Student Research Award in the Master's Degree Category, World Biomaterials Congress 2000, Kamuela, HI, May 15–20, 2000

Patterned poly(chlorotrifluoroethylene) guides primary nerve cell adhesion and neurite outgrowth

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Received 15 September 1999; revised 15 December 1999; accepted 30 December 1999

Abstract: Central nervous system (CNS) neurons, unlike those of the peripheral nervous system, do not spontaneously regenerate following injury. Recently it has been shown that in the developing CNS, a combination of cell-adhesive and cell-repulsive cues guide growing axons to their targets. We hypothesized that by mimicking these guidance signals, we could guide nerve cell adhesion and neurite outgrowth *in vitro.* Our objective was to direct primary nerve cell adhesion and neurite outgrowth on poly(chlorotrifluoroethylene) (PCTFE) surfaces by incorporating alternating patterns of cell-adhesive (peptide) and nonadhesive (polyethylene glycol; PEG) regions. PCTFE was surface-modified with lithium PEG-alkoxide, demonstrating the first report of metal–halogen exchange with an alkoxide and PCTFE. Titanium and then gold were sputtered onto PEG-modified films, using a shadow-masking technique that creates alternating patterns on the micrometer scale. PCTFE-Au regions then were modified with one of two cysteine-terminated laminin-derived peptides, C-GYIGSR or C-SIKVAV. Hippocampal neuron cell–surface interactions on homogeneously modified surfaces showed that neuron adhesion was decreased significantly on PEG-modified surfaces and was increased significantly on peptide-modified surfaces. Cell adhesion was greatest on CGYIGSR surfaces while neurite length was greatest on CSIKVAV surfaces and PLL/laminin positive controls, indicating the promise of peptides for enhanced cellular interactions. On patterned surfaces, hippocampal neurons adhered and extended neurites preferentially on peptide regions. By incorporating PEG and peptide molecules on the surface, we were able to simultaneously mimic cell-repulsive and cell-adhesive cues, respectively, and maintain the biopatterning of primary CNS neurons for over 1 week in culture. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 50, 465–474, 2000.

Key words: surface modification; nerve regeneration; poly(ethylene glycol); laminin-derived peptides; central nervous system

INTRODUCTION

Injuries associated with the central nervous system (CNS), such as spinal cord injury, often lead to permanent loss of sensory, motor, and reflex functions. These severe consequences result from the fact that CNS neurons, unlike most other cells in the body, lack the ability to spontaneously regenerate following injury. Several researchers have shown that the peripheral nervous system (PNS) and the embryonic CNS represent growth-promoting microenvironments for CNS regeneration.[1,2] Recently it has been shown that the adult CNS environment that lies beyond the inhibitory lesion area is capable of supporting regeneration.[3] Thus CNS regeneration should be possible if the axons are presented with a growth-permissive environment at the lesion site.

In the developing CNS, both diffusible and contact-mediated mechanisms are believed to guide axons toward their appropriate targets.[4] Several extracellular matrix molecules possessing either attracting or repulsing characteristics have been identified as contact-mediated cues.[5,6] By reproducing the effects of these

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Contract grant sponsor: Natural Science and Engineering Research Council of Canada
Contract grant sponsor: The Whitaker Foundation

cues on artificial substrates, we aim to guide axonal growth *in vitro.* Specifically, our objective was to direct nerve cell adhesion and neurite outgrowth *in vitro* by mimicking the effects of both cell-repulsive and cell-adhesive cues on 2-D polymeric surfaces using patterning techniques. Ultimately, we aim to extend our 2-D patterns to 3-D structures for guided nerve regeneration *in vivo.*

Patterning techniques[7–10] have been widely used for the spatial control of cells (biopatterning) in culture. In nerve regeneration research, patterning techniques have been used by us[11] and by others[12–14] to control nerve cell–surface interactions and to guide neuronal processes in a desired direction. In some studies, where patterned surfaces had some regions that were more adhesive than others,[12,15] the cells preferentially adhered to the more adhesive regions; however, biopatterning was achieved only in the presence of serum reflecting nonspecific cell–surface interactions. In other studies, where patterned surfaces had both adhesive and nonadhesive regions, cell–surface interactions were studied in a defined, serum-free medium.[13,14]

We previously demonstrated cellular patterning on glass surfaces,[11] yet the biopatterning was limited to a few days, probably due to limited surface coverage of the modified surfaces. In order to prepare well defined surfaces for cell patterning, we chose to work with poly(chlorotrifluoroethylene) (PCTFE), which has been used to culture a variety of cell types, including nerve cells.[16] Unlike perfluorinated fluoropolymers, such as poly(tetrafluoroethylene), PCTFE has a chlorine group on each repeat unit that can be used to accomplish specific chemistry.

In an attempt to mimic the cell-repulsive and cell-adhesive cues found *in vivo,* we modified PCTFE surfaces with alternating regions of nonadhesive polyethylene glycol (PEG) and cell-adhesive peptides. PEG-modified surfaces have been shown to exhibit low degrees of protein adsorption and cell adhesion.[17–19] The nonadhesive regions were prepared by reacting PCTFE films with lithium PEG-alkoxide, yielding PCTFE-PEG surfaces. These surfaces then were patterned using a shadow-masking technique in which gold is sputter-coated onto surfaces through micrometer-scale grids. The adhesive regions were prepared by modifying the gold regions with cysteine-terminated peptide sequences, C-GYIGSR and C-SIKVAV, which derive from the extracellular matrix protein laminin, and which have been shown to promote nerve cell adhesion and neurite outgrowth, respectively.[20–22] The interaction of embryonic day 18 (E18) hippocampal neurons with modified surfaces was studied because the hippocampal neurons represent a homogeneous and defined population of primary neurons. As will be shown, PEG was critical for the long-term biopatterning of cells on adhesive peptide surfaces.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Co. (Oakville, Ontario) unless otherwise specified. Water was distilled and deionized using Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, Massachusetts) at 18 MΩ of resistance, and all reactions were conducted at room temperature (RT). Aclar 33C (PCTFE) sheets (5 mL in thickness) were received from Allied Signal Inc. (Morristown, New Jersey). The sheets were cut into squares (1.5 ×1.5 cm) and extracted in refluxing dichloromethane for 2 h. They were dried under vacuum at RT for a minimum of 1 h before use.

All surfaces were characterized by measuring dynamic advancing and receding water contact angles using a telescopic goniometer (Ramé Hart, model 100-00, New Jersey). A total of three samples from each set of modified surfaces were analyzed, and five measurements were taken from each sample. All samples were characterized by X-ray photoelectron spectroscopy (XPS; Leybold LH Max 200) at take-off angles between the sample and detector of 15° and 90°. A MgKα source was used at an operating pressure of less than 10^{-8} Torr. Survey and low-resolution spectra were obtained for each surface, and angle-resolved XPS was performed to estimate the thickness and surface coverage of PEG, as previously described.[23] The thickness of the peptide-modified surface was estimated by ellipsometry (Rudolph Research AutoEL-II null reflection ellipsometer) at an operating wavelength of 632 nm and an incident angle of 70°. A total of three samples was analyzed, with six areas per sample measured. The index of refraction of the peptide overlayer was estimated to be 1.50, and the thickness was calculated using custom-built software. Iodine-125-radiolabeled tyrosine (Y) of CGYIGSR was quantified using a scintillation counter (LKB Wallac 1282-802 Universal γ-counter) with a 2×2 cm sodium iodide detector well of 80% efficiency.

Cell culture experiments were performed on both homogeneously modified surfaces—PCTFE, PEG, Au, CGYIGSR, CSIKVAV, and poly(L-lysine)/laminin positive control—and patterned surfaces—PCTFE/Au, PCTFE-PEG/Au, PCTFE/Au-peptide, and PCTFE-PEG/Au-peptide.

PEG modification (PCTFE-PEG)

All reactions were performed in an inert atmosphere. To a Schlenk flask containing a stirbar were added 20 mL of toluene, 10 mg of 1,10-phenanthroline,[24] and 1.5 mL of tert-butyllithium, yielding a dark red-colored solution with stirring. The solution was titrated[25] with a 0.3*M* solution of methoxy-terminated PEG-OH (750, 2000, or 5000 g/mol) in toluene until the solution became bright yellow in color. A slight excess of PEG was added to insure complete reaction with tert-butyllithium. The PEG-alkoxide solution then was transferred via cannula to a Schlenk tube containing PCTFE films. In some experiments, 5 mL of THF (Fisher, Nepean, Ontario) also were added to improve wetting of the films. The reaction was allowed to proceed for 1, 3, or 24 h under constant agitation. After the desired reaction time, the films were washed sequentially in toluene (once), methanol (three

times), water (three times), methanol (once), and dichloromethane (three times). They then were dried under vacuum overnight. Control experiments had PCTFE films immersed in PEG solution instead of PEG-alkoxide solution.

Gold patterning of PCTFE-PEG (PCTFE-Au)

PCTFE-PEG films were sputter-coated[26] under vacuum (1×10^{-4} Torr; 2400; Perkin Elmer, California) with titanium for 10 s, followed by gold for 150 s. The sputtering system was equipped with Argon gas at a flow rate of 12 sccm and a target bias of 1000 V (DC). Electron microscopy (EM) grids (Electron Microscopy Sciences, Pennsylvania) with bar sizes of 180 μm and hole sizes of 90 μm were used to create micrometer-scaled stripes on the films. The EM grids were positioned in the holes (2 mm) of a custom-made Teflon mask, and titanium and gold were sputtered through the mask onto PEG-modified films, creating 90-μm stripes of gold and 180-μm stripes of PEG.

Peptide modification of PCTFE-Au (PCTFE-peptide)

The peptides CGYIGSR and CSKIVAV (Procyon Biopharma, Ontario) were dissolved in a sodium acetate (BDH Inc., Toronto, Ontario)/acetic acid (ACP Chemicals Inc., Montreal, Quebec) pH 6-buffered solution at a concentration of 0.2 mg/mL. The gold-modified films were immersed in a 20-mL peptide solution for 24 h and then were rinsed with buffer solution and water (three times) before air-drying. As a control, PCTFE-PEG film samples were treated identically to PCTFE-Au film samples.

Peptide labeling and quantification

The tyrosine (Y) of CGYIGSR was radiolabeled with radioactive iodine (^{125}I) using sodium iodide, as previously described.[27] Briefly, 2 mg of CGYIGSR were dissolved in 5 mL of a pH 11 buffer solution containing 20 m*M* of sodium phosphate and 0.15*M* of sodium chloride. The solution was reacted with 0.07 mCi of carrier-free Na^{125}I (Amersham Life Science Inc., Arlington Heights, Illinois) in the presence of Iodobeads (Pierce, Rockford, Illinois) for 15 min. Free iodide ions were removed by successive passes of the peptide solution through columns packed with anion-exchange resin (Dowex 1-X8). Gold-modified films were immersed in labeled peptide solution, as described above, for 24 h and then were washed with buffer solution, followed by 10 m*M* of sodium iodide to desorb any trace amounts of ^{125}I before counting by scintillation.

Hippocampal cell culture experiments

All films were disinfected in sterile-filtered 70% ethanol (BDH Inc., Toronto, Ontario), rinsed with water, and air-dried for 1 h prior to cell plating.

E18 rat hippocampal cells (Southern Illinois University, School of Medicine, Springfield, Illinois) were dissociated from the tissue, as previously described.[28] Briefly, cells were dissociated from tissue by mechanical trituration and were suspended in a serum-free neurobasal medium (SFM, Gibco, New York) with B-27 supplement (2%, Gibco), chicken egg albumin (1 mg/mL), sodium pyruvate (0.1 mg/mL), glutamine (1%, Gibco), and penicillin–streptomycin (10,000 U/mL and 10,000 μg/mL, respectively). The cells were plated at concentrations of 2–4 $\times 10^4$ cells/cm^2 in 12-well plates (one film per plate) and were incubated at 37°C and 6% CO_2. After 3 h, the medium was removed and replaced with 1 mL of fresh SFM. The medium was replenished every 4 days. The positive control was prepared by coating the well surfaces of a 12-well plate with 1 mL of sterile 1 mg/mL poly(L-lysine) (PLL) solution for 24 h. The wells then were rinsed with sterile water and 1 mL of a 0.3-mg/mL laminin (mouse) solution was added to each well. The plate was incubated for 4 h, and the wells again were rinsed with water and left to air-dry prior to cell plating.

In order to test the specificity of cell–surface interactions on peptide-modified surfaces, a competitive cell adhesion assay was performed. Cells were pre-incubated for 30 min with SFM containing 0.1 mg/mL of soluble peptides (CGYIGSR and CSIKVAV) prior to cell plating. The cells then were plated in the SFM containing soluble peptides onto peptide-modified surfaces, and cell adhesion was quantified 1 day after cell plating.

Images from each surface were captured on days 1, 2, 4, and 7 after cell plating, using an inverted microscope (Axiovert S100, Zeiss, Germany) fitted with an analog video camera. Cell adhesion assays were performed on day 1 by counting the number of cells per field (25 mm^2) in ten random fields; averages and standard deviations are reported. Neurite outgrowth analysis was performed on day 2 by counting the number of neurites per cell of 50 random cells per surface; only neurites longer than the cell body length were counted and averaged. Using Northern Eclipse Software (version 5.0), the longest neurite per cell was measured for 50 random cells per surface on day 2.

RESULTS

PEG modification

PCTFE was surface-modified with PEG by reaction with lithium PEG-alkoxide. As summarized in Table I, both XPS (90° take-off angle) and contact angle measurements indicate PEG modification using PEG5000 for reaction times of 1, 3, and 24 h. As shown, PCTFE-PEG surfaces had greater percentages of carbon and oxygen accompanied by decreased percentages of both chlorine and fluorine. The outer layer attributed to PEG had carbon-to-oxygen ratios on the surface of approximately 2–2.5:1, which is in agreement with the expected 2:1 ratio for PEG. By XPS analysis, the percentage of oxygen and carbon on the surface had increased with increasing reaction time, suggesting in-

TABLE I
XPS and Dynamic Advancing (θ_A) and Receding (θ_R) Contact Angle Measurements for PEG5000-Modified PCTFE

Surface	Reaction Time	XPS Elemental Composition (90° Take-off Angle)	Contact Angle (θ_A/θ_R) (±1–3°)
PCTFE	—	$C_{32.0}F_{51.7}Cl_{16.0}$	93°/80°
PCTFE-PEG	1 h	$C_{34.5}F_{49.9}Cl_{15.0}O_{0.9}$	72°/63°
PCTFE-PEG	3 h	$C_{39.3}F_{44.0}Cl_{13.9}O_{2.8}$	72°/59°
PCTFE-PEG	24 h	$C_{40.2}F_{42.6}Cl_{13.8}O_{3.4}$	69°/55°
Control PCTFE	24 h	$C_{32.5}F_{50.9}Cl_{15.8}$	90°/78°

creased PEG surface modification. By contact angle analysis, the wettability of hydrophobic PCTFE (93°/80°) had increased with PEG modification (72°/63°). At longer reaction times the contact angles decreased, with a statistically significant decrease observed in the receding contact angle data after 24 h. Both XPS and contact angle data for control PCTFE film samples confirm chemical modification of PCTFE with PEG-alkoxide and no adsorption.

The PEG5000 experiments confirmed that it is possible to modify PCTFE with PEG using our protocol; however, there was a significant contribution from the underlying polymer in the XPS data that suggests low conversion and low surface coverage of PEG on PCTFE. In an attempt to create a more homogeneous surface by increasing PEG surface density, the 24-h modification was repeated with PEG2000 and PEG750. The XPS (15° take-off angle) and contact angle data for these surfaces are summarized in Table II. By decreasing the molecular weight of PEG, the surface concentration of oxygen increased from 4.3% for PEG5000 to 9.0% for PEG2000 to 9.2% for PEG750. The increase in oxygen was accompanied by an increase in carbon and decreases in both chlorine and fluorine. The results indicate that by decreasing PEG molecular weight, more PEG molecules are incorporated on PCTFE, likely resulting in increased surface coverage. The contact angle measurements showed significant decreases in θ_A and θ_R with decreasing PEG molecular weight from 5000 to 2000, but not from 2000 to 750. The XPS and contact-angle data indicate that the surface composition of PCTFE-PEG750 films are insignificantly different from PCTFE-PEG2000 films.

Angle-resolved XPS was used to estimate the thickness of the PEG modification on PCTFE. By analyzing PCTFE-PEG2000 films at four take-off angles (15°, 30°, 45°, and 90°) and using Andrade's single patchy overlayer model,[23] the thickness and surface coverage of PEG were estimated at 14 Å and 56%, respectively. It is important to note that the values obtained from XPS are representative of collapsed PEG molecules in the dry state; both surface coverage and thickness would be greater in aqueous systems where PEG molecules would be hydrated.

Peptide modification

Following gold deposition on PCTFE-PEG films, the composition of the underlying polymer no longer was detected, as evidenced by the absence of chlorine and fluorine in the XPS data (see Table III). The presence of carbon and oxygen on PCTFE-Au surfaces is due to the presence of adventitious carbon on the surface and accounts for the higher than expected contact angles (57°/48°). Others have shown that such weakly adsorbed carbon layers easily are desorbed by thiol modification.[29] Peptide modification of PCTFE-Au surfaces resulted in an increase in carbon and oxygen surface concentrations and the appearance of nitrogen, as shown in Table III. The contact angle data were consistent with the XPS results and decreased following peptide modification. The increased surface wettability was expected due to the hydrophilic nature of the peptides.

Ellipsometry was used to estimate the thickness of the peptide overlayer on gold. The thickness of CGYIGSR-modified films was estimated at 21 Å. Using radiolabeled tyrosine (Y) of CGYIGSR, the surface concentration of the peptide was found to be 18

TABLE II
XPS and Dynamic Advancing (θ_A) and Receding (θ_R) Contact Angle Measurements for PEG Modifications of PCTFE (PEG5000, 2000, and 750 g/mol)

Surface	Reaction Time	XPS Elemental Composition (15° Take-off Angle)	Contact Angle (θ_A/θ_R) (±1–3°)
PCTFE	—	$C_{31.3}F_{52.2}Cl_{16.0}$	93°/80°
PCTFE-PEG5000	24 h	$C_{42.1}F_{41.5}Cl_{12.1}O_{4.3}$	69°/55°
PCTFE-PEG2000	24 h	$C_{45.5}F_{34.7}Cl_{10.8}O_{9.0}$	61°/51°
PCTFE-PEG750	24 h	$C_{57.7}F_{25.4}Cl_{7.6}O_{9.2}$	61°/48°

TABLE III
XPS and Dynamic Advancing (θ_A) and Receding (θ_R) Contact Angle Measurements for the Peptide Modification of PCTFE-PEG-Au

Surface	XPS Elemental Composition (90° Take-off Angle)	Contact Angle (θ_A/θ_R) (±1–3°)
PCTFE-PEG-Au	$C_{19.5}O_{1.8}Au_{78.7}$	57°/48°
PCTFE-PEG-Au-CGYIGSR	$C_{44.8}O_{10.4}Au_{38.6}N_{6.0}$	45°/33°
PCTFE-PEG-Au-CSIKVAV	$C_{33.3}O_{5.4}Au_{58.4}N_{2.8}$	48°/31°
PCTFE-PEG (control)	$C_{44.8}F_{36.4}Cl_{11.7}O_{7.1}$	61°/50°

fmol/cm^2, which is similar to that found by others.[30] Since both peptide modifications are driven by a gold-thiol interaction, similar results were expected for CSIKVAV-modified films.

Hippocampal cell culture

Hippocampal neurons were plated on homogeneous surfaces and analyzed for cell adhesion and neurite outgrowth, as shown in figure 1. Unlike fluoropolymer surfaces, such as FEP,[31] PCTFE surfaces supported minimal nerve cell adhesion, which also has been observed by others.[16] Following PEG modification, the number of adherent cells decreased significantly ($p < 0.05$), confirming the utility of this surface for patterning. There was a dramatic increase in cell adhesion following peptide modification of gold. The CGYIGSR surface exhibited significantly more adherent cells than either CSIKVAV or PLL/laminin-positive control surfaces ($p < 0.05$).

The specificity of the hippocampal neuron–peptide interaction was tested with a competitive adhesion assay, as shown in Figure 2. Cell adhesion on both peptide-modified surfaces decreased significantly ($p < 0.05$) when cells were pre-incubated in medium containing soluble peptides. The results confirm that cell adhesion on CGYIGSR and CSIKVAV surfaces is predominantly receptor specific, as was found for other surfaces.[32]

To gain better insight into the cellular response to different surface chemistries, neurite outgrowth was quantified by counting the number of neurites per cell, as shown in Figure 3, and by measuring the longest neurite per cell, as shown in Figure 4, on day 2 after cell plating. There were more neurites per cell on peptide-modified surfaces than on unmodified PCTFE and significantly more neurites per cell on CGYIGSR surfaces than on CSIKVAV surfaces ($p < 0.05$). However, no significant differences were observed between either of the peptides and the PLL/laminin-positive control surface. Due to the limited number of adherent cells on PEG- and Au-modified surfaces, we were unable to quantify neurite extension and length by a statistically sound method and thus have not included these data in Figures 3 and 4.

As shown in Figure 4, significantly longer neurites were observed on peptide and PLL/laminin surfaces than on unmodified PCTFE surfaces ($p < 0.05$). While neurites on CSIKVAV were longer than those ob-

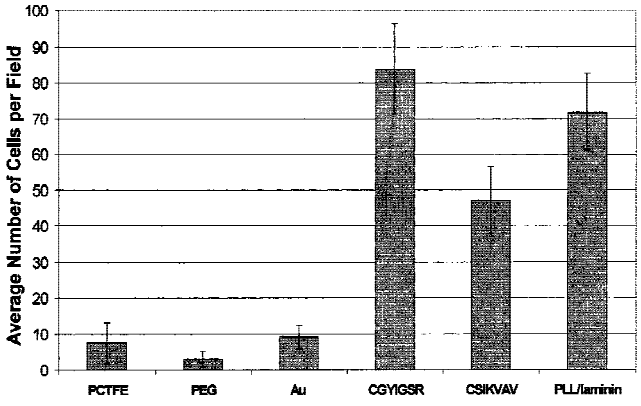

Figure 1. The cell adhesion assay correlated surface chemistry with the average number of cells/field (one field = 25 mm^2) for ten random fields per surface (±SD) on day 1 after plating (note that 4 ×10^4 cells/cm^2 were plated).

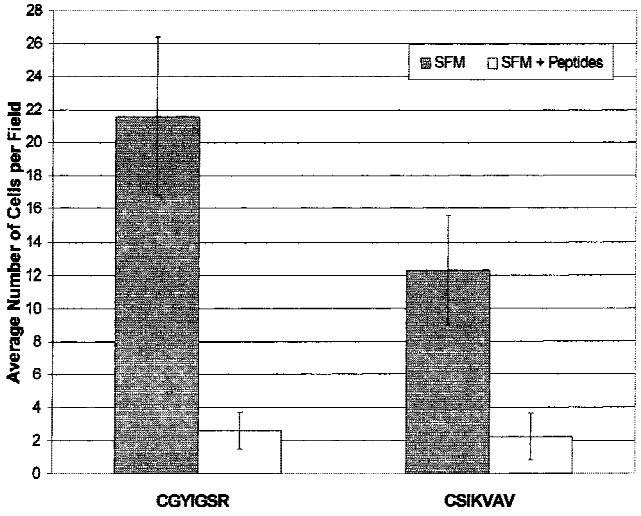

Figure 2. The competitive cell adhesion assay demonstrated a significant reduction in the number of cells per field when the cells were pre-incubated with peptides (SFM + peptides) prior to plating relative to plating cells in SFM alone. A total of ten random fields per sample were analyzed (±SD). Note that 2 ×10^4 cells/cm^2 were plated.

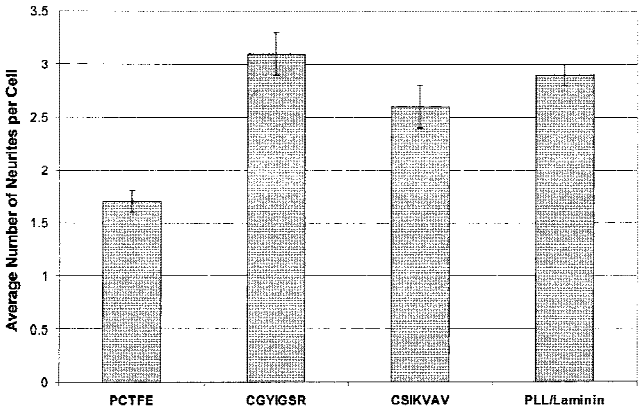

Figure 3. The average number of neurites per cell as compared for 50 random cells per surface (±SEM).

served on CGYIGSR on day 2, their surfaces were indistinguishable at longer time points. On average, the longest neurites were observed on the positive control; however, there were no statistical differences found between the CSIKVAV surface and the positive control surface ($p < 0.8$).

Patterned surfaces

In order to determine whether the combination of adhesive (peptide) and nonadhesive (PEG) regions were required to direct nerve cell adhesion and neurite outgrowth, cell culture experiments were performed on a series of patterned surfaces, representative images of which are shown in Figures 5–9. On PCTFE/Au-patterned surfaces (Fig. 5), a small number of adherent cells were found on both regions. It appeared that cells randomly encountered the patterned surface and adhered nonpreferentially to PCTFE and Au regions, resulting in no biopatterning. On PCTFE/peptide surfaces (Fig. 6), the majority of neurites elongated preferentially on the peptide regions. Most neurites extended within the peptide regions in the first 2 days of culture; however, after 4 days in culture, neurite outgrowth was observed on both the PCTFE and peptide regions. Similar results were observed on both PCTFE/CGYIGSR- and PCTFE/CSIKVAV-patterned surfaces (not shown).

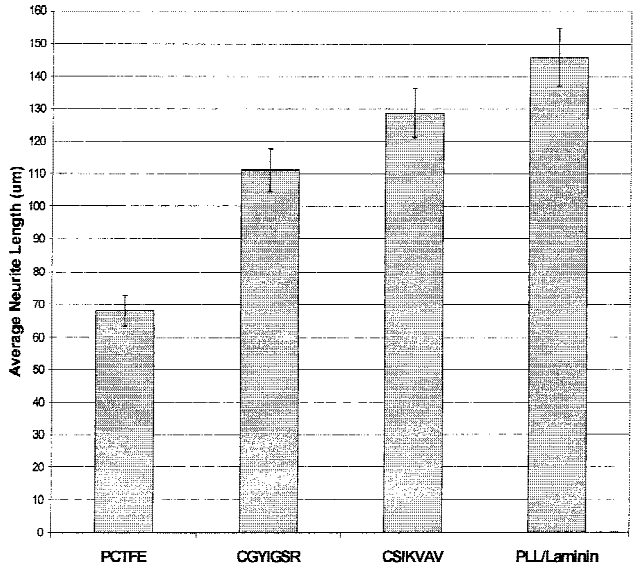

Figure 4. The average length of the longest neurite per cell was measured for 50 randomly selected cells per surface (±SEM).

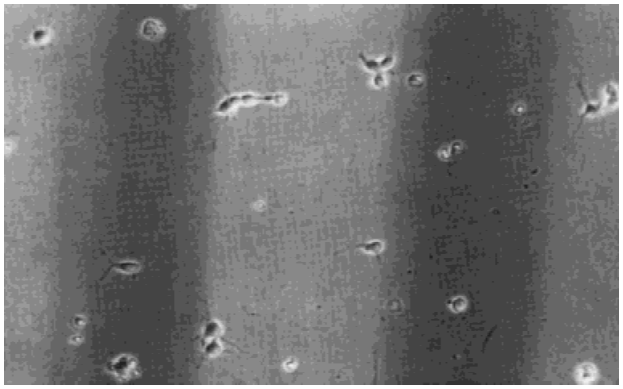

Figure 5. An image of hippocampal cells on a PCTFE (light)/Au (dark). Original magnification ×200.

On PEG/peptide-patterned surfaces (Fig. 7), after 24 h most of the cells adhered to the peptide regions. Neurite outgrowth remained true to the patterns with very few exceptions. After 4 days, we observed that long neurites extended within the peptide regions and changed direction upon encountering PEG areas (Fig. 8). The few neurites that grew beyond the peptide regions seemed to have their growth arrested within the PEG area. As shown in Figure 9, neurite outgrowth was directed within the peptide regions for at least 1 week in culture. Biopatterning seemed to be limited by the health of the cells and not by the sta-

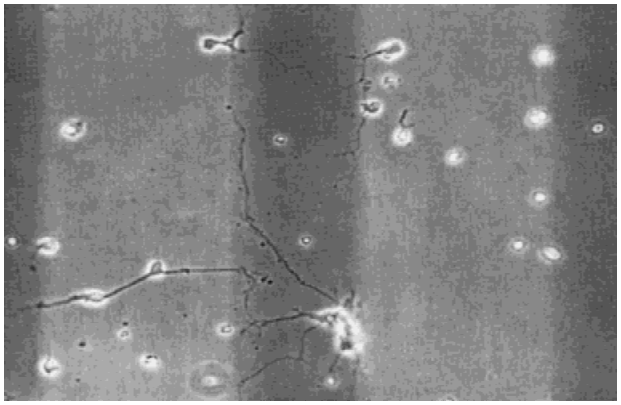

Figure 6. An image of hippocampal cells on a PCTFE (light)/CGYIGSR (dark)-patterned surface on day 4 after cell plating. Original magnification ×200.

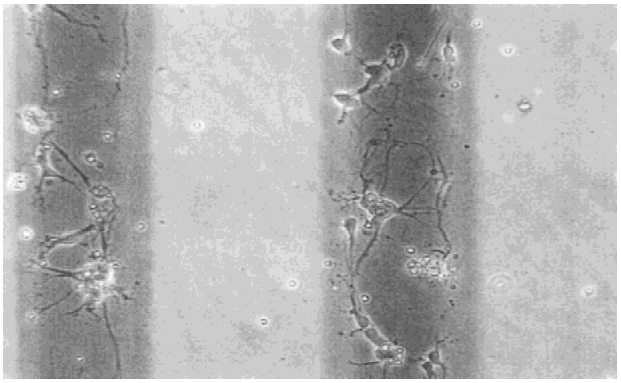

Figure 7. An image of hippocampal cells on a PEG (light)/CGYIGSR (dark)-patterned surface on day 4 after cell plating. Original magnification ×200.

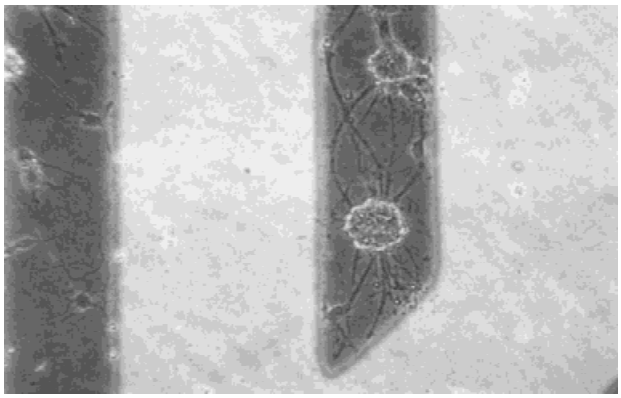

Figure 9. An image of hippocampal cells on a PEG (light)/CGYIGSR (dark)-patterned surface on day 7 after cell plating. Original magnification ×200.

bility of the pattern. Similar results were observed for both PEG/CGYIGSR- and PEG/CSIKVAV-patterned surfaces.

DISCUSSION

We successfully surface-modified PCTFE with PEG by a simple, one-step reaction. While others have functionalized PCTFE surfaces by well defined, albeit complicated, chemsitry,[33] no one has prepared PCTFE-PEG surfaces. Based on the research of Gombotz et al., who used PEG-alkoxide to modify the surface of cyanuric-chloride-activated PET films,[25] and McCarthy et al., who used alkyl lithium reagents to modify PCTFE,[33] we hypothesized that lithium PEG-alkoxide would react with PCTFE. At the outset, it was not clear whether PEG-alkoxide would react via a simple substitution reaction with chlorine of PCTFE or via a metal–halogen exchange followed by elimination of PCTFE.

We used the XPS data to propose a mechanism, which is based on that of McCarthy et al. for alkyl lithium,[34] as shown in Figure 10. For example, for PCTFE-PEG5000 (24 h), the surface composition is $C_{42}F_{41}Cl_{12}O_4$, as was shown in Table II (entry 2). This composition can be reorganized to separate PCTFE from PEG: $C_{24}F_{36}Cl_{12}$-[$C_{10}F_5$]-C_8O_4. The bracketed [$C_{10}F_5$] accounts for the unsaturated alkene that accompanies metal–halogen exchange. The XPS data of all surfaces supported this mechanism, indicating the unlikelihood of a substitution reaction. Furthermore, PEG-modified films were discolored, indicating the presence of conjugated carbon-carbon double bonds, which supports the proposed mechanism.

While PCTFE-PEG was successfully surface-modified with PEG5000, there was a significant amount of chlorine in the outermost surface, indicating that either: (1) the depth of modification was shallow, or (2)

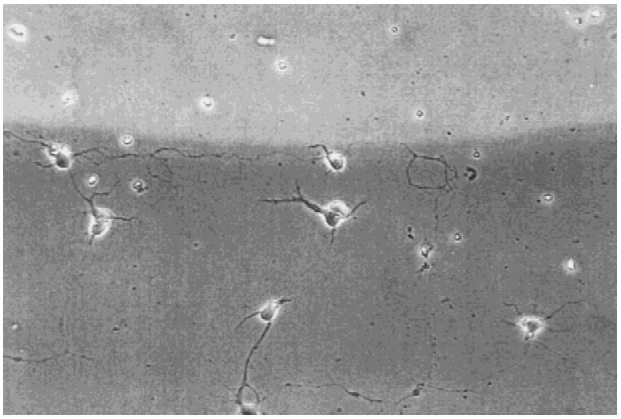

Figure 8. An image of hippocampal cells on a PEG (light)/CGYIGSR (dark)-patterned surface on day 4 after cell plating. Upon encountering the PEG regions, neurites often changed direction and continued to grow on the peptide surface. Original magnification ×200.

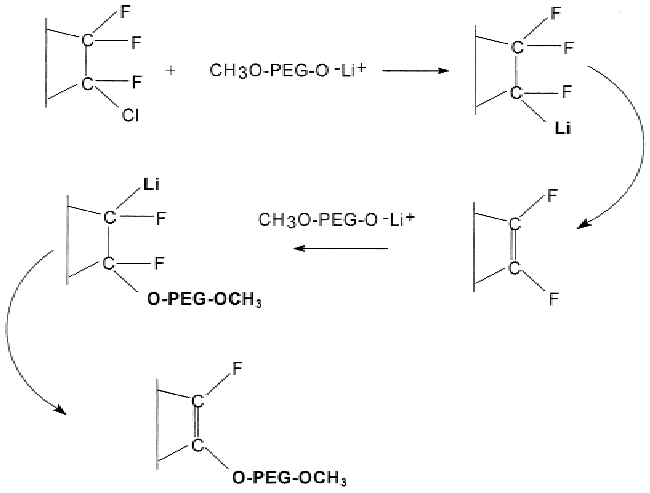

Figure 10. Proposed mechanism for the PEG modification of PCTFE–metal–halogen exchange followed by elimination of PCTFE by the lithium salt of PEG-alkoxide.

modification was patchy, resulting in a heterogeneous surface. The contact angle showed minimal hysteresis, indicating a shallow depth of modification. We repeated the modification reaction with lower molecular weight PEGs at constant time. Smaller PEG chains were expected to be more reactive due to a higher probability of exposed reactive terminal groups and faster diffusion to the surface. In addition, shorter grafted chains were expected to be less sterically hindered and to occupy less space on the surface, thereby allowing more PEG molecules to react in a given surface area. The XPS data (Table II) showed an increase in both oxygen and carbon concentrations as PEG molecular weight decreased from 5000 to 2000 and from 5000 to 750. Assuming that the depth of modification for all MW PEGs was the same (for a 24-h reaction), the XPS results indicated that surface coverage increased with decreasing PEG molecular weight. The XPS data showed that PEG2000- and PEG750-modified surfaces were not statistically different, indicating that either: (1) surface coverage was similar on both surfaces, or (2) the effects of the bulk material overshadowed any changes in surface coverage between the two surfaces. Based on these results, and those of Gombotz et al.,[35] who showed that the minimum PEG molecular weight for an effective hydration shell is 2000 g/mol, we used PCTFE-PEG2000 for cell culture assays.

PCTFE-PEG surfaces were further modified by sputter-coating with titanium and then gold, thereby providing a surface for reaction with thiol-terminated peptides. As has been shown for alkane thiols on gold,[26] the adsorbed layers are highly ordered, easy to prepare, and chemically stable. XPS and contact-angle data confirmed the peptide modifications. We detected only a very small percentage of sulfur by XPS on CGYIGSR-modified films, and others have reported similar results,[29] attributing the absence of sulfur to both its low concentration and the low cross-sectional area for X-ray detection.

Peptide and PEG modifications were performed to create cell-adhesive and nonadhesive surfaces, respectively. All cell culture experiments were performed in SFM in order to eliminate the effects of serum proteins on cell–surface interactions. PCTFE-PEG films supported minimal cell adhesion, as expected. The few cells that had adhered to PEG remained viable 5 to 7 days in culture, with no evidence of cytotoxicity. Following peptide modification of PCTFE-Au surfaces, a dramatic increase in both cell adhesion and neurite outgrowth was observed, with cells remaining viable for 1 week in culture. We saw better cell adhesion on the CGYIGSR surface than on the CSIKVAV surface, as has been observed by others.[21] While Huber et al.[21] saw better cell adhesion on PLL/laminin than on peptide-coated surfaces, we observed better cell adhesion on CGYIGSR surfaces than on positive controls. Since YIGSR (600 g/mol) occupies less than 0.1% w/w of laminin (800,000 g/mol), it is likely that there were more active and exposed YIGSR sites on CGYIGSR-modified surfaces than on PLL/laminin surfaces.

The number, length, and morphology of the neurites provided additional information on the cell–surface interactions. Neurites on CSIKVAV surfaces were of comparable length to those found on PLL/laminin. In comparing the two peptides, significantly longer neurites were found on CSIKVAV than on CGYIGSR, as was expected.[36] A significant amount of branching was observed on both CGYIGSR and positive control surfaces after 2–3 days in culture, which is indicative of cell development and important for the formation of a network of synaptic connections between cells.[37] By day 7, a highly interconnected network of neurites was observed on CGYIGSR, CSIKVAV, and positive control surfaces.

Cell culture experiments were conducted on a series of patterned surfaces: (1) PCTFE/Au, (2) PEG/Au, (3) PCTFE/peptide, and (4) PEG/peptide. Cell–surface interactions were poor on both PCTFE/Au and PEG/Au surfaces, with only minimal cell adhesion and neurite outgrowth on PCTFE regions. Some biopatterning was observed on PCTFE/peptide-patterned surfaces for up to 4 days in culture; however, the PCTFE regions were not effective in inhibiting cell adhesion and neurite extension. Similar results obtained by Corey et al. showed that hippocampal neurons remained true to the poly-D-lysine (PDL) regions of PDL/glass-patterned surfaces for up to 72 h.[15] After 72 h, however, the neurites migrated off the PDL regions and biopatterning was lost. PCTFE/peptide- and PDL/glass-patterned surfaces demonstrate "preferential adhesion"; that is, cells prefer one region to another, but their adhesion is not inhibited by the less adhesive surface. By modifying PCTFE with PEG, it was possible to create a surface that inhibited cell adhesion and neurite outgrowth. As a result, longer-term biopatterning was achieved on PEG/peptide-patterned surfaces; neurons adhered and extended neurites within the peptide regions and were inhibited from extending beyond these regions by PEG. Our studies did not extend beyond 1 week because the neural network was too complex for analysis.

CONCLUSIONS

We demonstrated for the first time covalent modification of PCTFE with a lithium alkoxide, specifically PEG alkoxide, and found a metal–halogen exchange–elimination reaction mechanism. We used PCTFE-PEG-modified films and gold sputter coating/peptide modification to create alternating regions of cell-nonadhesive (PEG) and cell-adhesive (peptide) re-

gions. Cell–surface interaction studies showed that cell adhesion was significantly reduced on PCTFE-PEG and dramatically increased on PCTFE-peptide surfaces. On patterned surfaces, hippocampal cell adhesion and neurite outgrowth were directed onto the peptide regions of the surface. The nonadhesive characteristic of PEG was critical in extending the biopatterning achieved in the first 2 days of culture to longer time periods. By incorporating PEG and peptide molecules on the surface, we were able simultaneously to mimic cell-repulsive and cell-adhesive cues, respectively, and to maintain biopatterning of primary CNS neurons for 1 week in culture. The strategies developed herein may ultimately be useful in the development of nerve guidance conduits for use in CNS regeneration studies.

The authors thank Ulrich Krull for the use of his ellipsometer, Greg Evans for the use of his scintillation counter, and Michael Sefton for reviewing this manuscript.

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