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Hydrogel Design for Supporting Neurite Outgrowth and Promoting Gene Delivery to Maximize Neurite Extension

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Abstract

Hydrogels capable of gene delivery provide a combinatorial approach for nerve regeneration, with the hydrogel supporting neurite outgrowth and gene delivery inducing the expression of inductive factors. This report investigates the design of hydrogels that balance the requirements for supporting neurite growth with those requirements for promoting gene delivery. Enzymatically-degradable PEG hydrogels encapsulating dorsal root ganglia explants, fibroblasts, and lipoplexes encoding nerve growth factor were gelled within channels that can physically guide neurite outgrowth. Transfection of fibroblasts increased with increasing concentration of Arg-Gly-Asp (RGD) cell adhesion sites and decreasing PEG content. The neurite length increased with increasing RGD concentration within 10% PEG hydrogels, yet was maximal within 7.5% PEG hydrogels at intermediate RGD levels. Delivering lipoplexes within the gel produced longer neurites than culture in NGF-supplemented media or co-culture with cells exposed to DNA prior to encapsulation. Hydrogels designed to support neurite outgrowth and deliver gene therapy vectors locally may ultimately be employed to address multiple barriers that limit regeneration.

Keywords

gene delivery; hydrogel; neurite outgrowth; dorsal root ganglia (DRG); three-dimensional

INTRODUCTION

Following spinal cord injury, the absence of stimulatory cues that promote regeneration is a factor that limits spontaneous regeneration of severed axons. Strategies that create a more permissive environment for neurogenesis and repair may protect surviving neurons from further damage and promote axonal growth (Thuret et al. 2006). Regenerating axons sample

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their environment for a repertoire of biological cues, such as neurotrophic factors (e.g., nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), or neurotrophin-3 (NT-3)) or adhesion sites on the matrix (Gunn et al. 2005; Schmidt and Leach 2003; Tessier-Lavigne and Goodman 1996; Yu and Shoichet 2005). Tissue engineering scaffolds are being designed to present a conducive environment for regeneration by providing physical support that promotes cell attachment and movement, while also serving as a vehicle for the delivery of therapeutic factors (Schmidt and Leach 2003).

Hydrogels are an attractive scaffolding material for spinal cord regeneration as their physical properties are similar to that of soft tissues, and these properties can be readily tailored (Andreopoulos et al. 1998; Lutolf et al. 2003; Peretti et al. 2006). Controlling the identity and density of adhesion sites incorporated into the hydrogel regulates neurite extension (Gunn et al. 2005; Mahoney and Anseth 2006; Schense et al. 2000). Additionally, modulation of the PEG content, which alters the hydrogel physical properties, impacts the extent of neurite outgrowth (Flanagan et al. 2002; Gunn et al. 2005; Leach et al. 2007). Adhesion and PEG content have been further modified with patterning technologies such as photolithography to orient neurite outgrowth through a defined topography or gradients in cell adhesion (Mahoney et al. 2005; Musoke-Zawedde and Shoichet 2006). Nevertheless, a biomaterial scaffold alone has been insufficient for functional regeneration, and additional interventions are required (Lu and Tuszynski 2008).

Gene delivery can be a versatile approach to provide localized and sustained expression of factors to promote nerve regeneration. Neurotrophic factors have been widely employed to promote neuron survival and neurite outgrowth following nerve injury (Burdick et al. 2006; Moore et al. 2006); however, delivery systems, such as osmotic pumps or the transplantation of genetically engineered cells, have been challenged by the need to maintain effective concentrations for long times. The delivery of genes encoding for neurotrophic factors can target accessory cells that act as bioreactors for the prolonged production of neurotrophic factors. *In vitro* models involving the co-culture of primary neurons with accessory cells on tissue culture plastic have indicated that the induced expression of neurotrophic factors by the accessory cells can promote neurite outgrowth, with neurite extension dependent on extent of transgene expression and localized protein gradients (Houchin-Ray et al. 2009b). These studies illustrated critical considerations in applying gene delivery to promote neurite outgrowth; however, the ultimate translation of gene delivery to promote regeneration requires a system that can deliver genes and support neurite outgrowth.

In this report, we have designed an *in vitro* co-culture system to investigate neurite extension as a function of the hydrogel parameters, and will test the hypothesis that the hydrogel design that maximizes neurite outgrowth results from balancing the design requirements for gene delivery with those for neurite outgrowth. The mechanisms of gene delivery within hydrogels have been linked to design parameters such as adhesion site density, degradation rate, and mechanics (Fukunaka et al. 2002; Quick and Anseth 2004; Shepard et al. 2010). Similarly, neurite outgrowth through hydrogels has been linked to the same properties (Flanagan et al. 2002.; Gunn et al. 2005; Mahoney and Anseth 2006; Schense and Hubbell 2000). Using photolithography, patterned channels having a width of 150 or 250 μm were fabricated, and enzymatically degradable PEG-based hydrogels were crosslinked within these channels (Fig. 1). Dorsal root ganglia (DRG) explants, a fibroblast cell line, and lipoplexes encoding for NGF were encapsulated within these degradable hydrogels. Fibroblasts were used for co-culture, as these cells have been observed at spinal cord injury sites and are transfected by released lipoplexes *in vivo* (De Laporte et al. 2009a; Yang et al. 2009). In the co-culture model, transfected fibroblasts act as bioreactors for the localized production of neurotrophic factors. The secreted neurotrophic factors induce neurite outgrowth by the encapsulated DRG, with both the neurons and fibroblasts secreting matrix

metalloproteinases that degrade the hydrogel crosslinks, which enables cell motility and neurite extension (Yoon et al. 2002; Zuo et al. 1998). The PEG content and density of RGD adhesion sites were investigated for their synergistic impact on neurite outgrowth (Gunn et al. 2005), and the gene delivery efficiency (Shepard et al. 2010). The channels were expected to provide physical guidance to extending neurites, and the width of the channels was also investigated for its topographical impact on neurite outgrowth. Hydrogels such as these that support growth and promote gene transfer may have numerous applications in nerve regeneration.

METHODS

Fabrication of PDMS patterns

The photoresist SU8-100 (approximately 6.5 g, Microchem, Newton, MA) was coated on silicon wafers (Wafer Reclaim Service, LLC, San Jose, CA). The wafers were prebaked and covered with film transparencies (CAD/Art Services, Inc., Bandon, OR) as a photomask. The photoresist was polymerized using a Quintel Q-4000 mask aligner (Quintel, San Jose, CA) followed by a post exposure bake cycle. Features were developed using propylene glycol monomethyl ether acetate (Sigma-Aldrich, St. Louis, MO).

Polydimethylsiloxane (PDMS) Dow Corning Sylgard 184 Elastomer kit (Krayden, Glenview, IL) was cured on the photoresist molds using previously established techniques (Xia et al. 1997). Briefly, curing agent was added to base in a 10:1 mass ratio. The liquid prepolymer of PDMS was degassed under vacuum, cast against a master, followed by further degassing under vacuum, and cured at 60°C overnight. PDMS patterns had raised (positive) features with thicknesses of 750 to 1,000 µm and widths of 150 or 250 µm were stored in 70% ethanol.

Hydrogel preparation

To form non-degradable, patterned hydrogel channels, four-arm poly(ethylene glycol) acryl (20 kDa) (SunBio, Orinda, CA) was dissolved in sterile 1x PBS, pH 7.4 at a concentration of 0.1 mg/mL and 600 mg/mL Igracure 2959 (I2959) (gifted by Ciba, Tarrytown, NY) in N-vinyl-2-pyrrolidone (PVP) was added to the PEG-acryl solution at final concentration of 0.05%. The precursor solution was injected into Teflon O-rings on a hydrophobic surface and covered with PDMS patterns. PEG substrates were photocrosslinked upon exposure to 365 nm UV light for 90 s, and washed overnight to remove unreacted monomers.

Degradable hydrogels were formed within the channel, using previously described techniques (Shepard et al. 2010). Briefly, four-arm poly(ethylene glycol) vinyl sulfone (PEG-VS) (20 kDa) was synthesized as previously described (Lutolf et al. 2003). To create 10% PEG hydrogels, stock solutions containing 50% PEG were functionalized with adhesion peptide Ac-GCGYGRGDSPG (custom synthesis at the IBNAM Core Facility at Northwestern University, Chicago, IL) within sterile 0.3 M triethanolamine (TEOA) pH 8.0. Hydrogels containing 7.5% PEG were similarly made to test multiple PEG conditions, and PEG contents greater than 10% would require changes significant changes to the PEG stock solution or lipoplex solution. After PEG-VS modification, HT-1080 cells (1.8×10^6 cells/mL final concentration), DRG explants, and lipoplexes were added to the precursor solution. Gelation occurred via Michael-Type addition upon addition of the crosslinking peptide GKCD-GPQG-IWGQ-DCKG at a stoichiometrically balanced molar ratio of thiol:VS. This peptide was dissolved in sterile 0.3 M TEOA pH 10.0 in accordance with previous studies encapsulating cells and DNA to maintain the reduction of the free thiols (Lei and Segura 2009; Shepard et al. 2010; Shepard et al. 2011). Gelation proceeded for 30 mins at 37°C in a

CO₂ environment. Hydrogels were washed immediately and at 1 h with supplemented DMEM to remove unreacted monomer.

Transfection profile quantification

The reporter plasmid *Gaussia* luciferase (pGLuc) (New England BioLabs, Ipswich, MA) containing the CMV promoter was produced in bacteria and purified using Qiagen maxiprep kits. Lipoplexes were formed by adding TransFast transfection reagent (Promega, Madison, WI) to pGLuc in a wt/wt ratio of 1:0.5. The HT-1080 cell line (generously gifted by Dr. J. Jones of Northwestern University, Chicago, IL) was split using trypsin with 0.05% EDTA and cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 10% FBS and 1% Pen/strep at 37° and 5% CO₂ environment. HT-1080 cells were co-encapsulated with lipoplexes (0.13 µg/µL) within degradable PEG-VS hydrogels as described above. Culture media was collected 1x/day of culture for 5 days to measure luciferase content in the media using a *Gaussia* luciferase assay kit (New England BioLabs, Ipswich, MA) and a luminometer (Turner Design, Sunnyvale, CA). Results were recorded in relative light units (RLUs).

In vitro neurite outgrowth

Primary neuron clusters were obtained by isolating intact DRG explants from E8 white leghorn chicken eggs (Michigan State University Poultry Center, East Lansing, MI) and maintained within Hank's buffered salt solution supplemented with 6 g/L glucose until the end of the isolation. Lipoplexes were formed as described above with plasmid encoding for full length mouse NGF in the RK5 vector backbone (pRK5-NGF) or an empty vector (pRK5) (gifted by Dr. Hiroshi Nomoto, Gifu Pharmaceutical University, Japan) as a negative control, both containing the CMV promoter.

Neurite outgrowth through the hydrogel and down the channel was assessed for three culture configurations. For each configuration, DRG explants were pipetted into the reservoir at the end of a channel, and the degradable hydrogel solution was added to the reservoir and filled the channel. Gelation of the solution encapsulated the DRG explants (Fig. 1). The culture configurations consisted of: i) NGF-supplemented media (i.e., no accessory cells), ii) accessory cells exposed to DNA prior to encapsulation, which will be referred to as pre-exposed cells, and iii) accessory cells and lipoplexes encoding NGF. Cultures lacking accessory cells (i) were the only conditions cultured in media supplemented with 20 ng/mL recombinant rat β-NGF (R&D Systems, Minneapolis, MN) as this concentration reflects the level of NGF concentration we have previously reported within these hydrogels following transfection (Shepard et al. 2011). For the remaining conditions (ii and iii), accessory cells were homogeneously distributed throughout the hydrogel filling the reservoir and channel. To pre-expose accessory cells, HT-1080 cells were incubated with 0.13 µg lipoplexes encoding for NGF for 1 h at 37°C. The lipoplex solution was removed from the cells by centrifugation with subsequent media replacement. The lipoplex-exposed HT-1080 cells were then encapsulated as previously described. For co-culture, HT-1080 cells and lipoplexes encoding for NGF (final concentration of 0.033, 0.067, or 0.13 µg DNA/µL) were added to the PEG precursor solution prior to gelation around DRG explants. The maximum dosage, 0.13 µg DNA/µL, approached the maximum dosage for the number of cells encapsulated, as greater dosages would hinder cell viability.

Following 4 days of culture, cells and DRGs were fixed with 4% paraformaldehyde and rinsed with PBS. PBS was replaced with a 0.1% Triton X-100 solution followed by 30 min blocking with 10% normal goat serum (Vector Labs, S-100) in PBS. DRG explants were stained using anti-neurofilament 200 (Sigma, N-4142) primary antibody diluted 1:300 in 1% normal goat serum for a 2 h and secondary antibody Alexa Fluor 546 goat anti-rabbit

(Invitrogen, A-11010) diluted 1:500 in 1% normal goat serum for 1 h. Stained neurons were imaged with a cooled CCD camera (Photometrics, Tucson, AR) on a Leica inverted fluorescence microscope.

Quantification of neurite extension

Multiple images were required to capture full neurite extension within the hydrogels and were stitched together. Normalized frequency distributions of the maximum length of neurites extending through the hydrogel within the channel was determined by tracing maximum extension of neurites across the hydrogel in black on a white canvas in Adobe Photoshop (Fig. 2). A Matlab program was implemented that calculates the maximum distance down the channel that neurite extension is observed for each pixel across the channel. The distance is measured from the initial leading edge of the explant to the end of the farthest extending neurite. The number of pixels reaching the specified distance was divided by the total number of pixels measured for each condition to generate a histogram for the fraction of maximum neurite extension that reached a given distance through the hydrogel and down the channel. Median values of distances reached by extending neurites are reported for all studies. Similar trends of median values and maximum distance reached were observed for all conditions unless otherwise noted, thus, comparisons are made across conditions based on the value of maximum distance reached by extension.

Statistics

Statistical analysis was performed using the statistical package JMP (SAS Institute, Cary, NC). Multiple comparison analysis was performed using analysis of variance (ANOVA) and a post-hoc *t*-test using a 95% confidence interval. Mean values with standard deviation values are reported for transfection profile data. All experiments were performed in quadruplicate.

RESULTS

Patterned hydrogel culture characterization

Culturing DRG explants within degradable hydrogels of varying RGD concentrations and NGF-supplemented media was initially employed to investigate hydrogel design parameters that support neurite extension. Degradable PEG hydrogels were formed within non-degradable hydrogel patterned substrates (Fig. 1), creating a hydrogel within a channel in which the channel walls can physically guide extending neurites. All RGD concentrations tested supported neurite outgrowth for each PEG content (7.5% and 10%), and an increasing RGD concentration increased the distance that neurites extended down the channel (Fig. 3a,b). As seen in the frequency distributions, explants extend neurites of varying lengths down the hydrogel-filled channel (Fig. 3c). The normalized frequency distributions of the maximum length of neurite extension were measured. Greater distances of extension were reached with increasing RGD densities for within both 7.5% and 10% PEG hydrogels. Maximum extension reached 1.0 mm for both 7.5% and 10% PEG containing 5 mM RGD. The 7.5% PEG, 5 mM RGD condition had a greater median distance of extending neurites (431 μ m) than the 10% PEG condition (362 μ m).

Transfection profiles of accessory cells cultured within patterned hydrogels

Transgene expression levels were monitored to determine the influence of hydrogel properties on transfection. A fibroblast cell line, HT-1080, was selected as fibroblasts are present at sites of spinal cord injury, and the localized release of DNA leads to transfection of fibroblasts *in vivo* (De Laporte et al. 2009a; Yang et al. 2009). Importantly, HT-1080 cells unexposed to DNA co-cultured with neurons do not induce neurite outgrowth, indicating

that these cells do not basally secrete neurotrophic factors. HT-1080 cells and rhodamine-labeled DNA were entrapped within the hydrogels, and were restricted to the channel following multiple days of culture for both 150 and 250 μm channels (Fig. 4). Expression levels approached maximal levels by day 1, were sustained throughout the 5-day culture for all conditions tested (Fig. 5), and increased significantly with increasing RGD concentrations for both 7.5% and 10% PEG ($p < 0.05$). Hydrogels with 5 mM RGD produced the greatest transfection for each PEG content. Expression levels were significantly greater for 7.5% PEG than 10% PEG on day 1 (Fig. 5b inset).

Neurite outgrowth within the three-dimensional co-culture model using gene delivery

Neurite outgrowth was subsequently measured as a function of the DNA loading within the hydrogel. Neurites reached greater distances down the channel with increasing dosage of DNA encoding for NGF (Fig. 6a). Maximum extension reached 0.7, 0.8, and 1.5 mm down the channel for 0.033, 0.067, and 0.13 μg DNA encapsulated/ μL , respectively. The location of extending neurites was independent of RGD concentration and PEG content with neurites observed in both the middle of the hydrogel (Fig. 6b) and near the outside edge, along the channel walls (Fig. 6c). Interestingly, neurite extension in cultures containing NGF-supplemented media (Fig. 3c) had a greater density of neurites relative to neurite extension within all conditions involving gene delivery, which appeared to have a smaller density of extending neurites that reached greater distances (Figs. 6b,c, 7b). Negative control studies encapsulating lipoplexes formed with the pRK5 empty vector did not induce neurite extension (data not shown). For control studies encapsulating pre-exposed accessory cells, which provide maximal yet transient NGF production, maximum distance of neurite extension reached 1.0 mm (Fig. 7a). Neurites from hydrogels with pre-exposed cells did not extend to the same distance down the channel as cultures with hydrogels co-encapsulating accessory cells and lipoplexes.

Maximal neurite outgrowth with gene delivery

The impact of physical guidance by the channel walls was investigated by comparing neurite extension within channels of 150 μm and 250 μm widths. Similar to the previous studies, increasing RGD concentration within the degradable hydrogels led to greater neurite lengths for both 150 and 250 μm channels (Fig. 8). Maximum distance of neurite extension reached 0.8 mm down the channel for both 150 μm and 250 μm hydrogel widths, however median values of distances reached by extending neurites was over two-fold greater for 250 μm widths (476 μm) than for 150 μm widths (207 μm).

Subsequently, neurite extension was investigated for a range of PEG contents (7.5% and 10%) and RGD concentrations (0.5, 2.5, and 5 mM) with 0.13 μg DNA encapsulated/ μL . At day 4 within the 10% PEG, neurite length increased with increased RGD concentration. Maximum distances of neurite extension reached 0.6, 1.2, and 1.5 mm down the channel for 0.5, 2.5, and 5 mM RGD, respectively (Fig. 9a). However for 7.5% PEG, intermediate concentrations of RGD (2.5 mM) maximum neurite extension reached greater distances (1.5 mm) than either 0.5 or 5 mM RGD, which reached 0.8 and 1.0 mm, respectively (Fig. 9b). For all conditions tested, 7.5% PEG and 2.5 mM RGD resulted in the highest fraction of neurite extension that reached the greatest distance down the channel (1.5 mm).

The cumulative frequency distribution of neurite extension that reached the specified distance was calculated to compare neurite outgrowth within hydrogels cultured in NGF-supplemented media and co-cultures encapsulated with lipoplexes encoding for NGF. Neurites reached greater distances with encapsulated lipoplexes and co-culture compared to the same hydrogel design condition cultured with NGF-supplemented media for all hydrogel

conditions tested (Fig. 10). The greatest length down the channel was observed for 7.5% PEG hydrogels modified with 2.5 mM RGD.

DISCUSSION

Hydrogels are an attractive biomaterial for nerve regeneration since their physical properties are similar to native tissues and the biochemical signals can be readily manipulated to control cell adhesion and migration, matrix degradation, and deliver bioactive factors. Hydrogels alone have been insufficient to support nerve regeneration, and their combination with delivery of therapeutic factors has the potential to significantly enhance regeneration (Lu and Tuszynski 2008). In this report, a commonly used culture model consisting of DRG explants and the neurotrophic factor NGF was implemented within a lipoplex-loaded hydrogel encapsulating accessory cells to determine the impact of hydrogel design on transfection and neurite outgrowth. DRG explants contain sensory neurons that are responsive to NGF, thus, alternative neurotrophins, such as BDNF, NT-3, and glial cell line-derived neurotrophic factor (GDNF) would be more appropriate for regeneration of motor nerves. These studies represent an initial step towards understanding the design of hydrogels to support both gene delivery and neurite outgrowth, and the target gene can readily be manipulated for *in vivo* nerve regeneration. In the spinal cord, drug delivery remains challenging as the cerebrospinal fluid can facilitate transport of therapeutic factors from the injury site. Catheter implantation and drug delivery to the spinal cord with an osmotic pump has been accomplished, however this administration requires additional surgery for catheter removal, and the catheter may clog or damage the cord (Bradbury et al. 1999; Xu et al. 1995). More recently, implantation of genetically engineered cells capable of locally secreting neurotrophic factors have been investigated, yet this approach remains challenging due to low cell survival following transplantation (Nakahara et al. 1996; Tuszynski et al. 2003). Localized delivery of gene therapy vectors from hydrogels could overcome these challenges by targeting endogenous cells for the production of neurotrophic factors.

The hydrogel properties that directly influence neurite outgrowth through the hydrogel included the density of adhesion peptides and PEG content. The cell-degradable, PEG-based hydrogels (Fig. 1) used herein are readily tailored to control the mesh size by altering the PEG content present during gelation and modified with the incorporation of RGD cell adhesion peptides. Our results with neurite extension in 3D and the dependence on adhesion and PEG content are consistent with previous reports on hydrogel surfaces (Gunn et al. 2005); however, degradation of the matrix is essential for extension through the hydrogel. These hydrogels have a reported mesh size of 25 nm, which is smaller than the size of cells, vector, and the diameter of neurites, yet larger than many diffusible proteins (Bengali et al. 2005; Raeber et al. 2005). The mechanism of cell migration and neurite extension through these gels occurs via degradation of the crosslinking peptides. The fibroblast and neurons secrete matrix metalloproteinases that cleave the peptide crosslinkers, thereby increasing the mesh size and creating space for neurite extension (Yoon et al. 2002; Zuo et al. 1998). The length of neurite extension within these hydrogels cultured in NGF supplemented media increased with increasing RGD concentration (Fig. 3), which was consistent with other reports (Gunn et al. 2005; Schense and Hubbell 2000). Additionally, substrate mechanics can impact transfection, with more rigid hydrogels facilitating greater levels of expression (Kong et al. 2005). The storage modulus of the hydrogels used herein ranges from 341 to 597 Pa (Shepard et al. 2010); however, greater levels of expression were observed within the less rigid, 7.5% PEG hydrogels. This greater level of expression was previously correlated to greater rates of cell migration through the hydrogels, which enhanced matrix degradation and increased cell contract with the liberated DNA. Substrate mechanics have also been reported to influence neurite outgrowth (Flanagan et al. 2002; Gunn et al. 2005). However,

within degradable hydrogels, crosslink density and matrix degradation significantly contribute to the distances reached by extending neurites.

The hydrogel design that maximizes neurite outgrowth reflects balancing the requirements for neurite outgrowth with those for effective gene delivery. The PEG content and adhesion peptide density, which impacted neurite extension as described above, also impacted gene delivery (Fig. 5), in part due to cell migration and release of entrapped lipoplexes (Gunn et al. 2005; Lutolf et al. 2003; Shepard et al. 2010). Neither of the conditions leading to the greatest levels of transgene expression, 7.5% nor 10% PEG with 5 mM RGD, resulted in the maximum distance of neurite elongation for the conditions tested (Fig. 9b), consistent with previous studies performed in 2D culture (Houchin-Ray et al. 2009b). Maximal transfection would be expected to produce the greatest concentrations of NGF within the hydrogel, which may not lead to directed neurite outgrowth. We previously reported extensively branched neurite outgrowth of neurons cultured on polystyrene with accessory cells that were maximally transfected, while relatively low levels of transfection resulted in long neurite projections (Houchin-Ray et al. 2009a; Houchin-Ray et al. 2009b). The long projections and minimal branching are consistent with directed neurite extension. Thus, neurite extension is modulated by both the hydrogel design that directly regulates neurite outgrowth and the transfection profile of encapsulated accessory cells.

Sustained expression of neurotrophic factors enhances neurite outgrowth within the hydrogels delivering DNA (Fig. 9). Transfection of cells by entrapped lipoplexes leads to a longer duration of expression within the hydrogel (Lei and Segura 2009; Shepard et al. 2010), which would maintain elevated levels of neurotrophic factors to promote neurite growth. In animal models, delivery of neurotrophic factors in the days following injury are critical to mitigate cell death, and their persistence can encourage regeneration. Previous reports indicate the duration of transgene expression is increased by the continued or repeated exposure of cells to DNA (des Rieux et al. 2009; Shepard et al. 2010). Overall, the short-term expression of NGF supports the initial outgrowth (Fig. 7); however, persistent expression achieved by delivering lipoplexes from within the gel led to greater neurite extension (Fig. 9).

The combination of the channel wall and the expression of neurotrophic factors within the hydrogel may have introduced a directional cue that leads to greater distances reached by extending neurites (Fig. 10). Physical guidance has been shown to play a role in the direction and orientation of neurite outgrowth (Gundersen and Barrett 1980; Houchin-Ray et al. 2009b; Mahoney et al. 2005; Rajnicek et al. 1997), and our *in vitro* model was based on bridges that have channels to support and direct neurite outgrowth *in vivo* (De Laporte et al. 2009b). Gene delivery within hydrogels localizes NGF expression, and would create the greatest concentration within the hydrogel (Shepard et al. 2011). Additionally, the channel walls provide topographical cues that increase neurite extension within hydrogels. For cells cultured on patterned surfaces or within open channels of bridge, contact guidance orients neurites parallel to the patterned surface (De Laporte et al. 2009b; Houchin-Ray et al. 2007a; Mahoney et al. 2005). In this report, channels of varying width, 150 and 250 μm , were employed to provide contact guidance within the hydrogel as these widths have been previously reported to direct neurite extension (Houchin-Ray et al. 2007a). Narrower widths were expected to facilitate longer extension due to topographical guidance by the hydrogel walls. However, in contrast to studies on 2D surfaces (Houchin-Ray et al. 2007b), extending neurites reached greater median distances when cultured within 250 μm wide channels compared to 150 μm wide channels (Fig. 8). Thus, while the hydrogel walls may provide topographical guidance, the PEG content, adhesivity, and transgene expression were more critical for facilitating nerve growth through the hydrogels.

In conclusion, neurite extension is enhanced using gene delivery within hydrogels, whereby the hydrogel provides physical support for neurite extension and persistent expression of NGF provides the biochemical stimulus. The degree of neurite extension increased with an increasing level and duration of transgene expression. However, maximum transfection levels did not maximize neurite outgrowth, indicating that the hydrogel design must effectively balance the requirements for supporting neurite outgrowth and promoting gene delivery. This system involving hydrogel-filled channels, gene delivery, and the co-culture of neurons with accessory cells may be a valuable system for investigating the barriers to nerve growth and for developing hydrogels that maximally support and promote neurite extension, which may ultimately lead to novel therapies for treating spinal cord injury.

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Fig. 1.

Channel fabrication. PEG templates containing channels are formed by overlaying a PDMS pattern on a precursor PEG solution containing photoinitiator followed by exposure to UV light (a). After crosslinking, the PDMS is removed leaving behind a PEG template containing a channel with a length (L) of 6 mm, reservoir diameter (D) of 500 μm , thickness (t) of 1 mm, and width (W) of either 150 or 250 μm . Note the dimensions in the fabrication schematic are not drawn to scale. A photograph of a patterned PEG template with channel width of 250 μm is shown in (b).

Fig. 2. Schematic illustrating the quantification method for neurite extension down the channel. The maximum distance of neurite extension was quantified by measuring the distance ($d(i)$) down the channel the extending neurites reach at each pixel (i) along the width of the hydrogel-filled channel. The distance in pixels is converted to millimeters.

Fig. 3. Neurite extension with exogenous NGF addition. Channels were filled with 7.5% (a) or 10% PEG hydrogels (b) containing 0, 2.5, or 5 mM RGD and overlaid with media supplemented with NGF. Neurite extension data represents the fraction of neurite extension within the degradable hydrogel reaching the specified distance from the reservoir down the channel. Median values of maximum distances reached by extension are reported for each condition. A representative image shows the morphology of neurite extension using NGF-supplemented media (c). The scale bar represents 250 μm .

Fig. 4. Patterned lipoplex deposition and cell encapsulation. HT-1080 cells and rhodamine-labeled DNA were encapsulated within degradable hydrogels inside channels having widths of 150 μm (a) or 250 μm (b). After 2 days of culture, the cell nuclei were stained with Hoechst, and both the cell nuclei and the encapsulated DNA were retained within the channel. The scale bar represents 250 μm .

Fig. 5.

Expression levels of HT-1080 cells with varying hydrogel PEG content and adhesion. Transfection within hydrogels containing 0, 2.5, or 5 mM, each encapsulating $0.13 \mu\text{g}/\mu\text{L}$ DNA encoding for *Gaussia* Luciferase, were monitored once per day up to 5 days for 7.5% (a) and 10% PEG (b). Statistical differences between conditions at each time point is indicated by different letters (A or B) based on ANOVA with $p < 0.05$. For example, for 7.5% PEG on day 3, 0 mM RGD is statistically different than 5 mM RGD, but neither is statistically different than 2.5 mM RGD. The inset denotes expression levels for 7.5% and 10% PEG each containing 5 mM RGD.

Fig. 6.

DNA dose response and neurite length. DRG explants and HT-1080 cells were encapsulated within hydrogels containing 10% PEG, 5 mM RGD, and 0.033, 0.067, or 0.13 µg DNA encapsulated/µL. For delivery of DNA encoding for NGF, neurite extension was quantified as the fraction of neurite extension reaching the specified distance (a). Median values of maximum distances reached by extension are reported for each condition. The morphology of neurite extension varied from culture-to-culture within the same condition, and representative images of neurite extension are shown in (b) and (c). The scale bar represents 250 µm.

Fig. 7.

Neurite extension with pre-exposed cells. Neurite extension within 10% PEG hydrogels containing 5 mM RGD and cells pre-exposed with lipoplexes (0.13 μg DNA encapsulated/ μL) encoding for NGF (a). Neurite extension data represents the fraction of neurite extension within the degradable hydrogel reaching the specified distance from the leading edge of reservoir down the channel. The median value of maximum distance reached by extending neurites is reported. A representative image shows the morphology of neurite extension upon encapsulating pre-exposed cells (b). The scale bar represents 250 μm .

Fig. 8.

Channel width and neurite extension. DRG explants, HT-1080 cells, and DNA encoding for NGF were encapsulated within 10% PEG hydrogels containing 5 mM RGD and a final concentration of 0.067 μg DNA encapsulated/ μL inside either 150 (a) or 250 μm (b) wide channels. Neurite extension was quantified as the fraction of neurite extension reaching the specified distance. Median values of maximum distances reached by extending neurites are reported for each condition.

Fig. 9.

Neurite extension within lipoplex-loaded hydrogels of varying PEG content and adhesivity. DRG explants, HT-1080 cells, and a final concentration of 0.13 μ g DNA encapsulated/ μ L encoding for NGF were encapsulated within hydrogels containing 0.5, 2.5, or 5 mM RGD. Neurite extension down the hydrogel was measured on day 4 for cultures within 10% PEG (a) and 7.5% PEG (b). Neurite extension data represents the fraction of neurite extension within the degradable hydrogel reaching the specified distance from the leading edge of reservoir down the channel. The median values of maximum distances reached by extension are reported.

Fig. 10.

The cumulative frequency distribution of neurite outgrowth within hydrogels with either culture with NGF supplemented media or co-cultured with cells and entrapped lipoplexes. The cumulative frequency distribution of the fraction of extension greater than the specified distance compares cultures containing NGF-supplemented media (NGF) with co-cultures encapsulating lipoplexes encoding for NGF (DNA).