Immobilized concentration gradients of nerve growth factor guide neurite outgrowth

Terri Adams Kapur,[1,2] Molly S. Shoichet[1,2,3]

[1]University of Toronto, Department of Chemical Engineering and Applied Chemistry, 200 College Street, Toronto ON Canada M5S 3E5

[2]Institute of Biomaterials and Biomedical Engineering, 4 Taddle Creek Road, Toronto, ON Canada M5S 3G9

[3]Department of Chemistry, 80 St. George Street, Toronto, ON Canada M5S 3H6

Abstract: Axons are guided to their targets by a combination of haptotactic and chemotactic cues. We previously demonstrated that soluble neurotrophic factor concentration gradients guide axons in a model system. In an attempt to translate this model system to a device for implantation, our goal was to immobilize a stable neurotrophic concentration gradient for axonal (or neurite) guidance. Nerve growth factor (NGF) was immobilized within poly(2-hydroxyethyl-methacrylate) [p(HEMA)] microporous gels using a gradient maker. The NGF was stably immobilized, with only ~0.05% of the amount originally incorporated into the gel released over an 8-day period. Immobilized NGF was bioactive: the percent of PC12 cells extending neurites on NGF-immobilized p(HEMA) gels was $16 \pm 2\%$, which was statistically the same as those exposed to soluble NGF ($22 \pm 6\%$). We were able to predict and reproducibly create stable NGF concentration gradients in the gel. At an NGF concentration gradient of 357 ng/mL/mm, PC12 cell neurites were guided up the gradient. The facile, flexible, and reproducible nature of this method allowed us to translate soluble growth factor gradient models to stable growth factor gradient devices that may ultimately enhance axonal guidance and regeneration *in vivo*. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 68A: 235–243, 2004

Key words: poly(hydroxyethyl methacrylate); pHEMA; PC12 cells; gradient; NGF; neurotrophins; spinal cord injury

INTRODUCTION

The events following spinal cord injury are complex and not well understood; however, recently, several methods to both understand and enhance axonal regeneration have been described and reviewed.[1–3] Although the molecules that stimulate regeneration have begun to be identified, what is lacking is a delivery vehicle for the most promising cellular and molecular therapies. Biomaterials promise to play an important role herein. Building on the entubulation strategy followed in peripheral nerve studies, where regeneration has been promoted in nerve guidance channels filled with extracellular matrix materials imbided with growth factors,[4–6] we (and others) have begun to test similar strategies in the spinal cord. To this end, we have designed and studied many of the components of a nerve regeneration device, including the tube itself[11,12] and the haptotactic[13,14] and chemotactic cues[15] that will fill it. Although we recognize that the spinal cord, unlike the peripheral nerve, will not spontaneously regenerate,[11,16,17] the entubulation strategy seems to be a good place to start—especially in light of the promising results obtained with Schwann cell-lined PAN/PVC guidance channels for spinal cord injury repair.[18] In this article, our focus is on the chemotactic cues required for regeneration, and specifically on the creation and utility of stable growth factor concentration gradients for neurite guidance.

After injury in the peripheral nervous system, axons have been shown to reinnervate their targets as a result of both stimulatory and inhibitory gradients, which together, provide the appropriate guidance signals.[19] A similar path-finding gradient is exhibited during embryogenesis where new neural tissue must properly differentiate and target-innervate. Stimulatory and inhibitory molecules have been studied in both surface bound and soluble forms,[20–35] and che-

motactic cues have been shown to retain their guidance potential whether matrix bound or in solution.[21] Although some of these studies demonstrate turning, neither the gradient required to elicit turning nor the maximum distance for guidance were quantified. In a previous study, we developed a model diffusion chamber that answered this question. We determined that a minimum concentration gradient of soluble nerve growth factor (NGF) was required for axonal guidance over a maximum distance.[36] Our goal here is to determine whether we can translate this soluble gradient to a stable immobilized gradient that will provide neurite guidance.

Our first objective was to find an appropriate material within which to immobilize the gradient and along which we could test our hypothesis of neurite guidance by an immobilized growth factor gradient. Although there are numerous biomaterials that have been tested and developed for tissue engineering applications,[37] we chose to use poly(2-hydroxyethylmethacrylate) [p(HEMA)] because it is used clinically and p(HEMA) gels can be created with the desired chemical, physical, and mechanical properties for soft tissue applications. Furthermore, numerous proteins, including NGF, have been stably encapsulated inside p(HEMA) for a variety of biomedical applications.[16,38–41]

Our second objective was to develop an appropriate method to create a stable, immobilized concentration gradient of growth factors in p(HEMA) gels. After trying several strategies, we found that adapting a commercially available gradient maker[42–44] was most suitable, and used this method to create a series of NGF concentration gradients. We chose to work with NGF because it has been well characterized, serves as a good model for other neurotrophic factors, and we had previously demonstrated PC12 cell neurite guidance with soluble NGF concentration gradients.

Our third objective was to test whether a stable, immobilized NGF concentration gradient could guide neurites as we previously demonstrated with soluble NGF. Specifically, we tested: the stability of the gradient, the bioactivity of immobilized NGF and the neurite guidance potential of immobilized gradients of NGF using a model neuronal cell line, pheochromocytoma (PC12) cells.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and used as received unless otherwise specified. Water was distilled and deionized using Millipore Milli-RO 10 Plus at 18MΩ. Polymerization reactions were conducted at room temperature and cell culture experiments at 37°C. Mouse NGF (2.5S NGF) was purchased from Cedarlane Laboratory (Hornby, Ontario, Canada). ELISA reagents were purchased as a kit from Chemicon International, Inc. (Temecula, CA). The adrenal rat PC12 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). All reagents for culture media, media additives, stain reagents and HANKS balanced salt solution (HBSS) were purchased from Invitrogen (Burlington, Ontario, Canada). Filamentous actin stain labeled with FITC was purchased from Molecular Probes (Eugene, OR).

Preparation of p(HEMA) microporous gels with immobilized nerve growth factor [p(HEMA)-NGF]

To determine bioactivity, NGF was immobilized in p(HEMA) gels at a homogenous concentration. P(HEMA) hydrogels were made by mixing 245 μL (or 35 wt %) of 2-hydroxyethyl methacrylate (HEMA), and 455 μL (or 65 wt %) water within which 6.5 μg/mL of NGF was dissolved. To this solution were added, 0.1 wt % ethylene dimethacrylate (EDMA) crosslinker, 0.07 wt % ammonium persulfate (APS) initiator, and 0.35 wt % tetramethyl ethylenediamine (TEMED) accelerator, where the latter three percentages were relative to that of the HEMA monomer. To create a gel with a homogenous concentration of NGF, each component was mixed thoroughly together, and then the entire ~700 μL mixture was injected into a long glass tube mold having a 2-mm inner diameter. The solution polymerized overnight and resulted in an 18 cm-long rod-shaped white gel, which was removed from the glass mold and then immersed in water overnight to remove any unreacted molecules. The gels were then cut into 0.5-cm sections using a razor blade and a dissection microscope, cut in half longitudinally, and finally placed into the bottom of a 96-well plate. These samples were prepared for cell culture by disinfecting them with 70% ethanol, rinsing with water, placing them in sterile 96 well plates, and then plating PC12 cells as described in more detail later. The immobilized NGF was assayed for bioactivity by examining PC12 cell neurite length.

Preparation of gradients of immobilized NGF in p(HEMA)

Gradients of immobilized NGF were prepared in p(HEMA) gels using a modified gradient maker (CBS Scientific, Temecula CA) as shown in Figure 1. The gel formulation was similar to that described above, with the exception that the NGF concentration was increased. The gradient maker required that the total prepolymer solution (HEMA, water, EDMA, APS, and TEMED) be separated into two solutions of equal volume, each of which contained a different concentration of NGF. For example, an NGF gradient gel with a total volume of 700 μL was prepared using a gradient maker with a combined chamber volume of 500 μL and a "dead volume" of 200 μL between the chambers. To form a gradient of NGF with concentration extremes of 71.4 μg/mL
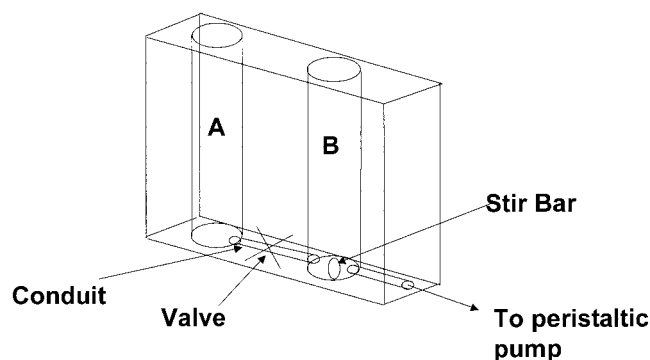
Figure 1. The gradient maker used to create NGF concentration gradients in p(HEMA) gels had chambers A and B separated by a conduit. With the center valve closed, a known concentration of NGF and prepolymer solution were added to chamber A while an NGF-free prepolymer solution was added to chamber B. The center valve was opened and the peristaltic pump and stir bar were activated. The volumes in both chambers were equally depleted and mixed, and the resulting solution was delivered into a mold and allowed to polymerize, resulting in a gradient of entrapped or immobilized NGF in p(HEMA).

and 0 μg/mL, the prepolymer solution was separated into solution A (250 μL of 200 μg/mL NGF) and solution B (250 μL of 0 μg/mL NGF). Two hundred microliters of the prepolymer solution (with no NGF) was added to the conduit connecting chambers A and B. The center valve was closed and then solutions A and B were added to chambers A and B, respectively. The stir bar in chamber B was activated, the center valve opened, and the peristaltic mini pump started. As the solution in chamber A was drawn through the conduit, it mixed completely with the solution in chamber B, and the volumes of chambers A and B were simultaneously and equally depleted. A linear NGF concentration gradient was created because the 200 μg/mL NGF solution in chamber A was mixed with an increasingly lower volume of 0 μg/mL NGF free solution in chamber B, and delivered out to the glass tubular mold. The resulting 18 cm-long rod-shaped, white gels were prepared for cell culture as described below. The gradient gels were separated into three groups for characterization, as described more fully below: (1) the stability of the immobilized NGF was determined by measuring the amount of NGF released from the gel by standard sandwich ELISA; (2) the slope of the NGF concentration gradient was determined by direct ELISA; and (3) the ability of the NGF concentration gradient immobilized in the gel to guide neurites was determined using PC12 cells.

Determination of immobilized NGF stability using sandwich ELISA

NGF-immobilized p(HEMA) rods that were prepared according to one of the procedures described above, were first rinsed with 1 mL of 0.1 mg/mL of bovine serum albumin (BSA) in HBSS buffer to remove any NGF not immobilized, and then immersed in 1 mL of a 0.1 mg/mL BSA in buffer

solution at 37°C for 8 days. A BSA solution was used for two reasons: (1) BSA is known to stabilize soluble NGF;[45] (2) BSA will coat the sample vials used, thereby reducing any NGF loss due to adsorption. The supernatant was collected and replaced with fresh BSA solution at 1 and 8 days. The collected samples were analyzed for NGF by a standard ELISA to determine the stability of immobilized NGF. To control for any loss of NGF bioactivity, a vial of NGF was prepared at a similar concentration (similar to the NGF concentration entrapped in the gel), kept under the same conditions, and assayed simultaneously with the gel supernatants each time the assay was performed.

Surface detection of NGF by direct ELISA

A direct ELISA method was used to detect NGF at the surface of the gels. The gels prepared in this section served two purposes: (1) to prepare a calibration curve to infer the amount of NGF immobilized in the bulk, and (2) to verify that the NGF at the surface of the p(HEMA) gels increased linearly as the bulk concentration increased. A series of homogenous immobilized p(HEMA)-NGF gel concentrations were prepared: 0, 2, 3, 5 and 10 μg/mL, each in triplicate as described earlier. For the direct ELISA, the primary antibody for NGF was incubated with the hemisected rods for 2 h, after which the rods were rinsed four times with rinsing buffer before adding the secondary IgG conjugated with horse radish peroxidase (HRP). After a 2-h incubation at room temperature, the rods were rinsed four 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. Finally, the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to the disks and incubated for 15 min. A deep blue color developed near the surface of the gel. After incubation, the stop solution was added resulting in a yellow solution. The solution was removed with a pipette, placed into fresh wells, and read immediately using a UV plate reader at 450 nm. The absorbance of each gel was plotted against the concentration of NGF originally added. This bulk NGF calibration curve was used to determine the amount (and then the slope) of NGF immobilized within the gradient gels.

Preparation of p(HEMA)-NGF gels for cell culture

To prepare the NGF gradient gels for cell culture, p(HEMA), which is nonadhesive to cells, was modified with poly(allylamine) (pAA), which is cell adhesive. PAA was modified with 6-(4-azido-2-nitrophenylamino)hexanoic acid N-hydroxysuccinimide ester (pAA-azido) as described elsewhere[25] and photo-immobilized on precut, NGF immobilized p(HEMA) rods. Briefly, 3 μL of pAA-azido (13.36 mg/mL in water) was added by pipette to the top of each p(HEMA) gel section and then dried under vacuum for ~15 min, resulting in an orange color on the surface of the dehydrated p(HEMA) rod. Each dehydrated rod was then exposed to a metal halide UV light source (~23,400 mW/

cm^2) for 1 s at a working distance of 1 cm using an Exfo spot curing system (Mississauga, Ontario). After exposure, the surfaces turned dark brown indicating that the reaction was complete. The rods were then rinsed with water overnight to remove any unreacted pAA-azido. P(HEMA)-NGF-pAA gels were disinfected with 70% ethanol, rinsed in water, placed in sterile 96-well plates, and then incubated with PC12 cells to determine the neurite guidance potential of immobilized NGF concentration gradients, as described in more detail in the next section.

Cell culture on gels with both homogenous concentrations of NGF and gradients of NGF

PC12 cells were maintained in T-25 cell culture flasks at 37°C in a 5% CO$_2$/air atmosphere. The cell culture medium consisted of RPMI 1640, with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 1% penicillin/streptomycin. Cell culture medium was changed every other day, and cells were subcultured once every week. PC12 cells were "primed" with 40 ng/mL for at least 24 h before they were plated on the gradients and controls. Cells were removed from the culture flasks by flushing with fresh culture medium and agitation, and then counted using a hemocytometer. PC12 cells were plated on disinfected p(HEMA)-NGF-pAA gels at a concentration of 7×10^4 cells/well. The following controls were used: tissue culture wells coated with collagen ($n = 6$), p(HEMA)-pAA rods (without immobilized NGF) ($n = 3$), and p(HEMA)-pAA homogenous NGF rods ($n = 3$). NGF was added to three of the collagen coated well controls at a concentration of 40 ng/mL. The collagen controls served as positive (with added soluble NGF) and negative (without NGF) controls for neurite length, whereas the p(HEMA)-pAA rods served as a negative control for guidance and the p(HEMA)-pAA-homogenous NGF as a control for guidance. Another set of controls had PC12 cells plated on p(HEMA)-pAA samples using media taken from the immobilized p(HEMA)-NGF-pAA samples. This last control assayed for the stability of immobilized NGF. Cells were plated on the same day at the same density on all surfaces. All cells were fixed after 3 days in culture and assayed for neurite length and guidance as described in more detail in the next two sections.

Cell assay for percent of cells responding on homogenous concentrations of immobilized NGF

After 3 days of culture, the media was removed and gels were rinsed with HBSS. Cells were fixed to the gels by immersion in 3.7% formaldehyde for 10 min at room temperature, rinsed in HBSS, and then incubated in 0.1% Triton X-100 for 90 s to permeate the cellular membrane and allow the cellular stain to enter the cells. Cells were rinsed with HBSS and incubated with 5 μL/mL of FITC actin phallotoxin for 30 min, which stains for filamentous actin (F-actin). Cells on the gels were visualized using an inverted fluorescence microscope (Zeiss Axiovert 100) using FITC excitation and emission filters and a mercury lamp. Images were cap-

tured using a digital camera (Diagnostic Instruments) and SPOT software. P(HEMA) rods were inverted and imaged upside down because they are opaque. At least 100 cells from each gel were imaged, and each gel was analyzed in triplicate to obtain statistically significant data. The SPOT software was calibrated with a stage micrometer. Each cell was counted, and the longest neurite was measured. A cell bearing a neurite longer than 20 μm (greater than one cell diameter) was deemed to be responding. The average number of percent responding cells/gel and the standard deviation of the triplicates in each treatment are reported.

Cell assay for neurite guidance on immobilized gradients of NGF

To determine if cells are guided by gradients of immobilized NGF, gradient gels of different NGF slopes were prepared by varying the concentration of NGF added to chamber A of the gradient maker and keeping the concentration of NGF in chamber B constant at 0 ng/mL. Cells were plated on gradient gels as outlined earlier. After incubation, cells were stained for F-actin, and images were captured as described in the section above. The direction of the gradient was noted, and the angle of the neurites extending from the cells was measured with respect to the direction of the gradient ($n = 50$). As previously reported,[31] the Rayleigh test of uniformity was performed to determine if the neurites were growing up the concentration gradient. This test consists of inputting the values for C and S into a simple vector addition [eq. (1)]:

$$R = (C^2 + S^2)^{1/2} \qquad (1)$$

to obtain a resultant vector (R), where C and S are given by eqs. (2) and (3):

$$C = \frac{1}{n} \sum_{i=1}^{n} \cos \theta_i \qquad (2)$$

$$S = \frac{1}{n} \sum_{i=1}^{n} \sin \theta_i \qquad (3)$$

where n is the number of neurites ($n > 50$) and θ is the angle measured between two imaginary lines: (1) a line drawn from the center of the cell towards the increasing gradient, and (2) a line drawn from the center of the cell to the end of the neurite. By definition, vectors give both directionality and magnitude information; therefore, the closer the neurites are to the direction of the gradient, the larger the magnitude of the R value. These calculated R values were compared to tabulated R values, at 99% confidence, that have been previously published.[46] When the calculated R was greater than the tabulated R, there was preferred neurite outgrowth. A chi square test was then performed to test whether the preferred neurite outgrowth direction was in the expected range. To perform this test, the mean angle was calculated according to eq. (4):

$$\phi = \arctan\left(\frac{S}{C}\right) \qquad (4)$$

By comparing ϕ to a Bastschelet chart, ϕ values that fell within 2 standard deviations of a 95% confidence interval demonstrated guidance. The mean and 95% confidence interval for ϕ are reported. Angles with standard deviations that overlapped with 0° demonstrated preferred directionality.

RESULTS AND DISCUSSION

We previously demonstrated that a minimum linear concentration gradient of soluble NGF was required to guide neurites over a maximum distance. These studies, performed in a model diffusion chamber, allow one to probe different factors and gradients for guidance potential, but the system is inherently limited to *in vitro* studies. In an attempt to begin to translate this model to a system that can be tested *in vivo*, we strove to create a stable immobilized concentration gradient of bioactive NGF that would guide neurites *in vitro.*

Determination of immobilized nerve growth factor stability

The stability of immobilized NGF in p(HEMA) gels was studied by analyzing the amount of NGF released over time. A p(HEMA)-NGF gel was immersed in HBSS and assayed for NGF over an 8-day period. By comparison to a calibration curve ($R^2 = 0.99$), the concentration of NGF released was determined by standard sandwich ELISA. P(HEMA) gels rinsed on day 0 had 74 ± 6 ng/mL of NGF in the supernatant, which indicates that $3.7 \pm 0.3\%$ of the growth factor that was initially added was never incorporated in the polymer gel matrix. The assay was repeated on days 1 and 8, where an average of 0.55 ± 0.06 ng/mL or ~0.05% was released at each time point. It is important to note that this concentration of NGF does not elicit neurite outgrowth. Given that all cell studies were terminated after 3 days, any cellular response to p(HEMA)-NGF gels was due to NGF in the gel and not NGF released from the gel. To ensure that the ELISA would accurately measure the concentration of NGF released, a known concentration of NGF was incubated at 37°C for the same period of time and assayed simultaneously with the supernatants. On day 1, the ELISA detected 85% of the initial NGF, whereas on day 8, it detected only 65% of the initial NGF. The NGF release numbers reported were appropriately adjusted taking the decrease in detection into account.

Surface detection of NGF by direct ELISA

The amount of NGF immobilized in the p(HEMA) gels was calculated by comparison to a calibration
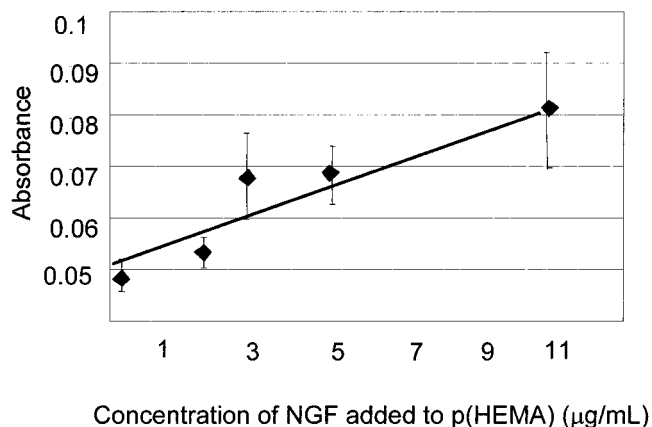

Figure 2. The calibration curve for pHEMA-NGF gels: $y = 0.0026x + 0.05$, $R^2 = 0.82$. ($n = 3$ for each point, the mean and standard deviation are reported.)

curve. The calibration curve was plotted using a series of p(HEMA) gels with different NGF concentrations entrapped and assayed by direct ELISA. The purpose of this experiment was twofold: (1) to determine if the bulk NGF concentrations could accurately estimate the surface concentration by direct ELISA; and (2) to obtain a calibration curve that could be used to estimate the amount of NGF incorporated in the bulk of the gradient gels. As shown in Figure 2, the calibration curve had a correlation coefficient (R^2) of 0.82, which indicates that NGF surface concentration is representative of NGF bulk concentration. The R^2 value of 0.82 may be low due to either error associated with the direct ELISA technique or changes in NGF conformation during immobilization. Notwithstanding the R^2 value, this data corroborates the validity of the direct ELISA assay to estimate the concentration gradient profiles prepared in p(HEMA)-NGF gels.

A series of NGF concentration gradient gels were prepared and the data of one of these is summarized in Figure 3. The concentration gradient was calculated by measuring the concentration of NGF by direct ELISA in the 5 mm p(HEMA) gel pieces at every 1–2 cm along the 18-cm rod length. The concentration gradient (or slope) of the NGF in the p(HEMA)-NGF gel was 129.2 ng/mL/mm as shown in Figure 3, which was similar to the predicted slope of 129.0 ng/mL/mm determined from eq. (5):

$$[(A + B)/V_{\text{tot}}]/L \qquad (5)$$

where A and B are the amounts of NGF added to chambers A and B, respectively, in ng; V_{tot} is the total volume in the gradient maker in mL (including the two chambers and the conduit), and L is the length of the resulting rod in cm. Specifically, for the gradient summarized in Figure 3, 81.25 µL of 200 µg/mL NGF was added to chamber A, and 0 µg of NGF was added to chamber B. The ELISA method described here dem-
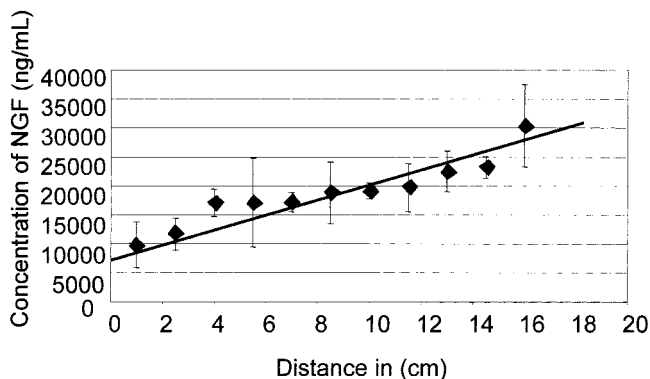
Figure 3. NGF concentration gradient in pHEMA gel: $y = 1292x + 7150$, $R^2 = 0.83$ ($n = 3$ for each point, the mean and standard deviation are reported). The slope of the p(HEMA)-NGF curve matches that of the predicted curve: $y = 1290x$.

onstrates that (1) NGF can be stably immobilized in a gradient; (2) this gradient is available at the surface and should be detected by the PC12 cells; and (3) the NGF bulk concentration gradient profile is predictable.

Cell assay for neurite length and percent of cells responding

The percentage of cells responding to NGF immobilized in p(HEMA) gels was determined by calculating the number of cells with neurites longer than one cell body length relative to the total number of cells. The PC12 cells were primed with NGF prior to plating to avoid the longer tissue culture time required for neurites to be extended in nonprimed cells. Longer culture times result in PC12 cell division, which can hinder accurate measurements of neurite length and directionality, while PC12 cells plated too sparsely may not survive. The neurite length data are summarized in Figure 4 relative to controls for both bioactivity and stability of immobilized NGF. Approximately the same number of cells responded to immobilized NGF in p(HEMA)-NGF-pAA rods as responded to positive controls, where PC12 cells were cultured on collagen in the presence of soluble NGF in the media. To ensure that PC12 cells on p(HEMA)-NGF-pAA gels were responding to immobilized NGF, and not to released NGF, the media from immobilized p(HEMA)-NGF-pAA gels was removed and used to culture fresh PC12 cells on p(HEMA)-pAA gels. The average number of cells responding to preconditioned media was statistically the same as the number responding to the negative control (i.e., PC12 cells cultured on collagen-coated wells in the absence of NGF). In contrast, the number of cells responding to p(HEMA)-NGF-pAA was statistically different from

those responding to media from p(HEMA)-NGF-pAA plated on fresh cells at $p < 0.05$, using a Students t-test. It is interesting to note that PC12 cells required an adhesive coating on p(HEMA)-NGF gels, such as pAA, for cell adhesion to test the guidance hypothesis. Although we did not measure the thickness of the pAA coating on the p(HEMA) gels, we anticipate a thin or patchy coating because PC12 cells responded to immobilized NGF.

Cell assay for neurite guidance

Primed PC12 cells were cultured on p(HEMA)-NGF-pAA gels having well-defined and stable NGF concentration gradients to determine whether a bound concentration gradient could guide PC12 cell neurites as we previously observed with soluble NGF concentration gradients. As summarized in Table I, a series of concentration gradients were created and tested for guidance by the Rayleigh test, as described in eq. (1). If a positive result was obtained for preferred directionality, the angle of the extended neurite relative to the concentration gradient was calculated by a chi square test, as was described in eq. (4). Of the eight
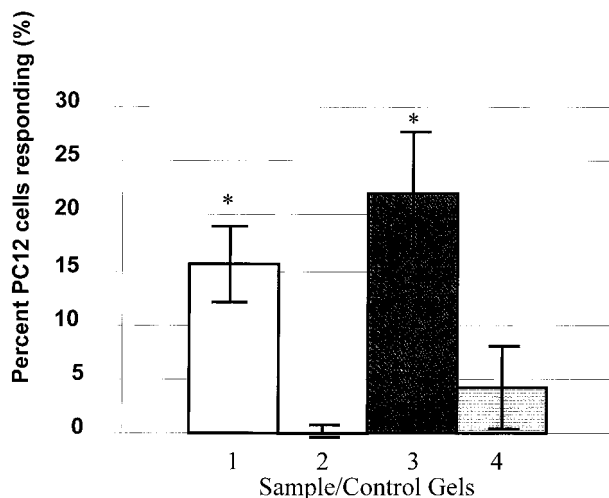
Figure 4. Primed PC12 cells express neurites in response to immobilized NGF: (1) Positive control: PC12 cells cultured on collagen coated wells in the presence of 40 ng/mL of soluble NGF added to the media; (2) negative control: PC12 cells plated on collagen-coated wells in the absence of NGF; (3) sample: 4.2 ng/mL of NGF immobilized in p(HEMA)-NGF-pAA gel. (4) Sample: media from (3) was added to PC12 cells cultured on p(HEMA)-pAA gels. For every sample/control, a minimum of 50 cells was counted. Note that (3) is statistically different from (4), demonstrating that the PC12 cells respond to immobilized NGF and not to NGF that may be diffusing out of the gel over the course of the experiment. The data represents the average of three samples/controls and their standard deviation. *Statistically significant difference from the negative control with Students t-test at $p < 0.05$.

TABLE I
Effect of Immobilized NGF Concentration Gradients on Primed PC12 Cell Neurite Outgrowth
($n > 50$, Mean ± 95% Confidence Interval)

Concentration Gradient (ng/mL/mm)	Preferred Direction of Neurite Outgrowth (ϕ, Degrees)
3.46	None
9.19	None
24.4	None
47.8	None
67.7	None
122	None
137	None
357	8.2 ± 17

concentration gradients tested, we found only the largest gradient of 357 ng/mL/mm passed the Rayleigh test of guidance. At this immobilized NGF concentration gradient, neurites were guided at an average angle of 8.2° within the 95% confidence interval of 17°. Thus, the minimum gradient for guidance with immobilized NGF is between 137 ng/mL/mm, where we saw no guidance and 357 ng/mL/mm, where guidance was observed. The immobilized NGF gradient was able to guide neurites in a similar manner as was observed for soluble NGF concentration gradients. In these experiments, the slope of the concentration gradients is expressed as concentration of NGF/distance in the gel because, although we investigated the cells at the PHEMA surface, the NGF gradient existed within the entire volume of the gel, which was then cut in half prior to cell culturing. Although one may contemplate expressing the gradient as the amount of NGF on the culture surface per distance, we chose to report the concentration/mm as this reflects the data obtained by ELISA. We recognize that the cells may have experienced a different NGF concentration because: (1) the NGF exposed to the cells may differ from that exposed to ELISA, and (2) surface NGF density may have been affected by PHEMA chemistry.

Representative images shown in Figure 5 demonstrate that PC12 cell neurites are guided by the immobilized NGF concentration gradient [Fig.5(a)–(c)] relative to control samples that have a homogenous concentration of NGF immobilized [Fig. 5(d)]. Interestingly, the PC12 cells cultured on immobilized NGF samples [Fig.5(a)–(d)] had thickened or engorged neurites compared to neurites of PC12 cells cultured with soluble NGF [Fig. 5(e)]. (Note that the scalebars represent 20 μm.)

The differences in PC12 cell neurite phenotype observed in the presence of soluble versus immobilized NGF may be explained by the availability of growth factors at the growth cone. NGF has been shown to be internalized by the neurites at the growth cone and

retrogradely transported to the soma, influencing the cell on many levels including gene expression and apoptosis.[22] However, other mechanisms of NGF action have been elucidated.[20,21,47–49] For neurite guidance, local phosphorylation events can cause expansive changes in the cell membrane, actin polymerization, different microtubule dynamics, calcium influx, and organelle recruitment; these events do not necessarily require uptake and transport of NGF to the soma.

In the soluble concentration gradient system,[36] NGF was taken up by the cell and transported to the soma. Although this depleted the concentration of NGF near the growth cone, the gradient was maintained by constant replenishment of the NGF source. Thus, the growth cone was exposed to a fresh supply of NGF over the course of the culture period. This repeated removal and replenishment of NGF may have increased the efficacy of the growth factor by presenting the cell with a constant NGF supply at the growth cone. In the case of the immobilized NGF concentration gradient system, the growth cone must expand its membrane and "reach" for the next bound factor.[50] If the next growth factor was too far away, the cell would not recognize it and may not be guided. By increasing the slope (and thus the surface density) of the NGF concentration gradient, the cell could sense more growth factor within its "reach," and expand its membrane along the direction of the gradient.

The phenomenon of thickened axons that was observed here was also previously observed for PC12 cells cultured with NGF-bound beads.[21] It was hypothesized that, after the extended neurite made contact with the NGF bound bead, the local active receptor caused calcium influx and organelle recruitment at the site of contact with the bead. It has also been reported that the NGF high-affinity TrkA receptor increases F-actin polymerization when activated.[47] The system reported here is different from the others because the entire cell is in contact with the immobilized NGF, and thus the thickening is found over the entire neurite. Our results, together with those of others, may begin to explain the hypothesis that neurite thickening is caused by constitutively active TrkA receptors that, in turn, cause excessive F-actin polymerization, Ca^{2+} influx, and organelle recruitment.

CONCLUSIONS

We have shown that stable, bioactive concentrations and concentration gradients of NGF can be immobilized within p(HEMA) gels. By modifying these p(HEMA)-NGF gels with pAA, PC12 cells adhere and respond to the immobilized NGF by extending neurites in a manner similar to that which is observed
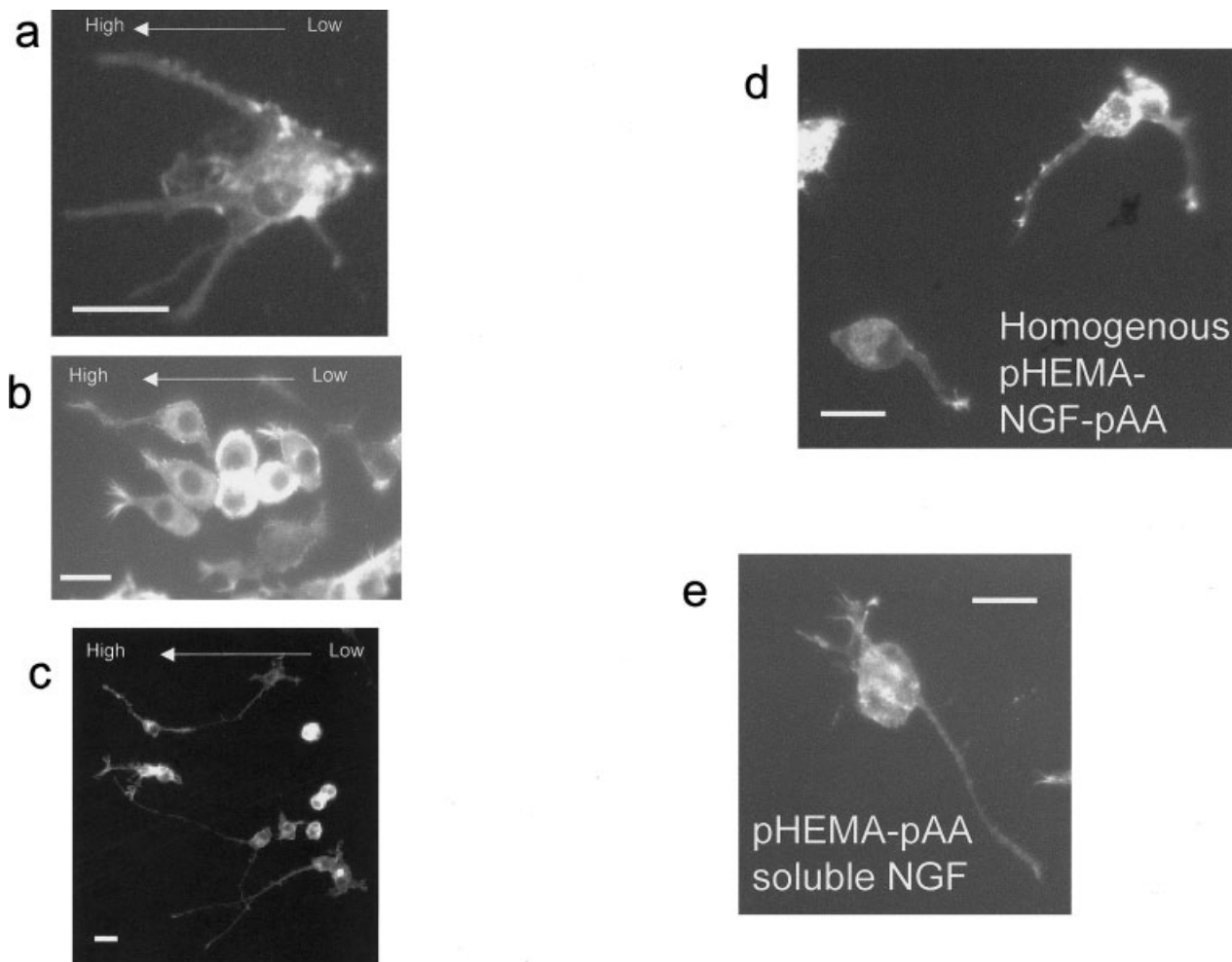
Figure 5. Representative fluorescent optical microscope images of primed PC12 cells extending neurites on (a), (b), and (c) p(HEMA)-NGF-pAA immobilized gradient gels versus (d) p(HEMA)-NGF-pAA immobilized homogeneous gels versus (e) p(HEMA)-pAA in the presence of soluble NGF. Note that directionality is observed in (a) and (b) and (c) versus (d) and (e). Thickened neurites are observed in cells cultured on immobilized NGF substrates, shown in panels (a), (b), (c), and (d) as compared to those growing on similar substrates with soluble NGF, as shown in (e). Note that the scalebars represent 20 μm.

with soluble NGF. PC12 cell neurites were observed to be thicker when cultured on immobilized NGF than when cultured in the presence of soluble NGF, likely reflecting different cellular mechanisms for stimulation and guidance.

The gradient methodology described herein is powerful in that it allows many growth factors and growth factor concentration gradients to be tested for their guidance potential with a multitude of cell types. Furthermore, using these well-defined systems, the receptor dynamics governing directionality and guidance may be more fully understood. In ongoing research, we aim to translate the gradient created in p(HEMA) microporous gels to gradients in cell-invasive or macroporous p(HEMA) scaffolds. Ultimately, we intend to test these scaffolds for guidance *in vitro* and *in vivo* for guided nerve tissue regeneration after injury in either the peripheral nerve or spinal cord.

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