Patterned glass surfaces direct cell adhesion and process outgrowth of primary neurons of the central nervous system

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Abstract: Glass surfaces were patterned with cell-adhesive regions of laminin adhesive peptides YIGSR, RGD, and IKVAV, and cell-repulsive regions of poly(ethylene glycol) (PEG). The patterns were created by sputter-coating titanium and then gold onto glass coverslips through electron microscope grids. Gold surfaces were modified with cysteine-terminated peptides to have approximately 450 fmol/cm^2 of peptide incorporated on the glass coverslips as determined with radiolabeled CGYIGSR. Amine-functionalized glass coverslips were prepared using an amine-functionalized silane and then further modified with PEG-aldehyde by a Schiff base reduction. All surfaces were characterized by X-ray photoelectron spectroscopy and water contact angles. Hippocampal neurons, plated from a serum-free medium, adhered preferentially to peptide-functionalized surfaces over PEG-modified surfaces. Cell adhesion and neurite outgrowth were limited to the peptide region, demonstrating that neurite outgrowth could be directed by a combination of cell-adhesive and cell-repulsive cues. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 42, 13–19, 1998.

Key words: cell–substrate interaction; cell adhesion; cell repulsion; guidance; poly(ethylene glycol); hippocampal neurons; patterning; peptides

INTRODUCTION

Axonal regeneration in the central nervous system (CNS), such as after spinal cord injury, has been most successful in peripheral nerve grafts[1,2] and in environments that mimic those of the peripheral nerve.[3] While the peripheral nerve graft presents a complex environment, the extracellular matrix (ECM) comprises an important component and has been shown to influence cell interaction and function during development, maintenance, and regeneration of the nervous system.[4] The ECM in the peripheral nerve consists, among other ECM molecules, of laminin, fibronectin, and collagen (I and IV). Laminin, in particular, has been shown to enhance the cell–substrate interaction and axonal extension.[5]

Integrins (cell-surface receptors) bind specifically to these ECM proteins, with particular amino acid sequences comprising the adhesive regions.[6] For example, the YIGSR* sequence on the B1 chain of laminin promotes neural cell adhesion and outgrowth,[7] while the IKVAV* sequence on the A chain of laminin promotes neurite outgrowth.[8] The RGD* sequence on both laminin and fibronectin influences numerous cell types, including neurons.[9,10] In an attempt to mimic the functional role of laminin, glass coverslips were surface-modified with these laminin adhesive peptides. While several methods have been pursued to link peptides to surfaces, the technique used here capitalizes on the gold-thiol bond, previously described,[11–13] using cysteine-terminated peptides: CGYIGSR, CSIKVAV, and CGRGDS.

In vivo, axons are guided to their targets by a combination of diffusible and membrane-bound cues that are both attractive and repulsive to growth cones.[14–17] This enables the growth cones to navigate axons along specific pathways to their correct targets with few if any errors. In an attempt to mimic both the contact attraction and contact repulsion cues that direct growth cones *in vivo,* glass coverslips were patterned

*Y = tyrosine; I = isoleucine; G = glycine; S = serine; R = arginine; K = lysine; V = valine; A = alanine; D = aspartic acid: C = cysteine.

to include both cell-adhesive and cell-repulsive regions. The laminin-adhesive peptides define the cell-adhesive regions, whereas poly(ethylene glycol) (PEG) defines the cell-repulsive regions.

The cell-repulsive regions were prepared by covalently attaching PEG-aldehyde to amine-functionalized glass surfaces by a Schiff base reaction. Surfaces have been modified with PEG by a number of techniques including adsorption, grafting, crosslinking, plasma deposition, and self-assembled monolayer[18] (SAM) techniques. In these studies, PEG-modified surfaces were shown to limit protein adsorption[19–23] and cell adhesion[24–26]; a recent review summarizes the efficacy of PEG-modified surfaces in these applications.[27]

The glass surfaces were patterned using the shadow-masking technique in which first titanium and then gold was sputter-coated onto modified glass surfaces[28] through either a centimeter-, millimeter-, or micrometer-scale grid. Micropatterned surfaces can also be prepared by photolithography,[29] photochemical,[30,31] and laser ablation.[32]

Figure 1 summarizes the methodology used to create cell-adhesive and cell-repulsive regions on activated glass substrates, demonstrating the versatility and facile nature of this technique. The cell-repulsive regions (PEG) were as important to the success of the directed cell outgrowth as were the cell-adhesive regions (peptides). Striped patterns of cell-adhesive regions on glass alone, where glass or gold served as the nonadhesive substrate, were insufficient to direct neural cell adhesion and neurite outgrowth.[33]

MATERIALS AND METHODS

Unless otherwise noted, water was distilled and deionized using Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) at 18 MΩ resistance, and all reactions were conducted at room temperature (RT).

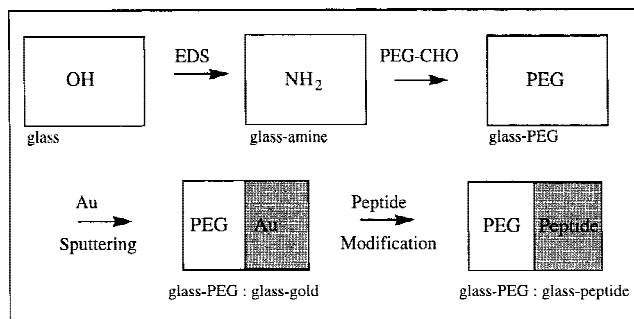All surfaces were characterized by dynamic advancing and receding water contact angles using a telescopic goniometer (Model 100-00; Ramé Hart, NJ). Three samples of each surface were analyzed and five measurements were taken for each sample. All samples were characterized by X-ray photoelectron spectroscopy (XPS) (Leybold LH Max 200) at takeoff angles between sample and detector of 20° and 90°. A MgKα source was used at an operating pressure $<10^{-8}$ Torr. XPS data were used to estimate both the thickness of the modified surfaces[34] and their surface coverage,[35] as previously described. Iodine-125-radiolabeled tyrosine (Y) of GYIGSR 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.

Preparation and activation of surface hydroxyl groups on glass coverslips (glass-OH)

Borosilicate glass coverslips (2.5 cm in diameter; 0.15 mm thick; Bellco, NJ) were activated as previously described.[29] Briefly, the coverslips were cleaned with an aqueous detergent solution, sonicated for 10 min (Branasonic 2210; Branson, CT), rinsed repeatedly with water, and then air-dried for 30 min. The dry coverslips were immersed in 20 mL of a solution containing nine parts of concentrated sulfuric acid (BDH, Ontario, Canada) and one part of 50% hydrogen peroxide in water (BDH). After 15 min, the coverslips were rinsed repeatedly with water and then air-dried.

Amine functionalization of activated glass (glass-NH$_2$)

The method to modify activated glass with an amine functionalized silane, using N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (EDS) (Aldrich, WI), was previously described.[36] Briefly, activated coverslips were immersed in a 1% solution of EDS/ethanol/water in a 1:95:4 v/v/v ratio. After sonicating for 2 min, the coverslips were rinsed repeatedly with ethanol, then dried at 105°C for 30 min.

PEG-aldehyde modification of glass-NH$_2$ (glass-PEG)

Glass-NH$_2$ coverslips were immersed in 30 mL of a pH 6-buffered aqueous solution containing 10% potassium sulfate (Aldrich, WI), 0.017% PEG-aldehyde (5 kg/mol; Shearwater Polymers, AL), and 0.003% sodium cyanoborohydride (Aldrich) for 40 h at 60 °C.[37] The modified coverslips were rinsed with pH 6 buffer (60 °C) and then with water (60°C) several times before they were dried (105°C, 1 h). The pH 6 buffer was prepared with 9 mg/mL sodium acetate (BDH) and 0.03% acetic acid (ACP Chemicals, Quebec, Canada).

Gold patterning on glass-PEG (glass-gold)

Glass-PEG coverslips were sputter-coated under vacuum (3×10^{-4} Torr; 2400; Perkin Elmer, CA) with titanium for 10 s followed by gold for 150 s.[28] The sputtering system was equipped with argon gas at a flow rate of 12 sccm and a target bias of 1000 V (DC).

Figure 1. Surface modification steps used to create a patterned glass surface: All patterns (centimeter, millimeter, and micrometer) were created using the same strategy.

Three different patterns were created on the glass coverslips using masking techniques: (a) one stripe on the centimeter scale where half of the coverslip was masked and the other half exposed; (b) multiple stripes on the millimeter scale where gold was sputtered through a custom-made Teflon mask with stripes between 0.8 and 1.2 mm; and (c) numerous stripes on the micrometer scale where gold was sputtered through an electron microscope grid (Electron Microscopy Sciences, PA) having a 200-μm bar size and a 50-μm hole size. The resulting micropattern had 50-μm stripes of gold, which were then further modified with peptides and separated from each other by 200 μm of PEG-modified glass. The opposite pattern with 200-μm gold stripes separated by 50-μm PEG stripes was also prepared using a grid with a 50-μm bar size and a 200-μm hole size.

Peptide modification of glass-gold (glass-peptide)

The peptides CGRGDS, CGYIGSR, and CSIKVAV (Vetrogen Corporation, Ontario, Canada) were dissolved in the pH 6 sodium acetate/acetic acid buffer solution at a concentration of 0.2 mg/mL. A glass-gold coverslip was immersed in 10 mL of the peptide solution for 24 h and then rinsed with the pH 6 buffer and water (three times) before air-drying.

Peptide labeling and quantification

The tyrosine (Y) of CGYIGSR was radiolabeled with radioactive iodine (^{125}I) using sodium iodide as previously described.[38] Briefly, 2 mg of CGYIGSR was dissolved in 5 mL of a pH 11 buffer containing 20 mM sodium phosphate and 0.15M sodium chloride, and reacted with 1 mCi of carrier-free Na^{125}I (ICN, Costa Mesa, CA) in the presence of Iodobeads (Pierce, Rockford, IL) for 15 min. Free iodide was removed by successive passes through columns packed with anion-exchange resin (Dowex 1-X8; Aldrich). The labeled peptide was coupled to gold surfaces as described above, and glass-peptide surfaces were rinsed with 10 mM sodium iodide to desorb any trace amounts of ^{125}I before counting by scintillation.

Hippocampal cell culture experiment

All modified glass coverslips were immersed in 70% ethanol (BDH), rinsed with water (three times), and air-dried for 1 h prior to plating hippocampal neurons.

Embryonic day 18 (E18) mouse hippocampal neurons were isolated as previously described[39] by dissociation with papain (Worthington Biochemical Corporation) and DNase (Sigma, Ontario, Canada) for 30 min and mechanical trituration in calcium-free Hank's buffered saline solution (HBSS). Hippocampal cells were suspended in a serum-free medium (SFM) of Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY) supplemented with chicken egg albumin (1 mg/mL; Sigma), sodium pyruvate (0.1 mg/mL, Sigma), B-27 supplement (2%; Gibco), penicillin G (100 U/

mL; Sigma), and streptomycin sulfate (100 μg/mL; Sigma). After 1 mL of 5×10^5 cells/mL (or 1×10^5 cells/cm^2) was added to the surface of each coverslip, they were incubated at 35°C, 5% CO$_2$ overnight. After 24 h, 1 mL of SFM and 5 μL of mitotic inhibitor solution made up of deionized water supplemented with fluorodeoxyuridine (8.0 mM; Sigma) and uridine (20 mM; Sigma) were added to each sample. Light micrographs were taken 1 and 4 days after plating. The number and relative length of neurite extensions were counted for 50 random cells on both the PEG and the peptide regions of each coverslip.

A positive control (glass-PLL/laminin) was prepared by coating a clean glass coverslip with a sterile, aqueous 1-mg/mL solution of poly(L-lysine) (PLL) (36.6 kg/mol) (Sigma) for 24 h. The PLL-modified coverslip was rinsed with sterile water and then coated with 0.5 mL of a 0.05-mg/mL sterile, aqueous laminin solution (Sigma). After 4 h, the coverslips were rinsed with sterile water and air-dried. The positive control was plated with cells and analyzed alongside sample surfaces.

RESULTS

Surface characterization

Glass coverslips were modified sequentially, as shown in Figure 1, and characterized after each step of modification in terms of both surface elemental composition by XPS (20° takeoff angle) and relative surface hydrophilicity by advancing (θ_A) and receding (θ_R) water contact-angle measurements, as shown in Table I.

EDS-silane modification of glass coverslips (glass-NH$_2$) resulted, as expected, in the presence of nitrogen, an increase in carbon, and decreases in silicon and oxygen in the surface composition, as determined by XPS. Correspondingly, the contact-angle data showed that the glass-NH$_2$ surface (58°/42°) was more hydrophobic than glass alone (31°/23°), as was expected after organosilane modification. PEG-aldehyde coupling to the glass-NH$_2$ surface was evident by the decreased nitrogen and silicon and the increased carbon and oxygen surface concentrations. The contact angles decreased (41°/36°), corresponding to the increased hydrophilic nature of PEG versus organosilane. After sputter-coating with gold, the contact angle (69°/56°) was greater than expected owing to the adsorption of adventitious carbon, which was confirmed by the XPS data. Similar impurities have been observed by others,[40] but since this adventitious carbon layer was weakly adsorbed, it was easily desorbed by thiol modification. As anticipated, peptide modification resulted in an increase in nitrogen, carbon, and sulfur (from cysteine), and a decrease in gold atomic compositions. Consistently, the contact angle of the peptide-modified surfaces was lower than the gold surface, confirming the gold–thiol interaction.

TABLE I
Surfaces of Glass-Modified Coverslips Characterized by XPS and Advancing (θ_A) and Receding (θ_R) Water Contact Angles

Surface	XPS Elemental Composition (20° Takeoff Angle)	Contact Angle (θ_A/θ_R)
Glass	$Si_{18.7}O_{42.9}C_{37.6}$	$31° \pm 2°/23° \pm 2°$
Glass-NH$_2$	$Si_{9.9}O_{21.1}C_{58.6}N_{6.5}$	$58° \pm 2°/42° \pm 2°$
Glass-NH$_2$-PEG	$Si_{4.9}O_{22.1}C_{68.3}N_{2.8}$	$41° \pm 2°/36° \pm 2°$
Glass-NH$_2$-PEG-Au	$Si_{2.6}O_{11.6}C_{43.0}N_{1.6}Au_{40.8}$	$69° \pm 2°/56° \pm 2°$
Glass-NH$_2$-PEG-Au-CGRGDS	$Si_{3.2}O_{19.3}C_{67.7}N_{3.9}Au_{4.7}S_{1.2}$	$36° \pm 2°/21° \pm 2°$
Glass-NH$_2$-PEG-Au-CGYIGSR	$Si_{0.9}O_{13.6}C_{62.6}N_{9.6}Au_{11.8}S_{1.5}$	$40° \pm 2°/24° \pm 2°$
Glass-NH$_2$-PEG-Au-CSIKVAV	$Si_{0.6}O_{13.6}C_{61.5}N_{12.3}Au_{11.2}S_{0.8}$	$54° \pm 2°/34° \pm 2°$

Using radiolabeled tyrosine of CGYIGSR, the amount of peptide on the gold surfaces was quantified at 447 ± 37 fmol/cm^2. Since the surface modification is driven by the gold–thiol interaction, we assume that similar amounts of CGRGDS and CSIKVAV as CGYIGSR were bound to the surface.

The XPS data acquired at a 90° takeoff angle for the same surfaces are summarized in Table II. Here, the surface selectivity of the reactions is clear, as bulk glass (i.e., silicon and oxygen) have a higher elemental concentration in all surfaces relative to those analyzed at the 20° takeoff angle. The data in Table II were used to calculate the relative thickness[34] and surface coverage[35] of each layer using formulas previously described. Figure 2 summarizes these data. For glass-NH$_2$ surfaces, the surface thickness and coverage were low at 4 Å and 8%, respectively. Of the amine-functional groups present, PEG modification resulted in a 19 Å-thick layer with a surface coverage of 16%. Sputter-coating gold on PEG resulted in a 39 Å-thick layer of 26% coverage. Peptide modification of the glass-gold substrate resulted in a thin peptide layer (1 Å) and 21% coverage.

Hippocampal cell culture

Hippocampal neurons were plated on three types of patterned surfaces and compared to positive controls for cell adhesion and neurite outgrowth. The cell-surface interactions were scored by both quantitative

TABLE II
XPS Data Acquired at 90° Takeoff Angle of Modified Glass Surfaces

Suface	XPS Elemental Composition
Glass	$Si_{26.7}O_{66.0}C_{7.3}$
Glass-NH$_2$	$Si_{19.4}O_{39.8}C_{27.2}N_{5.8}$
Glass-NH$_2$-PEG	$Si_{18.4}O_{45.4}C_{27.1}N_{2.5}$
Glass-NH$_2$-PEG-Au	$Si_{4.4}O_{19.3}C_{19.8}N_{0.8}Au_{55.6}$
Glass-NH$_2$-PEG-Au-CGRGDS	$Si_{0.0}O_{9.8}C_{34.0}N_{7.3}Au_{48.8}S_{0.2}$
Glass-NH$_2$-PEG-Au-CGYIGSR	$Si_{3.6}O_{11.0}C_{44.0}N_{6.4}Au_{34.3}S_{0.7}$
Glass-NH$_2$-PEG-Au-CSIKVAV	$Si_{11.2}O_{10.0}C_{39.5}N_{6.8}Au_{32.1}S_{0.4}$

and qualitative analyses. The relative number of hippocampal cells that adhered to the modified surfaces were compared. Figure 3 includes representative micrographs of the patterned surfaces taken after 1 day of plating.

Qualitative analysis of hippocampal cells on day 1 showed that cell adhesion could be directed on patterned surfaces (cf. Fig. 3). The cells observed under both ×10 and ×20 magnification showed preferential adhesion and neurite outgrowth on the peptide regions of the coverslip for patterned surfaces having either a 200- or 50-µm PEG gap. Few cells adhered and extended neurites on the PEG regions. Similar cell adhesion/repulsion was observed on CSIKVAV/PEG, CGRGDS/PEG, and CGYIGSR/PEG coverslips. Qualitatively, the hippocampal neurons interacted more favorably with the CSIKVAV-modified surfaces than either CGYIGSR- or CGRGDS-modified surfaces. Using the number and size of cell clusters as an indication of cell–material interaction, the fewest and smallest number of cell clusters were observed on CSIKVAV-modified surfaces. Large cell clusters may indicate that the cells interact preferably with each other over the surface.

Quantitatively, the peptide-modified surfaces were compared by averaging the number of neurites extending from 50 random cells on each surface and
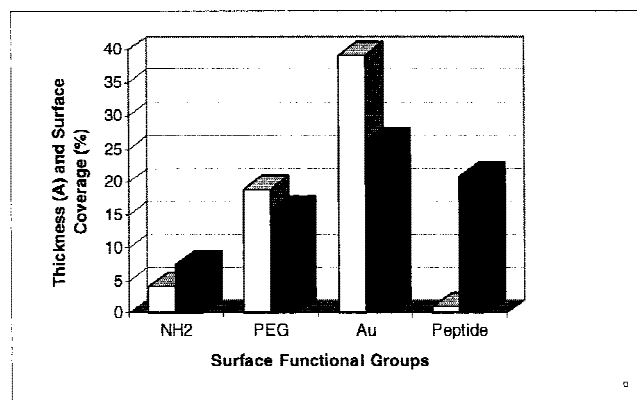

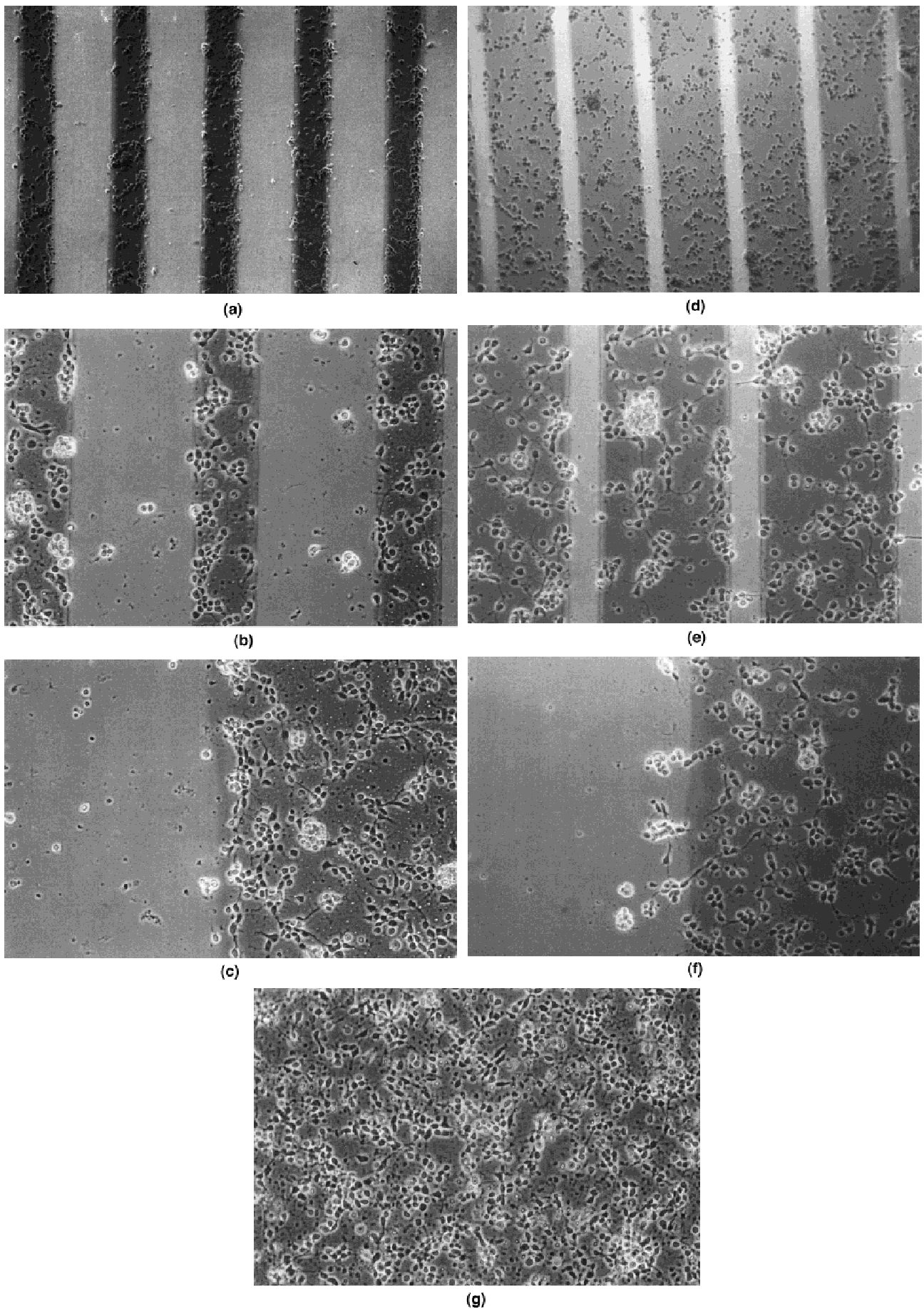**Figure 2.** Relative thickness (□) and surface coverage (■) of surface functional groups.

Figure 3. Light micrographs were taken 1 day after plating hippocampal neurons on surface-modified glass coverslips: CGRGDS (50-μm)/PEG (200-μm) micropatterned surfaces at (a) ×10 magnification, (b) ×20 magnification, and (c) ×20 magnification of the peptide–PEG interface of the millimeter pattern; CSIKVAV(200-μm)/PEG (50-μm) micropatterned surfaces at (d) ×10 magnification, (e) ×20 magnification, and (f) ×20 magnification of the peptide–PEG interface of the millimeter pattern; and (g) positive control (PLL/laminin) at ×20 magnification.

calculating the percentage of cells having one or more neurites longer than the cell body length. The data were collected after 1 day of plating and are summarized in Table III. As expected, the PEG region was nonadhesive to neurons and inhibited neurite extension. The positive control (PLL/laminin) had numerous adherent cells with an average of 2.2 neurites/cell body, and 68% of these cells had neurites longer than one cell body length. All of the peptide-modified surfaces showed similar (~3) neurite extensions per cell, and similar percentages (70–78%) of the cells had neurites longer than the cell body length. While it was difficult to distinguish between the peptide-modified surfaces quantitatively, all showed a hippocampal response greater than that observed on the positive control.

DISCUSSION

The series of sequential surface modifications of glass to glass-peptide, as outlined in Figure 1, were confirmed by both XPS and contact-angle data (cf. Table I). We assumed that the quantitative radiolabeled data for CGYIGSR were representative of all peptides. The XPS data of the three peptide-modified surfaces are similar; yet, the corresponding contact-angle data are dissimilar. Interestingly, the surface-modified CSIKVAV was significantly more hydrophobic (54°/34°) than either of those modified with CGRGDS (36°/21°) or CGYIGSR (40°/24°). The contact-angle data reflect the greater hydrophobicity of CSIKVAV over CGRGDS and CGYIGSR. In addition, the contact-angle data may imply that self-assembled monolayers formed on gold. Since the driving force for peptide modification is via the gold–thiol bond, all peptides are anchored to the surface via the cysteine (C) amino acid. Thus, the terminal amino acids sequences may have a greater influence on the contact angle than those closer to the glass surface. For example, ''VAV'' of CSIKVAV is more hydrophobic than ''GSR'' of CGYIGSR and ''GDS'' of CGRGDS.

The thickness and percent coverage data (shown in Fig. 2) for each surface functional group are more useful as relative comparisons between surface functional

groups than as absolute numbers, because the calculations were based on dry samples, evacuated to 10^{-8} Torr. These data represent an estimate, at best, of the hydrated surfaces which we would have expected to be both thicker and occupy a greater surface coverage. The gold-modified surface (which is independent of surface chemistry) had the greatest calculated thickness (40 Å) and coverage; yet fell short of the 500 Å-thick layer expected based on sputtering time and distance between gold target and sample. This order of magnitude difference indicates that the calculated values are only estimates. Assuming a self-assembled monolayer, the peptide-modified surface is also expected to be an order of magnitude greater than the calculated 1 Å value. The percent coverage data indicate that a significant percentage of the surface was left unreacted, and that the methods of modification may be improved. Atomic force microscopy may provide a more accurate description of *in situ* surface thickness and coverage.

Despite the apparently low thickness and surface coverage of PEG and peptide modifications, the adhesion and neurite outgrowth of E18 hippocampal neurons were directed by the combination of an adhesive interaction with the laminin adhesive peptides and a repulsive interaction from PEG. Quantitatively, the number of neurites and percentage of cells with neurites greater than the cell body length of each of the peptide-modified surfaces were indistinguishable from each other and the positive control (PLL-laminin). Qualitatively, fewer and smaller numbers of cell clusters were observed on glass-CSIKVAV surfaces. This indicated that of the three peptides studied, the cell interaction was best on CSIKVAV. Overall, CSIKVAV, CGYIGSR, and CGRGDS sufficiently mimicked the cell–laminin interaction. Substrate-bound laminin pathways have been shown to guide neural growth cones.[41,42]

The number and length of neurites were a direct reflection of the surface chemistry and may be less than what is often observed in neuronal tissue culture systems because this tissue culture medium was not supplemented with growth factors. It is widely accepted that growth factors such as nerve growth factor, brain-derived neurotrophic factor, fibroblast growth factor, etc., enhance neurite outgrowth.

This set of peptide-adhesive and PEG-repulsive cues mimics the *in vivo* guidance of axons to their targets.[14–17] This strategy may be useful in creating regenerative structures for axonal guidance from proximal to either distal or target tissues.

TABLE III
Quantitative Analysis of Cell–Surface Interactions after 1 Day of Plating

Surface	No. of Neurite Extensions/Cell (Average ± sem)	Relative No. of Cells with Neurites Longer than Cell Body Length (%)
PLL/laminin	2.2 ± 0.8	68
PEG	0.8 ± 0.5	0
CGRGDS	2.9 ± 0.9	76
CGYIGSR	3.1 ± 1.0	78
CSIKVAV	3.2 ± 1.0	70

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