Micropatterning gradients and controlling surface densities of photoactivatable biomolecules on self-assembled monolayers of oligo(ethylene glycol) alkanethiolates

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Background: Bioactive molecules that are covalently immobilized in patterns on surfaces have previously been used to control or study cell behavior such as adhesion, spreading, movement or differentiation. Photoimmobilization techniques can be used, however, to control not only the spatial pattern of molecular immobilization, termed the micropattern, but also the surface density of the molecules – a characteristic that has not been previously exploited.

Results: Oligopeptides containing the bioactive Arg–Gly–Asp cell-adhesion sequence were immobilized upon self-assembled monolayers of an oligo(ethylene glycol) alkanethiolate in patterns that were visualized and quantified by autoradiography. The amount and pattern of immobilized peptide were controlled by manipulating the exposure of the sample to a UV lamp or a laser beam. Patterns of peptides, including a density gradient, were used to control the location and number of adherent cells and also the cell shape.

Conclusions: A photoimmobilization technique for decorating surfaces with micropatterns that consist of variable densities of bioactive molecules is described. The efficacy of the patterns for controlling cell adhesion and shape has been demonstrated. This technique is useful for the study of cell behavior on micropatterns.

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Introduction

The covalent immobilization of bioactive molecules to surfaces has been used to control or study aspects of cell behavior such as adhesion, spreading, movement or differentiation [1–4]. The positional control of biomolecules by covalent or physicochemical immobilization of biomolecules (i.e. micropatterning) has been especially useful for experiments such as those used in the study of axon growth and pathfinding or those used to study the effects of cell shape on cellular function [5–8]. Micropatterning studies could be even more useful if both the position and *surface density* of biomolecules could be simultaneously controlled.

Photoimmobilization methods are useful for generating micropatterns. One advantage of these methods is that sophisticated photolithographic mask technology, developed for producing micropatterns on silicon computer chips, can be employed. Masks that are opaque except for micropatterns that transmit light can be produced. A mask is placed over a surface that is coated with photoactive molecules and it is exposed to light so that photoimmobilization can occur only underneath the micropatterned area of the mask. Another advantage of photochemical approaches is that the surface is altered only when a molecule is successfully immobilized. Furthermore, photoimmobilization can be performed from aqueous solution under mild conditions, reducing the potential for the denaturation of proteins.

Benzophenone is a particularly useful group for the photoactivation of biomolecules (for review, see [9,10]). It preferentially reacts with C–H bonds, even in water or in the presence of nucleophiles, making it a versatile reactant. Since it can be activated at relatively long wavelengths (>300 nm) where proteins do not absorb, only minimal damage occurs upon irradiation. Benzophenone is also reversibly excitable: in the absence of suitable reactants it relaxes back to its ground state. Benzophenone-containing compounds are stable to prolonged storage and exposure to ambient light, and are not affected by many of the reaction conditions that are commonly used for peptide synthesis or for modifying proteins (e.g. succinimidyl ester activation).

We have recently reported a photoimmobilization technique for creating patterns, including gradients, of molecules on surfaces [11]. In this approach, a model protein, the naturally fluorescent molecule phycoerythrin, was

conjugated with benzophenone. The conjugate was applied to polystyrene surfaces and irradiated with a focused 325 nm wavelength laser (4 mW; HeCd). The pattern and density of protein deposition were controlled by manipulating the pattern and duration of exposure of the specimen to the laser. This exposure was controlled by moving the specimen with a programmable, motorized stage. After irradiation, the surfaces were washed and the patterns were visualized using fluorescence microscopy.

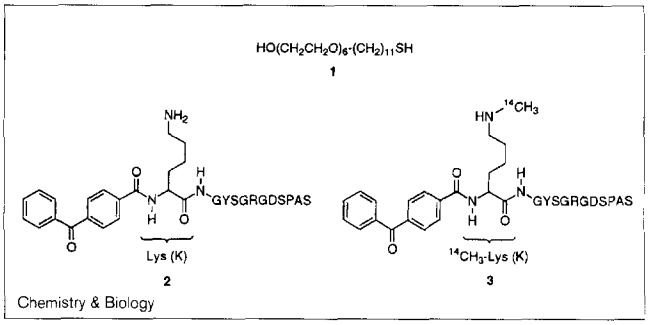
Studies of cells interacting with surface-immobilized biomolecules, including micropatterns, are complicated by the presence of nonspecifically adsorbed molecules. These can be photoactivatable molecules that are adsorbed without becoming covalently immobilized, or biomolecules that originate from cells or the cell culture medium. The adsorbed molecules can provide signals that compete with the covalently attached molecules and complicate the experimental interpretation. One approach for minimizing nonspecific protein adsorption has been to use hydrophilic materials such as hydrogels or poly(ethylene glycol) (for review, see [12]). Furthermore, the hydrophilic character of these materials is likely to minimize protein denaturation, a variable that affects cell–protein interactions [13]. A facile technique for producing surfaces that are uniformly coated with ethylene glycol is to create self-assembled monolayers of oligo(ethylene glycol) alkanethiolates on gold-coated materials (for review, see [14,15]). Alkanethiolates spontaneously adsorb to gold and form self-assembled monolayers that are stable in solution, even in the presence of proteins. A short oligo(ethylene glycol) segment that is grafted to the alkane does not prevent monolayer formation.

Previous studies have demonstrated the use of photoimmobilization techniques for making patterns of peptides on a variety of materials using photolithographically generated masks but they have not addressed the issues of controlling the peptide density or making patterns that contain variable peptide densities [8,10,16]. Here, we demonstrate a photoimmobilization technique for depositing biomolecules from aqueous solution in micrometer-scale patterns that contain different densities of immobilized molecules and we show that these techniques can be used to control the adhesion response of cells.

Results and discussion

Oligo(ethylene glycol)-coated surfaces were prepared by coating gold-covered silicone wafers with self-assembled monolayers of hexa(ethylene glycol)-undecanethiolate (HEG-UT; **1**, Figure 1). These surfaces resisted the adhesion of a variety of cell types, such as human foreskin fibroblasts (HFF), rat hepatocytes, mouse fibroblasts (3T3 cell line), or pheochromocytoma cells (PC12 cell line), when seeded at concentrations of up to 15,000 cells/cm^2 in a serum-free medium (data not shown). After a 24 h incubation with cells resting on the surfaces, only a few cells (typically less than 10 cells/cm^2) were adherent.

Figure 1

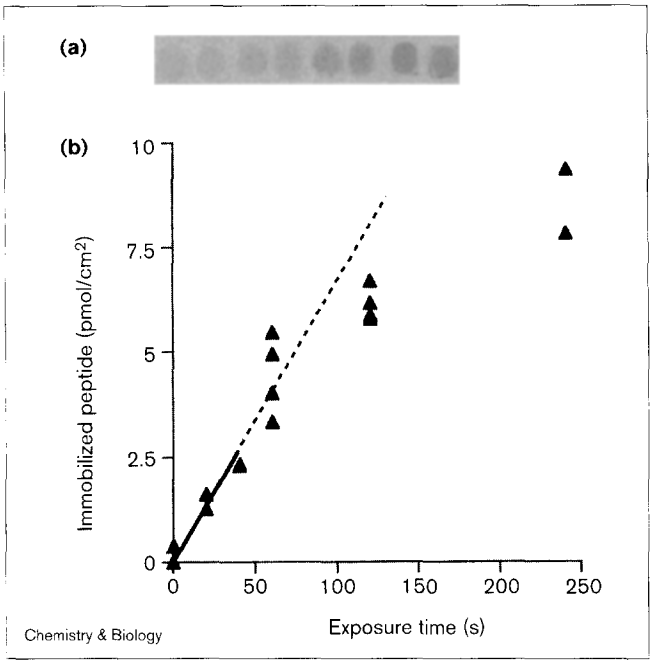


The compounds that are used in this paper to produce self-assembled monolayers and for photoimmobilization. **1**, hexa(ethylene glycol)-undecanethiolate (HEG-UT) is used to form self-assembled monolayers on gold surfaces; **2**, photoactivated RGD-containing peptide and **3**, radioactive label incorporated into **2**.

Photoactivatable peptides were attached to self-assembled monolayers of HEG-UT using either a laser or a UV lamp. A photoactivatable form of the peptide KGYSGRGDSPAS, which contains the bioactive Arg–Gly–Asp (RGD) sequence, was synthesized by reacting its amino terminus with an activated ester of benzophenone (**2**, Figure 1). A radiolabeled form of the photoactivatable peptide (**3**) was prepared by reductive amination of the ε-amino group of lysine with ^{14}C-formaldehyde. This material was used to quantitate the amounts of immobilized peptide. Self-assembled monolayers were covered with a drop of photoactivatable peptide solution (8 μM) and illuminated with UV light. Exposure to UV light for 10–230 s caused the immobilization of 1–10 pmol/cm^2 of **3** (Figure 2). Exposure to an estimated 4–20 J/mm^2 of 325 nm laser light (4 mW; HeCd) caused the immobilization of 0.6–2.2 pmol/cm^2 of peptide (Figure 3a,b). A linear relationship between the amount of light exposure and the amount of peptide immobilized was observed in both cases at peptide densities of less than 2.5 pmol/cm^2. The relationship between light exposure and immobilization became nonlinear when greater densities were immobilized using the UV lamp. This relationship was exploited to make patterns that had variable densities of peptides (see below). Pattern generation with the laser was more convenient than that with photolithographic techniques because time-consuming procedures for producing masks were unnecessary.

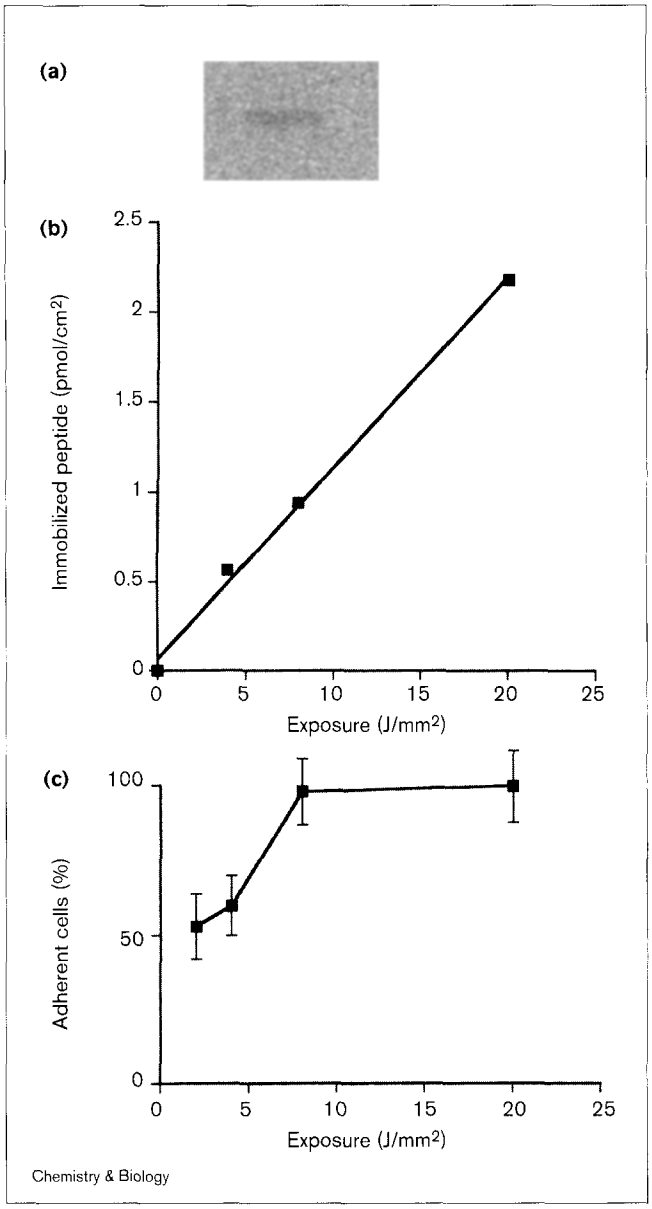
Cell adhesion was controlled by manipulating the immobilized density of RGD-containing peptide. Peptide (**2**)

Figure 2



Peptide immobilization by exposure to UV light. Peptide densities on the surfaces of self-assembled monolayers were proportional to exposure to UV light. **(a)** A digitized autoradiography image of duplicate self-assembled monolayer specimens (1 × 0.5 cm) with immobilized ^{14}C-labeled peptide. Specimen pairs (from left to right) were exposed to a UV-spectrum lamp for 20, 40, 60 or 120 s (0 s exposure not visible at this level of image contrast, not shown; peptide density is proportional to image darkness). **(b)** A plot of quantified images from two experiments shows a linear relationship between exposure time and peptide immobilization ($r^2 = 0.85$; $P < 0.05$, least squares regression). Peptides were immobilized from 8 μM aqueous solution onto self-assembled monolayers of HEG-UT.

Figure 3

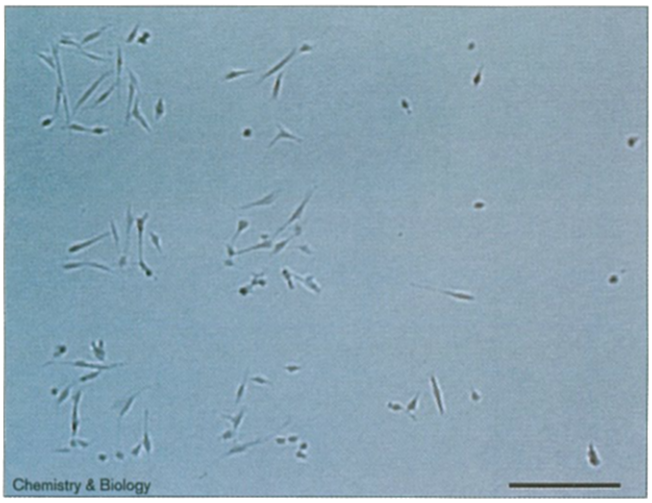


Laser-controlled peptide immobilization and the cell-adhesion response. **(a)** A digital autoradiography image of a rectangular pattern (4 × 1 mm) of immobilized peptide created by rastering a laser across a self-assembled monolayer of HEG-UT that is coated with photoactivatable radiolabeled peptide (325 nm). **(b)** The immobilized peptide density was linearly related to laser-beam exposure (least squares fit; $r^2 = 1.0$; $P < 0.05$, n = 1). Other conditions are as described in Figure 2. **(c)** The number of adherent cells was proportional to the immobilized peptide density until reaching a maximum at 1 pmol/cm^2 of peptide (8 J/mm^2 exposure). Data were taken before cells spread, 4 h after seeding. 100% = 172,000 cells/cm^2.

was immobilized in a pattern of four columns of three squares, 300 × 300 μm per square; each column contained a different peptide density. When cells were seeded at 15,000 cells/cm^2, enough cells (>75) typically were attached to each square in the first 4 h to essentially fill each square after spreading. The number of adherent cells increased as peptide density was increased up to 1.0 pmol/cm^2 but increased little more as peptide density was further increased (Figure 3c). When lower seeding densities were used (5000 cells/cm^2), few cells typically attached to squares containing less than 0.6 pmol/cm^2 of peptide (Figure 4).

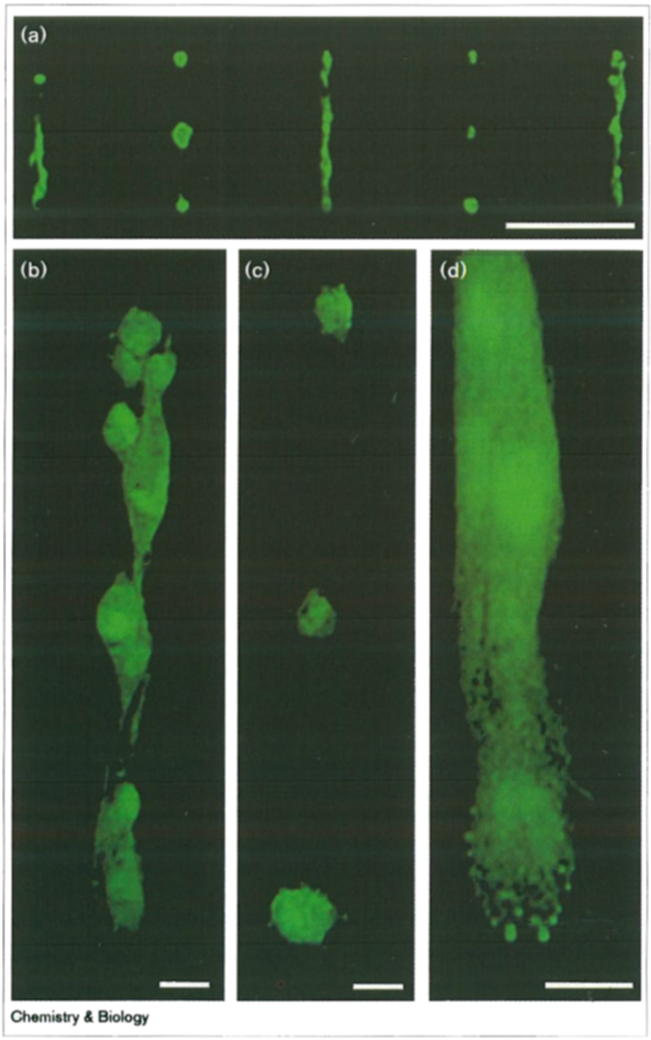
Cell shape, as well as the number or pattern of adherent cells, could be manipulated with these techniques by confining the cells to peptide patterns of lines or dots that were produced using a laser beam (Figure 5). Cells assumed an elongated shape on lines, but remained round on dots. Cells remained in this pattern in a serum-free medium for as long as a week without spreading beyond the boundaries of the lines or dots of peptides. It may be that the cells did not move from these patterns because the density or number of cells was too low to permit the accumulation of secreted surface-adsorbed factors. The control of cell density may therefore be an

Figure 4



Fibroblast adhesion to a pattern containing varying densities of RGD. Fibroblasts were seeded on a self-assembled monolayer patterned with three rows of squares (300 × 300 μm each) arranged in an array of four columns: from left to right, the squares in each column had 2.2, 1.0, 0.6 or 0.3 pmol/mm^2 of peptide (densities are based on the calibration shown in Figure 3; the last point is based on extrapolation). Cells fill the squares in the left-hand column, which contained the most peptide, but few cells were adherent to the squares in the right-hand column. Cells were seeded at a density of 5000 cells/cm^2 in a serum-free medium. The photomicrograph was taken 24 h after cell seeding. Scale bar = 300 μm.

Figure 5



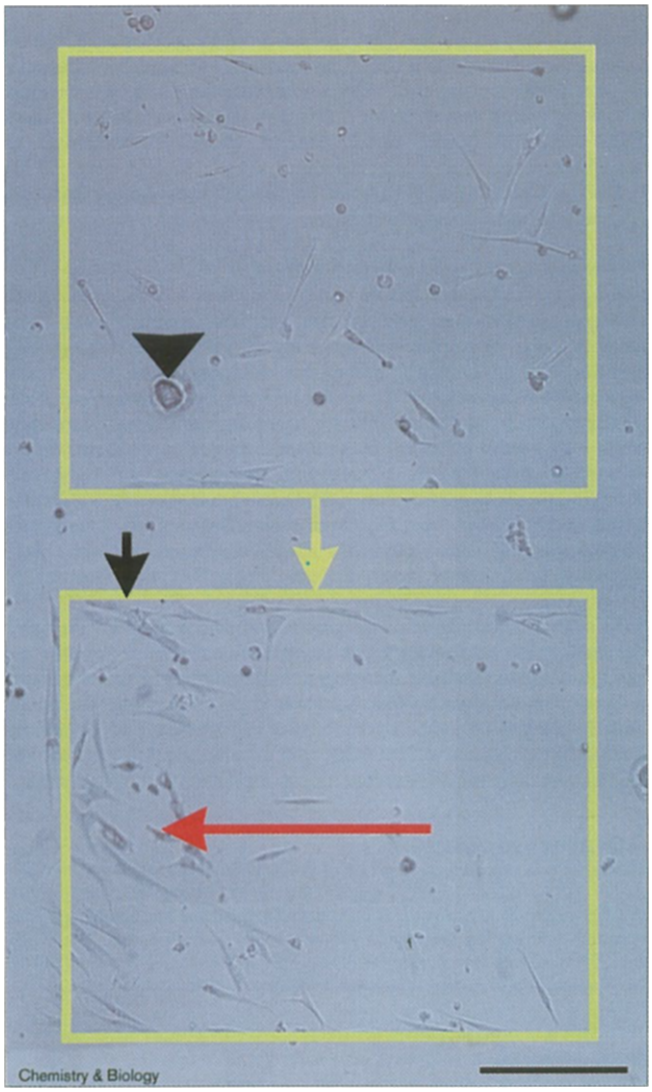
The control of cell shape using patterns of RGD-containing peptide. Fibroblasts were seeded on a pattern of RGD-containing peptides that were immobilized using a laser in 300 μm lines alternating with three 20 μm dots. **(a)** Fibroblasts occupied the pattern and typically assumed: **(b)** and **(d)** an elongated shape on lines or **(c)** a round shape on dots. Scale bar = 250 μm (a) or 10 μm (b, c, d). Cells were stained with fluorescein diacetate and photographed 24 h after seeding in a serum-free medium.

important strategy for studies involving the long-term maintenance of cells on patterned HEG-UT self-assembled monolayers. Initial cell-seeding densities were important for arranging cells in patterns: cells readily adhered and spread upon both patterned and unpatterned portions of HEG-UT self-assembled monolayers in a serum-free medium if high cell-seeding concentrations were used (>40,000 cells/cm^2, using HFFs or PC12s; data not shown).

Cells were also seeded on gradients of immobilized molecules that were generated with the laser-raster technique (Figure 6). A pattern was produced that contained two 1000 × 800 μm rectangles of RGD-containing peptides: one rectangle was a density gradient and the other rectangle was exposed to a uniform 4 J/mm^2 of light. The gradient was generated with a laser exposure ranging from 20 to 2 J/mm^2 by varying the laser speed from 50 to 500 μm/s in increments of 1.8 μm/s every 4 μm over a distance of 1000 μm. The exposure varied nonlinearly with distance: E = 1000/(50 + 0.45x) where x=distance in μm from the start of the rectangle and E = calculated exposure, J/mm^2. Thus, the first 170 μm of the gradient was exposed to more than 8 J/mm^2 of light, an exposure that was observed to elicit a near-maximal cell-adhesion response (Figure 3). Observation of the cells 24 h after seeding in a serum-free medium showed that cell adhesion was maximal in the region of the pattern that contained the most peptide (Figure 6). We are currently investigating the effect of gradients on cell movement.

It is likely that surface receptors on the cells interacted with the RGD-containing peptides via a specific receptor–ligand interaction; comparable immobilized ligand studies have shown that immobilized RGD or other peptides interact with their appropriate cell-surface receptors and that little cell-adhesion is mediated by immobilized

Figure 6



Cell adhesion to a density gradient of RGD-containing peptide. Fibroblasts were seeded on a laser-generated pattern of RGD-containing peptide. The upper portion (upper rectangle), with no gradient, was exposed to 4 J/mm^2 of light. The lower portion of the pattern (lower rectangle) was generated as a gradient with a laser exposure ranging from 20 to 2 J/mm^2 (from left to right). The density of the immobilized peptide increases from right to left (shown by the red arrow); a yellow arrow indicates the portion of the lower rectangle that was irradiated with the same exposure as the entire upper rectangle. The region of the pattern with the most immobilized peptide (black arrow, lower panel) had the greatest number of adherent cells. A black arrowhead (upper panel) indicates a defect in the gold coating. Scale bar = 250 μm.

control peptides [1,8]. Nonetheless, it may be that cell-adhesion effects were mediated by some nonspecific mechanism, such as the adsorption of molecules from the medium to patterned areas. Cells did not attach to control samples that were not exposed to UV light or that were exposed to UV light with no peptide present.

Significance

Here, we describe a photoimmobilization technique for decorating surfaces with micropatterns that contain variable densities of biomolecules. Methods for controlling the densities of immobilized factors within a given pattern have not been previously demonstrated. Photoactivatable peptides were attached to self-assembled monolayers of an oligo(ethylene glycol) alkanethiolate using either a laser or a UV lamp. Different periods of light exposure created different densities of immobilized peptide. We have shown that the technique can be used to manipulate cell adhesion and shape; the number of cells that adhered to the surface was shown to increase as peptide density increased and cells were also shown to adopt the shape of the peptide pattern. This technique can be readily adapted to a variety of surfaces and will be useful for creating experimental systems for the study of aspects of cell behavior such as adhesion and spreading.

Materials and methods

Synthesis and radiolabeling of photoactivatable peptides

The peptide sequence GYSGRGDSPAS was synthesized with 9-Fluorenylmethoxycarbonyl (Fmoc) chemistry on an amide resin and an amino-terminal lysine was added off-line to make the sequence KGYSGRGDSPAS. All standard peptide-synthesis chemicals were analytical reagent grade or better and purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher. Fmoc-4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy resin (sub. level = 0.52 mmol/g) was from Novabiochem (La Jolla, CA) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate from Richelieu Biotechnologies (St.-Hyacinthe, Quebec). Fmoc-amino acid derivatives were obtained from Novabiochem, PerSeptive Biosystems (Framingham, MA), or Advanced Chemtech (Louisville, KY). Amino acids are of the L-configuration (except for glycine). The synthesis of GYSGRGDSPAS was performed by Fmoc solid-phase methodology on an Applied Biosystems 431A Peptide Synthesizer using cycles described previously [17]. The peptide-resin was analyzed by Edman degradation sequence analysis [18] to evaluate the efficiency of assembly. Fmoc–Lys(Boc) was coupled to the peptide-resin by first washing with dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) then reacting threefold molar excesses of Fmoc-Lys(Boc)-OPfp and *N,N*-diisopropylethylamine in DMF for 6 h on an orbital shaker.

Activated benzophenone was prepared by coupling 4-benzoyl benzoic acid ($C_6H_5COC_6H_4CO_2H$) and N-hydroxy succinimide with 1,3-dicyclohexylcarbodiimide [11]. After washing the resin in DCM and DMF, the swollen resin was shaken for 6 h with a 3 × molar excess of activated benzophenone in DMF plus 3 × excess diisopropylethylamine (Fluka; Ronkonkoma, NY). The resin was washed twice for 15 min in a large excess of DMF, twice in DCM, and was air-dried. Deprotection and cleavage were accomplished by shaking the resin in 95% TFA solution for 4 h followed by precipitation and rinsing twice in anhydrous tert-butyl ether.

Radiolabeling was performed by reductive methylation of lysine epsilon amines according to published protocols [8,19]. In brief, 1.8 μmol peptide, 29 μmol $NaCNBH_3$ and 3.3 μmol ^{14}C-labeled formaldehyde (30 mCi/mmol, ICN Pharmaceuticals, Irvine, CA) were combined in 280 μl of 5 mM HEPES buffer, pH 7.5, and agitated for 4 h. A reverse phase chromatography column (Sep-Pak Classic, Waters; Milford, MA) was loaded with the solution, rinsed with 40 ml of deionized water (1 ml/min) and eluted with solutions of 5–55% acetonitrile in water. Fractions with a high radioactivity and more than 10 μg/ml peptide (determined by tyrosine absorbance) were pooled, dried and redissolved in deionized water; specific activity of the peptide was 18 mCi/mmol by liquid scintillation counting (Beckman LS-4000, Fullerton, CA).

Self-assembled monolayer preparation

HEG-UT was prepared as described in published protocols [20]. A Bal-Tec (Bedford, NY) thermal evaporator (BAE 080 T) equipped for dual sources was used to coat a methanol-cleaned silicon wafer with 75 Å chrome (coated tungsten rod source: R.D. Mathis, Inc., Long Beach, CA) and 1000 Å gold (Aldrich; Milwaukee, WI) at 3–4 Å/s (less than 5×10^{-6} Torr of pressure). Samples were cleaved from the wafer with glass cutters and immersed in 0.1 mM of HEG-UT in ethanol overnight. The samples were rinsed for 1 h in ethanol, 2 h in distilled water, and briefly under a gentle stream of distilled water. Samples were used immediately after cleaning.

Peptide immobilization

Peptides were dissolved in deionized water (10 μg/ml), aliquoted, and stored at −70°C until used. For UV photoactivation, 50 μl of peptide solution was pipetted onto a self-assembled monolayer and placed 25 cm beneath a 400 W UV-spectrum lamp for the time indicated (at this distance, 25 mW/cm^2 manufacturer reported spectral irradiance at 365 nm; model ELC4000, Electro-Lite Corp., Danbury, CT). The self-assembled monolayers were rinsed quickly with a gentle stream of 5 ml distilled water, placed inside a centrifuge tube with 40 ml distilled water, and rotated at 100 rpm overnight on a rotary shaker.

For laser-beam photoactivation, wafers coated with self-assembled monolayers were attached with double-sided tape to a petri dish and a drop of 10 μg/ml peptide solution was trapped between the self-assembled monolayer and a quartz coverslip (Chem Glass; Vineland, NJ); a liquid thickness of 1.0 mm on top of the self-assembled monolayer was maintained by resting the coverslip on supports attached to the petri dish. The dish was taped to a mobile programmable stage (Motion Master 2000 system; Newport/Klinger, Garden City, NY). A beam from a HeCd laser (4 mW, 325 nm, Omnichrome Corporation model 3056-4, Chino, CA) was passed through an electronic shutter (Newport/Klinger; controlled by the mobile stage software), through a 190 μm pinhole in a copper plate to eliminate uncollimated light, through a 5 × UV objective lens (Newport/Klinger), and onto the self-assembled monolayer positioned 4 cm below the objective. The stage and shutter were programmed to expose the specimen to the laser to create the patterns described. The relation between laser exposure (product of laser power and time) and peptide immobilization was measured from 4 × 1 mm rectangular patterns (n = 1) made with a raster pattern 4 mm wide with 250 perpendicular steps of 4 μm each: this step size was designed so that the 20 μm diameter beam would pass over every spot in the interior of the pattern 5 times (exposure of 4 J/mm^2 made by laser speed of 250 μm/s; 100% of laser power assumed to be focused on specimen).

Visualization of radiolabeled peptides

Peptides labeled with ^{14}C were visualized by quantitative digital autoradiography using a Molecular Dynamics phosphorimager (Sunnyvale, CA); the samples were exposed to the phosphorimaging screen for 60–82 h, scanned at a resolution of 88 μm per imaging screen pixel, digitized and analyzed with ImageQuant (Molecular Dynamics) software. The recorded counts were calibrated by including two sets of standards in each experiment. The first set was made by air-drying 10 μl drops of radioactive peptide solutions ranging from 0.1–1000 ng of peptide in tenfold dilutions onto polystyrene chips mounted in triplicate on a card. The activity of the second set of ^{14}C standards (ARC-146S; American Radiolabeled Chemicals) ranged from 0.002–3 μCi in twofold dilutions. Digital images of samples irradiated by UV were individually traced, their total area and activity recorded, background activity according to their area was subtracted and their activity reported per unit area. Images of laser-irradiated samples were treated similarly except that the counts of radioactivity were recorded from a rectangle corresponding to the calculated size of the pattern that was superimposed manually over the patterned area.

Cell culture media

Materials were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise specified. HFFs (passage 4–8), generously provided by R.T. Tranquillo, were maintained in Dulbecco's modified Eagle Medium (DMEM) plus 10% fetal bovine serum (Life Technologies, Grand Island, NY), 100 U/l penicillin and 100 mg/l streptomycin (Celox, Hopkins, MN). Serum-free defined medium [21] contained: Williams' Medium E, 0.2 U/l bovine-porcine pancreas insulin (Lilly Research Laboratories, Indianapolis, IN), 2 mM L-glutamine, 1 nM dexamethasone, 4 ng/ml glucagon, 5 ng/ml epidermal growth factor, 6.25 μg/ml transferrin, 50 ng/ml linoleic acid, 0.5 mg/ml bovine serum albumin, penicillin-streptomycin as described above and trace elements: 0.1 μM $CuSO_4 \cdot 5H_2O$, 3 nM H_2SeO_3, 50 pM $ZnSO_4 \cdot 7H_2O$ and 15 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES).

Cell seeding, visualization and adhesion

Laser-patterned self-assembled monolayers (0.5 × 1.0 cm) were placed in 35 mm petri dishes, sterilized 2 min in 70% ethanol, rinsed three times in sterile phosphate buffered solution (PBS; pH 7.3, 0.2 g/l KCl and KH_2PO_4, 8.0 g/l NaCl, 2.16 g/l $Na_2HPO_4 \cdot H_2O$), and covered with 3 ml of serum-free medium. Confluent HFF cells were trypsinized 5 min in 2 ml 0.25% trypsin plus 1 mM ethylenediaminetetraacetic acid, combined with 2 ml of maintenance medium, centrifuged, washed twice in serum-free defined medium, mixed into petri dishes containing self-assembled monolayers covered in 3 ml of serum-free medium and incubated at 37°C in a 5% CO_2 atmosphere. Cell-seeding density was 15,000 cells/cm^2 unless otherwise specified. Photomicrographs were taken with an Optronics digitizing video camera (TEC470; Goleta, CA) mounted on an Olympus microscope (BX-60; Lake Success, NY). Cells were made fluorescent when indicated by adding 10 μg/ml fluorescein diacetate to the medium for 2 min. To measure cell adhesion, cells were seeded on self-assembled monolayers patterned with a set of squares arrayed in three rows by four columns. Each column had a different peptide density as indicated in the text. The number of cells were counted on each square (300 × 300 μm) 4 h after seeding in serum-free medium. Average results reported for two experiments, n = 2 patterns.

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