

**IMMOBILIZING GRADIENTS OF
NEUROTROPHIC FACTORS FOR DIRECTED
PERIPHERAL NERVOUS SYSTEM CELL
MIGRATION GUIDANCE**

BY:

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ABSTRACT

Bioengineered nerve guidance conduits (NGCs) offer a less invasive treatment option to current surgical procedures for peripheral nerve repair following traumatic injury. Biochemical cues and topographical guidance have been shown to independently contribute to directing Schwann cell migration guidance and neuron axonal extension across large gap injuries, but little work has been done in looking at the effect of combining these two elements on cell response. In the present study, we report a number of methods to immobilize concentration gradients of neurotrophic factors onto the surface of aligned polycaprolactone (PCL) nanofibers and collagen microfibers using a simple diffusion-based microfluidic platform. Direct immobilization of molecules to PCL can be accomplished using a UV-activated crosslinker, and gradient parameters can be tuned by controlling UV irradiation time as well as loading concentration. A two-step method using EDC/NHS surface activation followed by streptavidin/biotin binding was found to be effective for immobilizing molecules to collagen. We propose the use of these immobilization protocols in future *in vitro* studies to investigate the synergy of neurotrophic factor gradient guidance and topographical alignment cues in a combinatorial platform with the goal of maximizing directed peripheral nervous system cell response and enhancing the functionality of NGCs.

Advisor: Hai-Quan Mao, Ph.D.

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1. Introduction

Millions of patients worldwide suffer from tissue damage or loss as a result of accidents, trauma, or cancer every year. For many tissue types, autologous transplantation, a surgery in which healthy tissue taken from the patient is transplanted to the site of the defect in the patient's body, remains the most common form of treatment despite complications such as donor site morbidity, donor site mismatch, and severe pain to patients as a result of the secondary surgery.¹ Artificial constructs with tunable properties that can aid in restoring and maintaining functional tissue promise to be a less invasive alternative to autotransplantation. As a case in point, peripheral nerves have incredible regenerative capacity following a transection injury, but in large gap injuries that cannot heal naturally or be treated with direct end-to-end repair, the clinical gold standard is the autologous nerve graft.² Tissue engineering efforts in the context of peripheral nerve repair attempt to recapitulate and restore the distal nerve stump and create a scaffold that aids regenerating axons reach from the proximal nerve stump to the distal end.³ Engineered nerve guidance conduits (NGCs) provide mechanical support to extending neurites and protect the regenerating nerve from the ingrowth of fibrous scar tissue. They must also provide a microenvironment that mimics the extracellular matrix (ECM)—a three dimensional network composed of proteoglycans, fibrillary proteins, and glycoproteins that provide physical, chemical, and mechanical cues to guide cellular processes⁴—and present biochemical signaling cues to promote cell adhesion and survival in order to guide regenerating nerves to the target site.⁵ Although no artificial NGC to date is as effective as the autologous nerve graft, extensive work in NGC design has been done in manipulating four essential NGC components to enhance the efficacy of

regeneration across large gaps to match that of the gold standard. These components are the growth substrate (often in the form of hydrogels or fibers), ECM proteins, neurotrophic factors, and glial or support cells.⁶ Other considerations for materials selection in scaffold design include three-dimensional geometry and porosity to allow tissue growth as well as nutrient transport and exchange, controllable biodegradation and resorption rates, mechanical properties that are comparable to the native tissue, and ease of processing.

Materials commonly used in NGCs include both synthetic and natural biodegradable polymers. Synthetic polymer materials, such as poly-L-lactic acid (PLLA), polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA), and polycaprolactone (PCL) offer tunable degradability and mechanical properties. Natural polymers such as collagen, fibrin, gelatin, and chondroitin sulfate offer greater biocompatibility than synthetic materials but more limited mechanical support.³ The incorporation of cells and growth factors is also critical to the success of engineered nerve grafts. Following a nerve injury, Schwann cells (SCs) clean up myelin debris and dedifferentiate into a proliferative phenotype. They also mediate peripheral nerve regeneration through their neurotrophic and neurotropic influence, secreting growth factors that promote axon regrowth and enhance cell survival in addition to expressing cell adhesion molecules and producing basal lamina components.^{3,7} Several neurotrophic factors (NFs) secreted by SCs that are of interest for NGCs have been identified. Glial cell line-derived neurotrophic factor (GDNF) protects motor neurons from cell death.⁸ Nerve growth factor (NGF) promotes SC migration, and along with neurotrophin-3 (NT-3) and brain-derived neurotrophic

factor (BDNF), promotes neurite elongation.^{9,10} The addition of exogenous NFs and ECM proteins, including laminin and Arg-Gly-Asp (RGD), Ile-Lys-Val-Ala-Val (IKVAV), and Tyr-Ile-Gly-Ser-Arg (YIGSR) peptide sequences¹ to a NGC for delivery to the target tissue, can supplement the role of endogenous NFs.

A successful NGC should mimic the microenvironment in native tissue, but current designs fail to provide spatial and directional cues for nerve growth. The organized formation of tissue relies on the spatial distribution of biochemical cues. Concentration gradients of biomolecules are one of the most critical evolutionarily-conserved signaling mechanisms for guiding a variety of cellular processes including growth, migration, and differentiation within living tissue. The precision of gradient signaling is crucial, as the cellular response to the gradient is dependent upon on the complement, the concentration, and the spatiotemporal specifications of the gradient exposed to the cell.¹¹ The importance of gradients of biochemical cues in the development of the nervous system is well-documented. In brief, the development of the nervous system begins with neural induction, in which ectodermal cells differentiate into the neural plate. Neural induction is followed by neurulation, in which cells in the neural plate migrate to form the neural tube—this will eventually develop into the central nervous system—and the neural crest cells, which give rise to the cells of the peripheral nervous system (PNS), including PNS sensory neurons, Schwann cells, postganglionic neurons, satellite cells for dorsal root ganglia (DRGs) and autonomic ganglia.¹² After a neural crest cell migrates to its final location, axonal growth to the target site begins, starting with the formation of the growth cone. The growth cone, located at the tip of the extending neurite, is highly sensitive to

concentrations of biomolecules and ECM molecules; axons will grow into areas rich in chemoattractant factors but avoid those rich in chemorepellent factors. Growth factors enable motor neuron synapse formation with skeletal muscle, and the secretion of neurotrophins, which include NGF, BDNF, and NT-3, are responsible for ensuring the survival of sensory neurons. The signaling molecules involved in this series of cell differentiation, migration, and growth steps are strictly regulated spatially and temporally, and gradient-induced signaling cascades often interact with one another.¹¹ Thus, multiple concentration gradients acting in conjunction, and the specific details of each one, play a major role in dictating cellular processes and should be taken into account while designing an effective NGC. In addition to chemical gradients, physical gradients, which are gradients in physical properties such as substrate stiffness, topography, or porosity, are of interest in tissue engineering applications for other tissue types including bone, cartilage, and cardiac tissues.⁴

The formation of concentration gradients requires consideration of a number of attributes: gradient dimensionality, gradient directionality, the length scale of the gradient, and the temporal dependency.¹³ The dimensionality of the gradient is dictated by the dimensionality of the matrix on or in which the gradient is placed. Gradients are inherently directional because some physical or chemical property varies in a particular direction across the substrate. Most gradient assemblies are unidirectional (or more simply, directional) because the property varies along one direction. However, many variations can be made to the unidirectional gradient platform by changing two different properties along a specified direction or by creating two gradients of the same type but

propagate in opposite directions. A more natural extension of the unidirectional gradient into multiple directions is the radial gradient in which the property changes radially from a central point. A multitude of multidirectional gradients can be developed by combining unidirectional gradients in different manners and in multiple dimensions. The length scale of the gradient is another important consideration. Obviously, the gradient possesses an intrinsic length scale—the overall length over which a given property is varied on the substrate—but at length scales much smaller than the inherent length, the property of interest may actually appear uniform. Most gradients are static, meaning that their properties are fixed once the gradients are formed, but in some applications, there may be a need for a gradient that can change properties at a later time in response to an external stimulus.

In the context of tissue engineering, cell response is dependent upon the concentration and the slope of the concentration gradient, thus the source concentration, the concentration range, and the gradient length are a critical design aspect for gradients *in vitro*.⁴ Since gradient presentation in native tissue depends on the binding of affinity of growth factors and adhesion molecules to the ECM, biochemical concentration gradients are classified as either immobilized or diffusible. In immobilized gradients, molecules are tightly bound to the ECM, whereas in diffusible gradients, soluble factors are not tightly bound to the ECM. Immobilized gradients are advantageous for peripheral nerve regeneration because they can remain stable for a longer time scale than diffusible gradients and therefore promote growth more efficiently over long gap injuries. Previous studies have shown that photoimmobilized gradients of laminin-1 (LN-1) in agarose gels

promote directional neurite extension and faster neurite extension rates in chicken embryo DRGs in comparison to uniform concentrations of the adhesion molecule.¹⁴ Furthermore, immobilized gradients of LN-1 and NGF work synergistically to promote enhanced axonal regeneration across large peripheral nerve gaps that cannot be achieved by a concentration gradient of a single molecule.¹⁵ A similar result has been shown for immobilized gradients of multiple NFs in poly(2-hydroxyethylmethacrylate) scaffolds to guide chick DRG neurite growth.¹⁶ The shortcoming of immobilized gradients is the challenge of bonding the protein or growth factor to the substrate.⁴ Not all proteins can be covalently bound to the substrate, and those that are may exhibit a loss of bioactivity due to certain functional groups being blocked by the chemical conjugation. This compromised bioactivity limits the functionality of the concentration gradient and potentially the therapeutic potential of a NGC. Furthermore, since cells cannot uptake growth factors immobilized to the scaffold, signaling pathways that depend on the uptake of the immobilized proteins may be affected, limiting the extent to which the scaffold mimics *in vivo* tissue. The advantage of diffusible gradients of soluble NFs is that cells are continuously surrounded by NFs and the NFs can be uptaken by the cell. Diffusible gradients incorporated into hydrogels have also been shown to guide neurite extension but they are less stable than immobilized gradients and dissipate in a few days.¹⁷ The greatest limitation of diffusible gradients is that spatiotemporal evolution of the gradient can be difficult to control and generated gradients are therefore less reproducible.

Traditional methods of generating concentration gradients include injecting known concentrations of the molecule of interest into the substrate or depositing arrays of

droplets on the substrate surface, pneumatically ejecting a known concentration of solution out of a micropipette tip into the ECM, Transwell assays, Zigmond chambers, and Dunn chambers.¹¹ More recent methods of gradient generation that employ microfluidic devices can produce much more complex and defined gradient environments that are predictable, reproducible, and quantitative.⁴ The simplest devices use diffusion to create a gradient between a source and a sink. Diffusion-based gradients may be maintained for several days as long as the source and sink volumes are sufficiently large, but gradient generation tends to be slow. Devices that incorporate fluid flow can generate gradients on much shorter time scales. The tree-like microfluidic gradient generator is a classic device that dilutes a solution at each successive branch in the microchannel network and uses diffusive mixing to form gradients across hundreds of microns in a few minutes.¹⁸ The disadvantage of the tree-like gradient generator is that the length of the gradients is limited to a few millimeters and the gradient remains only while the device is on, otherwise diffusion will equilibrate the concentration. Dynamic mixing devices that use multiple syringe pumps in parallel to pump fluids at controllable rates into a mixing chamber can generate gradients with controllable profiles. Convection-driven devices generate highly reproducible and well-controlled gradients over length scales in the centimeter range in timeframes on the order of seconds to minutes.^{19,20} The general protocol in such devices is to fill a channel with a baseline solution, load an outlet port with a second solution and draw it into the channel at a high flow rate, and subject the solutions to alternate flow by pumping it back and forth until a gradient forms. In general when using these techniques, the gradient is formed within the prepolymer solution and then gradient is embedded within the scaffold when polymer is crosslinked.

Alternatively, the gradient may be generated over a preformed substrate and the crosslinking process creates the gradient on the scaffold.

We use a diffusion-based method to generate gradients on various scaffold materials including PCL, collagen, and fibrin. We have developed a simple yet versatile microfluidic platform that uses a polydimethylsiloxane (PDMS) gradient channel of controlled height connected to a source well and a sink well. The gradient channel is placed over the substrate, and the channel is filled with a baseline solution. The source well gets sealed and the sink well is filled with the same baseline solution as the channel. The sink well gets sealed, excess baseline solution in the source well is removed, and the source well is filled with the highest desired concentration of NF. The source well is sealed once again, and the gradient is allowed to establish over the course of hours according to Fick's laws of diffusion. Our platform creates gradients that are highly reproducible and scalable. This platform also offers great flexibility in the choice and concentration range of NFs. Furthermore, since there is no fluid flow, gradient formation is gentle and can take place in the presence of cells, and we can easily monitor live cell gradient response.

A wide array of methods are available to immobilize molecules onto the surface of a substrate. In general, the chosen immobilization scheme should satisfy the following criteria: the method should be fast and efficient, selective to a particular functional group that is ideally already present on the substrate, and should avoid the use of harsh conditions that could disrupt the functional group.²¹ Common methods used to

chemically couple proteins to substrates include 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) reactions, Diels-Alder reactions, click chemistry, and Michael addition. The EDC/NHS reaction is a standard method to immobilize amine-containing groups onto carboxylated surfaces via the formation of an amide bond, thus it is suitable for coupling molecules to substrates composed of ECM proteins. Immobilization with EDC/NHS is advantageous due to its high conversion efficiency, mild reaction conditions, and its biocompatibility without disrupting bioactivity.²² The Diels-Alder cycloaddition reaction is used in a large number of applications, including conjugating proteins, peptides, and oligonucleotides onto various substrates. It has been shown that the Diels-Alder reaction is fast, mild, and chemoselective in aqueous conditions, which renders it useful for biomedical applications.²³ Due to its high selectivity and yield, click chemistry is a popular technique for functionalizing the surface of various biomacromolecules via a copper(I)-catalyzed Huisgen cycloaddition reaction.²¹ Michael addition can be used to conjugate thiol- or amine-containing molecules such as proteins to maleimide- or acrylate-functionalized surfaces. Even though Michael addition requires an extra functionalization step because most substrates for tissue engineering applications lack maleimide or acrylate groups, this chemistry works well because thiol groups are highly reactive and the coupling has good stability.²⁴ In addition to chemical coupling, physical methods such as photoimmobilization using ultraviolet (UV) activated groups may be used to immobilize molecules onto substrates. Biological interactions, such as streptavidin/biotin binding, may be employed as well, but may be limited by nonspecific interactions that disrupt cell or protein interactions with the surface.²¹

In this study, we functionalized our substrates with NHS-diazirine or activated them using the EDC/NHS reaction before immobilizing a concentration gradient of amine-biotin to the substrate surface. A streptavidin-conjugated molecule of interest was then added to allow streptavidin/biotin binding to occur and form a gradient of the molecule. NHS-diazirine is an amine-reactive UV-activated crosslinking molecule. Since it is amine-reactive, it can be used with a multitude of proteins, peptides, growth factors and other aminated molecules. Furthermore, diazirine-mediated crosslinking will not occur in the absence of UV light, so immobilization is controllable by tuning the exposure to UV irradiation. Diazirine-mediated coupling is also versatile and can be used on both synthetic and natural polymer substrates. The EDC/NHS reaction was selected because the chemistry is well-understood and can be used to couple several molecules to ECM substrates.

Electrospinning is a simple and versatile method to make random or aligned nanofibers in many tissue engineering applications. The general principle of electrospinning is to apply a high voltage to a polymer solution being extruded through a needle at a constant rate. Eventually, the electrostatic charge that builds up on the polymer droplet at the needle tip and the electric field overcome the inherent surface tension of the polymer, and a fine jet of polymer is ejected from the needle tip. The polymer jet whips and stretches before landing in a random nonwoven mesh on a grounded collection plate.²⁵ Rotating the collection plate during the spinning process results in meshes of aligned fibers. Fibrous scaffolds provide a higher surface area than

solid wall scaffolds on which proteins and cells can attach. Additionally, the fiber dimensions can be tuned by altering the parameters of the electrospinning process such as the polymer concentration, solution viscosity, solution flow rate, and collecting distance.^{26,27} Fiber diameter has been shown to affect both the directionality of neurite extension and SC migration; individual fibers that are too small cannot be detected by neurites and SCs, whereas larger fibers provide well-defined grooves in which cells can move.²⁸ Electrospinning can be done on a wide range of synthetic biodegradable polyesters such as PCL and PLGA, as well as natural polymers such as collagen, fibrin, alginate, hyaluronic acid, and gelatin. Nanofiber-based scaffolds not only resemble the morphology of the ECM but the surface can be functionalized to act as a delivery vehicle for protein coatings or signaling molecules and enhance the scaffold biofunctionality.²⁷

In this study, aligned PCL nanofibers were made using traditional electrospinning techniques. PCL is a hydrophobic and semi-crystalline polyester commonly used for drug delivery and tissue engineering applications. Like similar aliphatic polyesters, PCL offers tailorable degradation kinetics, mechanical properties, and porosity that make it a good candidate for tissue ingrowth and controlled drug delivery. Due to its long carbon chain, PCL has a longer degradation and resorption times in comparison to PLLA, PGA, and their copolymers, which makes it a suitable option for sustained drug release or long lifetime tissue scaffolding. Moreover, PCL possesses superior rheological and viscoelastic properties that make it easy to manufacture and manipulate into a variety of scaffolds for a relatively low cost.²⁹

In addition to traditional electrospinning, we employ a modified electrospinning technique to generate microfibers of hydrogel materials with internal alignment. Aqueous polymer solutions are ejected into a rotating collection bath where they are rapidly crosslinked into hydrogels. Fiber alignment is a result of both the applied voltage and mechanical stretching. The electric field induces alignment of individual polymer chains, and mechanical stretching occurs as the polymer jet lands in the rotating collection bath. As with dry electrospinning, fiber dimensions can be tuned by altering spinning parameters including the polymer concentration, collection distance, flow rate, and the rotation speed of the crosslinking solution. Uniaxial molecular alignment within the hydrogel fibers using our modified electrospinning method has been confirmed using scanning electron microscopy and small angle X-ray scattering.³⁰

We used our wet electrospinning method to form aligned sheets of collagen type I fibers. Collagen I is a fibrous protein found in the ECM and connective tissue.³¹ The basic structural subunit of collagen I is a 300 nm long triple-stranded helical molecule composed of three coiled α helix chains that contain repeating Gly-Pro-X motifs. Collagen I molecules pack together in a staggered array to form collagen fibrils with 50 nm diameter. Crosslinks between lysine and hydroxylysine residues at the ends of individual molecules stabilize the packed structure to generate strong fibrils that are several microns long. Collagen can be chemically crosslinked with glutaraldehyde to improve its stability against degradation, but more biocompatible alternatives to glutaraldehyde include cyanamide, EDC, and hexamethylene diisocyanate, or physical crosslinking methods such as UV or heat irradiation.³² Collagen scaffolds are clinically

available as corneal shields,³³ injectable dermal fillers,³⁴ tissue adhesives,³⁵ and skin coverings to heal burns and skin ulcers.³⁶ Collagen is intrinsically bioactive, so surface modification not necessary, but can be done to tune its biofunctionality.

Using our gradient generation platform, we aimed to study the synergy of aligned substrate topography and biochemical cues to enhance directed PNS cell response *in vitro* by immobilizing concentration gradients of neurotrophic factors to electrospun fibers. Previous work has shown that both aligned substrates in the absence of chemical guidance cues and gradients of growth factors in the absence of aligned topography promote the regeneration of axons across nerve gap injuries.^{37,38} Yet, little work has been done to investigate the combined effects of aligned topographical cues and biochemical gradients in promoting nerve regrowth and enhancing the functionality of engineered nerve guides. Additionally, the optimal presentation of biochemical cues in NGCs is yet to be understood. Both immobilized gradients³⁸ and diffusible gradients^{17,39} of soluble biomolecules have been effective in enhancing neurite guidance, but there is a gap in the knowledge regarding which form of gradient produces improved regeneration and functional outcome in the presence of topographical cues. We hope to develop a versatile and reliable method for immobilizing concentration gradients of growth factors onto micro- and nanofibrous polymeric substrates and elucidate the role of the synergistic effects of biochemical guidance and contact guidance in peripheral nerve regeneration.

2. Materials and Methods

2.1 Materials and reagents

Polycaprolactone (PCL, average M_n 80,000), *N,N*-dimethylformamide (DMF, anhydrous, 99.8%), rhodamine B, Poly(ethylene oxide) (PEO, average M_v 4,000,000), lysozyme, *N*-hydroxysuccinimide 98% (NHS), streptavidin-FITC conjugate, and streptavidin-Cy3 conjugate were purchased from Sigma-Aldrich. Succinimidyl 6-(4,4'-azipentanamido)hexanoate (NHS-LC-diazirine or NHS-diazirine) and EZ-Link amine-PEG₂-biotin (amine-biotin) and were purchased from ThermoFisher Scientific. Collagen I from rat tail (3 mg/ml) was purchased from Gibco. Dichloromethane (DCM, 99.5%) was purchased from Alfa Aesar. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Santa Cruz Biotechnology, Inc. Glycine was purchased from Invitrogen. Lightning Link FITC was purchased from Novus Biologicals. Dow Corning Sylgard 184 Silicone Elastomer Kit was purchased from Krayden, Inc. BT-402 secure medical adhesive was purchased from Factor II, Inc.

2.2 Electrospinning of aligned nanofiber sheets

7% w/w PCL was dissolved in 9:1 DCM/DMF by volume and placed in a 1 mL syringe fitted with a blunt-end 27 gauge needle tip. Glass coverslips were taped to the circumference of a grounded 40 cm diameter aluminum collecting wheel. An electrode was attached to the needle tip, the separation distance between the needle tip and the collecting plate was set to 6 cm, and an electric potential of approximately 15 kV was applied to the PCL solution to generate a positive charge at the needle tip. The syringe pump holding the PCL was set to a flow rate of 1 mL/hr and mounted on a linear stage

set to raster the syringe pump back and forth to control the deposition of the polymer over a specified area. The charged polymer jet was ejected onto the rotating collecting plate (60-70 rpm). The coverslips were removed from the collecting plate and the nanofiber sheets were glued to the coverslips with Factor II silicone adhesive.

2.3 Preparation of hydrogel films

Collagen type I films were polymerized in 500 μm polydimethylsiloxane (PDMS) frames with outer dimensions 14 mm (length) \times 8 mm (width) and inner frame dimensions 10 mm (length) \times 4 mm (width) to form gels with 500 μm thickness. 8 parts chilled collagen Type I (3 mg/ml) were mixed with 1 part chilled sodium bicarbonate (7.5% w/v) and 1 part chilled 10 \times PBS. The collagen solution was pipetted into separate frames and incubated at 37°C for one hour to polymerize.

2.4 Electrospinning of aligned hydrogel sheets

1 wt% collagen type I was dissolved in 0.2 wt% PEO and placed in a 1 mL syringe fitted with a blunt-end 27 gauge needle tip. 50 mL of a 40 mg/mL EDC collection solution was deposited on a grounded aluminum collection wheel rotating between 30-40 rpm. An electrode was attached to the needle tip, the separation distance between the needle tip and the collection wheel was set between 3-3.5 cm, and an electric potential of 4.0 kV was applied to the collagen solution. The syringe pump holding the collagen was set to a flow rate of 3.5 mL/hr. The charged polymer jet at the needle tip was ejected into and crosslinked in the rotating collection solution (**Figure 1**). The syringe pump was mounted on a linear stage set to raster the syringe pump back and forth (45-60 seconds

per pass) to form sheets of collagen microfibrils. The fiber sheet was given an additional 5 minutes to crosslink on the collection wheel after spinning. The collagen sheet was cut into 6-8 sections. Each section was wrapped on a polyethylene terephthalate (PET) frame with outer dimensions 14 mm (length) \times 8 mm (width) and inner frame dimensions 10 mm (length) \times 4 mm (width), and the sheets were stored in a 12-well plate filled with DI water at 4°C until use.

At the time of use, each collagen fiber sheet was glued between PET frames with outer dimensions 18 mm (length) \times 12 mm (width), and this PET-collagen-PET construct was glued between 500 μ m PDMS frames with the same dimensions.

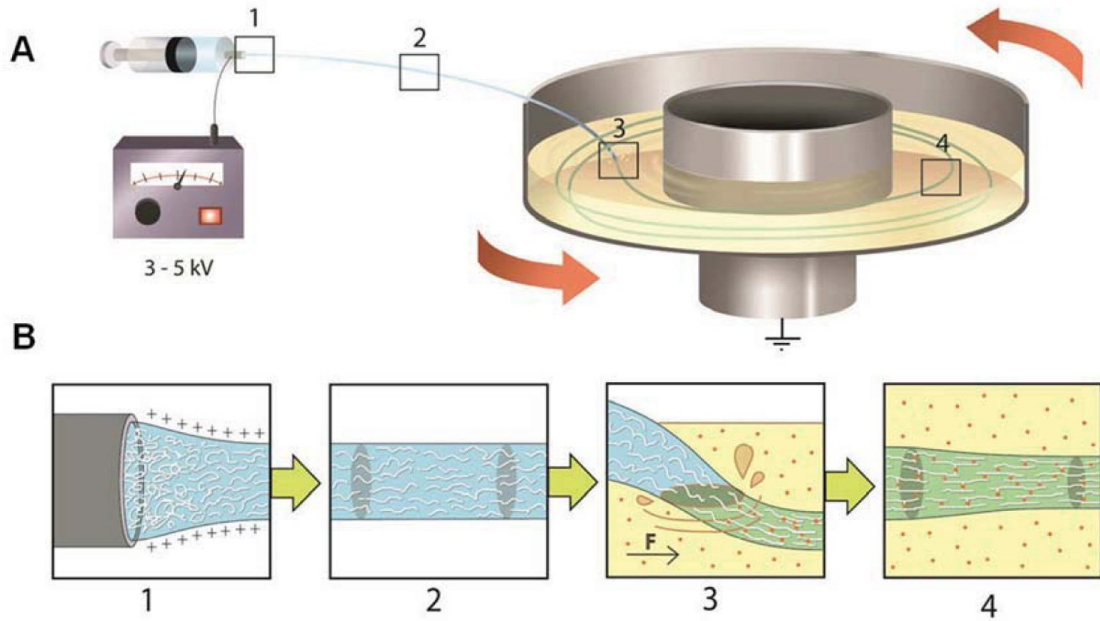


Figure 1: Hydrogel electrospinning platform by Zhang *et al.* Aqueous polymer solution is extruded from the needle tip and stretched electrically by an applied electric field and mechanically in the rotating bath of crosslinking solution.³⁰

2.5 Fabrication of microfluidics gradient devices

Silicone elastomer base and curing agent were mixed in a 10:1 ratio by mass to form a PDMS prepolymer solution. The mixed prepolymer solution was placed in a vacuum chamber for 1 hour to remove gas bubbles. A 2 mm thick layer of prepolymer solution was poured over a silicon master and baked at 80°C for 2 hours to form gradient channels with dimensions of 0.1 mm (height) \times 10 mm (length) \times 4 mm (width). The cured PDMS was removed from the silicon master and a 3 mm hole punch was used to cut out wells at the end of each channel.

PDMS seals with dimensions 2 mm (height) \times 4 mm (length) \times 4 mm (width) and PDMS frames of 500 μ m thickness with outer dimensions 18 mm (length) \times 12 mm (width) and inner frame dimensions 10 mm (length) \times 4 mm (width) were fabricated using the same prepolymer solution. The prepolymer solution was poured between two sheets of transparency film, clamped between two aluminum blocks to the desired thickness, and baked at 80°C for 2 hours. Cured PDMS was peeled off the film and cut to the desired dimensions. All PDMS gradient device components were stored in 70% ethanol until use.

2.6 Characterization of immobilization using NHS-diazirine

NHS-diazirine was dissolved in DMSO and diluted to 5 mg/ml in PBS. Rhodamine was dissolved in PBS and NHS-diazirine was added to the protein solution to yield a 10:1 molar ratio of rhodamine to NHS-diazirine. The rhodamine-diazirine solution was

allowed to react at room temperature for 1 hour and then aliquoted into 100 μ L aliquots and stored at -20°C until use.

Aligned PCL sheets were cut to dimensions of 10 mm (length) \times 4 mm (width) and glued to a glass coverslip with Factor 2 tissue glue. To characterize time-dependent immobilization, rhodamine-diazirine solution was thawed and diluted to 100 μ g/ml. 50 μ L of rhodamine-diazirine was placed on the prepared PCL to cover the entire surface and the sheets were exposed to 360 nm wavelength UV light for 0, 1, 2, 5, or 10 minutes. Excess rhodamine-diazirine was removed immediately after UV irradiation. Samples were washed in RO water 3 \times for 10 minutes. To characterize concentration-dependent immobilization, rhodamine-diazirine was diluted to 10, 50, and 100 μ g/ml. 50 μ L of rhodamine-diazirine was placed on prepared PCL to cover the entire surface and all samples were exposed to 360 nm UV light for 10 minutes. Excess rhodamine-diazirine was removed immediately after UV irradiation. Fibers were washed in RO water 3 \times for 10 minutes. Rhodamine immobilization was visualized using fluorescence microscopy.

2.7 Gradient generation and immobilization using NHS-diazirine

Rhodamine-diazirine solution was thawed and diluted to 100 μ g/ml. Aligned PCL sheets were cut to dimensions of 10 mm (length) \times 4 mm (width) and glued to a glass coverslip with Factor 2 adhesive. As shown in **Figure 2**, a PDMS gradient channel was placed over the fibers (A) and the channel was filled with PBS (B). The source well was sealed, and the sink well was filled with PBS and sealed (C). The source well was unsealed, any PBS overflow was removed (D), and the source well was filled with

rhodamine-diazirine (E). The source well was sealed and a gradient was allowed to establish over 1-2 hours (F). After gradient formation was complete, samples were irradiated with 360 nm wavelength UV light for 10 minutes. The gradient channel and was removed from the PCL. The fibers were flushed with 1 mL RO water to remove excess rhodamine-diazirine and then washed in RO water 3× for 10 minutes. Rhodamine gradient immobilization was visualized with fluorescence microscopy and characterized with ImageJ.

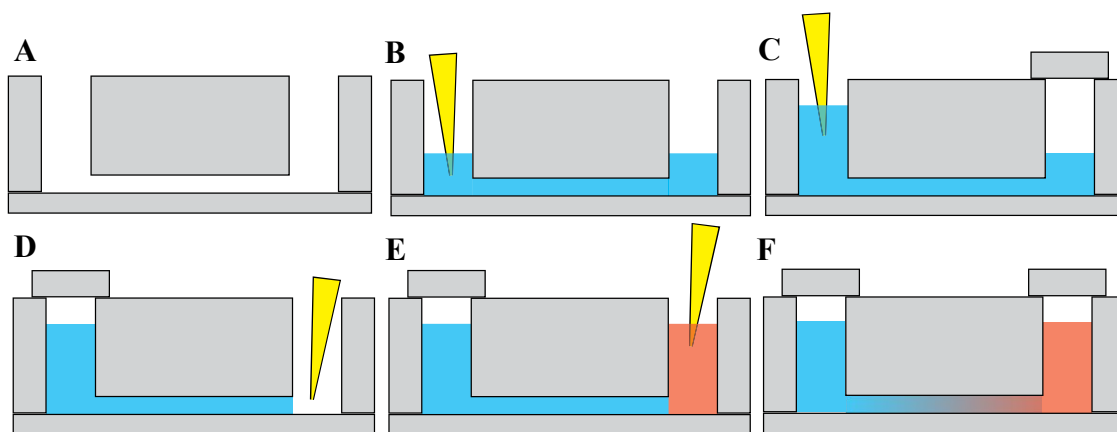


Figure 2: Diffusion-based gradient formation platform. (A) PDMS channel is placed over the substrate of interest. (B) Channel is filled with baseline solution. (C) Source well is sealed and sink well is filled with baseline solution. (D) Sink well is sealed and excess baseline solution is removed from source well. (E) Source well is filled with highest desired concentration of molecule of interest. (F) Source well is sealed and gradient forms over the course of hours. Image adapted from Kellin Krick.

2.8 Characterization of two-step immobilization using NHS-diazirine

NHS-diazirine was dissolved in DMSO and diluted to 5 mg/ml in water (pH 8). EZ-link amine-PEG2-biotin was dissolved in water (pH 8) and the reagents were mixed in a 1:1 molar ratio and reacted for 1 hour to yield a 1 mM biotin-diazirine solution in water. The biotin-diazirine solution was aliquoted into 100 μ L aliquots and stored at -20°C until use.

Sheets of PCL nanofibers were cut to dimensions of 10 mm (length) \times 4 mm (width) and glued to glass coverslips with Factor 2 adhesive. Biotin-diazirine stock solution was thawed and diluted to concentrations of 200, 400, 600, 800, and 1000 μ M. Fifty μ L of biotin-diazirine was placed on prepared PCL to cover the surface of the fibers. Fifty μ L of PBS was placed on a group of PCL fibers as a negative control. The fibers were irradiated with 365 nm UV light for 5 minutes. Fibers were flushed with 1 mL PBS to remove excess biotin-diazirine and then washed in PBS 3 \times for 10 minutes. 1 mg/ml stock solution of FITC-conjugated streptavidin was diluted to 100 μ g/ml. Fifty μ L of streptavidin-FITC was loaded onto the biotinylated PCL and allowed to react overnight at room temperature. Excess streptavidin-FITC was removed from PCL and washed 3 \times in PBS for 10 minutes. Biotin/streptavidin-FITC immobilization was visualized using a Typhoon Gel Reader and characterized with ImageJ.

2.9 Characterization of EDC/NHS-mediated surface functionalization

Collagen gels and collagen sheets were incubated in MES buffer (pH 6) for 30 minutes at room temperature. EDC and NHS were dissolved in MES in a 2:3 ratio by mass, respectively, and mixed for 15 minutes to yield a 1 mg/ml EDC/NHS solution. After removing the buffer, the collagen gels were incubated in the EDC/NHS solution overnight at room temperature. The gels were then washed in PBS for 10 minutes.

EDC/NHS-activated coupling was characterized with FITC-lysozyme as a fluorescently-labeled amine analog. FITC-lysozyme was diluted to concentrations of 100 μ g/ml, 50 μ g/ml, 25 μ g/ml, 10 μ g/ml, 5 μ g/ml, and 1 μ g/ml in PBS and placed on the

collagen to cover the surface and reacted at room temperature for 2 hours. Excess FITC-lysozyme was removed and the collagen was washed in PBS 3× for 15 minutes. FITC-lysozyme coupling to collagen was visualized using a Typhoon Gel Reader and compared to a standard. Fluorescence intensity was quantified with ImageJ.

2.10 Gradient generation and immobilization via EDC/NHS-mediated surface functionalization

Amine-biotin was dissolved in PBS to yield 100 µg/ml and 10 µg/ml solutions. Collagen gels and collagen sheets were placed in a 12-well plate and incubated in MES buffer (pH 6) for 30 minutes at room temperature. EDC and NHS were dissolved in MES in a 2:3 ratio by mass and mixed for 15 minutes to yield a 1 mg/ml EDC/NHS solution. The buffer was removed from the collagen. The EDC/NHS solution was added over the collagen and the samples were incubated overnight at room temperature. After surface activation, the EDC/NHS was removed and the gels were washed in PBS for 10 minutes. A PDMS gradient channel was placed over each collagen sample. The channel was filled with PBS. The source well was sealed with a PDMS seal, and the sink well was filled with PBS and sealed with a PDMS seal. The seal on the source well was removed, any PBS spillover was removed, and the well was filled with either 10 µg/ml amine-biotin or 100 µg/ml amine-biotin and resealed. A concentration gradient of amine-biotin was allowed to establish via diffusion over the course of 4 hours.

Gradient formation was halted by removing the seal and the contents of the source well, removing the seal from the sink well, and pulling out the remaining solution via the

source well. 20 mM glycine solution was flushed through the gradient channel to quench the reaction of amine-biotin with the activated collagen surface. Each channel was filled with 100 µg/ml streptavidin-Cy3 and sealed, and the reaction proceeded for 2.5 hours. The gradient channels were removed, and the collagen was rinsed 3× with PBS to remove excess streptavidin-Cy3 and washed in PBS overnight. Gradient formation was visualized using a Typhoon Gel Reader and fluorescence intensity was quantified with ImageJ.

3. Results

3.1 Direct gradient immobilization using UV-activated crosslinking

We hypothesized that under a uniform loading concentration of rhodamine-diazirine, the amount of rhodamine conjugated to the PCL would increase as the UV irradiation time was increased. To test this, NHS-diazirine was first reacted with rhodamine B to make a rhodamine-diazirine solution. A uniform concentration of the rhodamine-diazirine solution was placed on PCL nanofibers and groups PCL were exposed to UV light for 0, 1, 2, 5, or 10 minutes. After UV irradiation, excess rhodamine-diazirine was washed off in a series of soaks in RO water. All unreacted rhodamine-diazirine that penetrated into the fibers was removed such that PCL nanofibers that were not exposed to UV light appeared as they did prior to the addition of the rhodamine-diazirine solution. Groups that were exposed to UV light, on the other hand, were pink and the color appeared more intense with increasing UV exposure times. Time-dependent immobilization of rhodamine using the diazirine chemistry was further confirmed using an inverted fluorescence microscope. Fluorescence intensity was greatest in the group exposed to UV

light for 10 minutes, and intensity decreased incrementally as the exposure time went down (**Figure 3**).

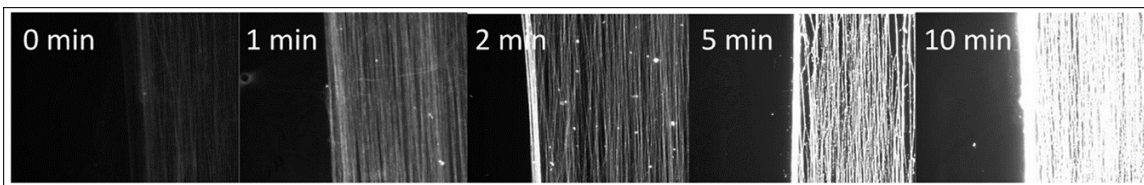


Figure 3: Time-dependent immobilization of rhodamine on PCL using NHS-diazirine as a crosslinker. Immobilization of rhodamine increases as UV irradiation time increases under a uniform loading concentration of rhodamine-diazirine.

We also hypothesized that under a uniform crosslinking time, the amount of rhodamine conjugated to PCL nanofibers would vary as the loading concentration of rhodamine-diazirine was altered. To test this, one group of PCL was loaded with 10 $\mu\text{g/ml}$ rhodamine-diazirine, another group was loaded with 50 $\mu\text{g/ml}$ rhodamine-diazirine, and a third group was loaded with 100 $\mu\text{g/ml}$ rhodamine-diazirine. All three groups were irradiated under UV light for 10 minutes. After irradiation, excess rhodamine-diazirine was washed off in a series of soaks in RO water. All groups appeared pink, indicating that rhodamine was conjugated to the PCL surface, and the intensity of the color increased with the loading concentration. Loading concentration-dependent immobilization of rhodamine with the diazirine chemistry was further confirmed using fluorescence microscopy. Fluorescence intensity was greatest in the group loaded with 100 $\mu\text{g/ml}$ solution and lowest in the group loaded with 10 $\mu\text{g/ml}$ solution (**Figure 4**).

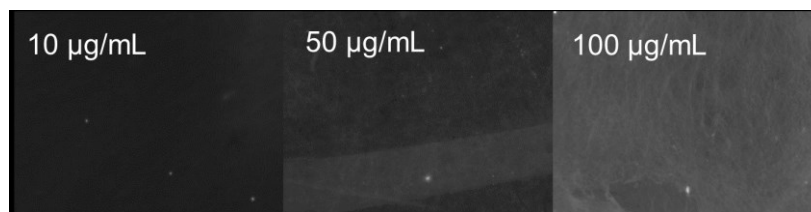


Figure 4: Concentration-dependent immobilization of rhodamine onto PCL nanofibers using NHS-diazirine as a crosslinking molecule. Under uniform UV irradiation time, immobilization of rhodamine increases as rhodamine-diazirine loading concentration increases.

Concentration-dependent immobilization was also tested by forming gradients of rhodamine-diazirine on the PCL. The source well of the gradient channel was filled with 100 µg/ml rhodamine-diazirine and the sink well was loaded with RO water. After the gradient had established, the device was exposed to UV light for 10 minutes. The PCL was washed in a series of soaks in RO water to remove unreacted rhodamine-diazirine solution. Gradient formation was confirmed with fluorescence microscopy, indicating that the rhodamine could be immobilized on the PCL in a graded manner, rather than in discrete concentrations, using diazirine as a crosslinking molecule (**Figure 5**).

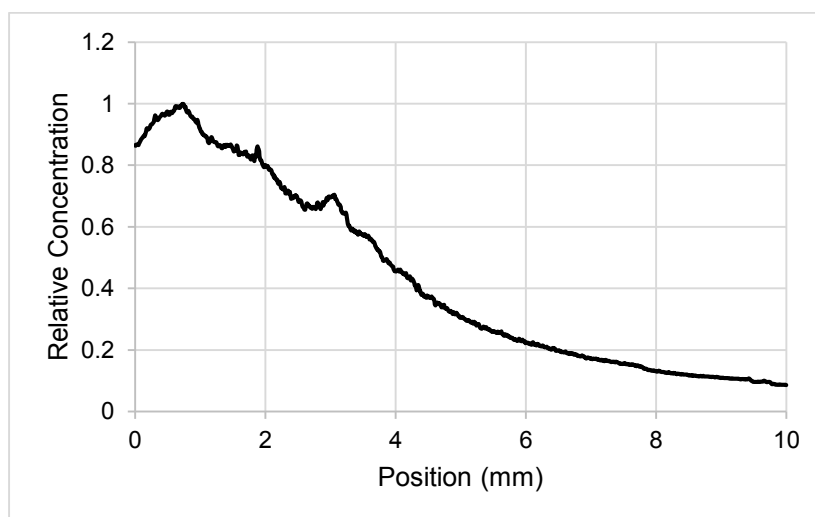


Figure 5: Representative gradient profile of rhodamine immobilized onto PCL using NHS-diazirine chemistry. Diffusion-based gradient generation in microfluidic platform results in well-defined gradients that establish in a few hours and the gradient remains intact on the PCL surface post-immobilization.

3.2 Indirect gradient immobilization using UV-activated crosslinking

To avoid the need to modify NFs directly with NHS-diazirine and expose the functionalized NFs to UV light, which could potentially cause denaturation and loss of bioactivity, an alternate method to immobilize molecules using NHS-diazirine as a crosslinker was developed. Amine-biotin was reacted with the NHS-diazirine to make a biotin-diazirine solution. We hypothesized that if we generated and immobilized a concentration gradient of biotin-diazirine and then added a streptavidin-conjugated molecule to the biotinylated surface, the streptavidin/biotin binding interaction would generate a gradient of the molecule of interest corresponding to the immobilized biotin-diazirine gradient on the substrate. To test this, sets of PCL fibers were loaded with 200, 400, 600, 800, or 1000 μM biotin-diazirine. A negative control was loaded with PBS. All groups were irradiated under UV light for 5 minutes. Following irradiation, excess biotin-diazirine was washed off and a uniform concentration of streptavidin-conjugated FITC was loaded onto the biotinylated fibers. After the streptavidin/biotin reaction was completed, excess streptavidin-FITC was washed off and fluorescence microscopy was used to check for immobilization. We expected to see the highest amount of FITC conjugation on the fibers that had been loaded with 1000 μM biotin-diazirine and a steady decline in conjugation as the biotin-diazirine loading concentration decreased. However, this two-step method using biotin-diazirine followed by streptavidin-FITC produced inconsistent immobilization at best. The addition of wash steps with SDS to remove excess biotin and blocking steps using BSA to prevent non-specific adsorption of streptavidin-FITC to PCL had a minimal effect in producing more consistent immobilization (**Figure 6**).

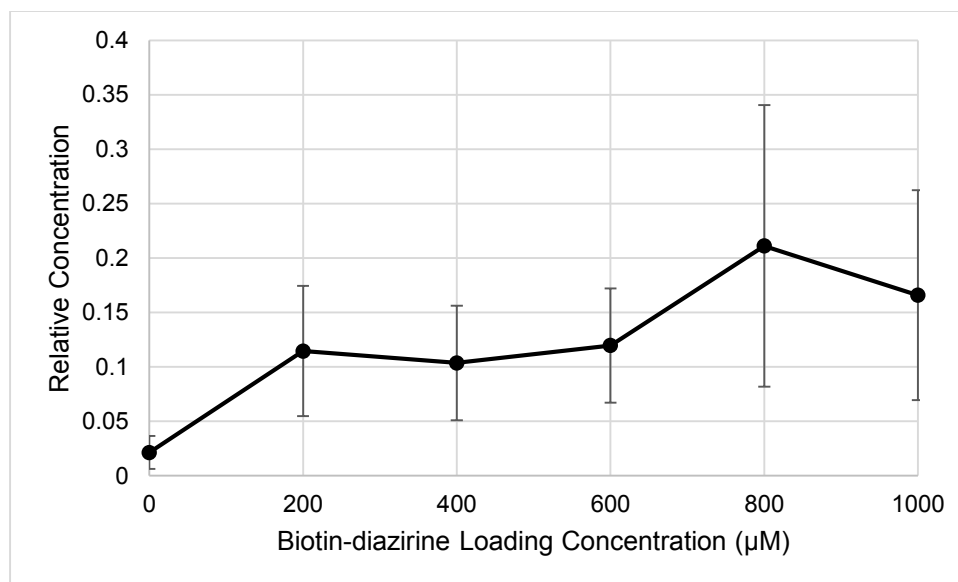


Figure 6: Streptavidin-FITC conjugation to biotin immobilized on PCL via NHS-diazirine chemistry. UV-mediated conjugation of biotin-diazirine followed by streptavidin/biotin binding led to inconsistent immobilization and no defined dependence on biotin-diazirine loading concentration.

3.3 Gradient immobilization using EDC/NHS surface activation

Carboxyl groups on the surface of collagen fibers were activated with EDC/NHS. We hypothesized that increasing the loading concentration of an amine-containing molecule on the activated surface would result in more amide bond formation. To test this, activated collagen fiber sheets and collagen films were placed in 1, 5, 10, 25, 50, or 100 $\mu\text{g/ml}$ FITC-labeled lysozyme solution. The FITC-lysozyme was removed and the collagen was washed in a series of soaks in PBS until unreacted FITC-lysozyme was no longer present in the PBS. The modified collagen was yellow and the intensity of the color was greatest in the 100 $\mu\text{g/ml}$ groups, suggesting that EDC/NHS-mediated immobilization varied with the concentration of an amine-containing molecule. Conjugation was found to be more effective on the fiber sheets than on the two dimensional (2D) gels, which is an outcome of the increased surface area for molecule attachment on microfibers. Surface immobilization corresponding to the loading

concentration of FITC-lysozyme was confirmed using a Typhoon Gel Reader and fluorescence intensity was quantified using ImageJ (**Figure 7**).

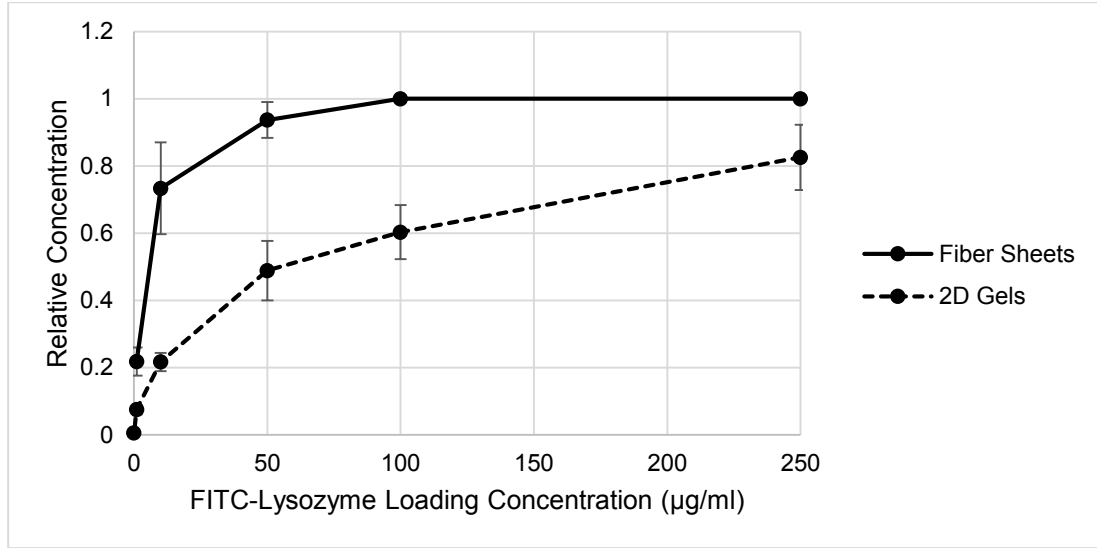


Figure 7: Immobilization of FITC-lysozyme to EDC/NHS-activated collagen fiber sheets and 2D collagen gels. Immobilization efficiency is higher on fibers sheets due to the greater surface area of fibers compared to the gels.

Following the success of immobilizing FITC-lysozyme to the surface of collagen fiber sheets and gels, gradients of amine-biotin were immobilized to 2D collagen gels. Rather than directly form a NF gradient on the activated collagen, we chose to generate a gradient of amine-biotin because its diffusion time is shorter than that of growth factors due to its small molecular weight. Additionally, steric hindrance is minimized by the use of a small molecule. To determine the time needed for amine-biotin gradient formation over a length of 10 mm, gradient formation was quenched at 3, 4, or 5 hours, and a uniform concentration of streptavidin-conjugated Cy3 was loaded onto the biotinylated surfaces. After streptavidin/biotin binding was complete, excess streptavidin-Cy3 was removed in a series of washes in PBS. Gradient formation was confirmed using a Typhoon Gel Reader and fluorescence intensity was quantified with ImageJ. In a gradient

channel of height 100 μm , a gradient of amine-biotin was found to form in 4 to 5 hours on 2D collagen gels. In an effort to determine the effect of concentration on gradient formation, amine-biotin gradients were generated over 4 hours on activated collagen gels with source well concentrations of 1, 10, or 100 $\mu\text{g/ml}$; the sink well was filled with PBS. It was found that over the course of 4 hours, gradient channels loaded with 1 $\mu\text{g/ml}$ amine-biotin equilibrated to a roughly uniform concentration over the length of the collagen sample. Gradients were formed in channels loaded with 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ amine-biotin, but gradients with a source well concentration of 10 $\mu\text{g/ml}$ were the most well-defined. Even though gradient formation is detectable, immobilization efficiency is low on collagen films (**Figure 8**).

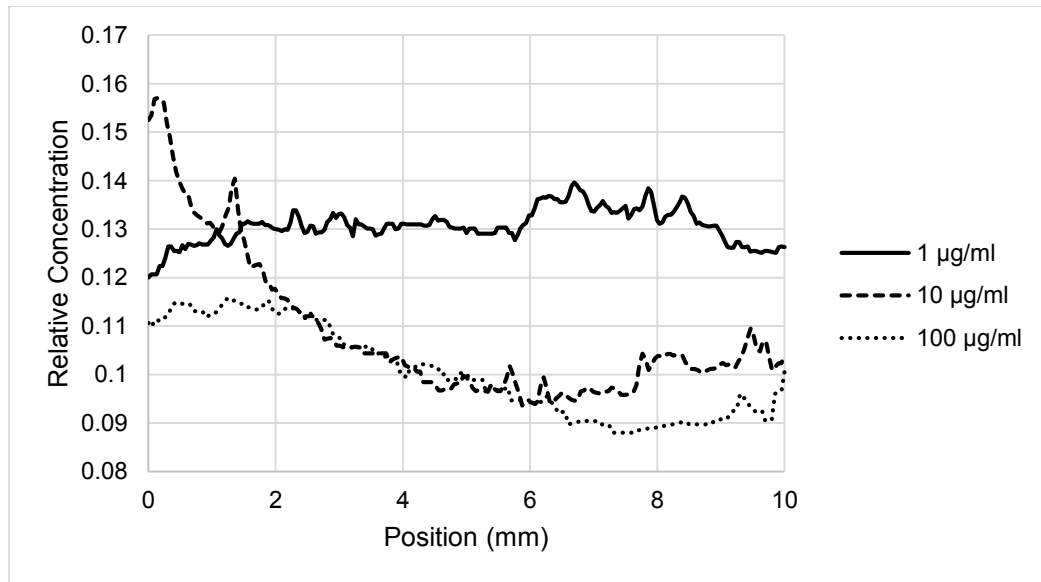


Figure 8: Amine-biotin/streptavidin-Cy3 gradient formation on 2D collagen gels. A relatively uniform distribution of amine-biotin formed across the collagen loaded with a source concentration of 1 $\mu\text{g/ml}$ after 4 hours. Gradients formed on collagen loaded with source concentrations of 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ with the 10 $\mu\text{g/ml}$ group forming the most defined gradient in 4 hours.

Gradients of amine-biotin were formed on EDC/NHS-activated sheets of aligned collagen fibers. We expected to see an improvement in conjugation efficiency of the

gradients due to the increase in surface area on fibers compared to 2D gels. In an effort to further enhance immobilization efficiency, 100 $\mu\text{g/ml}$ was selected as the source well loading concentration even though gradients generated with 10 $\mu\text{g/ml}$ amine-biotin in the source well exhibited the preferred gradient profile. Gradient generation was quenched with glycine after 4 hours and a uniform concentration of streptavidin-Cy3 was reacted on the biotinylated fibers for 3 hours. Excess streptavidin-Cy3 was removed and the collagen was washed in a series of soaks in PBS. Cy3-streptavidin/biotin binding was confirmed by the pink hue on the collagen. Gradient profiles were confirmed using a Typhoon Gel Reader and quantified with ImageJ. Gradient formation is detectable on collagen fibers, and the conjugation efficiency, as determined by fluorescence intensity, is improved over immobilization on 2D collagen gels. However, gradient profiles exhibit a significant amount of background due to non-specific adsorption of Cy3-streptavidin to the collagen surface and the uneven topography of the fibers (**Figure 9**).

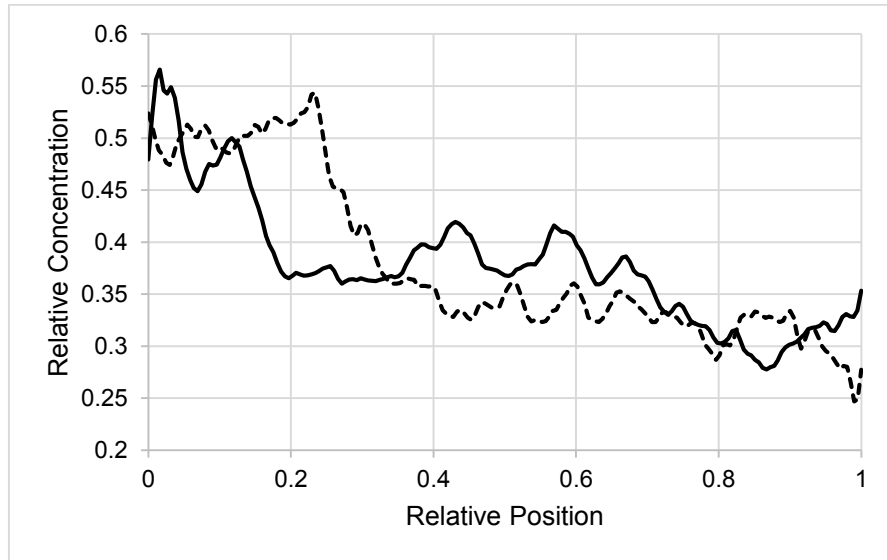


Figure 9: Representative profiles of amine-biotin gradients immobilized onto EDC/NHS-activated aligned collagen fibers. Conjugation efficiency of gradients on collagen fiber sheets is higher than gradients formed on 2D collagen in a comparable setup, but gradients exhibit much more noise due to sample topography and hydrogel swelling.

4. Discussion

We have demonstrated a number of methods that may be used to directly or indirectly immobilize gradients of neurotrophic factors onto the surface of polymer materials for nerve guidance conduits via a diffusion-based gradient platform. As characterized with a rhodamine-diazirine conjugate, modifying proteins, growth factors, or another molecule of interest with NHS-diazirine allows the molecule to be immobilized directly to a substrate under UV irradiation. Immobilized gradients may be generated by either altering the UV irradiation time with a uniform loading concentration of the molecule-diazirine conjugate or by varying the loading concentration of the molecule-diazirine conjugate with uniform exposure to UV light. Because of the ease with which we can form concentration gradients using our microchannel device, we find that the latter is the preferred approach for surface modification when using the diazirine chemistry. Controlling graded UV irradiation to form a linear requires complex setups to gradually expose the surface to the light, which results in low throughput and batch-to-batch variation. The disadvantage of using immobilizing NFs in this manner is the need to react the NF with the NHS-diazirine to form a NF-diazirine solution prior to photoimmobilization. The NHS-ester moiety is reactive to amine groups; the initial reaction between NHS-diazirine and the NF may not be efficient in proteins that have few exposed amine groups. This method also requires that NFs be exposed to UV light. Although UV irradiation at the recommended wavelength of 345 nm⁴⁰ should not induce protein denaturation, it does pose a risk. Since our gradient platform is diffusion-based, the molecular weight of the diffusing species greatly affects the time required for a linear concentration gradient to establish. We have simulated gradient formation for a molecule of comparable size to a neurotrophic factor—GDNF has a molecular weight around 15

kDa⁴¹ and NGF has a molecular weight of approximately 13 kDa⁴²—and found that a linear gradient establishes between 4 and 24 hours (**Figure 10**). This extensive gradient generation time is a drawback as NFs may exhibit a loss of bioactivity as the gradient establishes. To address these problems, we devised a two-step method to indirectly conjugate molecules to substrates. Immobilization via diazirine (step 1) was combined with the streptavidin/biotin binding interaction (step 2). This modification was not as effective as expected. Immobilization was inconsistent from batch to batch and exhibited no clear dependence on either the amine-biotin concentration gradient or the concentration of streptavidin-FITC loaded on the immobilized amine-biotin. The challenge of employing a multi-step immobilization scheme is that there are more steps at which problems may arise and in this case, the presence of a complication cannot be detected until the last step, so troubleshooting when the problem originates is difficult. With more time, we would have been able to further develop the optimal method to employ NHS-diazirine as a crosslinker for immobilizing growth factors and take advantage of its versatility on a wide selection of substrates for our NGC design.

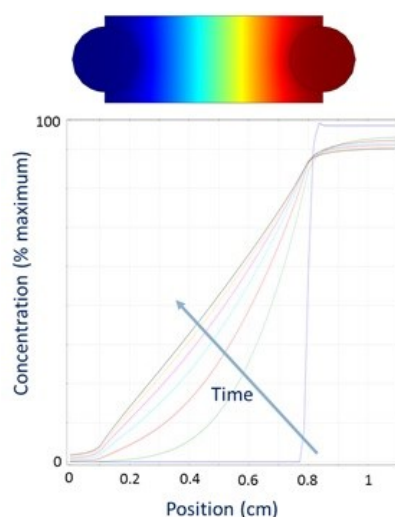


Figure 10: COMSOL modeling of gradient formation within PDMS microchannels. Linear gradients establish in 4-24 hours. Image adapted from Kellin Krick.

The second immobilization method that we developed was based on the well-established EDC/NHS activation of carboxyl groups for reactions with amines. EDC/NHS chemistry lacks the versatility offered by NHS-diazirine because it is only applicable to substrates that contain carboxyl groups. However, there is a demand to make NGCs with natural protein materials to circumvent biocompatibility issues associated with the use of synthetic polymer scaffolds. It was demonstrated with FITC-labeled lysozyme that amine-containing molecules can be directly immobilized in a graded concentrations to EDC/NHS-activated collagen, but we did not pursue gradient generation via direct immobilization of NFs using this chemistry. EDC/NHS activation is short-lived and the functionality would be lost by the time a gradient of NF solution establishes by diffusion. Instead, we established a two-step method using the streptavidin/biotin interaction. Gradients of amine-biotin were generated on collagen activated with EDC/NHS and then streptavidin/biotin binding was used to conjugate Cy3 to the collagen. Gradients were reproducible on 2D collagen gels as well as sheets of aligned collagen fibers, but further work is required to determine the optimal concentration range for efficient biotin immobilization. Another issue to be addressed in future work to optimize gradient generation and immobilization is the development of a protocol to prevent non-specific adsorption and absorption of the streptavidin-protein conjugate to the substrate surface, which prevents the formation of a clean, linear gradient profile. One potential strategy is to wash the substrate with bovine serum albumin (BSA) as a blocking step before the addition of the streptavidin-protein conjugate. Additionally, gradient profiles on collagen are inherently noisy due to the swelling of hydrated collagen. To minimize swelling, collagen fibers may be dehydrated in a

graded ethanol drying process prior to EDC/NHS surface activation. Dehydrating hydrogels also prevents disruption to the formation of the concentration gradient formation that may result from the presence of residual water in hydrated fibers. Apart from the minimal flexibility in the choice of substrate, the greatest limitation of this approach to immobilize NF gradients is the need to conjugate the desired NFs with streptavidin. This is far less of a concern than the need to conjugate NFs to NHS-diazirine and expose the proteins to UV light, but it is an additional factor that must be taken into consideration. Finally, regardless of the immobilization scheme, a rigorous method for quantifying conjugation efficiency is needed for future studies.

The motivation for the present study was to determine the synergy of aligned topography with the presence of gradients of biochemical cues on PNS cell response. With two methods available to immobilize gradients of molecules to the surface of scaffold materials, the next logical step for future work is to generate immobilized gradients of NFs onto the selected scaffold materials and conduct a series of *in vitro* cell studies. A preliminary study is needed to determine the optimal gradient conditions for different neurotrophic factors to elicit good cell response. This can be done with a cell proliferation experiment in which Schwann cells or neural stem cells (NSCs) are seeded on the desired substrate immobilized with different uniform concentrations of GDNF, NGF, or another growth factor of interest. The NF concentration that produces the most viable cells will be selected as the source concentration to be used in the gradient microchannel. The next set of studies will require substrates prepared in the following ways: isotropic (2D) topography with a uniform concentration of NF (negative control),

isotropic topography with an immobilized gradient (positive control), isotropic topography with a diffusible gradient (positive control), anisotropic (aligned) topography with a uniform concentration (positive control), anisotropic topography with an immobilized gradient, and anisotropic topography with a diffusible gradient. The effect of these groups on cell response will be determined with an analysis of live cell migration tracking of SCs and NSCs seeded on the substrates or quantification of DRG neurite extension. The proposed groups listed above use a combinatorial approach to cell guidance in which the physical topographical cues and the biochemical cues are presented in a single platform. A potential follow up experiment is to decouple the physical contact guidance provided by fiber alignment and biochemical guidance provided by gradient presentation: gradients will be immobilized on a 2D hydrogel, unmodified aligned polymer fibers will be placed over the hydrogel, and cells will be seeded on the fibers. Topographical guidance and biochemical guidance are both still accessible to the cells, but they are presented in separate elements.

Determining the most functional presentation of growth factors that can be generated with a simple, versatile, and reliable method opens up a world of possibilities for improving cell migration and axon regeneration for peripheral nerve repair and has the broader impact of being translatable to other tissue engineering applications as well. Once established, our gradient platform can potentially be modified to guide motor versus sensory neuron outgrowth, induce stem cell differentiation, or be used as a drug delivery platform. Individual aspects of the concentration gradient such as concentration range and concentration steepness can all be tuned in order to gain a deeper

understanding of physiological microenvironments involved in cell response and tissue regeneration and develop suitable therapies to aid the human body during the healing process.

5. Conclusion

Incorporating a combination of biochemical gradients and aligned topography into nerve guidance conduits offers a potentially powerful and synergistic platform to improve the outcome of peripheral nerve regeneration in large gap injuries. Gradient generation using a diffusion-based microfluidics device is easy, versatile, and scalable for high throughput. Direct UV-activated immobilization of gradients with NHS-diazirine is flexible with regards to substrate selection; EDC/NHS-activated immobilization of amine-biotin combined with streptavidin/biotin binding is a gentle, biocompatible, and suitable for protein-based substrates. PCL and collagen were selected as the substrates for the present studies and immobilization was characterized using various fluorophores, but the immobilization chemistry is applicable to virtually any biopolymer material that can be electrospun or synthesized into a fibrous morphology, and there are few limitations on the choice of growth factor for which immobilization works. Thus, the flexibility and versatility of this platform for diffusion-based formation of immobilized concentration gradients make it a promising candidate for use in future *in vitro* studies to better understand and improve upon the process of peripheral nerve regeneration.

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Biographical Sketch

Letitia Kai-Ling Chim was born November 1, 1992 in Houston, Texas. She began her undergraduate studies at the Johns Hopkins University in August 2011, pursuing a degree in Materials Science and Engineering with a concentration in Biomaterials. While at JHU, she worked under the guidance of Hai-Quan Mao, Ph.D., to develop an immobilized gradient generation platform for delivering neurotrophic factors to enhance peripheral nerve regeneration. She graduated with Departmental Honors and General Honors with a B.S. in Materials Science and Engineering from the Johns Hopkins University in May 2015. In August 2015, Letitia began her master's studies in Materials Science and Engineering at the Johns Hopkins University and continued her work under the mentorship of Hai-Quan Mao on gradient delivery for regeneration of peripheral nerve injuries. She will begin her doctoral studies in Bioengineering at Rice University in August 2016.