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Enhanced Schwann Cell Attachment and Alignment Using One-Pot “Dual Click” GRGDS and YIGSR Derivatized Nanofibers

Jukuan Zheng^{†,‡}, Dimitria Kontoveros^{†,§}, Fei Lin[‡], Geng Hua[‡], Darrell H. Reneker[‡], Matthew L. Becker^{‡,§}, and Rebecca K. Willits[§]

[‡]Department of Polymer Science, The University of Akron, Akron, Ohio 44325, United States

[§]Department of Biomedical Engineering, The University of Akron, Akron, Ohio 44325, United States

Abstract

Using metal-free click chemistry and oxime condensation methodologies, GRGDS and YIGSR peptides were coupled to random and aligned degradable nanofiber networks postelectrospinning in a one-pot reaction. The bound peptides are bioactive, as demonstrated by Schwann cell attachment and proliferation, and the inclusion of YIGSR with GRGDS alters the expression of the receptor for YIGSR. Additionally, aligned nanofibers act as a potential guidance cue by increasing the aspect ratio and aligning the actin filaments, which suggest that peptide-functionalized scaffolds would be useful to direct SCs for peripheral nerve regeneration.

Graphical abstract

INTRODUCTION

Polymeric nanofibers have been studied extensively for applications in tissue engineering and regenerative medicine.¹ When modified with bioactive peptides, nanofibers have found significant utility in bone,² neural,^{3–10} and vascular applications.^{11,12} The unique physicochemical properties of nanofibers have been found to significantly influence cell function, including cell morphology, contact guidance via confinement, and migratory properties.^{13,14} While functionalizing degradable polymers is challenging, several groups

[†]**Author Contributions:** These authors contributed equally to this work (J.Z. and D.K.).

Supporting Information

Polymer precursor synthesis and characterization methods is available. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interest.

have demonstrated the ability to derivatize nanofibers with bioactive species, including peptides and carbohydrates.^{15–19} Many strategies have focused on generating peptide–polymer conjugates prior to generation of the scaffold.²⁰ However, in most preconjugation methods, the specific electrospinning conditions must be varied for each formulation and the surface conductivity and solution properties are strongly influenced by the type of bioactive conjugate. In addition, a significant fraction of the bioactive species are buried within the nanofiber, and as such, are not bioavailable to the target cell population, limiting the translational and clinical potential. It is well established biologically that multiple bioactive molecules work synergistically in time- and concentration-dependent manners to regulate cellular functions.^{21–24} However, there are no reports describing the use of multiple bioactive groups attached to nanofibers to influence specific functions. Facile methods to create nanofiber scaffolds that can be controllably derivatized postfabrication with multiple bioactive species would advance our ability to probe fundamental interactions of these ligands on cellular behavior in vitro and in vivo.

Schwann cells (SC) are particularly important for appropriate regeneration in peripheral nerves. After a peripheral nerve injury, SC, the myelinating glial cells in the peripheral nerve system (PNS), traverse the injury site from both the proximal and distal nerve and assist fibroblasts in the breakdown and clean up process. In addition, SC perform a supportive role to regenerating nerves by presenting growth factors and organizing a basement matrix. Finally, the SC serve to myelinate and protect the regenerated nerve. In vivo, nerve regeneration only proceeds to the extent to which Schwann cells migrate within the conduit. Any material developed to assist nerve regeneration must provide a supportive environment for SC.

Both extracellular matrix (ECM) and topographical cues play an essential role in SC guidance of neurite outgrowth.^{25–29} Aligned materials investigated for nerve conduits have included fibers of collagen,^{30,31} fibronectin,³² chitosan,³³ and synthetic polymers^{34–36} and dimensions ranging from 50 nm to 150 μ m. Significantly, nanofibers that incorporate ECM molecules or peptide-based motifs, including collagen, laminin, and fibronectin, improve the resulting neurite outgrowth on the SC.^{25,37} Combinations of synthetic or natural biomaterials with neural-interacting laminin proteins have been shown to influence cellular attachment, migration, proliferation, and differentiation and are promising as an ECM platform for nerve regeneration.³⁸ Two common laminin-derived peptides, Tyr-Ile-Gly-Ser-Arg (YIGSR) and Ile-Lys-Val-Ala-Val (IKVAV), have been found to enhance SC adhesion³⁹ and neurite outgrowth⁴⁰ in vitro and in vivo.^{30,41,42} However, YIGSR-functionalized matrices impart similar or superior cellular effects to IKVAV, a more commonly studied laminin-derived peptide.^{30,43–48} GRGDS peptide is the most extensively used motif for modulating cell adhesion through integrin binding, which is essential to normal cell functions in a number of different cell types.^{49–54} Using combinations of metal-free click chemistry and oxime ligation reactions, we have fabricated novel nanofibers that are modified postfabrication to possess well-defined stoichiometries of GRGDS and YIGSR peptides.

EXPERIMENTAL SECTION

Electrospinning Conditions

The DIBO-terminated PCL-kPCL (5% ketone group) was dissolved in a 1:4 (v/v) *N,N*-dimethylformamide/dichloromethane solution to yield a clear, slightly viscous solution of 40% (w/v) concentration. The solution was shaken and left overnight to ensure homogeneity. A voltage of 10 kV was applied to the solutions, and the tip-to-screen distance was 15 cm. For random fibers, aluminum foil was used as the grounded collector. For aligned fibers, a special sheet of stainless steel collector was fabricated with elongated openings of 20 mm × 70 mm was used.²² The aligned fibers were collected on glass coverslips placed in the gaps of the stainless steel collector.

Scanning Electron Microscopy (SEM)

The fiber dimensions and alignment were evaluated by SEM (JSM-7401F, JEOL, Peabody, MA). The acceleration voltage was set at 1 kV. The fiber diameters and angles were calculated by measuring over 100 fibers using ImageJ.

Solid Phase Peptide Synthesis

The N₃-GRGDS and NH₂O-GYIGSR were synthesized using standard Fmoc conditions⁵⁵ on a CEM Discovery microwave peptide synthesizer. For N₃-GRGDS, the N-terminus was derivatized with 6-bromohexanoic acid as described previously.⁵⁶ The Br end group was substituted with an azide group in a 1:2 solution of methanol/water containing 18-crown-6 (0.05 equiv) stirred at 23 °C overnight. The azide-substituted peptide was purified by equilibrium dialysis and a white solid was obtained following lyophilization. The substitution was confirmed by ESI-mass spectra ($[M + Na]^+ = 630.6$ Da). NH₂O-GYIGSR was synthesized using Fmoc methods and capped with (Boc-aminooxy) acetic acid at the N-terminus. The functionalized peptide was deprotected using piperidine and cleaved from the resin with trifluoroacetic acid. The peptide was triturated, dialyzed and lyophilized. The desired peptide product was confirmed by ESI-mass spectra ($[M + Na]^+ = 725.4$ Da).

One-Pot Nanofiber Difunctionalization

The N₃-GRGDS and NH₂O-GYIGSR were dissolved in AcOH/NaAc pH = 4.5 buffer to yield a solution with concentration of 1 and 0.5 mg/mL, respectively. The glass slides covered with electrospun fibers were carefully dipped into the peptide solution three times and rinsed with buffer. The functionalized fibers were dried overnight and sterilized with ethylene oxide. The sterilized nanofibers were degassed for 3 days. The difunctionalized nanofiber was dissolved in DMF, and the resulting UV–visible absorbance spectrophotometry data were compared with both the pure polymer before difunctionalization at the same concentration and NH₂O-GYIGSR in DMF.

Lowry Assay

Peptide concentration on the fiber was measured using the Lowry assay as described previously.⁵⁷ Briefly, N₃-GRGDSfunctionalized fibers were fabricated according to methods mentioned above. Exactly 1.0 mg of N₃-GRGDS functionalized fibers was dissolved in 50

μL of DMSO. Then 5 μL of the solution was diluted by 45 μL of H_2O , 50 μL of reagent A, and 200 μL of reagent B for measurement. A standard curve was created by measuring solutions containing 0.67, 2, 4, 8, 16, and 30 $\mu\text{g/mL}$ of N_3 -GRGDS peptide. NH_2O -GYIGSR functionalized fibers were characterized using an identical procedure. Total protein within each sample was measured using a BCA protein assay (Biorad, Hercules, CA) according to manufacturers protocol. The contents of peptide were calculated to be 6.02 ± 2.13 and 14.82 ± 4.30 $\mu\text{g/mg}$ for RGD and YIGSR, respectively.

Cell Culture

A Schwann cell line (S16, ATCC), spontaneously derived from primary rat Schwann cells, was utilized for the studies described herein. The cells were maintained as prescribed by ATCC, using Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). Maintenance flasks were coated with 1.5 $\mu\text{g/cm}^2$ poly-L-lysine and cells were passed at least twice weekly. Cells were used between passages 5 and 10.

Cell Attachment

Six different scaffolds were examined for the cellular studies: nonfunctionalized aligned and random scaffolds, RGD-modified aligned and random scaffolds, and RGD+YIGSR aligned and random scaffolds. Cells (4×10^3 cells/sample) were seeded on glass coverslips or on a scaffold for 4 h in serum-free media (DMEM + 1X N2). Any cells that were not attached after 4 h were removed by rinsing with media. The nuclei were labeled with Hoechst 33342 for 15 min and scaffolds were imaged in their entirety. Nuclei were counted using ImageJ. The cell number on glass coverslips was used as the background and subtracted from the cell number on the scaffolds. At least 12 scaffolds of each type were seeded and imaged.

Cell Proliferation

Cell proliferation was examined using two methods. First, 4×10^3 cells were seeded in serum-free media as noted above, rinsed after 4 h, and then cultured in 10% FBS + DMEM media. The growth of S16 in serum-free media is limited, making the switch to serum-containing media necessary after seeding. Cell number was counted at 4 h and 2 days. A minimum of five samples from each group were examined at each time point. Additionally, 4×10^3 cells were seeded in serum-containing media and cultured for up to 7 days ($n = 3$). Cell number was measured at 4 h and 7 days using PrestoBlue Cell Assay kit (Invitrogen) according to manufacturer's instructions. The absorbance of each sample was compared to a standard curve of cell number versus absorbance to quantify the number of cells. Using the number at 4 h and the 7 day, doubling time of the SC was calculated for each condition.

Immunocytochemistry

Cells were seeded onto scaffolds as above and cultured in serum-containing media for 2 days (laminin receptor) or 4 days (vinculin). The scaffolds were then fixed using freshly prepared 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 for 10 min, quenched with 1 mg/mL sodium borohydride, and blocked with 2 mg/mL bovine serum albumin. Actin was then labeled using 100 nM Acti-stain 488 phalloidin (Life Technologies) and nuclei were labeled using 100 nM Hoechst 33342 solution for 15 min. Additionally,

antivinculin, to label for focal adhesions, or antilaminin receptor, to label for the YIGSR-cell receptor, was incubated with the scaffolds for at least 12 h at 20 °C. A least six samples of each type of scaffold were cultured and imaged.

Image Collection/Analysis

Images were collected using an inverted Olympus iX81 microscope with 40× objective in both phase and fluorescence. Phase images were used to align the fibers with the *x*-axis. Images (*n* = 3) were analyzed separately using an actin alignment procedure written in MATLAB as previously described.⁵⁸ This script uses edge detection and statistical analysis to measure percent alignment $\pm 10^\circ$ from the nanofiber orientation. To measure the aspect ratio of the cells, images with fluorescently labeled actin were analyzed using ImageJ. Each image was thresholded manually to maintain the integrity of the cell shape and then, using the *Analyze Particles* tool, the aspect ratio of each cell was measured.

Statistical Analysis

A *t* test was performed to determine statistical significance for all of the cellular assays and image analysis, with *p* < 0.05 considered significant.

RESULTS AND DISCUSSION

Polymer Synthesis

Random copolymers of P(CL-*co*-OPD) were prepared by ROP of ϵ -CL and 1,4,8-trioxaspiro-[4,6]-9-undecanone (TOSUO), followed by deprotection of the ketone groups using triphenylcarbeniumtetrafluoroborate. The successful polymerization, deprotection were characterized by ¹H NMR, UV–visible spectroscopy, and FTIR. The presence of the DIBO at the chain end was confirmed via ¹H NMR and UV–visible spectroscopy (Figures S2 and S3). A series of DIBO end-functionalized PCL polymers possessing mole fractions of 2-oxepane-1,5-dione ranging from 2 to 10% were synthesized. The amount of ketone in the copolymer trends closely with the respective feed ratio. The extent of ketone functionality in the P(CL-*co*-OPD) polymer backbone was quantified by ¹H NMR (Table 1 and Figures S2 and S3).

Nanofiber Fabrication and Functionalization

Random and aligned nanofibers were generated using a method described previously.²² The angle distribution of the aligned nanofibers was calculated by ImageJ. The oriented angle for the alignment was $91.1 \pm 4.6^\circ$. The distribution of nanofiber diameters was measured to be 382 ± 54 nm.

To demonstrate visually the one pot conjugation to the nanofibers, green and red fluorescence markers were used in a one-pot difunctionalization. The fluorescence based model reaction showed the potential of one pot method for attaching two different bioactive molecules (Figure 1). Chemo 488 azide ($4 \mu\text{g/mL}$) and AlexaFluor 568 ($2 \mu\text{g/mL}$) were dissolved in a sodium acetate/acetic acid buffer solution (1 mM) that was tuned to pH 4.5. Electrospun mats on glass coverslips were dipped into the solution for 5 min and washed in buffer and aqueous solutions. Following demonstration of the one-pot approach using

fluorescent dyes, two different peptides, GRGDS and YIGSR, were coupled to the nanofibers using identical buffer conditions. The extent of functionalization was confirmed using UV–visible spectroscopy. Figure 2 clearly shows the changes in the absorbance transitions at 306 and 282 nm comparing the polymer before modification, and following the attachment of NH₂O-GYIGSR and N₃-GRGDS peptides. In the difunctionalized nanofiber, there is a decrease at 306 nm compared with unmodified polymer, which is the reacted DIBO group. There is also an increase at 282 nm due to the loaded tyrosine from NH₂O-GYIGSR. Further, the percentage of reacted DIBO and ketone group were calculated to be about 9 and 16%, respectively, from a calibration curve of the DIBO and tyrosine signal.

Peptide Influence on Schwann Cell Response

Six different kinds of nanofiber scaffolds were generated to assess the cooperative influence of nanofiber alignment and peptide functionalization on SC attachment and proliferation. The nanofiber scaffold sets were random nonfunctionalized, random with GRGDS, random with both GRGDS and YIGSR (GRGDS + YIGSR), aligned nonfunctionalized, aligned with GRGDS peptide, and aligned with both GRGDS + YIGSR.

Attachment

Cells were cultured on each type of scaffold for 4 h with serum-free media to eliminate adsorbed protein interfering with the initial cell attachment. Without serum, cell attachment on scaffolds with peptides was significantly higher than scaffolds without peptide (Figure 3A). Increased cell attachment to the peptide-functionalized scaffolds demonstrates the retention of activity of the peptides during the fabrication process. SC attachment has been previously found to increase by 15% on RGD-modified PCL⁵⁹ over nonfunctionalized controls. We noted an almost 30% average increase for peptide-modified PCL, possibly due to differences in concentration of functional groups or peptides with our method. Additionally, YIGSR + RGD-functionalized scaffolds were found to significantly increase SC attachment over those scaffolds without peptide ($p = 0.02$). While YIGSR has been studied previously using SC, the results are generally similar to other peptides, such as RGD,³⁹ even though the two peptides interact with different cell surface receptors. Ultimately, without serum, the cells can interact with the peptides alone. When we included serum during the seeding process, the differences noted in attachment were negated (data not shown). Others that have studied SC have noted that modified, but nonfunctionalized PCL, showed only slightly lower results than those with peptide, and any differences were negated with time.³⁹ Overall, these attachment results clearly show that the peptides are active and the cells can use them to bind to the substrate, but protein adsorption from serum can interfere with the seeding. In translation to in vivo studies, the potential problem of protein adsorption fouling our surface has been considered. Key to using these materials in vivo translation will be control the fouling, which is a problem with many synthetic substrates, and will be the focus of future studies.

Proliferation

Scaffolds were then examined for their ability to support cell growth. In preliminary studies, cells were seeded and maintained in serum-free media, but cell growth was stunted (data not shown). Therefore, cells were seeded in serum-free media and then cultured in serum-

containing media. Doubling time was calculated to be ~60 h, and no differences were noted between the scaffolds or blank (glass coverslip, no scaffold). The low proliferation rate is likely due to the low cell number on each substrate. We then seeded and cultured in serum and determined the doubling time to be ~40 h (Figure 3B) on nonfunctionalized scaffolds and the blank. A significant decrease in doubling time was found on the RGD-modified scaffolds. RGD has previously been found to increase SC proliferation on other nanofibrous scaffolds, such as PCL,⁵⁹ peptide amphiphile,⁶⁰ or on polyhydroxyalkanoate³⁹ scaffolds. However, the presence of RGD does not guarantee a change in the proliferation rate, as nanofibers of PCL with RGD⁶¹ saw no difference in proliferation versus controls. Some of these differences are due to the controls for each study, as there are differences between the hydrophobicities of the substrates that likely cause differences in cell proliferation.

Cell Immunocytochemistry

The 67 kDa laminin receptor was not evident on cells cultured on scaffolds without YIGSR (Figure 3C). Labeling of the 67 kDa laminin receptor, which is specific for the YIGSR peptide,^{62,63} was enhanced on scaffolds with YIGSR. While others have shown that YIGSR binds to the 67 kDa laminin receptor, little was found regarding this interaction and regulation of expression. Further confirmation of the expression regulation would need to be performed to confirm these results. Vinculin, in combination with actin labeling, was used to characterize focal adhesions. The substrates with peptides show clear regions of focal adhesions (Figure 3D), with overlapping vinculin and actin labeling that are not found on SC seeded on nonfunctionalized scaffolds. As these cultures contained serum after the initial seeding, adsorbed protein may deposit to form binding sites for the nonfunctionalized scaffolds, but this cell binding did not result in focal adhesion plaques. Both YIGSR⁶⁴ and RGD^{59,65} have been previously shown to promote focal adhesion plaques with other cell types using both nonintegrin and integrin receptors. These types of interactions, however, have not been specifically noted with SC and peptides.

Nanofiber Influence on Schwann Cell Morphology

The nanofiber's influence on actin alignment was studied by culturing SC, labeling and imaging of actin fibers, and processing the images through Matlab.⁵⁸ Cells within $\pm 10^\circ$ from 0° , which was placed in alignment with the nanofibers, were averaged for alignment. Figure 4A is a sample image that was processed by Matlab to Figure 4B to determine actin fiber alignment. The percentages of actin alignment of all scaffold types are displayed in Figure 4D. SC showed statistically higher actin alignment on aligned fibers than randomly oriented fibers. Overall, an alignment within $\pm 10^\circ$ was quantified at ~40% on aligned nanofiber scaffolds, which was similar to the result of 50% alignment as observed by Kemeny and Clyne with $\pm 30^\circ$ alignment.⁵⁸ Endothelial cell alignment to shear stress was approximately 42% using similar measurements.⁶⁶ To further confirm the impact of the nanofibers on cell morphology, the aspect ratio of the nuclei of individual cells was determined (Figure 4E). Aligned scaffolds had a significantly increased aspect ratio over random scaffolds. Other studies with SC clearly demonstrate alignment of the cells to nanofibers through images,^{33,35,67} but do not quantify this cellular alignment with either techniques used here. Alignment of cells will direct cell migration and axonal regeneration in the appropriate direction, which is beneficial for neuron regeneration process.

CONCLUSIONS

This manuscript describes the synthesis and one pot difunctionalization of degradable polymer nanofibers with GRGDS and YIGSR. The approach is both highly versatile and enables the attachment of any pair of functional groups (peptides or carbohydrates) possessing an azide group or a hydroxylamine. The bound peptides are active, as demonstrated by SC attachment and growth, and the inclusion of YIGSR with GRGDS alters the expression of the receptor for YIGSR. Additionally, aligned fibers act as a potential guidance cue by increasing the aspect ratio and aligning the actin filaments. Overall, these results suggest that aligned, peptide-functionalized scaffolds would be useful to direct SCs for nerve regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

DIBO-initiated ring opening polymerization of ϵ -caprolactone and TOSUO results in an acetal-protected caprolactone copolymer where the stoichiometry trends with the monomer feed ratio. Upon deprotection, the resulting $\text{p}(\epsilon\text{CL-co-OPD})$ copolymer possesses a strained cyclooctyne and a reactive ketone group. Following electrospinning, both groups can be derivatized in a one-pot "dual-click" model reaction with Chemo 488 azide (green) and AlexaFluor 568 hydrazide (red) to yield nanofibers functionalized with two fluorescent species.

Figure 2.

Aligned and random nanofiber mats were fabricated using electrospinning with flat and patterned electrodes, respectively. SEM micrographs of the resulting matrices show the random and aligned fiber matrices have nearly identical dimensions (382 ± 54 nm). The scale bars are 10 μ m. A reactive DIBO-group at the initiating chain end enables precise concentration control of the end-labeled peptide as a function of molecular mass. Following electrospinning, the nanofiber-coated substrates were derivatized with N₃-GRGDS and NH₂O-GYIGSR peptide conjugates. UV–visible spectroscopy shows the successful difunctionalization of nanofibers by oxime ligation and copper-free click chemistry. The signal decreased at 306 nm was due to reacted DIBO, while the signal increased at 280 nm region was due to the tyrosine absorbance from the NH₂O-GYIGSR peptide. The extent of reacted DIBO and ketone groups were calculated to be 9 and 16%, respectively, from a calibration curve of the DIBO and tyrosine signal.

Figure 3.

Peptide influences Schwann cell response. (A) The initial attachment of Schwann cells in serum-free media was statistically increased on the peptide substrates over nonfunctionalized substrates ($p = 0.02$, $N = 12$). Additionally, any nanofiber scaffold statistically increased the Schwann cell attachment over the control glass (blank) substrate ($p = 0.0032$, $N = 12$). (B) GRGDS functionalized scaffolds reduced the doubling time of Schwann cells compared to both nonfunctionalized scaffolds and GRGDS and YIGSR functionalized scaffolds ($p = 0.02$, $N = 3$). (C) To investigate the Schwann cell interaction with YIGSR, the 67 kDa LN receptor was labeled. No labeling was noted with confocal microscopy on scaffolds without peptide and those with RGRGDS alone, however, LN receptor labeling was found on scaffolds with YIGSR along with GRGDS. (D) The GRGDS-containing scaffolds both showed clear vinculin plaques in coordination with actin labeling, which are indicative of focal adhesions, while the nonfunctionalized scaffolds had little plaque formation.

Figure 4.

Nanofiber orientation influenced the alignment and aspect ratio of the Schwann cells. First, a MATLAB code was utilized to determine actin alignment with the nanofibers. (A) An example image of actin on an aligned GRGDS-derivatized scaffold, was processed and (B) color coded based on orientation. The nanofiber scaffold was aligned in the direction of the nanofibers (assigned as 0°). (D) The results demonstrates a statistical increase in alignment for aligned nanofibers over random nanofibers ($p = 0.04$, $n = 3$ scaffolds). (E) Finally, for each sample, measurement of the cell aspect ratio was increased for cells on aligned scaffolds over those on random scaffolds ($p = 0.004$, $n = 6$ cells). These results demonstrate a clear impact of the fiber alignment of cell morphology. All results are presented as mean $\pm$ standard error.

Table 1

Summary of the Different Composition Copolymers Obtained by Different Feed Ratios of TOSUO Monomer^a

initiator (mmol)	A (%)	B (%)	catalyst (mmol)	time (h)	temp (°C)	M_n	M_w	D_m	ketone%
0.13	98.3	1.8	0.053	24	80	21200	24600	1.16	3.2
0.13	96.5	3.5	0.053	24	80	19500	23900	1.23	4.7
0.13	94.7	5.3	0.053	24	80	19600	23300	1.19	5.6
0.13	93.0	7.0	0.053	24	80	17300	20100	1.16	7.0

^aThe content of TOSUO feed is about the same as of that in the final polymer. INITIATOR-DIBO, A-CL, B-TOSUO, CAT-stannous octoate.