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Chemically-bound nerve growth factor for neural tissue engineering applications

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Abstract—In order to promote regeneration after spinal cord injury, growth factors have been applied *in vivo* to rescue ailing neurons and provide a path finding signal for regenerating neurites. We previously demonstrated that soluble growth factor concentration gradients can guide axons over long distances, but this model is inherently limited to *in vitro* applications. To translate the use of growth factor gradients to an implantible device for *in vivo* studies, we developed a photochemical method to bind nerve growth factor (NGF) to microporous poly(2-hydroxyethylmethacrylate) (PHEMA) gels and tested bioactivity *in vitro*. A cell adhesive photoreactive poly(allylamine) (PAA) was synthesized and characterized. This photoreactive PAA was applied to the surface of the PHEMA gels to provide both a cell adhesive layer and a photoreactive handle for further NGF immobilization. Using a direct ELISA technique, the amount of NGF immobilized on the surface of PHEMA after UV exposure was determined to be 5.65 ± 0.82 ng/cm^2 or 3.4% of the originally applied NGF. A cell-based assay was performed to determine the bioactivity of the immobilized NGF. Using pheochromocytoma (PC-12) cells, 30 ± 7% of the cell population responded to bound NGF, a response statistically similar to that of cells cultured on collagen in the presence of 40 ng/ml soluble NGF of 39 ± 12%. These results demonstrate that PHEMA with photochemically bound NGF is bioactive. This photochemical technique may be useful to spatially control the amount of NGF bound to PHEMA using light and thus build a stable concentration gradient.

Key words: Neurotrophins; bound NGF; PC12; pHEMA; gradient; spinal cord injury.

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INTRODUCTION

Spinal cord injury is a devastating event that affects patients physically, emotionally and psychologically. Thus spinal cord injury affects quality of life and productivity [1]. Initial injury is usually caused by either transection or compression of the spinal cord by one of the vertebrae. A secondary cascade of events follows which includes apoptosis and denervation at the main site of injury and can often expand superior and inferior to the injury site, causing a cyst or fluid filled cavity to develop and grow.

Scientists have applied soluble growth factors to arrest the injury and to entice regeneration through the wound site. Using mini-pump technology, growth factors were delivered to injured tissues to promote neurite re-innervation [2]. While providing insight into the guidance potential of a growth factor source for re-innervation, the mini-pump system is inherently limited to providing a soluble growth factor source that must be constantly replenished. In well-defined diffusion chambers, we demonstrated that there is a minimum concentration gradient of growth factors required to guide neurites over a maximum distance [3]. This model system provided us with greater insight into the importance of a stable gradient for guidance, but it too is limited by the use of soluble factors. In an attempt to translate the model system to a device that can be tested *in vivo*, we wanted to immobilize a growth factor concentration gradient to a scaffold that could be implanted.

We hypothesized that a stable, immobilized gradient would be able to guide neurites similarly to the soluble gradient because surface bound growth factors have repeatedly been shown to be biomimetic [4–8]. For example, during embryogenesis the central nervous system develops, in part, through patterned proteins and growth factors secreted by target cells. These proteins are often altered chemically after secretion through protease activity, which increases their affinity for the extracellular matrix and thus binds them to the substrate. For example, in the case of the sonic hedgehog protein (SHH) diffusible and bound gradients of SHH induce differentiation of cells in the ventral neural tube [9]. Depending on the concentration of SHH encountered by the cell in its local environment, it could differentiate into an interneuron, motor neuron or floor plate cell. In this example and others, the position of the SHH protein is strategically located along the tube so as to ensure that the cells differentiate properly during embryonic development. If the local concentration is too high or too low, mutations may develop such as spina bifida or limb deformities. Surface-bound stimulatory molecules are also important after injury in the peripheral nervous system, where regenerating neurites often locate their original innervation site by following surface-bound adhesive molecules from the degenerated distal stump [9]. Injured Schwann cells secrete growth factors that will also entice regenerating neurites to re-innervate the target through a concentration gradient mechanism.

In addition to examples of chemically tethered stimulatory molecules during embryogenesis, there are many examples of photochemically-bound growth factors in the literature [10]. Ito *et al.* have bound growth factor proteins to two-dimensional

solid substrates to mimic juxtacrine stimulation. These studies demonstrated that substrate bound and soluble growth factors can have similar effects. Further, the authors asserted that bound growth factors may be more potent due to long term receptor stimulation as in the example of bound epidermal growth factor (EGF). The authors demonstrated that the growth of mouse fibroblast and Chinese hamster ovary cells were enhanced when cultured on substrate-bound EGF. In other studies, Ito et al demonstrate that patterned NGF bound to polystyrene elicited neurite outgrowth in PC12 cells and further restricted the neurites to only those areas patterned with NGF [11].

Considering the biological phenomenon of surface-bound growth factor gradients for target innervation during embryogenesis and the substrate-bound growth factor studies, an implantible, bound gradient of NGF may be useful to direct neurite outgrowth. This in turn may increase regeneration in neural tissue engineering applications.

Prior to testing a gradient of immobilized growth factors for guidance, first we had to develop a method to immobilize bioactive growth factors. To this end, we developed a method to photochemically bind nerve growth factor (NGF) to a poly(2-hydroxyethyl methacrylate) (PHEMA) gel [12], building on previous photochemical reactions [13]. PHEMA-modified gels were characterized for both the amount of NGF immobilized using ELISA [14, 15] and bioactivity of the bound NGF using a cell-based assay. To bind NGF to PHEMA surfaces, azido-modified poly(allylamine) (PAA-azido) was used because it provided a reactive handle for NGF photochemical immobilization while at the same time rendering PHEMA gels cell-adhesive.

The physical characteristics of PHEMA gel formulations (including that described herein) have been extensively studied previously [16–19]. The formulation was constant throughout the entire study, therefore the physical properties of the PHEMA sponge were not studied as a variable.

MATERIALS AND METHODS

Materials

All chemicals for PHEMA and PAA preparation were purchased from Sigma-Aldrich (Oakville, ON, Canada) and used as received unless otherwise specified. Water was distilled and deionized using Millipore Milli-RO 10 Plus at 18 MΩ. 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, ON, Canada). Direct ELISA and standard ELISA reagents were purchased as a kit from Chemicon International (Temecula, CA, USA). The adrenal rat PC12 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). All reagents for culture media, media additives, stain reagents and Hanks' balanced salt solution (HBSS) were purchased from

Invitrogen (Burlington, ON, Canada). Filamentous actin stain labeled with FITC was purchased from Molecular Probes (Eugene, OR, USA).

Preparation of purified photoactive poly(allylamine) (PAA-azido)

Photoreactive PAA was prepared using a procedure published elsewhere [11] with modifications. Briefly, 15.79 mg PAA (molecular mass 70 kDa) was dissolved in 2.5 ml of HBSS, which was then added to 5 ml of dimethylformamide containing 7.34 mg of 6-(4-azido-2-nitrophenylamino)hexanoic acid *N*-hydroxysuccinimide ester (NHS azido). The mixture was stirred and allowed to react for 3 days at 4°C. To remove any unreacted NHS azido from the mixture, 1 ml of the mixture and 1 ml of HBSS were ultrafiltered using a 10 kDa molecular weight ultrafiltration device (Millipore, Etobicoke, ON, Canada). The mixture was washed four times with HBSS to remove all unreacted NHS azido. The filtrate was monitored at 467 nm at each rinsing step using a UV-VIS spectrophotometer to determine when all the NHS azido had been removed from the retentate. The number of primary amines of PAA that were modified with the NHS azido was determined by calculating the amount of free NHS-azido collected in the filtrate. The final concentration of PAA-azido after purification was determined using the ninhydrin reaction described below.

Characterization of PAA-azido

Ninhydrin reacts quantitatively with primary amines on poly(allylamine) (and not with amine groups of NHS-azido) and absorbs at 570 nm after conjugation [20]. From the 367 μl of purified PAA-azido synthesized, 50 μl was added to 1 ml of a 4 mg/ml aqueous ninhydrin stock solution and boiled for 30 s. The resulting solution was cooled to room temperature and the absorbance measured at 570 nm. To determine the concentration of PAA-azido lost in the filter during purification, the concentration of PAA-azido was calculated by comparison to a calibration curve, which was prepared using the unfiltered pAA-azido mixture. Since the amount of pAA added to the reaction mixture was known and the efficiency of reaction between pAA and NHS azido has been shown to be high [21], we assumed that there were very few un-reacted pAA molecules. The calibration curve was prepared by adding 1 ml of a 4 mg/ml aqueous ninhydrin solution to 5 vials, then the unfiltered PAA-azido NHS azido mixture was serially diluted 5 times, and finally each dilution was added to one of the ninhydrin-filled vials for use in the calibration curve. All the vials were boiled for 30 s and cooled to room temperature before measuring the UV absorbance at 570 nm. The resulting solutions turned deep purple.

Preparation of PHEMA microporous gels

PHEMA microporous gels were prepared by polymerizing 2-hydroxyethyl methacrylate (HEMA) in the presence of excess water, with ethylene dimethacrylate (EDMA) as the crosslinker and ammonium persulfate (APS)/tetramethylethylenediamine (TEMED) as the redox initiating system. Specifically, PHEMA hydrogels

were made by mixing 35 wt% HEMA, 65 wt% water, 0.1 wt% EDMA, 0.07 wt% APS and 0.35 wt% TEMED, with the latter three percentages being relative to the monomer (w/w). The formulation was added by pipette into a glass mold, which consisted of two glass plates separated by a rubber tubing spacer. The formulation polymerized overnight and resulted in a 2-mm-thick opaque white gel, which was rinsed in water overnight to remove any unreacted molecules. The gels were then cut using a hollow die punch with a diameter of 0.5 cm and placed into the bottom of a 96-well plate.

Immobilization of PAA and NGF on PHEMA microporous gels (PHEMA-PAA-NGF)

PAA-azido was photo-immobilized on PHEMA disks according to Fig. 1. As described: (1) 3 μl of PAA-azido (13.36 mg/ml in water) was added by pipette on top of each PHEMA disk and then dried under vacuum for approx. 15 min. This resulted in an orange layer on the surface of the dehydrated PHEMA disk; (2) 3 μl of 50 μg/ml NGF in water was applied on top of each gel and dried under vacuum; (3) each dehydrated disk was exposed to a metal halide UV light source (about 23 400 mW/cm^2) for 1 s at a working distance of 1 cm (using an exfo spot curing system). The surfaces turned dark brown indicating the reaction was complete. The disks were then rinsed with water overnight to remove any unreacted NGF and PAA-azido. The rinse buffer was collected to determine the amount of NGF released from

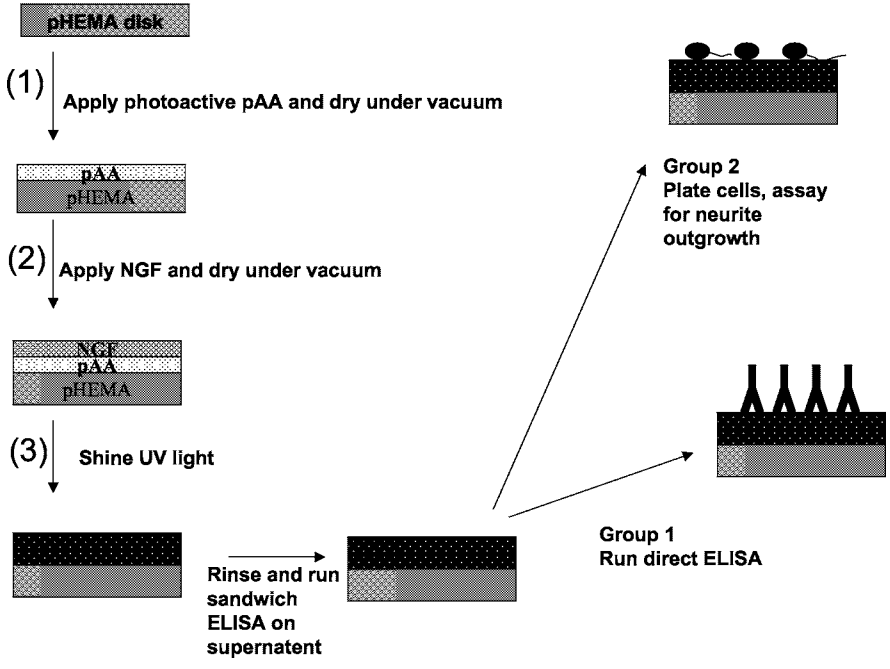


Figure 1. Experimental design for photochemically immobilizing NGF on p(HEMA).

the surface (see below) and the disks were separated into two groups. Group 1 was assayed for the concentration of NGF on the surface by ELISA, while group 2 was cultured with cells to determine the bioactivity of bound NGF. Prior to cell culture, disks were disinfected by immersion in 70% ethanol, rinsed in sterile HBSS and then placed in a 96-well plate.

Determination of surface-bound NGF on PHEMA-PAA-NGF using direct and standard ELISA

PHEMA-PAA-NGF disks were analyzed by ELISA to determine the concentration of NGF bound to the surface. Prior to characterizing the amount of bound NGF, the disks were rinsed over 3 days with 0.1 mg/ml BSA in HBSS to remove any non-bonded NGF. Buffer from each rinse step (100 μl) was assayed using a standard ELISA kit to determine the amount of non-bonded NGF. Next, the disks were placed into standard 96-well plates and the primary antibody against NGF was incubated with the disks for 2 h. Disks were rinsed four times with rinsing buffer and then the secondary IgG conjugated with horseradish peroxidase (HRP) was added to each dish and incubated for 2 h. The disks were rinsed again 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 concentration of NGF in the rinsing buffer was determined by comparison to the standards and methods outlined in the ELISA kit. The concentration of NGF on the surface of the gel was determined from a calibration curve also prepared by the standards and methods outlined in the ELISA kit and normalized to surface area.

Cell culture on PHEMA-PAA-NGF disks

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 sub-cultured once every week. Cells were removed from the culture flasks by adding fresh culture media over the cell sheet with a pipette and striking the side of the flask. Cells were then counted using a hemocytometer, diluted with the appropriate amount of cell culture media and plated on all samples (and controls) at 7×10^4 cells/disk. Tissue culture wells coated with collagen ($n = 9$) served as controls. For positive controls, soluble NGF was added to the complete media ($n = 3$) at a concentration of 40 ng/ml. For negative controls, cells were incubated ($n = 3$) with complete

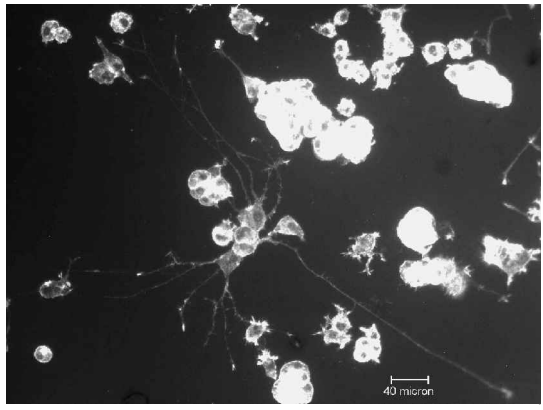
media only. For pre-cultured media controls ($n = 3$), PC12 cells were grown on collagen coated wells and incubated with media taken from the bound PHEMA-PAA-NGF disks. To test for the activity of adsorbed NGF, PC12 cells were cultured on disks prepared identically to PHEMA-PAA-NGF, except without UV irradiation ($n = 3$). All conditions were assayed after 3 days in culture for neurite length.

Cell assay for neurite length and percent of cells responding

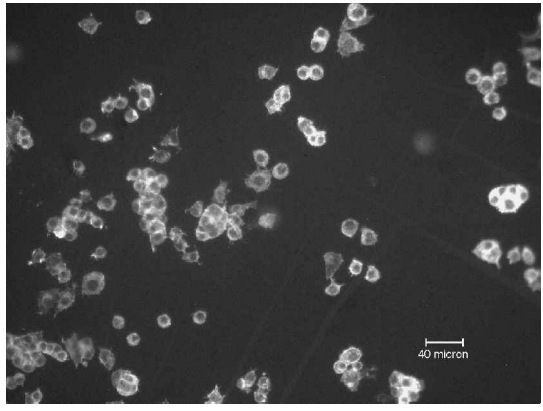
After the 3-day culture period, each disk was rinsed with HBSS to remove the media and serum proteins. The cells were fixed by incubating them in 3.7% formaldehyde for 10 min. To allow the stain to enter the cells, the samples were rinsed again in HBSS and then incubated in 0.1% Triton X-100 for 90 s. Finally, the cells were rinsed with HBSS and incubated for 30 min with 5 μl/ml FITC actin phallotoxin. After staining the cells, the control wells were visualized directly with a Zeiss Axiovert 100 inverted fluorescence microscope using FITC excitation and emission filters and a mercury lamp. Images were captured using a digital camera from Diagnostic Instruments running SPOT software using no binning. PHEMA disks were inverted and imaged upside down as they are opaque. At least 100 cells from each condition were imaged and each condition was analyzed in triplicate to obtain statistically significant data. The SPOT software was calibrated with a stage micrometer. Each cell was counted, and if present, the longest neurite was measured. A cell bearing a neurite longer than 20 μm (greater than 1 cell diameter) was considered to be responding. The average number of percent responding cells/well and the standard error of the triplicates in each group were calculated. The groups were compared for statistical significance using Student's t-test at 95% confidence intervals.

RESULTS AND DISCUSSION

The aim of this study was to develop a method to reproducibly bind NGF to PHEMA gels and to demonstrate the stability and bioactivity of bound NGF. While our ultimate goal is to immobilize a stable concentration gradient of growth factors that will promote axonal guidance and regeneration after injury to the spinal cord, we first had to develop a method to immobilize bioactive NGF. We chose to work with PHEMA because its mechanical properties can be adjusted to meet those of the spinal cord and it has been widely used in biomedical applications due to its ease of fabrication [19]. To render PHEMA cell adhesive, we applied a photoactive poly(allylamine) and then used that layer to covalently bind NGF. This system allowed us to create a cell adhesive surface with a stable concentration of NGF, which builds on our previous research of binding ovalbumin to agarose [22].



(A)



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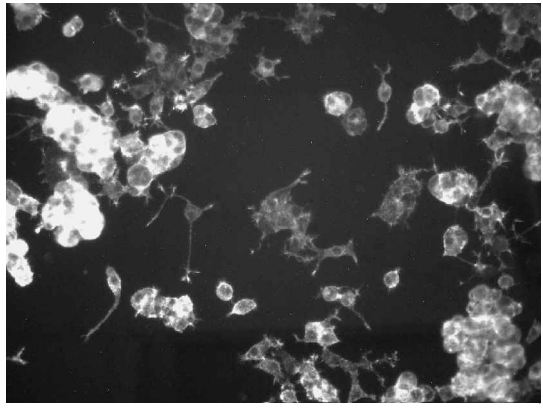

(C)

Figure 2. Representative images from the PC12 cell assay used to determine if photochemically-bound NGF was bioactive. (A) PC12 cells cultured on photochemically bound NGF (PHEMA-PAA-NGF sample); (B) PC12 cells cultured on collagen without NGF in the medium (negative control); (C) PC12 cells cultured on collagen with NGF added to the medium (positive control).

Characterization of PAA-azido

Photoactive PAA was synthesized by covalently binding azido to the primary PAA amine groups using NHS as the coupling agent. Two methods were used to quantify this reaction: (1) To determine the amount of azido groups that reacted with PAA, PAA-azido was repeatedly ultrafiltered until no NHS-azido was detected by UV-VIS absorbance at 467 nm. By comparison to a calibration curve for NHS-azido ($y = 13.6x + 0.002$, $R^2 = 0.99$), the amount of NHS-azido in the filtrate was calculated. The amount of NHS-azido in the filtrate decreased from approx. 0.04 mg after the first rinse to below the detection limit of the UV-vis spectrophotometer after the fourth rinse, which was considered sufficient to remove most of the unreacted NHS-azido (data not shown). A total of 0.05 mg of NHS-azido was recovered in the filtrate, which, by subtraction, indicated that 83 azido groups had reacted with PAA. Thus of the about 1228 primary amine groups of PAA available, 83 or 6.8% were modified with azido, leaving the remaining primary amines available for cell interaction. (2) To determine the concentration of PAA-azido (and the percent loss due to filtration), PAA-azido was assayed with ninhydrin, which colorimetrically labels primary amine groups. By comparison to a calibration curve, 0.15 mg, or 7% of PAA-azido was lost during purification. The calibration curve data was fitted with the line $y = 0.55x + 0.02$, $R^2 = 0.96$.

Determination of surface-bound NGF using direct and standard sandwich ELISA

To determine the stability of the bound NGF, the supernatant (or rinsing solution) was assayed for NGF by standard ELISA. By comparison to a calibration curve, ($R^2 = 0.97$) the amount of NGF in the supernatant rinse was determined. We found an average and standard deviation of 3 measurements of 27.65 ± 4.24 ng of NGF in the first rinse. Over the next 3 days, 1.5 ± 0.06 ng/ml was detected daily, indicating that an insignificant amount of NGF was released from the surface after the initial rinse. NGF at this concentration elicits minimal (if any) neurite outgrowth.

A direct ELISA technique was used to quantify the amount of NGF bound to the surface of the gel [23]. By comparison to a calibration curve we report an average and standard deviation of 3 measurements of 5.07 ± 0.82 ng or 3.4% of the applied NGF was bound to the surface. This translates to a surface coverage of approx. 25 ng/cm^2 on each pHEMA disk (assuming the NGF was mostly bound to the surface of the disks).

Overall, we were only able to account for 30 ng, of the 150 ng of NGF applied, by ELISA. There are several reasons that may explain the loss of NGF: (1) The UV light used to immobilize NGF is very intense and may have damaged NGF, causing a lack of recognition by the monoclonal primary antibody used in the ELISA assay. (2) To increase the efficiency of the photochemical reaction, NGF was dried onto the PHEMA surface and this may have compromised the protein's ability to develop H-bonding interactions and thus adopt the appropriate 3-dimensional conformation for recognition by the primary antibody used in the ELISA assay.

(3) The photochemical reaction may bind the active site of NGF itself, decreasing the amount detected by ELISA [24]. (4) Although NGF was collected in 0.1 mg/ml BSA and BSA modified plasticware, some NGF may have been lost to adsorption.

Cell assay for neurite length and percent of cells responding

PC-12 cells were cultured on PHEMA-PAA-NGF surfaces to determine the bioactivity of bound NGF. The cells were cultured for 3 days on these disks and stained with a fluorescent tag to visualize the neurites. Representative images are shown in Fig. 2. The PC-12 cell neurites were quantified, as summarized in Fig. 3 where a cell with a neurite longer than one cell diameter (20 μm) was considered to be responding.

The same number of cells responded to bound PHEMA-PAA-NGF disks as responded to positive controls, where soluble NGF was added to the medium of PC12 cells cultured on collagen. To ensure that the cells were not responding to NGF de-bonding from the surface, the media from bound PHEMA-PAA-NGF disks was removed and used to culture fresh cells on PHEMA-PAA surfaces. The average number of cells responding to pre-conditioned medium was statistically the same as those responding to the negative control at $P < 0.05$. In contrast, a statistically significant difference was calculated for the number of cells responding to PHEMA-PAA-NGF vs. pre-conditioned medium from PHEMA-PAA-NGF plated on fresh cells cultured on PHEMA-PAA disks at $P < 0.05$. Interestingly, when NGF is adsorbed to a PHEMA surface but not irradiated, there is minimal neurite

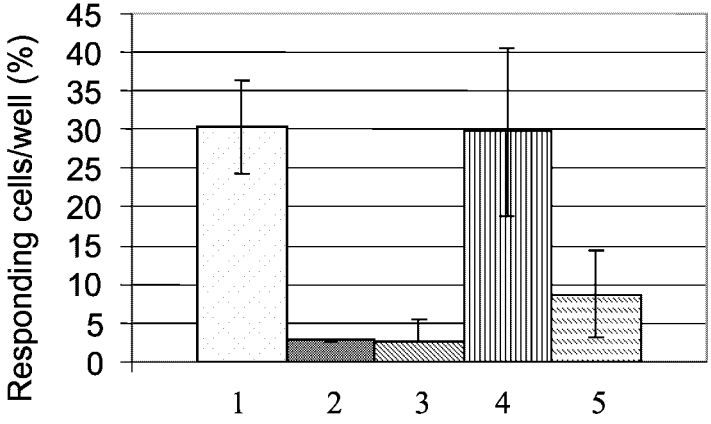

Figure 3. The percentage of responding PC12 cells when cultured on: (1) bound PHEMA-PAA-NGF samples; (2) collagen without NGF (negative control); (3) PHEMA-PAA with adsorbed NGF (i.e. not irradiated, control for bound *versus* adsorbed NGF); (4) collagen with NGF added to the medium (positive control); (5) PHEMA-PAA using pre-conditioned media from PHEMA-PAA-NGF (control for the stability of bound NGF). Statistical significance was determined by Student's t-test at $P < 0.05$. The mean and standard deviation are reported ($n = 3$). Conditions 1 and 4 were statistically significantly different from conditions 2, 3 and 5. This data demonstrates that the NGF is both bound and bioactive on PHEMA-PAA-NGF samples.

outgrowth. This result indicates that the NGF on the PHEMA-PAA-NGF disks is bound to the surface by irradiation and not by adsorption phenomena.

CONCLUSIONS

Using the photochemical method described herein, NGF was immobilized on PHEMA-PAA disks. Bound NGF was stable and shown to be bioactive using a PC-12 cell assay. We postulate that light can be used to vary the amount of bound NGF and thus covalently bound gradients of growth factors can be created for axonal guidance. For example, if we assume that light exposure modulates the concentration of NGF bound to the surface, then irradiating the surfaces through a chrome on quartz gradient mask may lead to surface-bound NGF gradients. We demonstrated that chemically-bound NGF induces neurite outgrowth on three dimensional surfaces and we hypothesize that similar methods can be used to create well-controlled gradients to guide neurites over long distances for neural tissue engineering applications.

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REFERENCES

1. M. Ramer, G. Harper and E. Bradbury, *Spinal Cord* **38**, 449 (2000).
2. M. Zheng and D. Kuffler, *J. Neurobiol.* **42**, 212 (2000).
3. X. Cao and M. Shoichet, *Neuroscience* **103**, 831 (2001).
4. R. Shirasaki, M. C. M. Tessier-Lavigne and F. Murakami, *Neuron* **17**, 1079 (1996).
5. T. Serafini, S. Colamarino, E. Leonardo, H. Wang, R. Beddington, W. Skarnes and M. Tessier-Lavigne, *Cell* **87**, 1001 (1996).
6. S. Retaux, L. McNeill and W. Harris, *Neuron* **16**, 63 (1996).
7. M. Matise, M. Lusting, T. Sakurai, M. Grumet and A. Joyner, *Development* **126**, 3649 (1999).
8. E. Leonardo, L. Hinck, M. Masu, K. Keino-Masu, A. Fazeli, E. Stoeckli, S. Ackerman, R. Weinberg and M. Tessier-Lavigne, *Cold Spring Harbor Symposium Quant. Biol.* **62**, 467 (1997).
9. T. M. Jessell and J. R. Sanes, in: *Principles of Neural Science*, E. Kandel, J. Schwartz and T. Jessell (Eds). McGraw-Hill, New York, NY (2000).
10. Y. Ito, *Mater. Sci. Eng. C* **6**, 267 (1998).
11. Y. Ito, *Jpn. J. Artif. Organs* **27**, 541 (1998).
12. X. Cao and M. Shoichet, *Neuroscience* (2003) (submitted).
13. Y. Ito, M. Hayashi and Y. Imanishi, *J. Biomater. Sci. Polymer Edn* **12**, 367 (2001).
14. T. Horbett and M. Schway, *J. Biomed. Mater. Res.* **22**, 763 (1988).
15. C. Zettler, *J. Neurosci. Res.* **46**, 581 (1996).

16. T. Chirila, Y.-C. Chen, B. Griffin and I. Constable, *Polymer Int.* **32**, 221 (1993).
17. N. Clayton, T. Chirila and P. Dalton, *Polymer Int.* **42**, 45 (1997).
18. A. Clayton, T. Chirila and X. Lou, *Polymer Int.* **44**, 201 (1997).
19. X. Lou, P. Dalton and T. Chirila, *J. Mater. Sci. Mater. Med.* **11**, 319 (2000).
20. E. Curotto and F. Aros, *Anal. Biochem.* **211**, 240 (1993).
21. Y. Ito, in: *Fifth Foresight Conference on Molecular Nanotechnology*, Palo Alto, CA (1997).
22. X. Cao and M. Shoichet, *J. Biomater. Sci. Polymer Edn* **13**, 623 (2002).
23. P. P. Leung, M. Khadre, T. H. Shellhammer and A. E. Yousef, *Lett. Appl. Microbiol.* **34**, 199 (2002).
24. A. Kruttgen, J. Heymach, P. Kahle and E. Shooter, *J. Biol. Chem.* **272**, 29222 (1997).