Peptide surface modification of poly(tetrafluoroethylene-*co*-hexafluoropropylene) enhances its interaction with central nervous system neurons

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Abstract: Poly(tetrafluoroethylene-*co*-hexafluoropropylene) (FEP) film surfaces were chemically surface modified to introduce one of three laminin adhesive peptides: GYIGSR, GRGDS, or SIKVAV. FEP film surfaces were first reduced with sodium naphthalide to introduce surface carbon–carbon double bonds at two reaction conditions: 20 min at –78°C, and 3 h at 25°C. Scanning electron microscopy and atomic force microscopy indicated that surface topography was unaffected by the reaction conditions. Reduced FEP film surfaces were further modified to introduce hydroxyl groups via hydroboration/oxidation or carboxylic acid groups via oxidation. The hydroxyl (FEP-CH_xOH) and carboxylic acid (FEP-COOH) functionalized surfaces provided reactive handles for peptide coupling using tresyl chloride. Surface elemental composition data, determined from X-ray protoelectron spectroscopy, indicated that equivalent amounts of GYIGSR, GRGDS, and SIKVAV were introduced. Two additional coupling reagents, SMCC and TSU, were compared to tresyl chloride for the coupling of radiolabeled tyrosine of GYIGSR. Between 8 and 150 fmol/cm^2 of peptide was introduced to the hydroxyl and carboxylic acid functionalized surfaces, with the tresyl coupling reagent showing the greatest amount of peptide incorporated. The tresyl-coupled peptide-modified surfaces were compared in terms of the response of primary, embryonic hippocampal neurons plated from serum-free medium for 4 days. The number and length of neurites extending from the cell bodies were averaged over 50 cells after 1 and 4 days FEP-CH_xO-peptide surfaces had either a greater or equivalent hippocampal neuron interaction than the corresponding FEP-COO-peptide surfaces. All peptide-functionalized surfaces had a greater hippocampal neuron interaction than the corresponding FEP-CH_xOH, FEP-COOH, and FEP controls after 4 days underlying the importance of the peptides over hydrophilic or hydrophobic surfaces. After 4 days differences in neurite extension were evident among the peptide-functionalized surfaces, with the longest neurites observed on the SIKVAV-functionalized surfaces. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 42, 85–95, 1998.

Key words: fluoropolymer surface modification; peptides; hippocampal neurons; regeneration; central nervous system

INTRODUCTION

Central nervous system (CNS) neurons, unlike those of the peripheral nervous system, do not regenerate spontaneously following injury; however, CNS neurons can regenerate in a peripheral nerve graft[1–4] or in an environment that mimics that of the peripheral nerve.[5] A synthetic graft must provide the adhesion molecules that enhance cellular interaction and regeneration, such as those of the basal lamina of the peripheral nerve graft.[6]

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Cell adhesion molecules, consisting of diverse cell-surface glycoproteins and extracellular matrices (ECMs), affect cell interactions during the development, maintenance and regeneration of the nervous system.[7] Specific cell-surface receptors[8] (i.e., integrins) adhere to ECM proteins, such as laminin and fibronectin.[9] Particular amino acid sequences of these naturally occurring ECM proteins have been identified as binding domains. For example, arginine-glycine-aspartic acid (RGD) has been found to be important for the binding of numerous cell types to fibronectin and laminin[10,11]; tyrosine-isoleucine-glycine-serine-arginine (YIGSR), on the B1 chain of laminin, has been shown to be active in epithelial cell attachment[12] and in promoting neural cell adhesion and outgrowth[13]; and isoleucine-lysine-valine-alanine-valine (IKVAV), on the A chain of laminin, has been found to promote neurite outgrowth of PC12 cells.[14]

Several investigators have found that peptide-

modified surfaces enhance cell adhesion. RGD promotes the interaction of fibroblasts and endothelial cells with, for example, poly(acrylamide),[15] poly(vinyl alcohol),[16] polystyrene,[17] and glass beads.[18] YIGSR, IKVAV, and RGD enhance the interaction of neuronal cells with agarose,[19] and YIGSR enhances the adhesion of neuronal cells lines with poly(tetrafluoroethylene-*co*-hexafluoropropylene) (FEP) that is pretreated with albumin.[20,21]

Fluoropolymers such as expanded poly(tetrafluoroethylene) (ePTFE) have a history of *in vivo* use, including those in vascular grafts[22] and peripheral nerve repair.[23] Since fluoropolymers are inert in the CNS,[24] peptide-modified fluoropolymers may lead to a cleaner delineation of the effect of immobilized oligopeptide bioavailability on axonal outgrowth than other substrates. Unlike plasma modification, which introduces a diverse set of functionalities, chemical modification of the normally inert fluoropolymers allows discrete functional groups to be introduced to defined depths of reaction and topographies.[25,26]

In this article, we describe the chemistry required to surface-modify FEP with laminin-derived adhesive peptides and the interaction of primary CNS neurons with the modified surfaces. By modifying the fluoropolymer surfaces with cell-adhesive peptides (YIGSR, IKVAV, or RGD), we aim to determine which surface best mimics the interaction normally observed between laminin and peripheral neurons, thereby preparing the optimum surface for enhanced cell interaction and process outgrowth in the CNS. To date, the interaction of primary CNS neurons with peptide-functionalized polymer surfaces has received limited attention. The interaction of primary hippocampal neurons with three peptide-modified surfaces, which required no pretreatment, is compared quantitatively in terms of neurite outgrowth and qualitatively in terms of cell adhesion and viability.

Herein, FEP film surfaces were chemically modified by a series of reactions to introduce either hydroxyl or carboxylic acid functional groups which were subsequently coupled to cell-adhesive peptides using one of three coupling agents. FEP film samples were surface-modified by sodium naphthalide reduction at several reaction conditions (20 min at −78°C, 0°C, and 25°C, and 3 h at −78°C, 0°C, and 25°C) to determine whether surface chemistry can be modified independently from surface morphology. The two extremes (i.e., 20 min at −78°C vs. 3 h at 25°C) of the reduced films (FEP-C) were converted to either hydroxyl (FEP-CH_xOH) by hydroboration/oxidation or carboxylic acid (FEP-COOH) by oxidation. As shown in Figure 1, the hydroxyl and carboxylic acid groups provide two different synthetic routes to introduce peptides, GYIGSR, GRGDS, or SIKVAV, to the surface. The amount of peptide incorporated onto each surface is calculated by coupling radioactive, iodinated tyrosine of GYIGSR

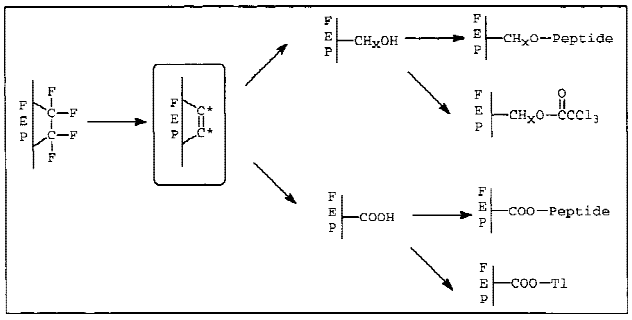

Figure 1. Poly(tetrafluoroethylene-*co*-hexafluoropropylene) (FEP) film samples were surface modified to either hydroxyl or carboxylic acid groups which were in turn either labeled with trichloroacetyl chloride or thallous ethoxide, respectively, or coupled with cell-adhesive peptides.

to the functionalized surfaces; the coupling efficiencies of three different reagents are compared in Figure 2. The surfaces are characterized in terms of relative hydrophilicity, atomic composition, and morphology. The response of hippocampal neurons to the modified surfaces is compared quantitatively in terms of the number and relative length of extended neurites per cell.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. Tetrahydrofuran (THF) (Fisher, Nepean, Ontario, Canada) was distilled from sodium benzophenone dianion and stored under nitrogen in Schlenk flasks. Hank's balanced salt solution (HBSS), neurobasal medium, B27 supplement, and phosphate-buffered saline (PBS) were sterile-filtered with 0.22-μm cellulose acetate filters (all from Gibco BRL, Burlington, Ontario, Canada). All peptides were purchased from Vetro-

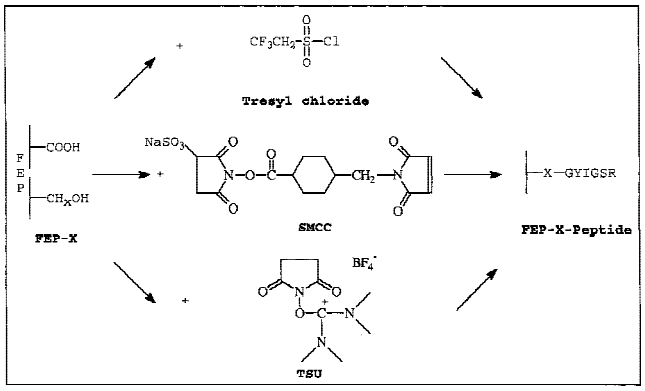

Figure 2. Three coupling agents (tresyl chloride, SMCC, and TSU) were used to couple the GYIGSR peptide to functionalized FEP (FEP-X) film surfaces, yielding FEP-X-peptide.

gen (Ontario, Canada) and used as received. FEP films (5 mil thickness, received from DuPont and cut into 2 × 2-cm samples) were Soxhlet-extracted in THF for 24 h. All reactions were done under inert nitrogen atmosphere unless otherwise indicated. Deionized distilled water was obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) and used at 18 MΩ resistance.

X-ray photoelectron spectroscopy (XPS) data were collected on a Leybold LH Max 200 using a MgKα X-ray source at 15 kV and 20 mA emission current. An aperture size of 13 × 7 μm was used to collect data at takeoff angles of 20° and 90° between sample and detector. Advancing and receding water contact angles were obtained on a Ramé-Hart NRL telescopic goniometer. Values reported represent the average and standard deviation of five measurements. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were obtained on a Mattson Galaxy 5400 spectrometer using a germanium crystal (45°). Scanning electron microscopy (SEM) micrographs were taken on a Hitachi S4500 field emission microscope at 1 kV acceleration voltage. Atomic force microscopy (AFM) micrographs were obtained on a Digital Instrument Nanoscope 3 using tapping mode imaging. Iodine-125–radiolabeled tyrosine 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. Embryonic mouse (CDI Type; Charles River, Ontario, Canada) hippocampal neurons that were cultured on all surfaces were photographed under normal light or filters for fluorescently stained cells at ×20 magnification under an Axiovert 150 microscope.

Reduction of FEP, FEP-C

Poly(tetrafluoroethylene-*co*-hexafluoropropylene) film surfaces were reduced as previously described[26,27] in 20 mL of a 0.16*M* sodium naphthalide solution in THF for either 3 h at 25°C (referred to as 25°C from here forward) or 20 min at −78°C (referred to as −78°C from here forward). The films were then rinsed three times each with THF, dichloromethane, and THF before drying under vacuum (pressure = 0.01 mm Hg).

FEP functionalization, FEP-X (X = –COOH, –CH_xOH)

FEP-COOH film samples were prepared as previously described[26] by oxidizing FEP-C films with 0.16*M* potassium chlorate in sulfuric acid in a beaker for 2 h at room temperature (RT). Films were then rinsed five times each with water, methanol, and dichloromethane prior to drying under vacuum. Unmodified FEP film samples were used as controls.

FEP-COO-Tl film samples were prepared as previously described[26] by immersing FEP-COOH film samples in 10 mL of thallous ethoxide for 60 s at RT and then rinsing six times with ethanol prior to drying under vacuum. Unmodified FEP film samples were used as controls for this labeling reaction.

FEP-CH_XOH film samples were prepared as previously described[27] by hydroboration of FEP-C in 10 mL of a 0.5*M* borane/THF solution for 18 h followed by oxidation in 10 mL of a 30% hydrogen peroxide solution and 20 mL of a 0.18*M* aqueous sodium hydroxide solution for 3 h in a beaker. Films were then rinsed five times each with 20 mL dilute sodium hydroxide solution, dilute hydrochloric acid solution, water, and THF prior to drying under vacuum.

FEP-CH_XO-C(O)CCl_3 films were prepared as previously described[27] by reacting FEP-CH_XOH film samples with 0.5 mL trichloroacetyl chloride in 19 mL THF and 1 mL pyridine for 12 h at RT. The film samples were rinsed three times each in THF, methanol, and dichloromethane prior to drying under vacuum. Unmodified FEP film samples were used as controls for this labeling reaction.

Peptide coupling to functionalize FEP, FEP-X-peptide

Three peptides (GYIGSR, SIKVAV, and GRGDS) were coupled to FEP-X film surfaces using the trifluoroethanesulfonyl chloride (tresyl chloride) coupling agent. In addition, GYIGSR was coupled to FEP-X film surfaces using one of three different coupling reagents, as shown in Figure 2: tresyl chloride, *O*-(*N*-succinimidyl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TSU), or sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). Unmodified FEP film samples were used as controls for the coupling reactions.

FEP-X film samples were immersed in a solution containing 200 μL of tresyl chloride in 1 mL pyridine/19 mL THF for 20 min at RT and then added to a beaker containing 10 mL of a 0.2*M*, pH 10, sodium carbonate-buffered solution and 0.1 μg/mL of peptide (i.e., GYIGSR, GRGDS, or SIKVAV) for 24 h.[28] The films samples were rinsed five times each with the buffer solution, dilute hydrochloric acid, water, and THF prior to drying under vacuum.

FEP-X film samples were immersed in 20 mL of dimethyl formamide (DMF) containing 1.3 m*M* TSU and 1.3 m*M* diisopropylethylamine for 4 h at RT. Film samples were then added to a beaker containing 10 mL of a 0.2*M*, pH 10, sodium carbonate-buffered solution containing 0.1 μg/mL of GYIGSR for 24 h.[29] The film samples were rinsed five times each with the buffer solution, water, and THF prior to drying under vacuum.

FEP-X were immersed in a beaker containing 30 mL of a 0.2*M* sodium bicarbonate-buffered solution at pH 10 and containing 2 mg of SMCC. After 4 h, the activated film samples were immersed in a 0.2*M*, pH 10, buffered sodium bicarbonate solution containing 0.1 μg/mL of cysteine-terminated CGYIGSR for 24 h at RT.[30] The film samples were rinsed five times each with the buffer solution, dilute hydrochloric acid, water, and THF, and then dried under vacuum.

Radioactive labeling of tyrosine

Tyrosine (Y) of GYIGSR was labeled as previously described[29] with radioactive iodine (^{125}I). Briefly, 2 mg of CGY-

IGSR was dissolved in 5 mL of a pH 11 buffer containing 20 m*M* sodium phosphate and 0.15*M* sodium chloride and then 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 FEP-X as described above and FEP-*X*-peptide films were rinsed with 10 m*M* sodium iodide to desorb any trace ^{125}I before counting by scintillation.

Hippocampal neuron–fluoropolymer interaction

FEP-X-peptide films and controls (FEP, FEP-C, and FEP-X) were immersed in 70% ethanol for 1 h and then rinsed four times with sterile distilled water before air-drying. Positive control surfaces were prepared by coating glass coverslips with 1 mL of an aqueous 1 mg/mL solution of poly(L-lysine) (PLL; Sigma, MW = 37,000 g/mol) for 24 h at 37°C and then with 10 μL of a 1 mg/mL aqueous solution of laminin (Gibco) for 2 h at 37°C. PLL-laminin–coated glass coverslips were rinsed with sterile, distilled water and air-dried prior to plating the hippocampal neurons. Embryonic day 18 (E18) mouse hippocampal neurons were isolated as previously described[31] by dissociation with papain (Worthington Biochemical) and DNase (Sigma) for 30 min and mechanical trituration in calcium-free HBSS. Then, 1 mL of hippocampal neurons was plated at 1×10^6 cells/mL in serum-free medium (SFM) on each film sample (or 2.5×10^5 cells/cm^2). The SFM was made with 2 mL B27 supplement, 100 mg chicken egg albumin (Sigma), 10 mg pyruvic acid (Sigma), 1 mL glutamine (Sigma), and 1 mL penicillin–streptomycin (Gibco) (10,000 U/mL and 10,000 μg/mL, respectively) in 100 mL neurobasal medium. The antimitotic agent, fluorodeoxyuridine/uridine (Sigma), was added after 12 h. The cells were incubated at 37°C in 5% CO_2 for 4 days. The cell–material interaction was assessed after 1 and 4 days in terms of the number and length of extended neurites. At each time point, 50 cells were chosen at random and the number of neurites per cell and the number of cells having neurites longer than one cell body were quantified. Phase contrast micrographs were taken after 1 and 4 days. To assess cell viability at 4 days samples were incubated for 30 min at 37°C with 150 μl of stock viability assay solution (20 μL ethidium bromide and 5 μL calcein AM in 10 mL PBS; Molecular Probes, Eugene, OR) and examined under fluorescent filters of the optical microscope.

Statistics

For every surface, on which 50 cells were evaluated, triplicate experimental data sets were subjected to statistical analysis using SAS (SAS Institute, Cary, NC) on an SGI Challenge L Unix-based computer system. One-way analysis of variance (ANOVA) assuming a constant variance and a 95% confidence interval was used to determine statistical differences of various data sets. Results are reported as the mean ± standard error of the mean, and statistically significant differences are labeled in the figures with an asterisk.

RESULTS

Reduction of FEP to FEP-C and subsequent functionalization to FEP-X

Poly(terafluoroethylene-*co*-hexafluoropropylene) film samples were surface modified to introduce hydroxyl[27] or carboxylic acid[26] functionalities and subsequently labeled with trichloroacetyl chloride or thallous ethoxide, respectively, or coupled with cell adhesive peptides GYIGSR, GRGDS, and SIKVAV (cf. Fig. 1). Tables I and II summarize the advancing (θ_A) and

TABLE I
Water Contact Angle Data of Surface-Modified FEP Film Samples

Film Type	Contact Angle ($^\circ\theta_A/^\circ\theta_R$)	Contact Angle ($^\circ\theta_A/^\circ\theta_R$)
Clean FEP	120 ± 2/101 ± 2	
	Reduced for 20 min at –78°C	Reduced for 3 h at 25°C
FEP-C	50 ± 2/19 ± 1	52 ± 4/24 ± 3
Hydroxyl-functionalized films		
FEP-CH_XOH	58 ± 4/25 ± 3	58 ± 4/23 ± 4
FEP-CH_XO-CO-CCl_3	91 ± 2/58 ± 4	90 ± 1/53 ± 3
FEP-CH_XO-tresyl-GYIGSR	66 ± 4/23 ± 4	72 ± 3/16 ± 3
FEP-CH_XO-tresyl-GRGDS	52 ± 3/14 ± 3	64 ± 3/31 ± 5
FEP-CH_XO-tresyl-SIKVAV	72 ± 3/18 ± 3	68 ± 3/15 ± 2
Carboxylic acid–functionalized films		
FEP-COOH	96 ± 4/44 ± 4	91 ± 4/53 ± 3
FEP-COO-Tl		
FEP-COO-tresyl-GYIGSR	85 ± 3/38 ± 2	80 ± 3/23 ± 2
FEP-COO-tresyl-GRGDS	83 ± 3/40 ± 3	82 ± 3/19 ± 2
FEP-COO-tresyl-SIKVAV	74 ± 3/31 ± 3	77 ± 3/30 ± 3

Data represent the average ± standard deviation of the mean; n = 5/sample × 3 samples/film type.

TABLE II
XPS Data Showing Atomic Composition of FEP and Surface-Modified Films at Takeoff Angle of 20° between Sample and Detector

Film Type	Atomic Composition (%)	Atomic Composition (%)
Clean FEP	$C_{31.1}F_{68.9}$	
	Reduced for 20 min at −78°C	Reduced for 3 h at 25°C
FEP-C	$C_{91.7}F_{4.4}O_{3.8}$	$C_{84.7}F_{9.7}O_{5.2}$
Hydroxyl-functionalized films		
FEP-CH_XOH	$C_{59.7}F_{22.1}O_{18.2}$	$C_{74.5}F_{3.3}O_{22.2}$
FEP-CH_XO-CO-CCl_3	$C_{62.2}F_{13.5}O_{16.3}Cl_{8.0}$	$C_{68.0}F_{2.6}O_{17.1}Cl_{12.4}$
FEP-CH_XO-tresyl-GYIGSR	$C_{75.2}F_{3.5}O_{17.1}N_{3.4}$	$C_{72.9}F_{2.7}O_{18.6}N_{4.5}$
FEP-CH_XO-tresyl-GRGDS	$C_{73.2}F_{3.0}O_{20.0}N_{2.8}$	$C_{73.4}F_{4.1}O_{19.1}N_{2.5}$
FEP-CH_XO-tresyl-SIKVAV	$C_{76.2}F_{3.3}O_{17.7}N_{2.1}$	$C_{70.3}F_{3.0}O_{19.7}N_{6.1}$
Carboxylic acid–functionalized films		
FEP-COOH	$C_{33.3}F_{64.1}O_{2.6}$	$C_{31.5}F_{67.0}O_{1.5}$
FEP-COO-Tl	$C_{35.3}F_{60.0}O_{3.8}Tl_{0.5}$	$C_{37.2}F_{56.5}O_{4.6}Tl_{1.7}$
FEP-COO-tresyl-GYIGSR	$C_{49.4}F_{40.3}O_{7.8}N_{2.2}$	$C_{53.2}F_{35.1}O_{8.6}N_{3.0}$
FEP-COO-tresyl-GRGDS	$C_{46.9}F_{42.1}O_{8.5}N_{2.3}$	$C_{45.0}F_{45.7}O_{7.0}N_{2.0}$
FEP-COO-tresyl-SIKVAV	$C_{57.8}F_{29.2}O_{10.2}N_{2.7}$	$C_{58.4}F_{28.3}O_{9.6}N_{3.6}$

receding (θ_R) water contact angles and the XPS atomic composition data, respectively.

The FEP film samples were first reduced with sodium naphthalide in THF (to FEP-C) at the two reaction conditions (−78°C and 25°C) to determine if surface chemistry could be modified independently of surface morphology. Scanning electron micrographs for FEP and FEP-C were identical and smooth, having no topographical features. Since no differences in surface morphology were observed between FEP and FEP-C (at both times/temperatures) by either SEM or AFM (data not shown), we determined that surface chemistry alone accounted for the differences observed in the subsequent surface modifications and interactions with hippocampal neurons.

The FEP-C film samples were modified to produce hydroxyl or carboxylic acid functionalized surfaces by either hydroboration/oxidation or oxidation, respectively. The large decrease in both advancing and receding contact angles from FEP (120°/101°) to FEP-C (50°/19°) and then to FEP-CH_XOH (58°/25°) reflects the increased hydrophilic nature of the modified surfaces. FEP-CH_XOH is apparently less hydrophilic than FEP-C, likely a result of the depleted surface fluorine concentration of FEP-C. Similarly, FEP-COOH (96°/44°) is more hydrophilic than FEP, yet less than FEP-C, owing to the oxidative removal of the reduced layer,[26] producing carboxylic acid functionality at the fluoropolymer chain ends. The XPS data corroborate the contact angle data, demonstrating an increase in oxygen functionality in FEP-CH_XOH ($C_{59.7}F_{22.1}O_{18.2}$) and FEP-COOH ($C_{33.3}F_{64.1}O_{2.6}$) with respect to FEP ($C_{31.1}F_{68.9}$) and FEP-C ($C_{91.7}F_{4.4}O_{3.8}$). ATR-FTIR spectra confirmed the presence of the hydroxyl functional group with a peak at 3660 cm^{-1}; the carboxylic acid functional group was not easily detected by ATR-FTIR owing to its low surface concentration and the inherent surface selectivity of this reaction.

To determine the percentage of oxygen functionality attributed to either hydroxyl or carboxylic acid functional groups, the hydroxyl-functionalized surfaces were labeled with trichloroacetyl chloride, while the carboxylic acid functionalized surfaces were labeled with thallous ethoxide. The XPS and contact angle data in Tables I and II reflect the presence of chlorine [on FEP-CHO-C(O)CCl_3] and thallium (on FEP-COO-Tl), indicating the presence of alcohol and carboxylic acid, respectively. ATR-FTIR data confirm that covalent bonds were formed with the disappearance of the hydroxyl stretch of FEP-CH_XOH and the appearance of a carbonyl stretch at 1740 cm^{-1} of the newly formed ester.

Peptide coupling to functionalized FEP (FEP-X-peptide)

The hydroxyl and carboxylic acid functional groups on FEP film surfaces serve as reactive handles for further chemical modification. Using tresyl chloride as the coupling reagent, three peptides were coupled to the functionalized surfaces, the data of which are summarized in Tables I and II. The advancing contact angles of the hydroxyl functionalized surfaces increased after peptide coupling, whereas the receding contact angles remained the same or showed a slight decrease. The increase in hysteresis reflects the chemical heterogeneity of the peptide-modified surfaces.[32] The contact angles of the peptide-modified surfaces were for the most part, indistinguishable from each other owing to their similar composition. The XPS data confirmed that the change in contact angle data resulted from peptide modification. For all peptides, the presence of nitrogen, the increase in carbon, and the decrease in fluorine confirmed the peptide cou-

pling reaction. While there were slight differences in the amount of nitrogen observed between the different peptides, these differences were not seen for carbon and fluorine, indicating that a similar amount of GYIGSR, GRGDS, and SIKVAV was coupled to the hydroxyl-functionalized surfaces.

Peptide modification of the carboxylic acid–functionalized FEP surfaces was confirmed by decreased advancing and receding water contact angles with respect to FEP-COOH, and the presence of nitrogen, the relative increase in carbon, and relative decrease in fluorine. Unlike the hydroxyl-functionalized surfaces where the differences among peptides were insignificant, the XPS and contact angle data implied a greater concentration of SIKVAV than GYIGSR and GRGDS incorporated onto the FEP-COOH surface. By comparing the nitrogen concentration for peptide-modified FEP-X films that were reduced under the two conditions, –78°C, and 25°C, a comparison of the relative amount of peptide introduced to the surface could be made. The XPS data indicated that greater amounts of GYIGSR and SIKVAV were introduced to films reduced at 25°C than those reduced at –78°C. The data for GRGDS showed an insignificant difference for films reduced under these two reaction conditions.

Three reagents, tresyl, TSU, and SMCC, were compared quantitatively by scintillation counting and XPS in the coupling of GYIGSR to hydroxyl- and carboxylic acid–functionalized surfaces. (A cysteine-terminated peptide, i.e., CGYIGSR, was used for SMCC coupling.) As summarized in Table III, tresyl-activated FEP-CH_XOH (reduced at 3 h and 25°C) showed the highest concentration of GYIGSR with 150 ± 60 fmol/cm^2. Tresyl-activated FEP-COOH resulted in a lower (<8 fmol/cm^2) coupling yield, likely a result of fewer surface carboxylic acids relative to surface hydroxyl groups. With SMCC coupling, the concentration of GYIGSR bound to the surface was between 5 and 30 fmol/cm^2, whereas with TSU, it was between 7 and 80 fmol/cm^2.

The XPS data summarized as the nitrogen to fluorine surface concentration ratio in Table III indicate that the greatest amount of peptide was introduced with tresyl chloride coupling to the hydroxyl-functionalized surfaces, thereby confirming the data obtained from radiolabeling experiments. Together, the data obtained from radiolabeling experiments and XPS analysis indicate that both the surface functional group (i.e., hydroxyl vs. carboxylic acid) and the coupling agent affect the amount of peptide introduced. Perhaps the greatest contribution to the amount of peptide incorporated was the surface functional group, with the FEP-CH_XOH surface (prepared from FEP-C at 25°C) repeatedly having the most peptide incorporated. Of the three coupling agents, tresyl chloride seemed to be most effective at coupling the peptide to the functionalized surface, followed by TSU and SMCC.

Hippocampal neuron–fluoropolymer interaction

Hippocampal neurons isolated from embryonic day 18 rats were plated from serum-free neurobasal medium onto the peptide-modified film samples. After 1 and 4 days, the number of neurites extending per cell of 50 cells were counted, with the means and standard error of the mean summarized in Figure 3. The positive control of PLL/laminin-coated glass coverslips had 2.44 and 2.80 neurites/cell after 1 and 4 days, respectively, while the unmodified FEP control had only 0.96 and 0.06 neurites/cell at the same time points. Since hippocampal neurons are anchorage-dependent cells, they cannot survive on FEP. The hy-

TABLE III
Comparison of Surface Peptide Concentration on Peptide-Modified FEP-X Films (FEP-X-Peptide, Where X Is Either Hydroxyl CH_XO or Carboxylic Acid COO) as Determined from γ-Radiation Count of Radiolabeled GYIGSR Peptide, and Surface Nitrogen to Fluorine Atomic Ratio, as Determined from XPS Data

Coupling Agent	Peptide Concentration (fmol/cm^2)		Nitrogen/Fluorine XPS Atomic Ratio	
FEP-C Reduction	–78°C, 20 min	25°C, 180 min	–78°C, 20 min	25°C, 180 min
Tresyl chloride				
FEP-CH_XO-GYIGSR	6.6 ± 1.8	150 ± 60	0.06 ± 0.02	0.80 ± 0.07
FEP-COO-GYIGSR	7.9 ± 1.5	7.8 ± 1.9	0.03 ± 0.00	0.03 ± 0.01
SMCC				
FEP-CH_XO-GYIGSR	15 ± 13	32 ± 6	0.20 ± 0.08	0.50 ± 0.20
FEP-COO-GYIGSR	4.8 ± 1.8	6.0 ± 2.3	0.03 ± 0.00	0.04 ± 0.01
TSU				
FEP-CH_XO-GYIGSR	11 ± 8.3	81 ± 20	0.07 ± 0.04	0.20 ± 0.10
FEP-COO-GYIGSR	7.0 ± 2.0	22 ± 15	0.03 ± 0.01	0.02 ± 0.01

The GYIGSR peptides were coupled to FEP-X using tresyl chloride, SMCC, or TSU. The means ± standard errors of the mean were tabulated.

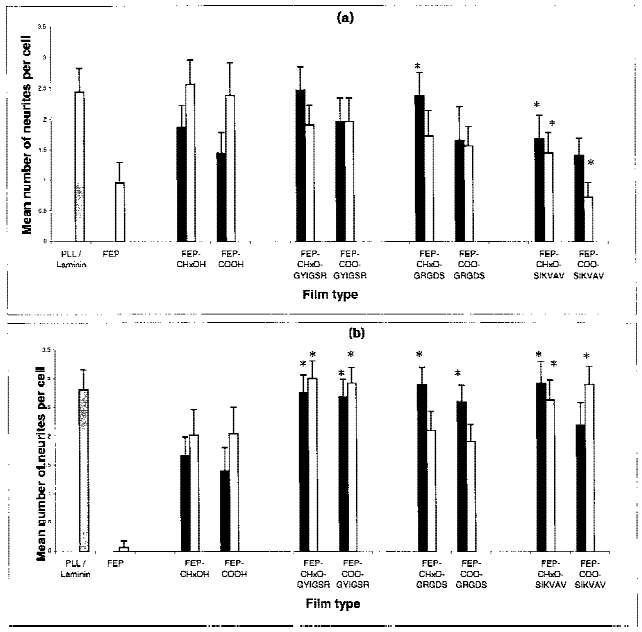

Figure 3. The number of neurites per cell (averaged over 50 cells) were compared using embryonic day 18 hippocampal neurons cultured in serum-free medium on PLL/laminin-coated glass coverslips, FEP, and FEP-X-peptide (X = COO or CH_xO; peptide = GYIGSR, GRGDS, SIKVAV) surfaces after (a) 1 day and (b) 4 days of incubation. Surfaces were prepared from FEP-C by reducing FEP for either 20 min, −78°C (■) or 3 h, 25°C (□). The mean number of neurites per cell with the standard error of the mean was reported for 50 cells, repeated three separate times. *Statistically significant differences in data (calculated using one-way ANOVA with $p < 0.05$) with respect to FEP-X controls of the same condition.

droxyl- and carboxylic acid–functionalized surfaces served as additional controls for the peptide-modified surfaces. For films reduced at −78°C, the peptides GYIGSR and GRGDS on FEP-CH_XOH showed greater interaction with the hippocampal neurons than did SIKVAV and underivatized FEP-CH_XOH at 1 day. By 4 days the peptide-modified surfaces were indistinguishable from each other and the positive control, but were statistically significantly different ($p < 0.05$) from the FEP-CH_XOH control. For films reduced at 25°C, none of the FEP-CH_XOH-peptide films were distinguishable at 1 day; however, at 4 days; the greatest neural cell interaction was observed on GYIGSR- and SIKVAV-modified surfaces.

For films reduced at −78°C, the peptide-modified carboxylic acid–functionalized film surfaces showed either the same or slightly less hippocampal neuron interaction than similar FEP-CH_XOH peptide-modified film surfaces. The peptide-modified FEP-COOH film surfaces were indistinguishable from each other and the control FEP-COOH at 1 day; however, after 4 days; the peptide-modified film surfaces were statistically different from FEP–COOH, yet indistinguishable from the positive control (PLL/laminin). For films reduced at 25°C, GYIGSR- and GRGDS-modified film surfaces showed the greatest hippocampal neuron interaction at 1 day; however, by 4 days, GYIGSR and SIKVAV showed the greatest interaction and were distinguishable from GRGDS and FEP-COOH control film samples. Interestingly, the number of neurites decreased between 1 and 4 days on all control FEP films samples (i.e., FEP, FEP-CH_XOH, and FEP-COOH), whereas the number of neurites increased on all peptide-modified surfaces, indicating these surfaces enhance prolonged hippocampal neuron interaction.

Since neurite length is indicative of the extent of interaction of the cell with the substrate, the functionalized surfaces were compared with control surfaces in terms of the length of the neurites, as shown in Figure 4. Of the 50 random cells counted, the number of cells having at least one neurite longer than the cell body was determined. FEP, FEP-CH_XOH, and FEP-COOH controls had a lower percentage of cells with longer neurites than either GYIGSR or SIKVAV-functionalized surfaces after 4 days, thereby further demonstrating the enhanced interaction of hippocampal neurons with peptide-functionalized surfaces over control surfaces. Figure 5 includes representative optical fluorescent micrographs taken at 4 days of FEP-X-peptide, FEP, and PLL/laminin control surfaces. The relative lengths of neurites confirmed the enhanced interaction on peptide-modified film samples.

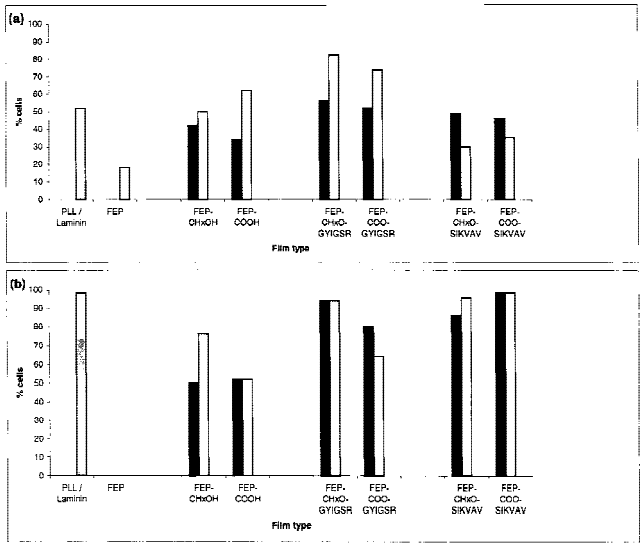

Figure 4. The percentage of cells with neurites longer than one cell body length were compared for 50 embryonic day 18 hippocampal neurons cultured in serum-free medium on PLL/laminin-coated glass coverslips, FEP, and FEP-X-peptide (X = COO or CH_xO; peptide = GYIGSR, GRGDS, SIKVAV) surfaces after (a) 1 day and (b) 4 days of plating. Surfaces were prepared from FEP-C by reducing FEP for either 20 min, −78°C (■) or 3 h, 25°C (□).

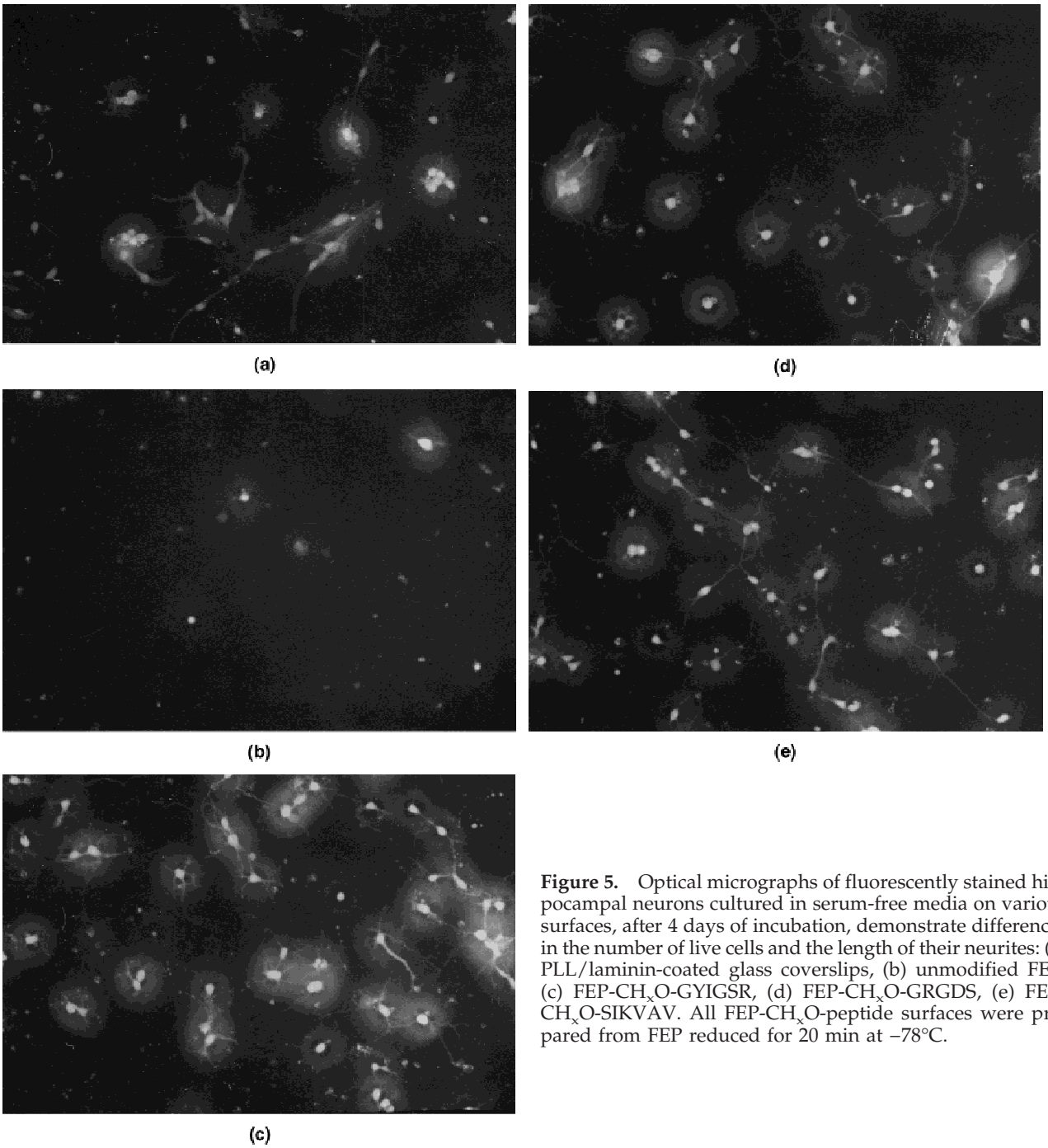

Figure 5. Optical micrographs of fluorescently stained hippocampal neurons cultured in serum-free media on various surfaces, after 4 days of incubation, demonstrate differences in the number of live cells and the length of their neurites: (a) PLL/laminin-coated glass coverslips, (b) unmodified FEP, (c) FEP-CH_xO-GYIGSR, (d) FEP-CH_xO-GRGDS, (e) FEP-CH_xO-SIKVAV. All FEP-CH_xO-peptide surfaces were prepared from FEP reduced for 20 min at −78°C.

Of the three peptides, SIKVAV-functionalized surfaces had the greatest proportion of longer neurites.

DISCUSSION

The reduction of FEP to FEP-C followed by hydroboration/oxidation to FEP-CH_XOH or oxidation to FEP-COOH was used as a means to introduce peptides to the normally chemically inert FEP surfaces. FEP-C samples readily oxidized when exposed to air, requiring that care be taken to limit its contact with air.

The reduction of FEP to FEP-C was conducted under two reaction conditions to determine whether subsequent surface modification would be affected. The number of hydroxyl groups per FEP repeat units were estimated from XPS data; the ratio of OH:CF_2CF_2 repeat units was 1:1 and 1:0.1, and similarly, the ratio of COOH:FEP repeat units were estimated at 1:18 and 1:30 for samples reduced −78°C and 25°C, respectively. The greater percentage of hydroxyl per FEP repeat unit versus carboxylic acid per FEP repeat unit

reflects the surface depletion of fluorine groups in the top ~10 Å of the FEP-CH_XOH film surfaces. The FEP-CH_XOH surfaces were labeled with trichloroacetyl chloride, producing the trichloroacetate FEP-CH_XO-$C(O)CCl_3$ to determine the percentage of oxygen functionality attributable to hydroxyl. Similarly, the FEP-COOH surfaces were labeled with thallium ethoxide, producing the thallium carboxylate salt FEP-COO-Tl, to determine the percentage of oxygen functionally attributable to carboxylic acid.[33] The ratios of chlorine to oxygen determined via XPS allowed us to conclude that 33% and 47% of elemental oxygen were present as hydroxyl functionality for reductions at −78°C and 25°C, respectively. Similarly, from the ratios of thallium to oxygen, 42% and 74% of elemental oxygen were present as carboxylic acid for reductions at −78°C and 25°C, respectively.

The yields for the peptide-coupling reactions were calculated relative to the number of hydroxyl and carboxylic acid functional groups present on FEP-CH_XOH and FEP-COOH, respectively, as shown in Equation (1).

$$\text{Yield} = \text{No. peptides}/\text{No. functional groups} \quad (1)$$

where No. peptides was calculated from the XPS nitrogen composition, taking into account the number of nitrogen atoms per peptide, and the No. functional groups was calculated from the XPS oxygen composition after subtracting the oxygen concentration associated with the peptide.

While there was some variability in the data, the yields for peptide coupling were consistently higher on FEP-COOH–modified surfaces than FEP-CH_XOH surfaces. For film surfaces reduced at −78°C, FEP-COO-peptide had an average yield of 35% (between 22% and 52%), whereas FEP-CH_XO-peptide had an average yield of 7% (between 5% and 12%). Similarly, for film surfaces reduced at 25°C, FEP-COO-peptide had an average yield of 26% (between 15% and 51%) whereas FEP-CH_XO-peptide had an average yield of 8% (between 2% and 25%). The yields for surfaces modified at −78°C were consistently higher than those modified at 25°C, indicating that the chemistry was more easily controlled at the lower time and temperature.

Although the yields are higher on FEP-COO-peptide–coupled surfaces than on FEP-CH_XO-peptide surfaces, the amount of peptide incorporated on FEP-CH_XOH surfaces is greater as shown by the radiolabeled GYIGSR data (cf. Table III) because there are more hydroxyl than carboxylic acid groups available. The amount of peptide covalently bound to FEP-functionalized surfaces falls within the range previously reported for peptide-surface modification of hydroxyl functionalized poly(ethylene terephthalate) and hydroxyl-functionalized poly(tetrafluoroethylene).[34]

Peptide-modified surfaces were prepared in an attempt to mimic the interaction normally observed between neurons and laminin to determine whether the peptide-functionalized surfaces could serve as replacement substrates in CNS injury repair strategies. Hippocampal neurons were plated on peptide-modified and control film samples to assess the impact of the peptides on CNS neuron interaction. By using E18 mice, a relatively homogeneous population of neurons were dissociated.[35,36] Fluorodeoxyuridine/uridine was added to the medium to inhibit the proliferation of any mitotically active cells such as astrocytes that were plated with the neurons. In an attempt to determine an optimal surface for further investigation with CNS neurons, two reduced surfaces (i.e., −78°C and 25°C), two reactive handles (i.e., hydroxyl and carboxylic acid functionalities) and three peptides (i.e., GYIGSR, GRGDS, and SIKVAV) were investigated in terms of the hippocampal neuron interaction. As was expected, unmodified FEP supported neither cell adhesion nor neurite outgrowth. Perhaps unexpectedly, FEP-CH_XOH and FEP-COOH both supported cell adhesion and neurite outgrowth after 1 day; however, the number of neurites decreased after 4 days. Only FEP, FEP-CH_XOH, and FEP-COOH control film surfaces showed a decrease in the number of neurites over time, indicating the importance of the peptide on these modified surfaces.

For all peptide-modified surfaces, the hippocampal neuron interaction was either greater or equivalent on FEP-CH_XO-peptide surfaces than the corresponding FEP-COO-peptide surfaces. This corresponds to the radiolabeled data that show more peptide on FEP-CH_XOH than on FEP-COOH surfaces. FEP-CH_XO-peptide surfaces that had been prepared from FEP-C at −78°C showed hippocampal neuron interaction greater than or equivalent to those prepared from FEP-C at 25°C. While it was difficult to differentiate among GYIGSR, GRGDS, and SIKVAV-functionalized surfaces, GYIGSR and GRGDS showed greater interaction than SIKVAV after 1 day of plating which may indicate that SIKVAV is less adhesive to neurons than the other peptides. While SIKVAV-modified surfaces were expected to provide the greatest neurite interaction (i.e., number and length of neurites), this effect was not observed until 4 days after plating. The representative optical micrographs included in Figure 5 demonstrate the qualitative differences observed between GYIGSR, GRGDS, and SIKVAV surfaces, where GYIGSR had the greatest number of adherent cells and SIKVAV had the greatest proportion of longer neurites. The hippocampal neurons responded equivalently to PLL-laminin and all peptide-modified surfaces alike, indicating that the peptide-modified surfaces sufficiently mimic the interaction of laminin and hippocampal neurons.

CONCLUSIONS

We demonstrated that chemical surface modification of fluoropolymers, specifically FEP, could be used to control the concentration of cell-adhesive peptides introduced without affecting the surface topography. By isolating surface chemistry from surface morphology, the relative importance of surface functionality on the hippocampal neuron cellular response was assessed. Of all the surfaces analyzed, the greatest concentration of peptide was achieved with tresyl chloride activation of hydroxyl-functionalized FEP that had been reduced at 25°C for 3 h. After 4 days of incubation with hippocampal neurons, the peptide-functionalized surfaces were indistinguishable from each other and the positive PLL/laminin control yet distinguishable from the controls—FEP, FEP-CH_XOH, and FEP–COOH—thereby underlying the importance of substrate adhesion molecules for an enhanced cellular response. Despite the differences in surface peptide composition and concentration, the hippocampal response was not easily differentiated. While initially unexpected, this result agrees with recent findings by Condic and Letourneau,[37] who demonstrated that for neurons, total integrin expression decreases with decreased ligand (i.e., laminin) availability, yet surface integrin expression increases. Thus, despite a lower concentration of available ligands, neuronal cell adhesion and neurite outgrowth increase. To better understand the differences between YIGSR-, IKVAV-, and RGD-functionalized surfaces in terms of the hippocampal neuron response, future studies will probe cell adhesion and total integrin expression. To further enhance the cellular response, mixed peptide surfaces will be prepared and compared to monofunctionalized peptide and PLL/laminin surfaces in terms of cell adhesion, neurite outgrowth, and total integrin expression.

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