. In a previous study using a similar functionalization scheme, we showed that the grafting process does not significantly affect fiber diameter, porosity, or mechanical strength of the scaffold [24]. However, as the long-term use of this scaffold is as a biomaterial therapy, the rate of proteolytic degradation of the material should also be preserved by functionalization.

The enzymatic degradation of the functionalized collagen was compared to native collagen with a collagenase assay, where the intensity of liberated fluorescently labeled collagen was sampled over time. The results in Fig. 1 show the increase in fluorescence intensity over time following collagenase treatment for both samples. There were no significant differences between the measured fluorescence in the native and glycomimetic grafted conditions during the 48 h of collagenase exposure. Therefore, the proteolytic stability and degradation rate of the collagen hydrogel is not affected by the grafting process.

Table 1
Summary of experimental conditions. 'n' represents the number of independent experiments per hydrogel condition. * Denotes a disulfide linkage between cysteine residues.

Condition (Sequence)	Sensory		Motor		Glial	
	Explant Extension	Dissociated Extension	NSC-34 Extension	SCN Extension	RSC-96 Proliferation	RSC-96 Extension
Native	$n = 12$	$n = 6$	$n = 3$	$n = 4$	$n = 4$	$n = 3$
Scramble (TVFHFRLL)	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 4$	$n = 3$
PSA (SSVTAWTTG)	$n = 9$	$n = 6$	$n = 3$	$n = 4$	$n = 4$	$n = 3$
cyc PSA (CSSVTAWTTGC*G)	$n = 3$	N/A	N/A	N/A	N/A	N/A
rev PSA (GTTWATVSS)	$n = 7$	N/A	N/A	N/A	N/A	N/A
HNK (FLHTRLFV)	$n = 3$	$n = 6$	$n = 3$	$n = 4$	$n = 4$	$n = 3$
PSA/HNK	N/A	$n = 6$	$n = 3$	$n = 4$	$n = 3$	$n = 3$
Soluble PSA (SSVTAWTTG)	$n = 3$	N/A	N/A	N/A	N/A	N/A
Soluble rev PSA (GTTWATVSS)	$n = 3$	N/A	N/A	N/A	N/A	N/A

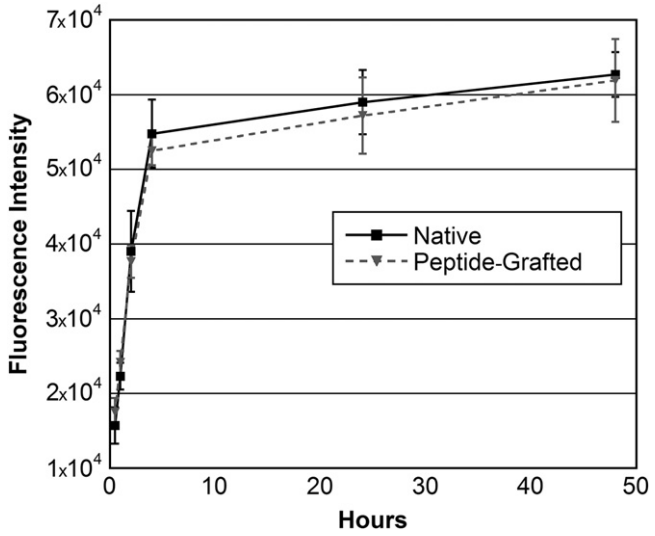

Fig. 1. Proteolytic stability of glycomimetic peptide-grafted hydrogels. FITC-tagged collagen was used to fluorescently spike native and peptide-grafted hydrogels. After collagenase exposure, the supernatant from the hydrogels was collected and the fluorescence intensity measured, which correlated with the amount of liberated collagen. The intensity of liberated collagen did not differ between the native and peptide-grafted hydrogels.

3.2. Sensory neurons

3.2.1. Whole explant DRGs

Whole explant studies were performed to assess the bioactivity of the peptides in grafted form and reduce the number of conditions in subsequent studies with other cultures (Fig. 2a). Similar to previous studies [31], native and peptide-grafted collagen hydrogels supported the adhesion, extension, and migration of cells from chick DRG explants which contained neurons, Schwann cells, and fibroblasts. Thus, the native collagen substrate is suitable for neural culture and our modification of the matrix does not affect this biocompatibility.

DRG neurite extension was not significantly different on HNK-grafted hydrogels when compared to native or scramble-grafted conditions, whereas extension on the grafted linear PSA was significantly different than that on native, scramble-, and HNK-grafted conditions ($p = 0.0002$, $p = 0.0039$, and $p = 0.0123$, respectively). These results are in agreement with previously reported studies that demonstrate that HNK-1, whether in the carbohydrate or the glycomimetic form, do not affect outgrowth of sensory neurons [16].

In the initial studies by Torregrossa et al., which discovered and assessed the bioactivity of the PSA mimotope, DRGs were incubated with media supplemented with the cyclic, linear, or reverse version of the peptide sequence. Only the cyclic version of the peptide significantly increased neurite outgrowth. The response of the glycomimetics when grafted to collagen was substantially different than when in solution. Neurite extension on hydrogels grafted with cyclic PSA (cycPSA) was not significantly different than on the native or other peptide-grafted hydrogels. However, the linear PSA-grafted glycomimetic significantly increased neurite extension compared to native and scramble-grafted conditions. Interestingly, the grafted linear reverse PSA (revPSA) sequence also significantly increased extension compared to native hydrogels ($p = 0.0323$), though not when compared to other peptide-grafted hydrogels. The conjugation of a scrambled peptide to the collagen backbone did not significantly improve or inhibit neurite extension compared to native collagen. Thus, the effects of peptide-grafted collagen on neuronal extension are sequence specific, and the grafting process itself does not significantly affect outgrowth.

Tethering the linear and reverse sequences to collagen limits the conformational changes available to the peptides when compared to the soluble form, perhaps similar to the constraints imparted by cyclizing the peptide. We suspect that the stable conformations of the soluble, linear peptides mask the active binding sites, but that these conformations are unavailable in the grafted or cyclized peptides. While it was unexpected that the linear reverse sequence of PSA would impart any effect, previous studies have reported the bioactivity of the reverse sequence of peptides. In a study by Ratcliffe et al. that evaluated the guiding behavior of laminin, both YIGSR, a bioactive motif on laminin, and its reverse sequence RSGIY significantly affected the guidance behavior of sensory axons. Notably, in a manner similar to our results, the reverse sequence was not as effective [32]. The linear and linear reverse sequences

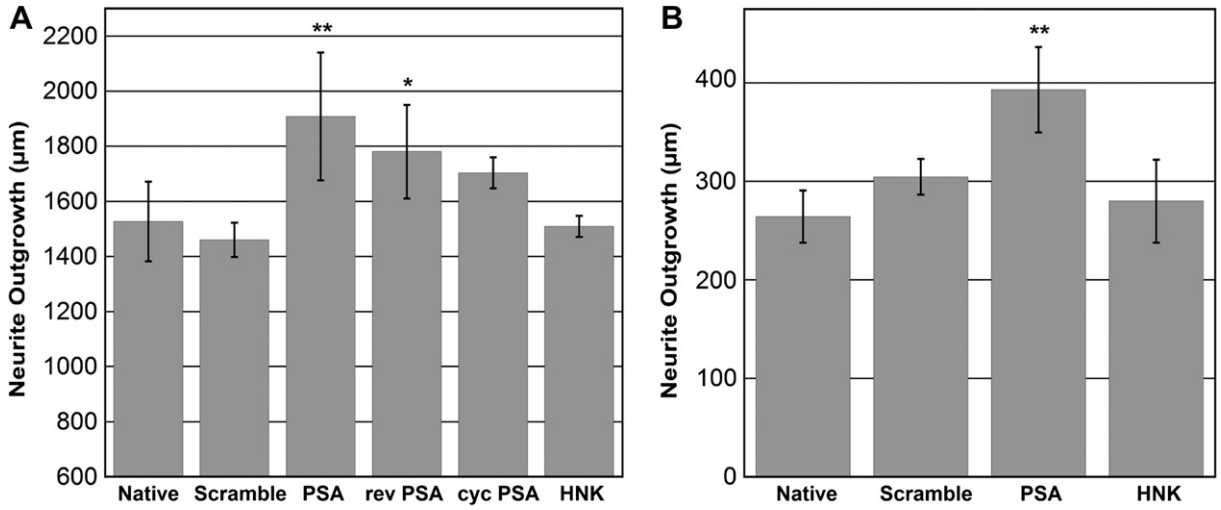

Fig. 2. Neurite outgrowth from sensory neurons on glycomimetic peptide-grafted hydrogels. Sensory neurite extension from (A) dorsal root ganglia explants and (B) dissociated dorsal root ganglia neurons on native and peptide-grafted collagen hydrogels. Neurons respond best to the hydrogels grafted with the linear form of the PSA-, and did not respond to the scramble-, cyc PSA-, or HNK- grafted hydrogels. While rev PSA-grafted hydrogels yielded significantly longer neurites from explants than native, they were not statistically different from other conditions. Mean values ± SD are shown. *Denotes statistically significant difference ($p < 0.05$) from native condition. **Denotes statistically significant difference ($p < 0.05$) from native, scramble grafted, and HNK-grafted hydrogels.

were also tested in soluble form with DRGs on native collagen hydrogels. Neurite extension was not significantly different between cultures incubated with and without the soluble peptide (Supplemental Fig. 1). Thus, the linear and linear reverse sequences appear to only affect neurite extension when immobilized, such as through conjugation to collagen.

There is also evidence to suggest that immobilized cyclic peptides may require a spacer arm to achieve optimal biological effects. Kantlehner et al. showed that an adequate spacer arm was required for osteoblast adhesion and proliferation on poly(methyl methacrylate) surfaces functionalized with cyclic RGD [33]. Without this spacer, the grafted molecule may not be effectively available for interaction with ligands on the cell surface [34]. Generally, however, the necessity for a spacer in a functionalization scheme must be examined case-by-case [35]. There is also the potential for limited grafting efficiency with the cyclic version of the peptide due to the disulfide bridge creating steric hinderance on the carboxyl end of the peptide. Thus, the cyclized peptide may not be readily activated by EDC or may not have sufficient access to free primary amines on the collagen backbone. However, the EDC can readily activate the carboxyl end of linear and linear reverse nonamer sequence, and this peptide likely has more access to the lysine and arginine residues on collagen. Unfortunately, the difficulty in synthesizing a FITC-tagged cyclic PSA molecule precluded any quantitative comparisons. The influence of peptide sequence, structure, conformation, and mode of presentation may merit future investigation to render scaffolds with optimal bioactivity. However, given our objective to develop a biomaterial that elicited specific behaviors from different neural cells, the remainder of the PSA studies were performed with only the linear form of the peptide mimic.

3.2.2. Dissociated DRG cultures

With dissociated DRG cultures, we can visualize the neuron in its entirety and have less experimental variability between cultures (Fig. 3). More so than whole explants, these cultures have been noted to be particularly sensitive to the presentation of neurotrophic factors, and the cultures can be purified to limit the influence of Schwann cells and fibroblasts on neurite extension [36]. As shown in Fig. 2b, neurite extension on native and scramble-grafted hydrogels was not significantly different from one another. However, neurite extension was significantly increased on PSA-functionalized hydrogels compared to native, scrambled-, and HNK- grafted conditions ($p < 0.0001$, $p = 0.0148$, and $p = 0.0003$, respectively). Similar to the whole explant cultures, HNK-grafted conditions were not significantly different than scramble grafted or native conditions. Interestingly, in dissociated cultures the percent difference in neurite extension between native and PSA-grafted hydrogels was approximately 2 times more than in the whole explant cultures (48.7% and 24.9% respectively), which may be a function of the level of interaction between the sensory neuron and the substrate. In dissociated cultures, the neuron grows directly on the functionalized surfaces, whereas neurites in explant cultures are more likely to interact with migrating Schwann cells and fibroblasts.

3.3. Motor neurons

3.3.1. NSC-34

As with sensory neurons, the linear grafted PSA glycomimetic significantly increased neurite extension when compared to native and scramble-grafted hydrogels ($p < 0.0001$ and $p = 0.0002$). HNK-functionalized hydrogels also increased motor neuron extension compared to native and scramble-grafted hydrogels ($p = 0.0001$ and $p = 0.0048$). However, neurite extension was not significantly different between the PSA- and HNK- functionalized conditions. No statistical differences were noted in neurite outgrowth from NSC-34 cells between native and scramble-grafted collagen hydrogels (Fig. 4a).

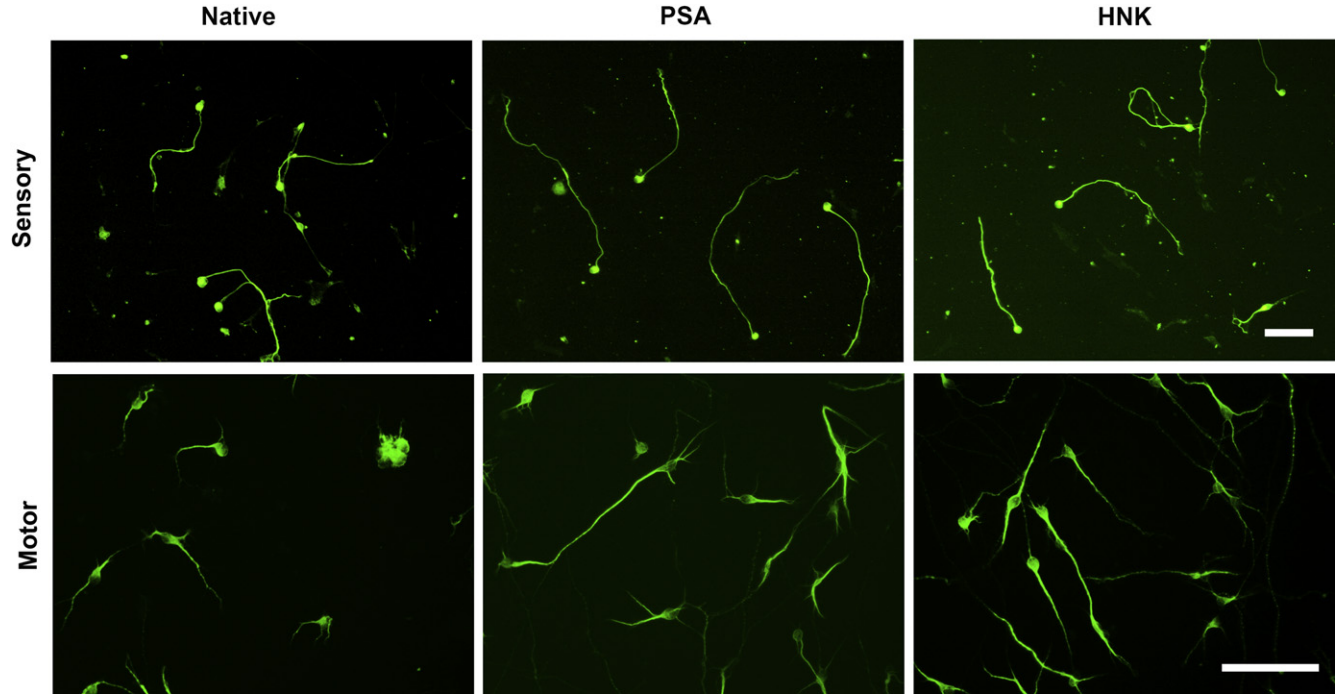

Fig. 3. Neuronal phenotypic specificity of glycomimetic peptide-grafted hydrogels. Representative images of NF-200 immunolableled dissociated DRG neurons (top panel), which extended significantly longer neurites on hydrogels grafted with PSA compared to native and HNK-grafted conditions. Representative images of MAP-2 immunostained dissociated spinal cord neurons (bottom panel), which extended significantly longer neurites on both PSA- and HNK-grafted hydrogels compared to native collagen. Scale bar represents 100 μm.

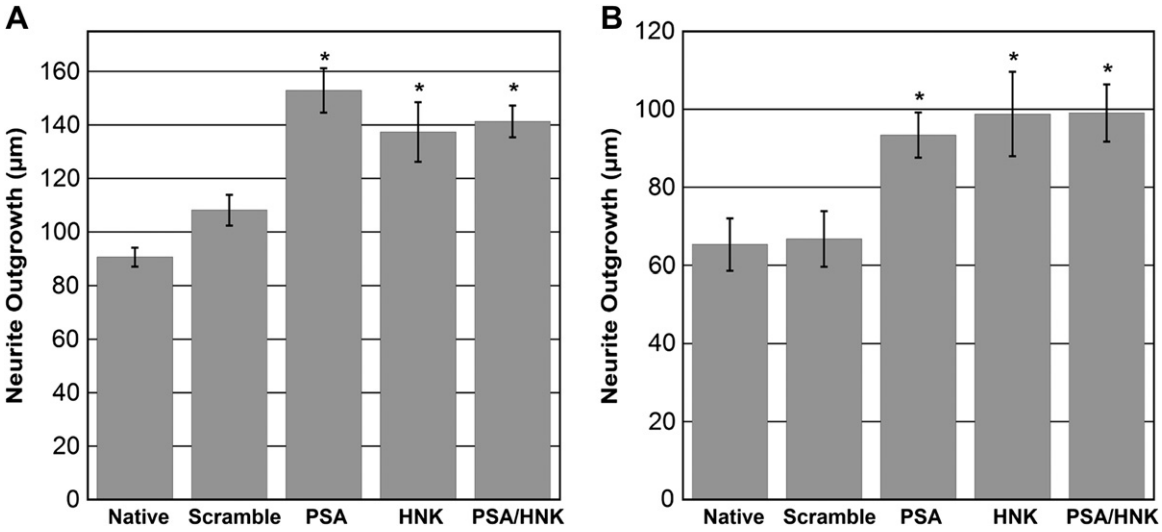

Fig. 4. Neurite outgrowth from motor neurons on glycomimetic peptide-grafted hydrogels. (A) NSC-34 neurite outgrowth and (B) dissociated spinal cord neuron outgrowth on native and peptide-grafted hydrogels. PSA- and HNK- grafted hydrogels induced significantly longer neurite extension compared to native and scramble- grafted conditions. Additionally, a PSA/HNK composite hydrogel, made from a 50/50 mix of the individual glycomimetic collagen solutions, yielded significantly longer neurites than native and scramble-grafted hydrogels. However, none of the glycomimetic peptide-grafted conditions were different from one another. Mean values ± SD are shown. * Denotes statistically significant difference ($p < 0.05$) from native and scramble-grafted hydrogels.

When considering the results of both neuronal populations, HNK-functionalized hydrogels encouraged motor neuron outgrowth, but not sensory. Previously reported studies have demonstrated the glycan HNK-1 is expressed selectively on Schwann cells associated with motor axons [5]. In addition, when presented in solution, both the carbohydrate and the glycomimetic were shown to enhance neurite outgrowth from motoneurons, however outgrowth from sensory neurons was unaffected [5,16]. Thus, conjugation to collagen does not appear to affect the modality specific responses to the HNK-1 mimotope.

As these molecules in their carbohydrate form interact with distinct ligands, including NCAM, L1, P0, and myelin associated glycoprotein (MAG) [2], we also applied a combination of the peptides to perhaps induce a synergistic increase in motoneuron outgrowth. The amount of each of the glycomimetics in the composite hydrogel was 50% of that of the full strength grafted glycomimetics. Although the combined peptides significantly increased neurite extension compared to native and scramble-grafted conditions ($p < 0.0001$ and $p = 0.0019$), there was no statistical difference between the composite substrate and those grafted with either of the glycomimetics alone.

3.3.2. Primary rat SCNs

We also investigated the functional response in primary spinal cord neuron-enriched cell cultures (Figs. 3 and 4b). The grafted scrambled peptide did not significantly improve or inhibit neurite extension from primary SCNs compared to native hydrogels. Similar to NSC-34 cultures, we observed that the neurites on the PSA-functionalized hydrogels were significantly longer than native and scramble-grafted collagen hydrogels ($p = 0.0013$ and $p = 0.004$). HNK-grafted hydrogels yielded significantly longer neurites compared to native and scramble-grafted hydrogels as well ($p = 0.0002$ and $p = 0.0008$). Additionally, while the PSA/HNK composite hydrogel significantly increased neurite extension compared to native and scramble-grafted hydrogels ($p = 0.0002$ and $p = 0.0007$), there were no significant differences between the composite hydrogel and the individual glycomimetic peptide-grafted hydrogels. It is possible that our composite hydrogels did not further improve neurite extension in either of our motoneuron cultures because there was an inadequate amount of each glycomimetic. Future studies will investigate various ratios of the PSA/HNK composite hydrogels to find the critical amount of each required.

3.4. Schwann cells

PSA, HNK-1, and their glycomimetics have been shown to affect glial cell behaviors. As neuron/glial apposition is crucial to nervous system regeneration, the ability to manipulate this interaction may be of importance. Thus, we assayed how our grafted biomaterials affect the proliferation (Fig. 5a, b) and process extension (Fig. 5c,d) of Schwann cell populations. There were no significant differences in the percentage of proliferating Schwann cells between native and scramble-grafted hydrogels. Additionally, HNK-grafted glycomimetics did not significantly affect RSC-96 proliferation rate. Interestingly however, only hydrogels functionalized with PSA significantly increased RSC-96 proliferation compared to native (PSA $p = 0.0005$; PSA/HNK $p = 0.0035$), scramble grafted, and HNK-grafted conditions. There were no significant differences in process extension among native, scramble-grafted, and HNK-grafted hydrogels. The only significant differences in extension were in PSA-containing hydrogels (PSA; PSA/HNK), compared to native (PSA $p = 0.0017$; PSA/HNK $p = 0.0001$), scramble-grafted (PSA $p < 0.0001$; PSA/HNK $p < 0.0001$), and HNK- grafted (PSA $p = 0.0145$; PSA/HNK $p = 0.0011$) conditions. Both experiments are in agreement with a previous study that demonstrates the ability for the PSA glycomimetic in solution to increase Schwann cell proliferation rates and process extension length [19].

Schwann cells have been categorized into motor or sensory phenotypes as dictated by the type of axons with which they interact [37]. As the glycan HNK-1 is reported to be expressed on Schwann cells associated with motor axons only [5], there may be phenotypic-specific effects of the glycomimetic counterparts on Schwann cells. It is possible that the cell line that we have chosen in our study does not adequately represent the motor Schwann cell phenotype, which would explain the lack of activity of HNK-grafted hydrogels. Unfortunately, isolating and maintaining a motor Schwann cell population is not trivial. Evidence suggests that many of the phenotypic specific markers, like the glycan HNK-1, are lost upon removal from in situ conditions. For example, a recent study

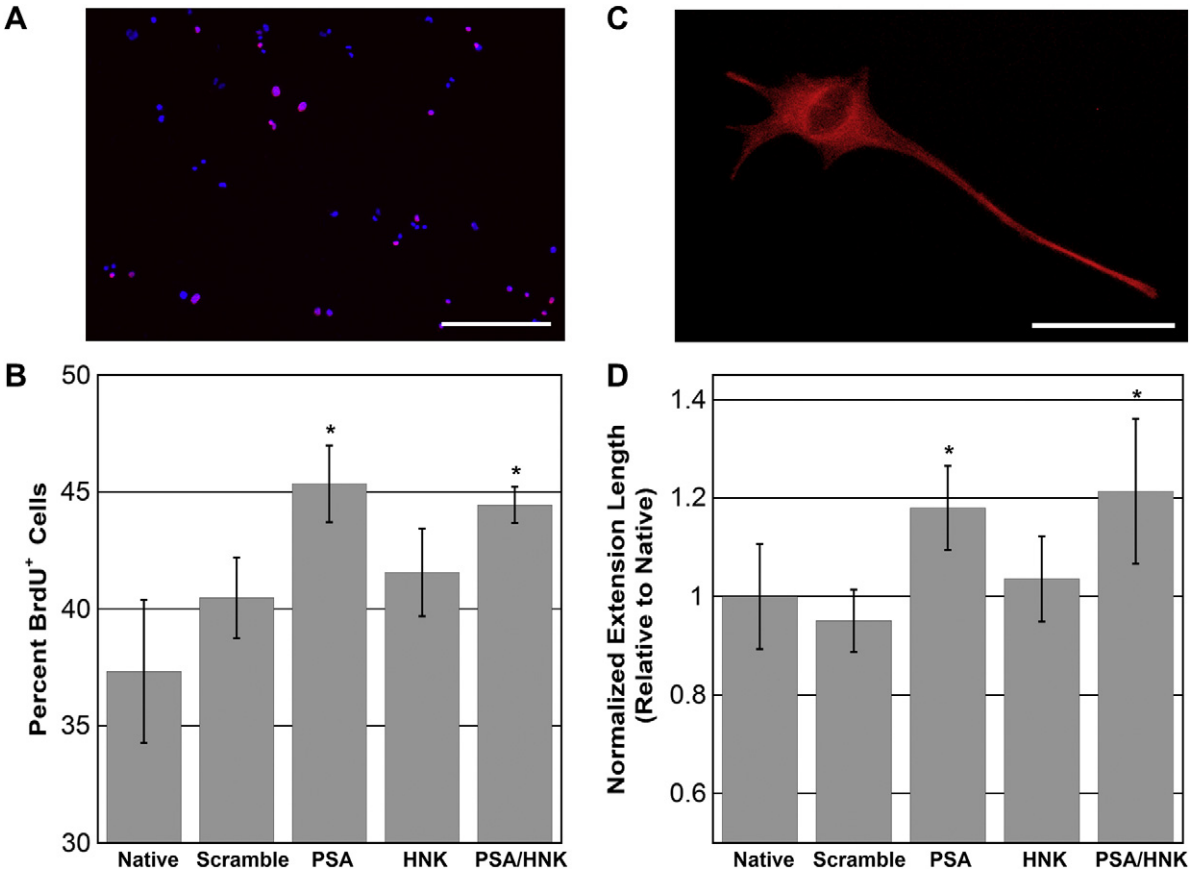

Fig. 5. Schwann cell response on peptide modified hydrogels. (A) Representative image of DAPI (blue) and $BrdU^+$ (red) stained Schwann cells on hydrogels. Scale bar = 200 μm (B) Percentage of proliferating Schwann cells on native and peptide-grafted hydrogels. (C) Representative image of phalloidin stained Schwann cell cultured on a hydrogel. Scale bar = 50 μm (D) Process extension length of Schwann cells on native and peptide-grafted hydrogels normalized to the average response on native collagen. Schwann cell proliferation and process extension was significantly affected by grafted gels that included PSA peptides. Mean values ± SD are shown. * Denotes statistically significant difference ($p < 0.05$) from native, scramble-grafted, and HNK-grafted conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by Bock et al. showed that the glycan HNK-1 is expressed in situ on adult canine Schwann cells but are immunonegative for the glycan upon in vitro culture [38]. Future studies will evaluate the glial response of HNK-grafted scaffolds in vivo.

3.5. Applications of glycomimetic functionalized collagen

The phenotypic specificity of glycomimetic functionalized biomaterials may be of particular importance in the PNS. Despite significant progress in encouraging axonal growth following PNS injury, functional recovery is still incomplete, which may be due in part to the malrouted reinnervation of emerging neurites. PSA, HNK-1, and their glycomimetics have been implicated in promoting preferential motor reinnervation, a phenomenon by which motor axons reconnect with their appropriate motor targets thereby increasing the accuracy of regeneration [39]. While the glycomimetic in solution has been shown to encourage motor axon targeting following PNI [20], this strategy may not suffice for more challenging injury models. Thus, inclusion of these cues in a biomaterial therapy that localizes and sustains their presentation may have important implications for large, clinically relevant gap sizes.

Recent work has shown that growth cones are responsive to gradients of conjugated cues including laminin and NGF, and that these anisotropic cues can often accelerate the rate of regeneration in vitro and in vivo [40–42]. When presented in solution, the ability to control the presentation of the glycomimetics is not trivial. However, once conjugated to collagen we may be able to pattern the glycomimetics and thereby motivate directional preference in outgrowth. For example, we have previously shown the ability to generate linear gradients of IKVAV and YIGSR grafted collagen scaffolds within microfluidic devices which results in a significant growth bias over isotropic presentation [31].

We note that while other materials, synthetic or natural in origin, can be functionalized with this peptide, the use of collagen provides some notable benefits. The native collagen hydrogel in our study supported the adhesion, extension, and proliferation of all neural populations that were interrogated. This innate bioactivity is likely attributed to the available ligands on collagen, including RGD and GFOGER. The potential does exist that these ligands work in combination with the grafted glycomimetic peptides to elicit or magnify the observed behaviors. Further, the use of collagen allows for the manipulation of the mechanical and structural properties of the scaffold. While we used collagen as a hydrogel in this study, the oligomeric collagen can easily be formed into a sponge or a longitudinally aligned fiber to provide an additional guidance field [22]. Additionally, the hydrogel can self-assemble in situ allowing for complete void filling of an injury-induced gap or use as an intraluminal filling within a nerve guidance conduit.

4. Conclusion

Despite their role as important regulators of cell and tissue fate in the nervous system, particularly following injury, the therapeutic potential of glycans has been largely unrealized due to difficulties in

synthesizing and purifying carbohydrates and their quick degradation in vivo. However, the recent discovery of glycomimetics has provided opportunities to utilize this class of molecules for neural repair. While peptide mimetics of two glycans, PSA and HNK-1, have been used in models of peripheral and spinal cord injury, presentation in solution results in relatively short residence times and limited functional recovery. In this study, we have functionalized oligomeric type I collagen with PSA and HNK-1 peptide mimics. We show that HNK-grafted collagen hydrogels encourages motor neuron outgrowth, while grafted PSA encourages sensory and motor neuron outgrowth and enhances Schwann cell proliferation and extension. The phenotypic specificity of these molecules may be of particular importance in encouraging preferential motor reinnervation in the PNS and targeting regeneration in the CNS. To the best of our knowledge, this work provides the first successful use of a glycomimetic functionalized biomaterial, and creates a novel, promising approach for nervous system repair.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.10.013.

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