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Immobilized Concentration Gradients of Neurotrophic Factors Guide Neurite Outgrowth of Primary Neurons in Macroporous Scaffolds

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ABSTRACT

Neurotrophic factors present as concentration gradients are neurotropic cues that direct axonal growth toward their targets. Multiple factors work together *in vivo* to ensure axons reach the proper targets, likely interacting with one another via intracellular signalling pathways. Nerve growth factor (NGF) and neurotrophin-3 (NT-3) are neurotrophins known to guide axons as well as promote axonal growth following injury to both the spinal cord and peripheral nerve. These molecules interact with neurons through different tyrosine kinase receptors. In this study, the receptors for these growth factors were shown to be co-localized on E10 chick dorsal root ganglion (DRG) cells, providing an opportunity for synergism. Well-defined concentration gradients of NGF and NT-3 were immobilized for the first time in a cell-penetrable, cell-adhesive scaffold of poly(2-hydroxyethyl-methacrylate) and poly(L-lysine). An NGF concentration gradient of 310 ng/mL/mm was required to guide chick DRG neurites. A lower concentration gradient of 200 ng/mL/mm of NGF was shown to elicit guidance when an NT-3 concentration gradient of 200 ng/mL/mm was also present, indicating a synergistic response in the DRG neurons. These gradient scaffolds may be useful for guided regeneration following injury to the spinal cord or peripheral nerve and may also elucidate the mechanism for intracellular signaling of neurotrophic factors.

INTRODUCTION

DURING DEVELOPMENT OF THE NERVOUS SYSTEM, axons are guided to their targets by the combined actions of haptotactic and chemotactic signaling molecules to which the growth cone responds.[1,2] Growth cones are highly motile structures, capable of receiving multiple types of guidance cues and transducing them into directed axonal growth.[3–6] The sensory and decision-making procedures that occur within the growth cone are believed to be dependent on concentration gradients of many guidance cues, where the relative steepness of the gradient is more important than the mean concentration of the guidance molecule.[7–11]

Neurotrophic factors are stimulatory molecules that have a positive effect on axonal growth and play an important role in the development and maintenance of the nervous system. Many neurotrophic factors have also been shown to be neurotropic and are able to guide growing axons.[11–14] The family of neurotrophins includes the first neurotrophic factor discovered, nerve growth factor (NGF), as well as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5).[14–18] NGF has been shown to elicit neurite outgrowth on its own,[4,11,12,19–21] as have NT-3[13,19,22,23] and BDNF.[13,22,23] In addition, the effects of combinations of some of these growth factors on neurite outgrowth have

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also been demonstrated.[24] Gradients of neurotrophins have been shown to induce turning of growth cones toward increasing concentrations of NGF, NT-3, and BDNF.[11,13,20,23,24] These attractive responses can be reversed by changing the level of cyclic nucleotides (e.g., cAMP, cGMP) within the neuron, suggesting second messenger modulation of responses to guidance cues.[6,23,25]

Neurotrophins bind two different types of cell-surface receptors, a tumor necrosis factor (TNF) receptor-like p75 neurotrophin receptor and the three Trk tyrosine kinase receptors. The p75 receptor binds all neurotrophins with similar low affinity and is believed to play a role in programmed cell death, while the Trk receptors enhance neuronal survival and have more specific neurotrophin interactions. NGF binds only TrkA, BDNF and NT-4/5 bind TrkB, and NT-3 preferentially binds TrkC, but can also interact with TrkA and TrkB with much lower affinity.[18,26–29] The co-localization of at least two of these Trk receptors in DRG neurons has been reported, which may provide an opportunity for synergistic interaction.[30,31]

In this study, we immobilized concentration gradients of neurotrophins in a cell-penetrable, cell-adhesive scaffold for the first time and studied the response of chick DRG cells cultured in the scaffold. We first demonstrated that a concentration gradient of immobilized NGF was required for guided neurite outgrowth. We then demonstrated, for the first time, that a lower concentration gradient of immobilized NGF was required for neurite outgrowth when combined with an immobilized concentration gradient of NT-3. By demonstrating that both TrkA (for NGF) and TrkC (for NT-3) were co-localized on the same DRG neurons, we determined that the lower gradients of NGF and NT-3 acted synergistically and not simply additively. While we have previously demonstrated synergism of combined *soluble* concentration gradients,[24] this is the first example of synergism of *immobilized* concentration gradients and the *first* example of immobilized gradients in a cell-invasive scaffold versus a noninvasive hydrogel.[20] These scaffolds may be useful tools to clarify the debate on the intracellular signaling mechanism of neurotrophic factors.[32] Moreover, the immobilized concentration gradient scaffolds may be useful as implants to guide and promote regeneration after injury to either the spinal cord or peripheral nerve.[33,34]

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and used as received unless otherwise noted. Water was distilled and deionized using Millipore Milli-RO 10 Plus at 18 MΩ. Mouse nerve growth factor (2.5 S NGF) was purchased from Cedarlane Laboratory (Hornby, Ontario). Recombinant human neurotrophin-3 (NT-3) was purchased from Peprotech (Ottawa, Ontario). Filamentous actin stain (Alexa Fluor 488 phalloidin), anti-rabbit IgG, and anti-goat IgG Alexa Fluor dyes were purchased from Molecular Probes (Eugene, OR). Rabbit anti-TrkA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and goat anti-TrkC was obtained from Research Diagnostics (Flanders, NJ). All reagents for culture media, media additives, and Hanks balanced salt solution (HBSS) were purchased from Invitrogen (Burlington, Ontario). Chick eggs were purchased from Curtis Chicks (Port Hope, Ontario). All polymerization reactions were performed at room temperature, and all cell culture experiments were conducted at 37°C in a 5% CO_2 incubated environment.

DRG cell culture

Chick dorsal root ganglia (DRG) were dissected from embryonic (E10) chicks according to an established procedure.[35] Dissected DRG tissues were digested with trypsin (0.0375% in HBSS) for 20 min at 37°C. Individual DRG cells were obtained by centrifugation (1500 rpm for 5 min), followed by immersion in cell culture medium and dissociation by gentle trituration. The dissociated DRG cells were cultured in minimum essential medium (MEM) containing 1% penicillin/streptomycin, 10% horse serum, and 0.2 M glutamine. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air.

Co-localization of neurotrophin receptors

E10 chick DRG cells were cultured overnight in 96-well plates precoated with poly-L-lysine (PLL). Cells were cultured in N2-supplemented medium (not in the presence of growth factors) to prevent selection of a particular neuron subpopulation. The N2-supplemented, serum-free medium supports postmitotic neuron growth and expression and does not support non-neuronal cell growth. Double-immunolabeling was performed to determine the co-localization pattern of the two growth factor receptors, TrkA and TrkC, as previously described.[11,20,24] Specifically, the cells were incubated overnight at 4°C with goat anti-TrkC primary antibody (1:200 dilution in PBS) to localize the TrkC receptors. The cells were then washed twice with PBS and incubated with the fluorescently labeled Alexa Fluor 488 donkey anti-goat IgG secondary antibody (5 μg/mL in PBS) at room temperature with shaking for 10 min. To localize the TrkA receptors, the cells were then washed twice with PBS and incubated overnight at 4°C with rabbit anti-TrkA primary antibody (1:200 dilution in PBS). The cells were washed twice with PBS and incubated with fluorescent Alexa Fluor 594 donkey anti-rabbit IgG secondary antibody (5 μg/mL in PBS) with shaking at room temperature for 10 min, followed by two final rinses with

PBS. A Zeiss Axiovert 100 inverted fluorescence microscope was used to visualize the co-localization of the receptors using different filters in the excitation of the labeled antibodies. Controls for auto-fluorescence and nonspecific labeling ($n = 3$) were prepared by following the double-immunolabeling procedure, but with the omission of the primary antibody labeling steps.

Synthesis of cell-adhesive, macroporous scaffolds with a homogeneous concentration of growth factors

Macroporous scaffolds of poly(2-hydroxyethylmethacrylate) were synthesized by polymerizing 15 wt% 2-hydroxyethylmethacrylate (HEMA) in 85 wt% water with 0.1 wt% ethylene dimethacrylate (EDMA) crosslinker, 0.25 wt% ammonium persulfate (APS) initiator, and 0.2 wt% tetramethylethylenediamine (TEMED) accelerator, with the latter three percentages being relative to the monomer. To increase the cell adhesion of the scaffold, a 0.01% solution of poly-L-lysine (PLL) was included in the prepolymer mixture at a concentration of 83.3 μL PLL/mL of monomer solution. NGF was also added to the mixture at a concentration of 10 μg/mL. All components were mixed together, with the initiator added last. The solution (total volume of 5 mL) was injected into a glass tube with an inner diameter of 8 mm and allowed to polymerize at room temperature. This resulted in scaffolds that fit snuggly inside the wells of a 96-well plate used for cell culture. The polymerized scaffolds were then stored at 4°C for at least 1 h to facilitate the removal of the scaffold from the glass mold. Upon removal from the mold, the scaffolds were stored in water overnight to remove any unreacted molecules, unincorporated growth factor, or PLL. The scaffolds were characterized for depth of penetration of cells, overall morphology using the scanning electron microscope (SEM), and stability of the immobilized NGF. The identical experiment was repeated with NT-3 to assess stability of immobilized NT-3. (Stability studies were conducted with scaffolds made from identical formulations and morphologies, but with smaller volumes, and thus collected in smaller, 1.8 mm diameter molds.)

Cell penetration assay

For the cell penetration assay, scaffolds were disinfected by immersion in 70% ethanol, followed by a quick rinse in HBSS. The scaffold was cut into 0.5–1 cm thick sections that were placed into a 96-well plate with a small amount of sterile vacuum grease on the bottom of the well to fix the scaffold in place and prevent floating. The scaffolds were soaked in 200 μL of serum-free MEM overnight, which was then removed and replaced with 200 μL of cell culture medium containing 5 μg/mL GF to promote neurite outgrowth into the scaffolds, 15 μg/mL each of uridine and 5-fluorodeoxyuridine to suppress the growth of non-neuronal cells, and E10 DRG cells at a concentration of 7.6×10^4 cell/mL. Cells were fed every other day with this medium for the first week and with the same medium (without the uridine/5-fluorodeoxyuridine) for the second week. After 2 weeks, cells were rinsed with HBSS, fixed for 10 min in a 3.7% formaldehyde solution, rinsed again in HBSS, incubated with Triton X-100 for 90 s to permeabilize the cell membrane, and rinsed again with HBSS. Next the cells were stained by incubation for 20 min in a 5 μL/mL solution of phalloidin stain, followed by a final rinse with HBSS. Scaffolds were then embedded in 5% agarose (Type 1-A, low EEO) and cut with a vibratome (Vibratome Series 1000-Pelco 101, Technical Products International, St. Louis, MO) into 250 μm slices. The slices were viewed under a Zeiss Axiovert 100 inverted fluorescence microscope, Jena, Germany) to determine to what depth the cells had invaded the scaffold. The percentage of scaffolds that stained positively for cells was calculated-that is, at every 250 μm depth, scaffolds were analyzed for the presence of cells and the percentage of scaffolds having cells reported.

Scaffold morphology

For overall morphology, the scaffold was characterized using the SEM. For SEM imaging, the samples were cut into pieces between 1–2 cm in length, quenched in liquid nitrogen, and then freeze-dried. Dried scaffolds were then broken into smaller pieces for mounting, attached with double-sided tape to microscopy sample studs, and sputter-coated with gold for 60 s. The samples were placed on the SEM stage (Model S-570, Hitachi, Tokyo, Japan) and imaged at a working distance of 15 mm and an accelerating voltage of 20 kV.

Stability of immobilized neurotrophins in scaffolds

For stability of the immobilized NGF, scaffolds were rinsed overnight in 1 mL of 0.1 mg/mL of bovine serum albumin (BSA) in Hanks buffer to remove any unreacted reagents, including NGF that had not been immobilized, and assayed over time for any released NGF, using a method previously described for non-cell invasive rods.[20] Briefly, the scaffolds were immersed in 0.5 mL of a 0.1 mg/mL BSA in buffer solution and incubated at 37°C for up to 28 days. After 1, 8, and 28 days, the supernatants were exchanged with fresh BSA solution and analyzed for any NGF that had diffused from the scaffold (and had therefore not been immobilized) using a standard sandwich ELISA kit for NGF. The NGF ELISA data allowed us to calculate the amount of NGF released over time from the p(HEMA) scaffold. To account for any loss of NGF activity with time, a vial of NGF prepared at a similar concentration was also kept at 37°C and analyzed si-

multaneously with the supernatants derived from the hydrogel scaffolds. The data reported are normalized for any loss of activity with time. The identical experiment was repeated with NT-3.

Synthesis of immobilized growth factor concentration gradients in cell-adhesive, macroporous p(HEMA) scaffolds

The polymer formulation was similar to that described above except that different growth factor concentrations were used, the overall volume reduced, and the mold in which the scaffold was collected had a diameter of 1.8 mm, identical to that used for the growth factor stability study. Concentration gradients were immobilized in cell-invasive scaffolds, as previously described for noninvasive scaffolds[20] using a gradient maker (CBS Scientific, Temecula, CA). The gradient maker (Fig. 1) required 200 μL each of solution in chambers A and B and the conduit between these two chambers. Chamber A contained pre-polymer mixture (i.e., HEMA, water, PLL, APS, and TEMED) with the growth factor(s) while chamber B and the conduit contained pre-polymer mixture with no growth factors. A series of gradients were prepared with different concentrations of growth factors in chambers A (and B), as summarized in Table 1. For example, a 23 cm gradient of 200 ng/mL/mm of NGF was made with 200 μL of pre-polymer solution containing 46.0 μg of NGF in chamber A, and 200 μL each of pre-polymer solution without NGF in both the conduit and chamber B. A glass mold was attached to the end of the tubing, the spin bar activated, the peristaltic mini-pump turned on, and then valve 2 opened, followed immediately by valve 1. The solutions were drawn out of chambers A and B simultaneously, and the solution from chamber A was mixed with a continuously decreasing volume of solution without growth factor in chamber B, which resulted in the formation of a gradient that was collected in the glass cylindrical mold. The solution was allowed to polymerize at room temperature, followed by storage at 4°C for at least 1 h to facilitate scaffold removal. Once the scaffold was removed, the direction of the gradient was noted and the scaffolds were stored in water overnight.

Three types of gradients were studied for neurite guidance with E10 chick DRG neurons: 1) single concentration gradients of either NGF or NT-3; 2) combined concentration gradients of both NGF and NT-3; and 3) single concentration gradients of one neurotrophin (i.e., NGF or NT-3) with a homogeneous concentration of the second growth factor (i.e., NT-3 or NGF). These combinations of one *gradient* neurotrophin plus another *homogenous* neurotrophin served as controls for the dual gradient of neurotrophins that was used to study synergism. Dual concentration gradients combining NGF and NT-3 were prepared identically to single neurotrophin gradients except that both growth factors were added to the pre-polymer solution in chamber A at the same concentration, with the extra growth factor replacing some of the water volume used in the solution. For the single gradient combined with a homogeneous concentration of the second growth factor, the first growth factor was added only to the chamber A solution, as previously described. The second growth factor, however, was included at equal concentrations in the solutions of both chambers A and B and the conduit. All three types of gradients were assayed for guidance of DRG neurites.

To confirm that linear concentration gradients of neu-

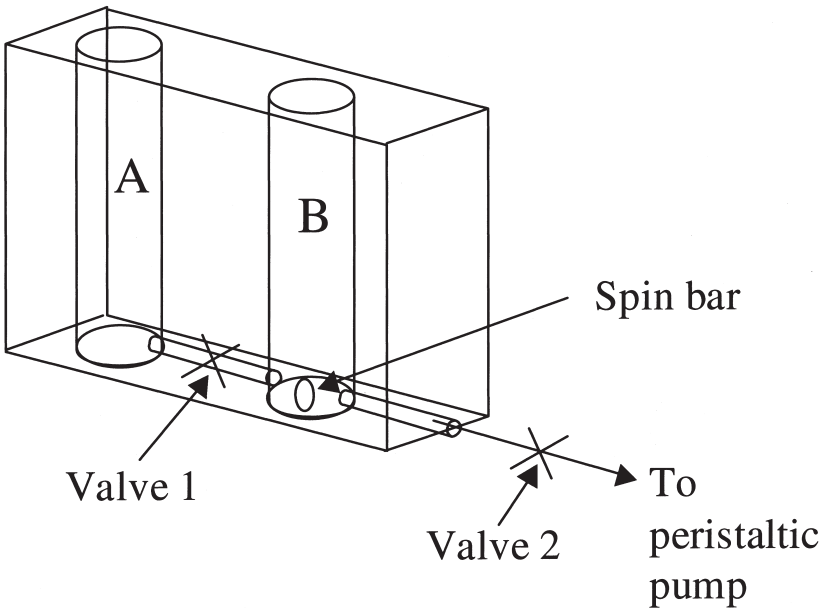

FIG. 1. Schematic of a gradient maker used to create gradients of NGF and NT-3 in p(HEMA) scaffolds. The monomer formulation is added to both chambers A and B and the conduit that connects the two. For neurotrophin gradients, the neurotrophin is added to chamber A only, with the concentration of neurotrophin added impacting the resultant gradient.

rotrophins were created in pHEMA scaffolds, a formulation of 20% HEMA in 80 wt% water with 0.1 wt% ethylene dimethacrylate (EDMA) crosslinker and 20 wt% (4-benzoylbenzyl) trimethyl ammonium chloride photoinitiator was added to both chambers of the gradient maker and with varying amounts of NGF added to chamber A, as described above. The gel was collected in a mold that was mounted horizontally in the chuck of an RZR-1 dual-range, variable speed stirring drill (Heidolph, Schwabach, Germany) and tumbled at a low speed (~40 rpm) for 1 h during which time it was exposed to a metal halide UV light source (~23,400 mW/cm^2, exfo spot curing system). Based on the initial concentration of NGF added to chamber A, scaffolds with theoretical concentration gradients of 214, 262, 310, and 357 ng/mL/mm of NGF were created. Using a direct ELISA technique, which included creating a calibration curve with homogenous immobilized NGF concentrations, the actual concentration gradients were measured. Calibration curves were prepared by entrapping homogeneous concentrations of 0, 2, 5, 8, 11, 13, 15, 17, and 20 μg/mL of NGF inside of p(HEMA) gels, which were each prepared in triplicate, cut into 5 mm sections, and placed inside a 96-well standard culture dish. The standard NGF ELISA procedure was followed using the gels as the primary substance. Briefly, the primary antibody for NGF was incubated with the sections for 2 h, after which the rods were rinsed 5 times with the rinsing buffer provided. Then the secondary IgG, conjugated with HRP, was added to the sections and incubated for 2 h at room temperature. The sections were then rinsed 5 times with the rinsing buffer, removed from their wells with clean forceps, and placed into a fresh 96-well plate to ensure that any secondary antibody adsorbed to the walls of the plate would not cause an erroneous signal. Lastly, the HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to the sections and incubated for 15 min, resulting in a deep blue color developing on the sections. The stop solution was added, turning the blue color into a yellow solution. This solution was removed from each well with a pipette, placed into fresh wells, and read immediately using a UV plate reader at 450 nm. The absorbance of each section was calculated and an average found for each concentration.

Neurotrophin immobilization

To understand the mechanism of neurotrophin immobilization, NGF-immobilized scaffolds were swelled in organic solvents and the supernatant examined for released NGF to determine whether NGF was chemically immobilized or physically entrapped. Specifically, p(HEMA) scaffolds were synthesized, as described in the preceding section with 50 μg of NGF, removed from the glass molds, rinsed once with a solution of bovine serum albumin, and then placed in 5 mL ethanol and left to swell overnight. After 24 h, the solvent was evaporated, the remaining scaffold removed, and the vial refilled with 1 mL HBSS and stirred at room temperature for 1 h to dissolve any eluted NGF. These solutions were analyzed for NGF by the standard sandwich ELISA method.

Cell assay for neurite guidance in immobilized neurotrophin concentration gradient scaffolds

Scaffolds of p(HEMA) containing immobilized concentration gradients of neurotrophic factors were prepared under sterile conditions and tested for guidance of DRG neurites using single and dual concentration gradients of neurotrophins. The concentration gradient of NGF alone required for guidance was first identified. To test for synergistic effects, combined concentration gradients of NGF and NT-3 (200 ng/mL/mm each) were prepared, using an NGF concentration gradient lower than the minimum gradient required for NGF alone. Controls included single gradients of each growth factor at 200 ng/mL/mm. Additional controls for the importance of the dual gradient included single gradients at 200 ng/mL/mm of one growth factor (i.e., NGF or NT-3) combined with a homogeneous concentration of the second growth factor (i.e., NT-3 or NGF) at a concentration equal to that at the high end of the gradient of 46 μg/mL. These scaffolds were used to determine whether the second neurotrophin simply needed to be present to elicit a synergistic response or whether the second neurotrophin had to be present as a gradient for synergism to be observed.

Cell culture on all gradients was identical. The scaffold was cut into sections 5 mm in length and then cut again longitudinally to provide a surface on which to culture the cells. Three sections were placed in a 96-well plate with small amounts of sterile vacuum grease on the well bottoms to prevent floating, flipping, or turning. The direction of the gradient was carefully noted for all scaffolds. Scaffolds were rinsed in water for 1 day to remove any nonimmobilized growth factor and then incubated in 100 μL of serum-free medium for at least 2 h prior to plating the cells. The medium was removed and replaced with 200 μL of medium containing serum, uridine/5-fluorodeoxyuridine, and cells at a concentration of 6×10^4 cell/mL. (The only neurotrophin present was that immobilized in the scaffolds.) Cells were cultured for 2 days at 37°C and 5% CO_2, then rinsed in HBSS and fixed and stained with phalloidin as described in the cell penetration assay.

Scaffolds were inverted and visualized using a Zeiss Axiovert 100 fluorescent microscope. Guidance was determined as previously described.[11,20] Briefly, angles were measured between an imaginary line drawn between the center of the cell and the end of the neurite and another imaginary line between the center of the cell and

the gradient. Perfect guidance would have the line between the center of the cell and neurite overlapping the line between the center of the cell and the concentration gradient: perfect guidance would have an angle of 0°. A minimum of 100 neurites per gradient were measured, and only neurites greater than 20 μm in length (estimated at one cell body length) were included in the directional analysis. The neurite orientation data were first analyzed with the Rayleigh test of uniformity to determine whether there was a preferred direction or simply a random distribution of neurite outgrowth. Those samples that failed the Rayleigh test were noted as having no preferred directionality ("none" in Table 1). Only those samples that passed the uniformity test were further analyzed to ascertain the mean preferred direction of neurite outgrowth relative to the direction of the concentration gradient. The mean angle $\pm$95% confidence interval data are reported in Table 1, where an angle of 0° reflects perfect guidance, and a 95% confidence interval overlapping 0° reflects that 95% of the data overlap the area around perfect guidance.

RESULTS

The goals of this research were to immobilize concentration gradients of neurotrophins within a cell-penetrable scaffold and determine whether concentration gradients of NGF alone or NGF and NT-3 together could guide neurite outgrowth. The mechanism for synergism of NGF and NT-3 combined gradients was examined with Trk receptor co-localization.

Receptor co-localization

Double immunolabeling was used to show the co-localization of both TrkA and TrkC receptors on the E10 chick DRG cells used in this study. The expression of TrkA and TrkC was widespread in E10 DRGs, as evidenced by the strong immunostaining seen in all images. The co-expression pattern of the two receptors was also quite extensive, as observed by comparing the immunostains in Figure 2. Cells were stained by both anti-TrkA and anti-TrkC antibodies. The anti-TrkC stain (green) had a slightly higher background staining compared with the anti-TrkA stain (red). No significant immunostaining was observed in negative controls where the primary antibodies were omitted to account for nonspecific labeling and auto-fluorescence. These results confirm previously published results.[24]

Cell penetration studies

Cells were cultured on large scaffold pieces for 2 weeks, then fixed and stained for F-actin with phalloidin. The scaffolds were embedded in 5% agarose, cut into 250 μm slices with a vibratome, and studied under a fluorescent microscope to check for the presence of cells. Since the purpose of this study was to determine the ability of cells to penetrate the scaffolds, we did not differentiate between cell type within the scaffold and only quantified cell presence at different depths. On the second slice–that is, 250–500 μm from the top of the scaffold–cells were observed on at least 65% of the scaffolds. Cells were also plentiful on slices between 500 and 750 μm, with 50% of the scaffolds exhibiting some cell staining. On approximately 25% of the scaffolds, cells could be observed on slices as far as 1000 μm from the top of the scaffold, although with lower cell density than at the more shallow depths. The decreased number of cells at 1000 μm may reflect the presence of "dead ends" in the scaffold or the duration of the penetration experiment. However, as observed in the SEM micrographs (Fig. 3), scaffolds had an open pore morphology, allowing facile communication between the pores and suggesting a lack of "dead ends," which is important for ultimate *in vivo* applicability and guidance across the scaffold.

NGF immobilization

To determine whether NGF is physically entrapped or chemically bound to p(HEMA) scaffolds, a swelling study was done and the supernatant examined for dissolved NGF by ELISA. Using this method, 40 $\pm$ 2.1 ng or $\sim$80% of the NGF was recovered from the gel that was swelled in ethanol, suggesting that the mechanism of NGF immobilization is primarily physical entrapment within the crosslinked mesh of the p(HEMA) scaffold.

Neurite Guidance in immobilized neurotrophin concentration gradient scaffolds

The stability of immobilized neurotrophins was studied over a 28-day period, where the concentration of both neurotrophic factors released from the scaffold was measured by ELISA. As was shown previously for NGF over an 8-day period[20] and here for both NGF and NT-3 over a longer, 28-day period, there was minimal neurotrophin released. Specifically, at 1, 8, and 28 days, there was 8, 1.5, and 0.2 ng/mL of NGF released into the medium from the scaffold, respectively, and 8, 2, and 0.3 ng/mL of NT-3 released into the medium from the scaffold, respectively, as determined by ELISA. These data were normalized to account for NGF and NT-3 degradation over time, respectively. While 8 ng/mL of either NGF or NT-3 may elicit neurite outgrowth, all scaffolds were rinsed for 1 day prior to cell seeding, thereby removing this soluble NGF and NT-3 and overcoming any effects on neurite outgrowth associated with soluble NGF and NT-3 in the

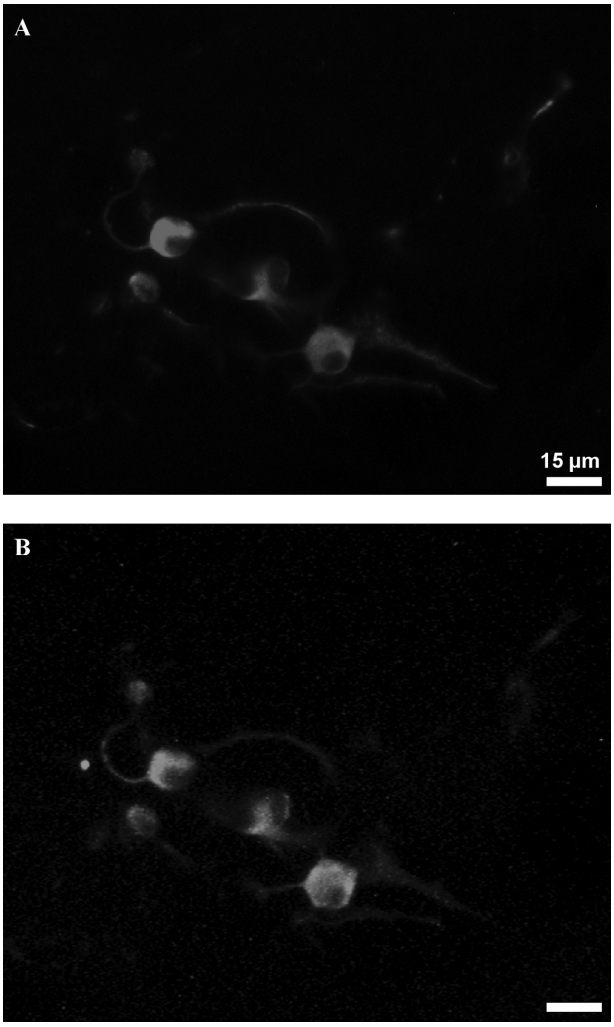

FIG. 2. Immunostaining of TrkA and TrkC receptors of E10 chick DRGs. Note the coexpression of receptors by comparing the staining in the two images taken of the same frame. **(A)** Immunostaining of TrkA receptor for NGF (red) and **(B)** immunostaining of TrkC receptor for NT-3 (green). scale bars, 15 μm. (Color images are available online.)

medium. Thus the amount of NGF and NT-3 released from the scaffolds over the 2-day cell culture experiment was insufficient to elicit neurite guidance.[11]

Linear concentration gradients of neurotrophic factors are important to neurite guidance[11] and were previously demonstrated for NGF immobilized in noninvasive hydrogels using the gradient maker.[20] Using a similar methodology, we calculated the experimental concentration gradients in these new macroporous, cell-penetrable pHEMA scaffolds (Fig. 4), achieved using the calibration curve that we created for the direct ELISA (where $y = 0.0062x + 0.0021$ and $R^2 = 0.85$).

As shown, the R^2 values for the concentration gradient curves were high, with values between 0.93 and 0.99, indicating the linearity of the produced gradients. Moreover, these data demonstrate that a series of immobilized, linear neurotrophin concentration gradients could be prepared. In a separate experiment, a series of concentration gradient scaffolds were prepared with gradients of neurotrophin of 168, 241, 298, and 350 ng/mL/mm, which deviated by 22, 8, 4, and 2%, respectively, from the theoretical concentrations of 214, 262, 310, and 357 ng/mL/mm. The differences between theoretical and experimental concentrations suggest that some neurotrophin was not incorporated into the scaffold and some neurotrophin was not detected by ELISA, which is recognized as an inexact technique. While the neurotrophin used in this study was NGF, we are confident, based on the similar structure, molar mass, and pI of NGF and NT-3, that NT-3 concentration gradients identically synthesized were also linear. Together with the stability data and immobilization data, we can conclude that stable immobilized concentration gradients were created, with the neurotrophin likely entrapped in the polymer network that comprises the wall of the scaffold.

The cellular response of DRG cells from E10 chick embryos to different gradients of immobilized neurotrophic factors was studied in terms of the direction of neurite outgrowth relative to the concentration gradient. DRG cells were cultured on p(HEMA) scaffolds containing combinations of neurotrophin concentration gradients of NGF alone and of NGF and NT-3 together to

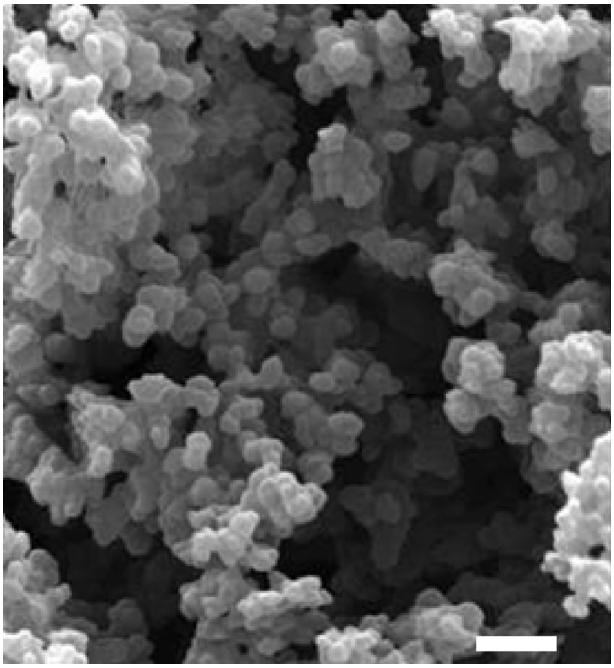

FIG. 3. Representative scanning electron micrograph image of a p(HEMA) scaffold used in this study. scale bar, 5 μm

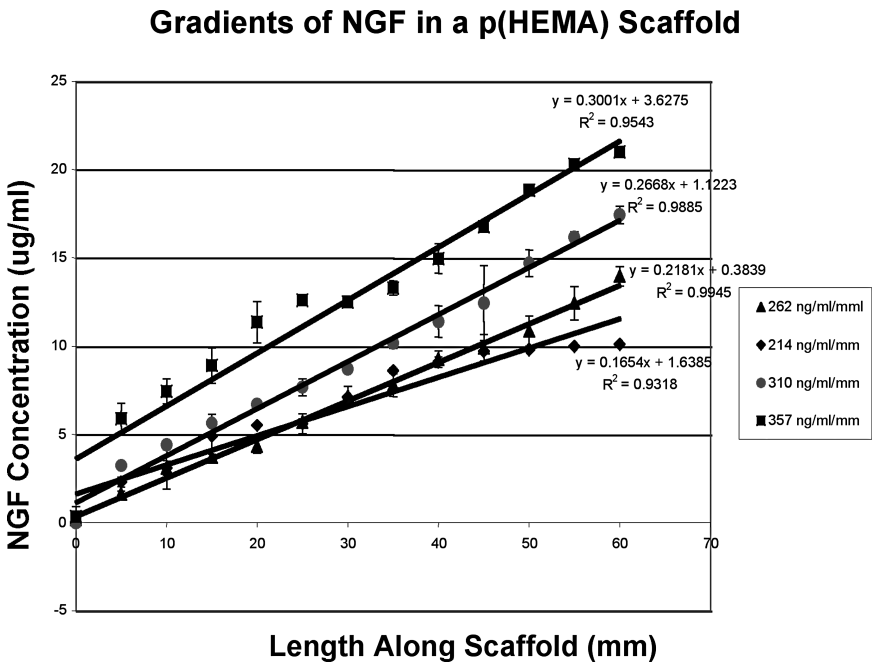

FIG. 4. A series of NGF concentration gradients were immobilized in macroporous p(HEMA) scaffolds (mean ± standard deviation, $n = 3$).

examine possible synergistic effects of the two growth factors. As shown in Table 1, no neurite guidance was observed for concentration gradients of NGF alone until a theoretical gradient of 310 ng/mL/mm was achieved. Neurite guidance was neither observed at single theoretical concentration gradients of NGF at 200 ng/mL/mm nor at single theoretical concentration gradients of NT-3 at 200 ng/mL/mm. However, guidance was observed in scaffolds that had dual theoretical concentration gradients of NGF and NT-3, both at 200 ng/mL/mm. Given that guidance was neither observed in scaffolds with a concentration gradient of 200 ng/mL/mm of NGF plus a high homogenous concentration of NT-3 nor at a concentration gradient of 200 ng/mL/mm of NT-3 plus a high homogeneous concentration of NGF, we can conclude that it is the dual concentration gradients of NGF and NT-3 that are critical for guidance. Thus simply having both neurotrophins present, even if one is present as a gradient, is insufficient for guidance. This demonstrates the synergism of the immobilized neurotrophin concentration gradients for guidance and mirrors previous results where synergism was observed for soluble neurotrophin concentration gradients[24]; however, as is discussed further below, the immobilized gradients required for guidance were greater than those of the soluble gradients.

TABLE 1. EFFECT OF IMMOBILIZED NEUROTROPHIC FACTOR CONCENTRATION GRADIENTS ON THE DIRECTION OF DRG NEURITE OUTGROWTH (IN ABSOLUTE VALUES)

Neurotrophic factor concentration gradient (theoretical, ng/mL/mm)	*Neurotrophic factor concentration added to chamber A (μg/mL)*	*Neurotrophic factor concentration added to chamber B and conduit (μg/mL)*	*Angle of preferred direction of neurite growth (°)*†*
0	0	0	None
310 NGF	71.3 NGF	0	0.01 + 18
200 NGF	46.0 NGF	0	None
200 NT-3	46.0 NT-3	0	None
200 NGF/+ constant NT-3	46.0 NGF + 46.0 NT-3	46.0 NT-3	None
200 NT-3/+ constant NGF	46.0 NT-3 + 46.0 NGF	46.0 NGF	None
200 NGF/200 NT-3	46.0 NGF + 46.0 NT-3	0	6.5 ± 20

*Determined by χ^2 test ($\alpha = 0.01$, $n > 100$ neurites).

†Mean ± 95% confidence interval.

Representative images of E10 chick DRGs demonstrate the guidance of neurites for cells grown on scaffolds with immobilized dual concentration gradients (Fig. 5A and B) compared with the lack of neurite guidance for those cells grown on scaffolds with a single concentration gradient (Fig. 5C and D).

DISCUSSION

The first step in demonstrating synergism between gradients of NGF and NT-3 was to show the presence of the two receptors, TrkA and TrkC, on the same cell. For any interactions to occur between the signaling pathways initiated by these two receptors, they must both coexist on a single cell; otherwise the effects that we observed may be confused with an additive effect versus a synergistic effect. During embryonic development, the expression pattern of the tyrosine kinase receptor family undergoes dramatic changes.[16,31,36] As such, the coexpression of the TrkA and TrkC receptors for NGF and NT-3, respectively, had to be shown for the E10 chick DRG cells used in these studies. Using double immunolabeling techniques first described by Farinas *et al.,*[37] the coexpression of the TrkA and TrkC receptors was evident on E10 chick DRGs, which validated the use of this particular cell population for this study of synergism.

The porous beaded polymer structure typical of phase-separated hydrogel sponges was expected to allow neurites to invade into the p(HEMA) scaffold. This was important since the scaffold created here may be incorporated into a device to promote regeneration in the spinal cord or peripheral nerve and thus must act as a support for regenerating neuronal tissue. The scaffold required large contiguous pores that would allow the neurites to grow through the scaffold to reach the severed nerve stump at the other end. Previous reports indicated that p(HEMA) scaffolds with monomer concentrations of both 10 and 20% were able to support invasion and proliferation of human fibroblasts up to depths of 500 μm.[38] Another study showed the invasion of astrocytes, macrophages, and oligodendrocytes into 20% monomer p(HEMA) scaffolds which were implanted *in vivo.*[39] In the current study, cells were observed at depths as great as 1000 μm after 2 weeks of culture, with the majority of cells found between 250 and 750 μm. This indicated that our 15% monomer p(HEMA) scaffold allowed cell penetration. While the cells likely reached these depths

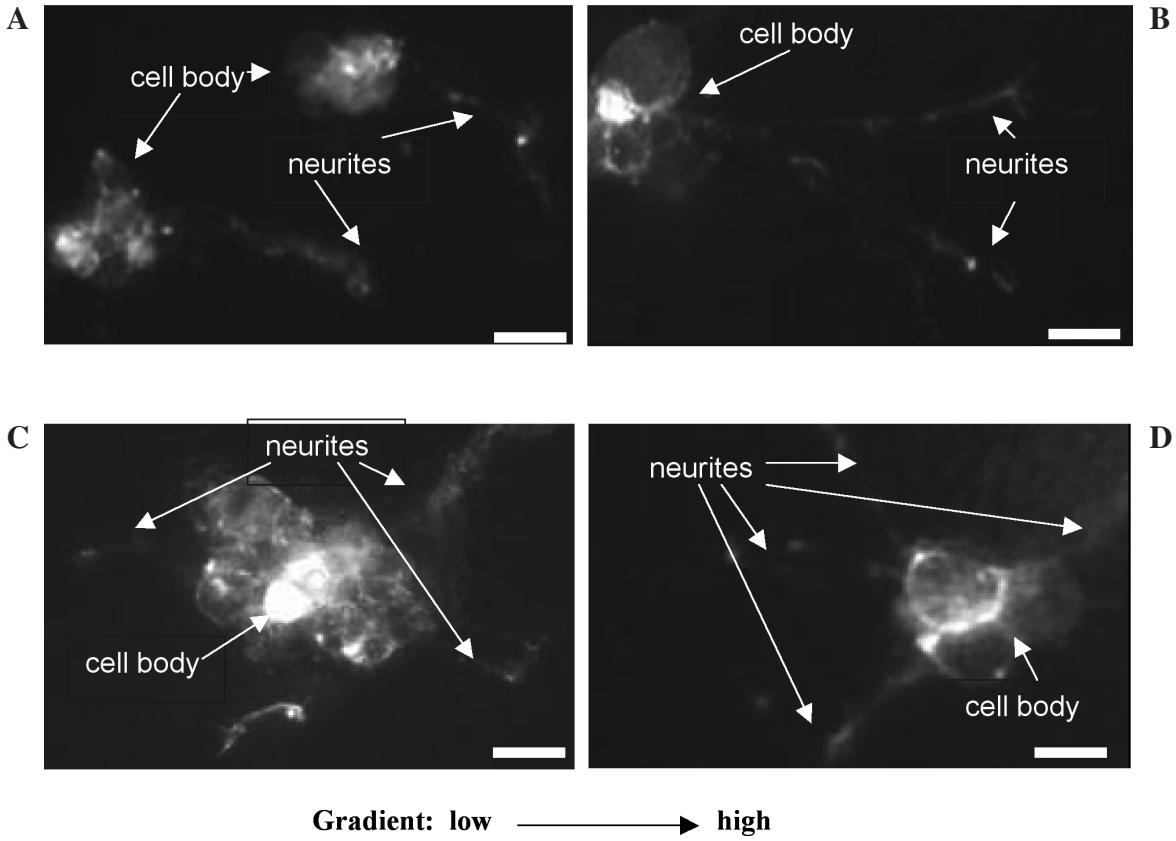

FIG. 5. Representative images of E10 chick DRG cells. Note that cells in **A and B,** exposed to combined gradients of NGF and NT-3, show neurite guidance when compared to cells in **C and D,** which were exposed to a gradient of NGF alone. The gradient direction is shown. The cell body and neurites are identified in each image. scale bars, 20 μm

aided by gravity, the scaffold morphology allowed for cell and neurite penetration. These results, taken together with the previous reports, are a strong indication that these p(HEMA) scaffolds are cell penetrable and may be useful to support axonal regeneration in the damaged spinal cord or peripheral nerve. These cell penetration data, combined with the guidance demonstrated on the macroporous scaffolds (and not throughout due to difficulties in quantifying neurite guidance within the scaffold), suggest that guidance will be mirrored throughout the 3D scaffold and appropriate for future examination *in vivo* where a cell-invasive scaffold provides the structure through which adherent neurites can extend. Achieving neurite guidance by a combination of chemotactic cues (as described herein) with haptotactic cues within a longitudinally oriented scaffold morphology[40] may promote greater regeneration and merits examination.

Gradients of *soluble* NGF have been shown to guide neurite outgrowth of both PC12 cells and primary DRG neurons and immobilized NGF gradients have been shown to guide neurite outgrowth of PC12 cells.[11,20,24] The NGF immobilized here was physically entrapped in the gel, but did not diffuse out of the gel over a 28-day period, demonstrating that the immobilization mechanism was effective in terms of maintaining a stable, bioactive gradient. We demonstrated here that an *immobilized* gradient of 310 ng/mL/mm NGF alone in cell-invasive scaffolds was required to guide neurite outgrowth of primary DRG neurons, which, interestingly, is similar to that previously observed for the PC12 cell line in a noninvasive gel. A lower gradient of 200 ng/mL/mm of NGF alone, however, was unable to elicit guidance, with the same result observed for an equivalent gradient of NT-3 alone. Only when the two growth factors were combined at gradients of 200 ng/mL/mm each was guidance of DRG neurite outgrowth achieved. This result suggested that the two immobilized neurotrophic factor gradients had a synergistic effect on guidance, given that no guidance was observed when one neurotrophin was presented at a gradient and the second at a homogeneous concentration. These data reflect those of soluble concentration gradients where synergism of NGF and NT-3 was also observed for DRG neurite guidance.[24] The possibility of receptor saturation for cells grown on scaffolds with the various gradient configurations was dismissed because the average neurite lengths calculated for cells on all of the scaffolds were not statistically different, as is obvious from the average ($\pm$95% confidence level) measurements: 37 $\pm$ 5 μm (on the dual NGF/NT-3 gradient scaffold); 30 $\pm$ 3 μm (on the single NGF gradient scaffold); 35 $\pm$ 5 μm (on the single NT-3 gradient scaffold); 35 $\pm$ 6 μm (on the NGF gradient scaffold with homogenous NT-3); and 35 $\pm$ 4 μm (on the NT-3 gradient scaffold with homogenous NGF). This, combined with receptor co-localization, demonstrated that the differences in the capacity for guidance were based on the specific gradients, and not on receptor saturation or simply stimulation of more cells.

A comparison of the immobilized neurotrophin concentration gradient system and the soluble neurotrophin concentration gradient system reveals that higher concentration gradients for both NGF alone (310 ng/mL/mm immobilized *vs* 133 ng/mL/mm soluble) and NGF/NT-3 combined (200 ng/mL/mm immobilized *vs* 80 ng/mL/mm soluble) were required for guidance.[11,24] Growth cones in the soluble system were surrounded by a continually replenished source of neurotrophins, which the cell could take up and transport to the cell body. In the immobilized system, cells were probably unable to take up the growth factor due to its immobilization within the scaffold. If, however, the cell could actively take up the neurotrophins, there would be no replacement of the growth factor in the immediate surroundings. In either case, the growth cone would have to expand to sample the environment, reaching to detect the next immobilized neurotrophin. The steeper gradient provides an increase in the amount of neurotrophin present within the growth cone's reach, which can be an area as large as 20 μm in diameter,[41] and may thus account for the greater gradient required for guidance in the immobilized system. Alternatively, the differences in concentration gradients may be related to differences in bioactivity of the NGF in the soluble versus immobilized systems. While the NGF in the soluble system was continually renewed (every 6 h), the immobilized NGF was not renewed and may have lost some bioactivity due to the immobilization process itself; however, this is difficult to quantify as extraction of NGF from the scaffold (by, for example, swelling with ethanol) invariably impacts bioactivity. While differences in soluble and immobilized neurotrophin concentration gradients cannot be easily resolved, the focus of this study was not to determine the minimum concentration gradient required for guidance, but to determine whether a gradient could be immobilized in a macroporous scaffold, whether this gradient could be used to guide neurite outgrowth, and whether a synergistic response could be observed for NGF and NT-3. Neurotrophin signaling pathways are highly complex, with multiple possibilities for synergistic interactions, especially in the case of NGF and NT-3 signaling. The high affinity receptors for NGF and NT-3, TrkA and TrkC, respectively, are believed to function via two distinct cytoplasmic signaling pathways.[6,25,42] The existence of two independent signaling cascades allows for possible crosstalk and synergistic interactions between the two pathways, possibly involving second messenger crosstalk.

A second method by which NGF and NT-3 may interact is through the TrkA receptor itself. Although TrkA is known as the high affinity receptor for NGF, it is also

capable of interacting with NT-3, albeit with much lower affinity than for NGF.[14,15,17,28] The high affinity of the TrkC receptor for NT-3 greatly minimizes any interaction of NT-3 with TrkA, as does the presence of the p75 pan-neurotrophin receptor, which has been shown to inhibit the activation of TrkA by NT-3 significantly.[43] Thus, the interaction of NT-3 with TrkA is an unlikely source of the synergy observed in this study, although it cannot be ruled out.

Combined neurotrophin concentration gradients are advantageous over single neurotrophin concentration gradients because neurite guidance can be achieved over greater distances due to the activation of the p75 death receptor at higher neurotrophin concentrations. Thus neurites should be guided over longer distances by lower neurotrophin concentration gradients, such as those achieved with the combined NGF/NT-3 gradients. This hypothesis is supported by recent data by Odzinler *et al.*,[44] who demonstrated that localized sources of NGF can direct axon outgrowth from dorsal root ganglion, which preferentially grow toward lower doses of localized NGF and grow away from higher concentrations at earlier stages of development, but do not show this response later. This data implies that at earlier stages of development, the axonal response to NGF is mediated by p75NTR signaling through TrkA expressing axons.

CONCLUSIONS

Immobilized neurotrophin gradients were shown to guide neurite outgrowth of primary DRG neurons in cell-penetrable, macroporous p(HEMA) scaffolds: successful gradients included single NGF gradients of 310 ng/mL/mm and dual NGF and NT-3 gradients of 200 ng/mL/mm each, where a synergistic response was observed. The combination of two gradients of neurotrophic factors takes a step toward more closely mimicking the *in vivo* environment of guiding axons where multiple cues work together to guide regenerating nerves back to their intended targets. These immobilized gradients will be incorporated in a device for implantation into the damaged peripheral nerve or spinal cord to promote regeneration after injury. In on-going studies, we are investigating the intracellular signaling pathways with immobilized neurotrophin concentration gradients in an attempt to better elucidate the mechanisms for guidance.

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