

Using Mixed Self-Assembled Monolayers Presenting RGD and (EG)₃OH Groups To Characterize Long-Term Attachment of Bovine Capillary Endothelial Cells to Surfaces

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Abstract: This paper describes surfaces that promote the ligand-directed binding of cells and resist the cellular deposition of adhesive proteins. These surfaces are based on self-assembled monolayers (SAMs) of alkanethiolates on gold that present mixtures of arginine-glycine-aspartate (RGD), a tripeptide that promotes cell adhesion by binding to cell surface integrin receptors, and oligo(ethylene glycol) moieties, groups that resist nonbiospecific adsorption of proteins and cells. Surface plasmon resonance (SPR) spectroscopy was used to measure the adsorption of carbonic anhydrase and fibrinogen to mixed SAMs comprising RGD groups ((EG)₆OGRGD) and tri(ethylene glycol) groups ((EG)₃OH); SAMs having values of the mole fraction of RGD ($\chi_{\text{RGD}} \leq 0.05$) adsorbed nearly undetectable levels of carbonic anhydrase or fibrinogen. Bovine capillary endothelial cells attached and spread on SAMs at $\chi_{\text{RGD}} \geq 0.00001$, with spreading of cells reaching a maximum at $\chi_{\text{RGD}} \geq 0.001$. These mixed SAMs reduced the deposition of proteins by attached cells relative to both fibronectin adsorbed on SAMs of hexadecanethiolate on gold and RGD peptide coated on glass. After allowing cells to attach for 2 or 4 h to any of these surfaces presenting RGD groups, addition of soluble GRGDSP to the medium contacting the adherent cells rapidly released them from the surfaces. However, if cells were allowed to attach to surfaces for 24 h, only those cells attached to the mixed SAM presenting (EG)₆OGRGD and (EG)₃OH groups could be released using the soluble GRGDSP at a rate comparable to cells attached to fibronectin for 2 h. These results demonstrate that RGD alone is sufficient for adhesion and survival of cells over 24 h.

Introduction

Adhesion of cells to the extracellular matrix (ECM) influences the shape, growth, viability, differentiation, migration, and metabolism of cells.^{1–6} It has been difficult, however, to characterize the biological activities of specific constituents of the ECM (e.g., fibronectin, laminin, vitronectin, collagens, and proteoglycans), primarily because within hours after plating cells onto substrates presenting specific ECM proteins, cells can deposit a new, uncharacterized layer of ECM; this process of depositing adhesive proteins is called “remodeling”. We hypothesized that mixed self-assembled monolayers (SAMs) that presented specific ECM moieties in an “inert”, nonadsorbing interface would provide a surface that would promote attachment through specific cell adhesion receptors while preventing the remodeling of the substrate. Here, we used mixed SAMs and

surface plasmon resonance (SPR) spectroscopy⁷ to study attachment and spreading of bovine capillary endothelial cells on -Gly-Arg-Gly-Asp (GRGD) presented in a background of (EG)₃OH groups that resists the deposition of extracellular matrix by the cell (Figure 1).

In the mid 1980s, Pierschbacher and Ruoslahti found that many extracellular matrix proteins contain the tripeptide RGD and that this short peptide is an important ligand for cell adhesion.^{8,9} It was subsequently found that cells employ a large family of transmembrane proteins called the integrins to recognize this and other ligands of the ECM.^{10–12} This finding was followed by many reports that demonstrated substrates modified with the RGD peptide alone supported the adhesion and spreading of mammalian cells. Brandley and Schnaar, for example, used polyacrylamide gel substrates that were modified with the RGD peptide to study cell adhesion.¹³ This early work demonstrated a model system for studies of cell adhesion but had the limitation that the immobilized ligands were presented

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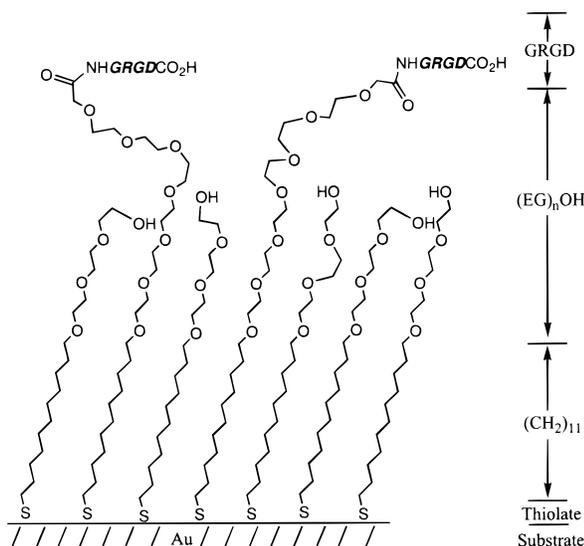


Figure 1. Diagram of self-assembled monolayer of alkanethiolates on gold presenting (EG)₆OGRGD and (EG)₃OH groups.

in the heterogeneous environment of the gel; this heterogeneity made it impossible for the authors to determine the density of functional ligand. Massia and Hubbell prepared alkylsiloxanes presenting RGD peptides for studies of cell adhesion and spreading.¹⁴ Unlike the polyacrylamide gels, the modified alkylsiloxane surfaces presented the RGD ligands in a homogeneous environment. Consequently, Massia and Hubbell were able to quantitate the relationship between the density of peptide ligands and the efficiency of cell attachment and spreading. The modified alkylsiloxane surfaces, however, were prepared using a synthetic strategy that employed the random attachment of the peptides fragments directly to the siloxane surface. As a result, the characterization of these modified alkylsiloxane surfaces was nontrivial. Drumheller and Hubbell improved the biospecificity of cell-substrate interactions by developing a surface made of poly(ethylene glycol) in a cross-linked matrix of acrylic acid and trimethylolpropane triacrylate grafted with RGD peptide.¹⁵ The adhesive properties of this surface remained unchanged by the presence of adhesion proteins in solution. However, these studies did not address the issue of whether these modified surfaces prevented cells from remodeling the interface.

Surfaces fabricated to date have not been shown to resist the deposition of additional adhesive ligands expressed by the attached cells; within hours, the molecular composition of the surface, and therefore the spectrum of cell surface receptors being engaged, change uncontrollably. Although the use of protein synthesis inhibitors to block synthesis and deposition of new ECM proteins may be useful for a few hours,¹⁶ this approach severely impairs many processes within cells and is lethal to them within 8–24 h.^{17,18} As a result it is difficult to assess the effects of specific ligand-cell interactions on many biological processes, including adhesion itself.

Using SAMs of alkanethiolates on gold, we have previously demonstrated that surfaces presenting oligo(ethylene glycol)

moieties prevent the adsorption of protein.^{19–21} In this study we develop surfaces that promote cell attachment by the specific interaction of RGD with cell surface integrin receptors and resist significant deposition of cell-derived matrix components. We prepare the mixed SAMs using solutions that contain both (EG)₆OGRGD and (EG)₃OH terminated alkanethiolates. By using this approach, we generate structures that are well-defined surfaces; the most important unresolved issues concern the 2-D distribution of the RGD groups and the nature and distribution of defects in the SAMs. The important point is that these surfaces present RGD groups at an otherwise “inert”—that is, nonadsorbing—interface. Using this model system, we demonstrate that RGD alone is sufficient to maintain long-term biospecific attachment and survival of cells; the deposition of proteins expressed by cells during remodeling is not essential to the cells in the first 24 h.

Experimental Procedure

Preparation of Alkanethiol 1 (see Figure 2):

Synthesis. A convergent synthetic strategy was used to prepare alkanethiol **1**. Each of the amide linkages was achieved using diphenylphosphoryl azide (DPPA) to activate the terminal carboxylic acids *in situ*.

Alcohol **5**, converted to the sodium alkoxide, was reacted with *tert*-butylbromoacetate to give ester **6**. The ester was then transformed into carboxylic acid **7** using trifluoroacetic acid (TFA).

The GRGD segment **4** was prepared from dipeptides **2** and **3**. Hydrogenation of **4** provided the terminal amine which was subsequently coupled with **7** to afford alkene **8** and complete the block portion of the synthesis.

The conversion of alkene **8** to thioester **9** was achieved by the radical addition of thioacetic acid across the terminal olefin. Finally, all protecting groups were removed using an aqueous mixture of TFA, ethanedithiol, and thioanisole to give the target molecule **1**.

Materials and Methods. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm silica gel plates (E. Merck). Column chromatography was performed using silica gel-60 (particle size 0.040–0.063) (E. Merck). All reactions in nonaqueous solvents were executed under nitrogen.

***N*-[(Phenylmethoxy)carbonyl]glycyl-*N*⁵-[[[(3,4-dihydro-2,2,5,7,8-pentamethyl-2*H*-1-benzopyran-6-yl)sulfonyl]amino]iminomethyl]-*L*-ornithyl Acid (**2b**).** To a solution of *N*-[(phenylmethoxy)carbonyl]glycyl-*N*-hydroxysuccinimide (3.81 g, 12.4 mmol) in DMF (40 mL) cooled to 0 °C was added *N*⁵-[[[(3,4-dihydro-2,2,5,7,8-pentamethyl-2*H*-1-benzopyran-6-yl)sulfonyl]amino]iminomethyl]-*L*-ornithyl acid **2a** (5.0 g, 11.3 mmol) followed by dropwise addition of diisopropylethylamine (DIPEA) (7.48 mL, 43 mmol). The reaction mixture was stirred at 0 °C for 1 h, allowed to warm to room temperature and stirred at room temperature, for an additional 3 h. Product **2b** precipitated from cold H₂O after acidification with 1 N aqueous HCl (26 mL). The obtained precipitate was filtered, washed with cold H₂O, and dried *in vacuo* to afford **2b** (6.7 g, 94%). ¹H NMR (500 MHz, CDCl₃): δ 1.27 (s, 6 H), 1.55 (bs, 2 H), 1.75 (m, 3 H), 1.85 (bs, 1 H), 2.06 (s, 3 H), 2.49 (s, 3 H), 2.50 (s, 3 H), 2.56 (bs, 2 H), 3.14 (bs, 2 H), 3.89 (bs, 2 H), 4.48 (bs, 1 H), 5.00 (s, 2 H), 6.15–6.50 (m, 4 H), 7.24 (m, 5 H), 7.61 (bs, 1 H).

***L*-Aspartic Acid, Glycyl-Bis(1,1-dimethylethyl) Ester (**3b**).** To a solution of *Z*-NH-G-ONHS (6.87 g, 22.4 mmol) in DMF (40 mL) cooled to 0 °C was added H₂ND(OtBu)-OtBu **3a** (5.0 g, 20.4 mmol) followed by dropwise addition of DIPEA (7.48 mL, 43 mmol). The reaction mixture was stirred at 0 °C for 1 h, allowed to warm to room temperature, and stirred at room temperature for an additional 3 h. The reaction mixture was added dropwise to cold H₂O and left on ice for 2 h. The obtained precipitate was filtered, washed with cold H₂O, and

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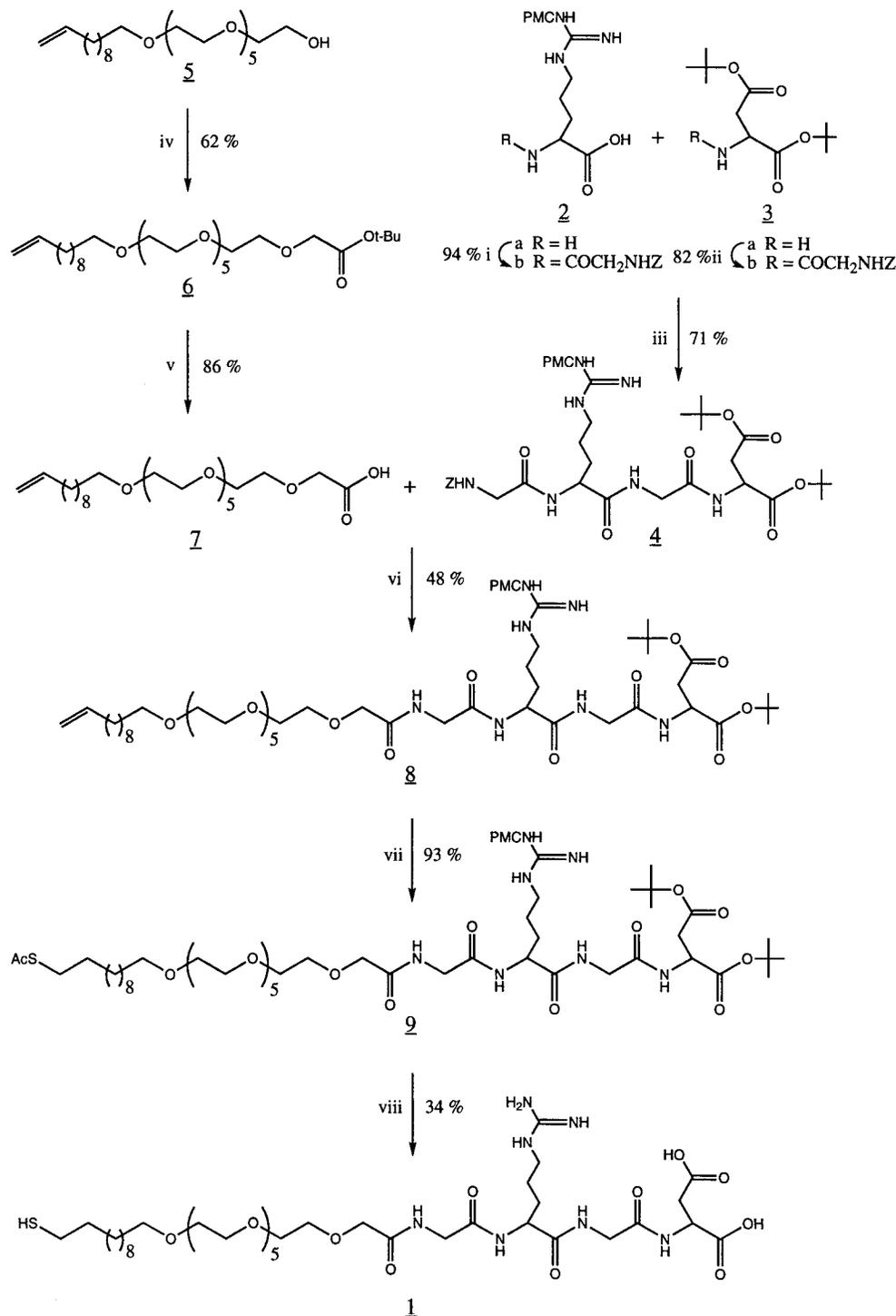


Figure 2. Synthesis of GRGD hexaethyleneglycol alkanethiol (**1**). (i) $\text{ZNHCH}_2\text{CONHS}$, DIPEA; (ii) $\text{ZNHCH}_2\text{CONHS}$, DIPEA, DMF; (iii) DPPA, DIPEA, DMF; (iv) NaH, DMF then $\text{BrCH}_2\text{COOt-Bu}$; (v) TFA, CH_2Cl_2 ; (vi) 10% Pd/C, EtOH on **4** then DPPA, DIPEA, DMF; (vii) CH_3COSH , AIBN, THF with UV irradiation; (viii) TFA, PhSMe, $\text{HS}(\text{CH}_2)_2\text{SH}$, CH_2Cl_2 .

dried in vacuo to afford **3b** (7.3 g, 82%). ^1H NMR (400 MHz, CDCl_3): δ 1.41 (s, 9 H), 1.43 (s, 9 H), 2.71 (dd, $J = 3.48, 17.11$ Hz, 1H), 2.88 (dd, $J = 4.17, 17.11$ Hz, 1H), 3.92 (m, 2 H), 4.68 (dt, $J = 4.29, 8.36$ Hz, 1H), 5.12 (s, 2 H), 5.38 (bs, 1 H), 6.85 (d, $J = 7.90$ Hz, 1H), 7.29–7.36 (m, 5 H).

L-Aspartic Acid, *N*-[(Phenylmethoxy)carbonyl]glycyl-*N*⁵-[[[(3,4-dihydro-2,2,5,7,8-pentamethyl-2*H*-1-benzopyran-6-yl)sulfonyl]amino]iminomethyl]-L-ornithylglycyl-, Bis(1,1-dimethylethyl) Ester (4**).** Compound **3b** (1.46 g, 3.35 mmol) was hydrogenated in EtOH (30 mL) over 10% Pd/C (0.3 g) until the TLC (33% EtOAc in hexanes) indicated that **3b** had been consumed. The reaction mixture was filtered through Celite and concentrated in vacuo to give the crude amine that

was used in the next step without further purification. The flask containing crude amine from **3b** was purged with N_2 , acid **2b** (2.3 g, 3.65 mmol) and dry DMF (20 mL) were added, and the stirred solution was cooled to 0 °C. Diphenylphosphoryl azide (DPPA) (0.94 mL, 4.38 mmol) was added, followed by a solution of DIPEA (0.76 mL, 4.38 mmol) in DMF (5 mL), and the stirring was continued at 0 °C for 10 h. The mixture was diluted with EtOAc (100 mL) and washed successively with H_2O (3×20 mL), 5% aqueous NaHCO_3 (20 mL), and brine (2×20 mL). The organic phase was dried (MgSO_4), and the solvent was removed in vacuo to give the residue which was chromatographed (eluting with 10% EtOH in EtOAc) to give product **4** (2.23 g, 71%). ^1H NMR (500 MHz, CDCl_3): δ 1.26 (s, 6 H), 1.36

(s, 9 H), 1.38 (s, 9 H), 1.54 (m, 2 H), 1.63 (m, 2 H), 1.75 (t, $J = 6.78$ Hz, 2 H), 1.85 (m, 2 H), 2.05 (s, 3 H), 2.50 (s, 3 H), 2.51 (s, 3 H), 2.57 (t, $J = 6.72$ Hz, 2 H), 2.65 (dd, $J = 3.36, 17.00$ Hz, 1 H), 2.78 (dd, $J = 4.20, 17.00$ Hz, 1 H), 3.18 (bs, 2 H), 3.81–4.01 (m, 4 H), 4.50 (m, 1 H), 4.63 (m, 1 H), 5.03 (s, 2 H), 6.10–6.40 (m, 4 H), 7.25 (m, 5 H), 7.43 (m, 1 H), 7.77 (m, 1 H). HRMS (FAB) calcd for $C_{44}H_{65}N_7O_{12}^-SNa$ ($M + Na$) 938.4310, found 938.4310.

3,6,9,12,15,18,21-Heptaaxadotriacont-31-enoic Acid, 1,1-Dimethylethyl Ester (6). To a solution of alcohol **5**^{20,21} (1.04 g, 2.4 mmol) in dry DMF (5 mL) cooled to 0 °C was added NaH (144 mg of 60% suspension in oil, 3.6 mmol). The mixture was stirred at 0 °C for 10 min, *tert*-butylbromoacetate (532 μ L, 3.6 mmol) was added in one portion, and the mixture was allowed to warm to room temperature. After the mixture was stirred for 6 h additional *tert*-butylbromoacetate (532 μ L, 3.6 mmol) was added, and the stirring was continued at 40 °C for 10 h. After the mixture cooled to room temperature, EtOAc (50 mL) was added, organic phase was washed with H₂O and brine, dried (MgSO₄), and concentrated in vacuo. Column chromatography (3% MeOH in CH₂Cl₂) afforded product **6** (0.81 g, 62%). ¹H NMR (400 MHz, CDCl₃): δ 1.25 (bs, 10 H), 1.29–1.41 (m, 2 H), 1.45 (s, 9 H), 1.55 (m, 2 H), 2.01 (m, 2 H), 3.42 (t, $J = 6.80$ Hz, 2H), 3.56 (m, 2 H), 3.60–3.73 (m, 22 H), 4.00 (s, 2 H), 4.89 (m, 2 H), 5.73–5.84 (m, 1 H). MS (FAB) calcd for $C_{29}H_{56}O_9Na$ ($M + Na$) 571, found 571.

L-Aspartic Acid, N-[(1-oxo-3,6,9,12,15,18,21-heptaaxatriacont-31-en-1-yl)glycyl-N⁵-[[[(3,4-dihydro-2,2,5,7,8-pentamethyl-2H-1-benzopyran-6-yl)sulfonyl]amino]iminomethyl]-L-ornithylglycyl-, Bis-(1,1-dimethylethyl) Ester (8). To a solution of **6** (376 mg, 0.69 mmol) in CH₂Cl₂ (5 mL), was added TFA (5 mL) and the mixture was stirred for 3 h. After concentration in vacuo and column chromatography (10% MeOH in CH₂Cl₂) heptaaxadotriacont-31-enoic acid **7** (290 mg, 0.59 mmol, 86%) was obtained. Z-Protected tetrapeptide **4** (507 mg, 0.65 mmol) was hydrogenated in EtOH (10 mL) over 10% Pd/C (0.1 g). After filtration and concentration in vacuo the crude amine was obtained. The amine and carboxylic acid **7** were combined, the flask was purged with N₂, dry DMF (3 mL) was added, and the stirred solution was cooled to 0 °C. DPPA (150 μ L, 4.38 mmol) was added, followed by a solution of DIPEA (120 μ L, 4.38 mmol) in DMF (1 mL), and the stirring was continued at 0 °C for 10 h. The mixture was diluted with EtOAc (10 mL) and washed successively with H₂O, 5% aqueous NaHCO₃, and brine. The organic phase was dried (MgSO₄), and the solvent was removed in vacuo to give a residue which was chromatographed (5% MeOH in CH₂Cl₂ \rightarrow 10% MeOH in CH₂Cl₂) to give product **8** (355 mg, 48%). ¹H NMR (500 MHz, CDCl₃): δ 1.25 (s, 10 H), 1.28 (s, 6 H), 1.30–1.36 (m, 2 H), 1.39 (s, 9 H), 1.40 (s, 9 H), 1.48–1.63 (m, 4 H), 1.68–1.75 (m, 1 H), 1.78 (t, $J = 6.80$ Hz, 2 H), 1.89–1.95 (m, 1 H), 2.01 (q, $J = 6.85$ Hz, 2 H), 2.07 (s, 3 H), 2.53 (s, 3 H), 2.55 (s, 3 H), 2.60 (t, $J = 6.70$ Hz, 2 H), 2.67 (dd, $J = 4.75, 16.94$ Hz, 1 H), 2.79 (dd, $J = 5.01, 16.99$ Hz, 1 H), 3.22 (m, 2 H), 3.40 (t, $J = 6.85$ Hz, 2 H), 3.52–3.55 (m, 2 H), 3.58–3.64 (m, 22 H), 3.66–3.71 (m, 2 H), 3.88 (dd, $J = 5.68, 16.62$ Hz, 1 H), 3.95 (dd, $J = 5.64, 14.47$ Hz, 1 H), 3.98–4.05 (m, 2 H), 4.01 (s, 2 H), 4.46 (q, $J = 7.61$ Hz, 1 H), 4.63 (dt, $J = 4.86, 8.04$ Hz, 1 H), 4.88–5.00 (m, 2 H), 5.73–5.83 (m, 1 H), 6.36 (bs, 2 H), 7.11 (d, $J = 8.06$ Hz, 1 H), 7.43 (bs, 1 H), 7.63 (bt, $J = 5.29$ Hz, 1 H), 7.90 (t, $J = 5.76$ Hz, 1 H). MS (FAB) calcd for $C_{61}H_{105}N_7O_{18}SNa$ ($M + Na$) 1278, found 1278.

L-Aspartic Acid, N-[(1,34-Dioxo-3,6,9,12,15,18,21-heptaaxa-33-thiapentatriacont-1-yl)glycyl-N⁵-[[[(3,4-dihydro-2,2,5,7,8-pentamethyl-2H-1-benzopyran-6-yl)sulfonyl]amino]iminomethyl]-L-ornithylglycyl-, Bis(1,1-dimethylethyl) Ester (9). A mixture of **8** (650 mg, 0.52 mmol), thioacetic acid (742 μ L, 10.4 mmol), and AIBN (20 mg) in THF (10 mL) was irradiated by UV. After 2 h additional AIBN (20 mg) was added, and irradiation was continued for 3 h. The mixture was concentrated in vacuo and chromatographed (5% MeOH in CH₂Cl₂ \rightarrow 10% MeOH in CH₂Cl₂) to give thioacetate **9** (641 mg, 93%). ¹H NMR (500 MHz, CDCl₃): δ 1.26 (bs, 12 H), 1.28 (s, 6 H), 1.28–1.33 (m, 2 H), 1.39 (s, 9 H), 1.40 (s, 9 H), 1.47–1.64 (m, 6 H), 1.68–1.75 (m, 1 H), 1.78 (t, $J = 6.80$ Hz, 2 H), 1.88–1.96 (m, 1 H), 2.07 (s, 3 H), 2.28 (s, 3 H), 2.53 (s, 3 H), 2.54 (s, 3 H), 2.60 (t, $J = 6.68$ Hz, 2 H), 2.68 (dd, $J = 4.80, 16.94$ Hz, 1 H), 2.78 (dd, $J = 5.10, 16.96$ Hz,

1 H), 2.82 (t, $J = 7.36, 2$ H), 3.23 (m, 2 H), 3.40 (t, $J = 6.85$ Hz, 2 H), 3.52–3.55 (m, 2 H), 3.58–3.64 (m, 22 H), 3.66–3.71 (m, 2 H), 3.87 (dd, $J = 5.70, 16.64$ Hz, 1 H), 3.95 (dd, $J = 5.68, 14.48$ Hz, 1 H), 3.98–4.05 (m, 2 H), 4.01 (s, 2 H), 4.45 (bq, $J = 7.52$ Hz, 1 H), 4.63 (dt, $J = 4.90, 8.18$ Hz, 1 H), 6.36 (bs, 2 H), 7.14 (d, $J = 8.07$ Hz, 1 H), 7.52 (bs, 1 H), 7.71 (bs, 1 H), 7.94 (t, $J = 5.78$ Hz, 1 H). MS (FAB) calcd for $C_{63}H_{109}N_7O_{19}S_2Na$ ($M + Na$) 1354, found 1354.

L-Aspartic Acid, N-[(32-Mercapto-1-oxo-3,6,9,12,15,18,21-heptaaxatriacont-1-yl)glycyl-L-arginylglycyl- (1). A mixture of CF₃COOH/PhSMe/HS(CH₂)₂SH/H₂O 37:1:1:1 (v/v) was used for cleavage of the protective groups in **9**. A solution of **9** (320 mg, 0.25 mmol) in CH₂Cl₂ (2 mL) was cooled 0 °C, and the cleavage mixture (10 mL) was added. The obtained solution was stirred at 0 °C for 1 h, allowed to warm to room temperature, and stirred for 2 h at room temperature. The mixture was concentrated in vacuo, CH₂Cl₂ (10 mL) was added, and the mixture was concentrated in vacuo again. The residue was dissolved in a minimum amount of CH₂Cl₂, cold Et₂O (50 mL) was added dropwise into the flask with vigorous stirring, and the obtained precipitate was filtered and dried in vacuo to afford AcS(CH₂)₁₁(OCH₂CH₂)₆OCH₂C-(O)NHGRGDH (195 mg, 0.20 mmol, 80%) as a white solid. MS (FAB) calcd for $C_{41}H_{75}N_7O_{16}SNa$ ($M + Na$) 976, found 976. The obtained thioacetate was dissolved in absolute MeOH (10 mL), and the solution was cooled 0 °C. NaOMe (1.54 mL of 0.39 M in MeOH, 0.60 mmol) was added, and the mixture was stirred at 0 °C for 3 h. The mixture was neutralized with Dowex exchange resin (H-form), filtered, and concentrated in vacuo. Repeated precipitation (3 times) from CH₂Cl₂ with cold Et₂O afforded pure **1** (63 mg, 34%). ¹H NMR (500 MHz, CD₃OD): δ 1.27–1.46 (m, 12 H), 1.52–1.61 (m, 4 H), 1.63–1.71 (m, 2 H), 1.72–1.81 (m, 2 H), 1.92–2.00 (m, 2 H), 2.48 (t, $J = 7.15$ Hz, 2 H), 2.82 (t, $J = 5.41$ Hz, 2 H), 3.20 (t, $J = 6.76$ Hz, 2 H), 3.46 (t, $J = 6.62$ Hz, 2 H), 3.57 (m, 2 H), 3.61–3.66 (m, 18 H), 3.68 (m, 2 H), 3.71 (m, 2 H), 3.84–4.02 (m, 4 H), 4.06 (s, 2 H), 4.40 (t, $J = 7.32$ Hz, 1 H), 4.63 (t, $J = 5.50$ Hz, 1 H); ¹³C NMR (125 MHz, CDCl₃): δ 24.97, 25.94, 27.20, 29.41, 30.22, 30.57, 30.65, 30.72, 35.22, 37.62, 42.02, 43.13, 43.39, 50.77, 54.33, 71.14, 71.19, 71.33, 71.43, 71.47, 71.52, 72.08, 72.37, 158.63, 171.06, 171.58, 173.74, 174.17, 174.92, 175.02. HRMS (FAB) calcd for $C_{39}H_{73}N_7O_{15}SNa$ ($M + Na$) 934.4783, found 934.4805.

Preparation of SAMs. Substrates were prepared as previously described,^{20,21} beginning with evaporation of titanium (1 nm) and gold onto glass slides (38 nm of Au for SPR experiments; and 12 nm of Au for cell culture).²² The slides were immersed in ethanolic solutions containing mixtures of HSC₁₁(EG)₆OGRGD and HSC₁₁(EG)₃OH (2 mM total thiol) in ethanol for 4 h. Ellipsometric measurements of mixed SAMs of HSC₁₁(EG)₆OGRGD and HSC₁₁(EG)₃OH resulted in thicknesses ranging from 22.8 to 33.7 Å; these values are in good agreement with those expected for a well-packed SAM containing trans-extended alkanethiolates.²¹ In reporting the composition of the system in terms of the parameter, χ_{GRGD} , we assume the relative mole fractions of (EG)₆OGRGD and (EG)₃OH groups on the surface and in solution to be the same (eq 1). This assumption may be incorrect in detail, but the relative trends will certainly be very similar.²³

$$\chi_{GRGD} = \frac{[EG_6OGRGD]}{[EG_6OGRGD] + [EG_3OH]} \quad (1)$$

Fibronectin (FN) coated substrates, used as a positive control for adhesion assays, were prepared by immersing metallized substrates in hexadecanethiol (2 mM) in ethanol for 4 h; then, coated substrates were placed in a solution of fibronectin in PBS (Collaborative Biomedical, 50 μ g/mL) for 1 h. Another control substrate using a 20 amino acid fragment containing RGD (Telios) coated on glass (RGD-glass) was prepared by immersing glass coverslips in a solution of the peptide (50 μ g/mL) in sodium bicarbonate (100 mM, pH 9.4) overnight to generate maximal coating density.

(22) SPR spectroscopy requires a reflective gold surface (38 nm of Au). In contrast, a transparent surface (12 nm of Au) is required for the study of cells.

(23) Prime and Whitesides (refs 17 and 18) found that the thickness of mixed SAMs of (EG)_n alkanethiolates increases almost linearly with composition.

Surface Plasmon Resonance Spectroscopy. We used the Biacore 1000 for all studies described here. We modified the manufacturer's chips to accept our substrates, as described previously.²¹ Phosphate-buffered saline (P3813), fibrinogen (F4883; 94% clottability), and carbonic anhydrase (C3934) were purchased from Sigma and used as received. Solutions of proteins were filtered through 0.22 μm filters immediately before use.

Cell Culture. Bovine capillary endothelial (BCE) cells were isolated from adrenal cortex and cultured as described previously.²⁴ Cells were dissociated with trypsin-EDTA, washed in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% bovine serum albumin (1% BSA/DMEM), and plated onto substrates in chemically defined media (10 $\mu\text{g}/\text{mL}$ high-density lipoprotein, 5 $\mu\text{g}/\text{mL}$ transferrin, 5 ng/mL basic fibroblast growth factor in 1% BSA/DMEM).¹ Cells were incubated in 10% CO_2 at 37 $^\circ\text{C}$.

Assessment of Efficiency of Cell Attachment. A fixed number of cells were plated onto substrates (15000 cells/cm²) containing varying amounts of RGD peptide. After 4 h, substrates were gently washed in PBS and fixed with 4% paraformaldehyde in PBS for 30 min. The number of cells attached per field was determined from photographs taken of samples on a Nikon Axiophot microscope at 200X magnification.

Measurement of *de novo* Matrix Deposition. Cells were pre-incubated in 95% cysteine-free, methionine-free medium (DMEM/cm-) containing 1% dialyzed fetal calf serum for 24 h. After dissociation with trypsin-EDTA, cells were washed in DMEM/cm containing 1% BSA and plated for 4 or 24 h onto substrates in the presence of ³⁵S-methionine and ³⁵S-cysteine (50 $\mu\text{Ci}/\text{mL}$, Amersham: Promix). Substrates were washed three times with PBS, and cells were gently extracted with 0.1% ammonium hydroxide, leaving the deposited matrix on the substrate as previously described.²³ Deposited matrix was then removed from substrates with radioimmunoprecipitate assay (RIPA) buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2).²⁶ Sodium hydroxide and hydrogen peroxide were added (to 1 M and 2% w/w respectively) and incubated at 37 $^\circ\text{C}$ for 10 min. Samples were precipitated in ice cold 25% trichloroacetic acid containing 2% casein proteolytic fragments (Amersham: casamino acids) and filtered through glass fiber filters (Whatman). After rinsing the precipitate on the filters three times in 5% trichloroacetic acid and once in acetone, samples were dried and counted in a scintillation counter (Wallac).

Detachment of Cells Using Soluble GRGDSP Peptide. Cells were plated onto substrates for 2, 4, or 24 h, after which the samples were transferred to an Omega RTD 0.1 stage heating ring mounted on a Nikon Diaphot inverted microscope. Cells were then immersed in defined media containing soluble GRGDSP²⁷⁻²⁹ (1 mg/mL, Peninsula Laboratories). Photographs were taken of the cells over time to document the effects of the soluble peptide. Projected cell areas were determined using these photographs with image analysis software (Oncor:BDS).

Results

Mixed SAMs of (EG)₃OH and (EG)₆OGRGD Resist the Nonspecific Adsorption of Proteins. We used SPR to measure the amount of carbonic anhydrase and fibrinogen that adsorbed to mixed SAMs containing different mole fractions of (EG)₆OGRGD and (EG)₃OH (eq 1). SPR is an optical technique that measures the angle of minimum reflectivity of polarized light incident on a gold-coated glass slide; the displacement in

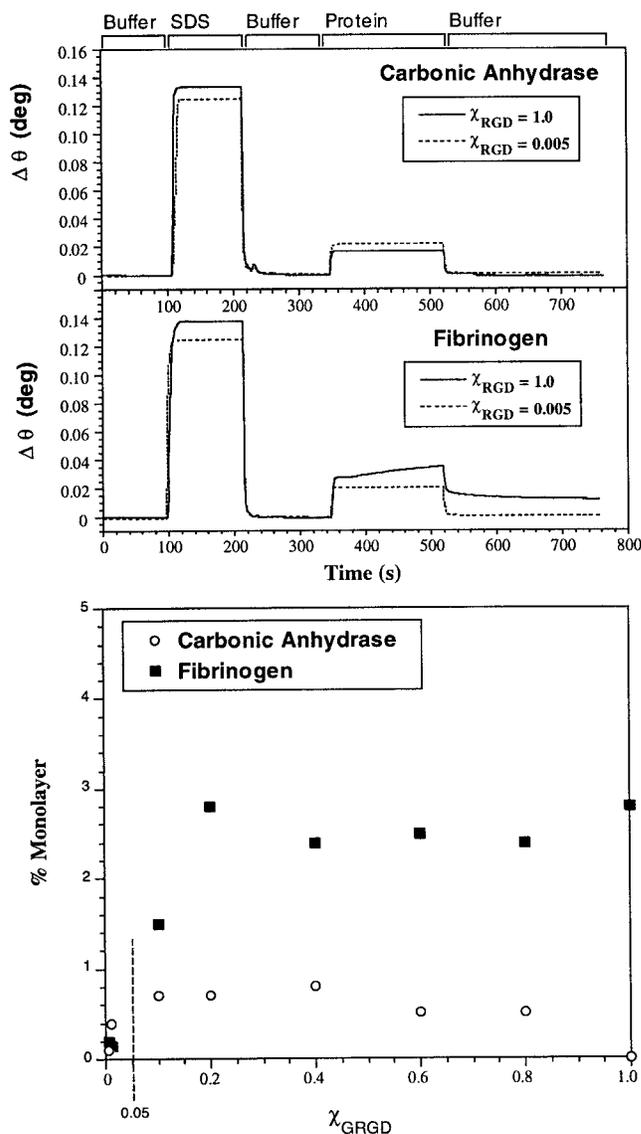


Figure 3. Percentage of a monolayer of protein irreversibly adsorbed to mixed SAMs presenting RGD groups and tri(ethylene glycol) groups. SPR response curves were obtained for the adsorption of carbonic anhydrase and fibrinogen to SAMs containing different mole fractions of RGD groups (top, middle panels, respectively). From these curves, steady-state adsorption of proteins was determined as a function of χ_{RGD} . Steady state changes in resonance angles ($\Delta\theta$) were normalized to previously measured values for a full monolayer of these proteins ($\Delta\theta$ of 0.15 $^\circ$ and 0.45 $^\circ$, respectively);²⁷ values of χ_{RGD} are indicated on the plot.

the angle is related linearly to the change in refractive index—and therefore the mass of protein—in the interfacial region. Because the protein-containing solution has a higher refractive index than does the buffer, this solution will cause an increase in $\Delta\theta$ that is not due to adsorption. Previous studies using SPR have determined that a monolayer of carbonic anhydrase and fibrinogen adsorbed on the surface of the SAMs of hexadecane alkanethiolates gives $\Delta\theta$ values of 0.15 $^\circ$ and 0.45 $^\circ$, respectively.²⁹ In the experiments reported here, buffer was allowed to flow over the monolayer followed by a solution of detergent to remove adsorbed impurities, and then a solution of protein was allowed to flow over the monolayer to observe adsorption followed by a solution of buffer to observe any desorption. Our results, repeated twice on separate occasions, show that SAMs having values of the mole fraction of RGD ($\chi_{\text{RGD}} \leq 0.05$) adsorb nearly undetectable amounts of carbonic

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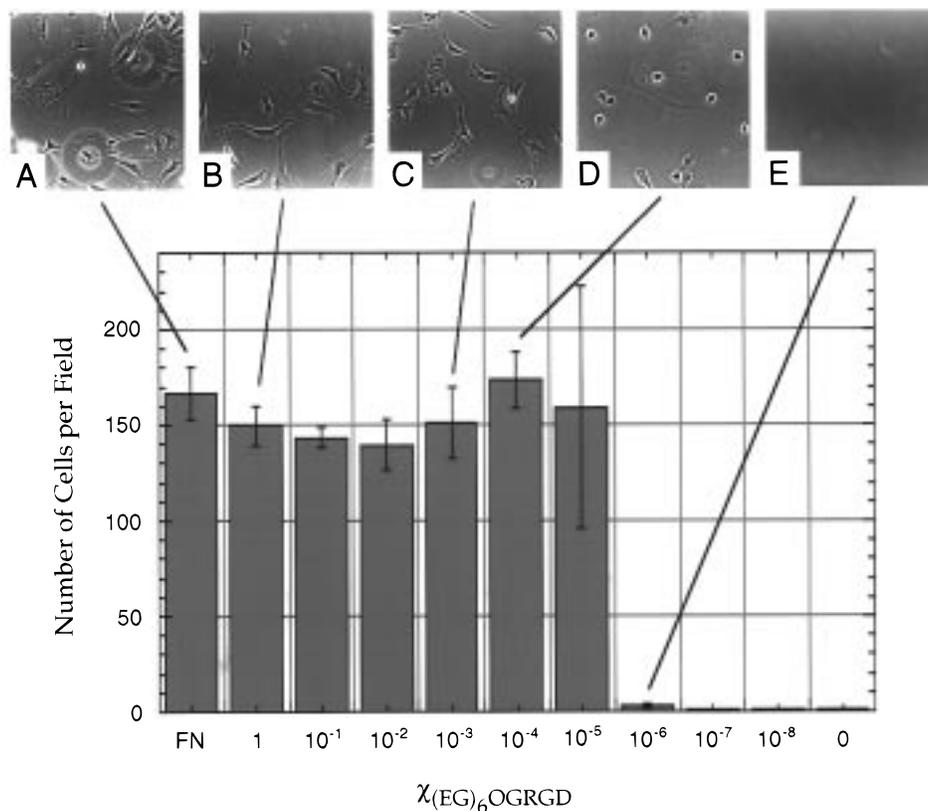


Figure 4. Adhesion of endothelial cells to mixed SAMs presenting RGD groups and tri(ethylene glycol) groups; nominal values of χ_{RGD} are indicated on the plot. The number of cells attached per field are indicated on the vertical axis. The photographs above the bar graph illustrate the amount of cell attachment and cell spreading observed for a given SAM: (A) cells on fibronectin-coated on $(CH_2)_{15} CH_3$ SAM; (B) cells are attached and spread; (C) cells are attached and spread; (D) cells are attached but do not spread; (E) cells are unable to attach. Error bars indicated standard error of the mean.

anhydrase or fibrinogen (<0.5% of maximum, fully adsorbed monolayer of each protein, respectively), while mixed SAMs having values of $\chi_{RGD} > 0.05$ adsorbed less than 5% of a monolayer of these proteins (Figure 3).

Cells Adhere and Spread to RGD SAMs in a Concentration Dependent Manner. Figure 4 shows the density of cells attaching to SAMs presenting different mixtures of $(EG)_6OGRGD$ and $(EG)_3OH$ groups. SAMs having $\chi_{RGD} \leq 1 \times 10^{-6}$ effectively resisted attachment of endothelial cells (approximately 3 ± 1 cells per field versus 150 ± 10 for $\chi_{RGD} = 1$). Cells readily attached to SAMs having $\chi_{RGD} \geq 1 \times 10^{-5}$, but maximal cell spreading was only observed when $\chi_{RGD} \geq 1 \times 10^{-3}$. On SAMs having $\chi_{RGD} \geq 1 \times 10^{-4}$, for example, cells attached but remained rounded and did not spread. At least six fields were counted per experiment, and the experiment was repeated on three different occasions.

Engineered Surfaces Reduce the Deposition of Extracellular Matrix by Cells. Although SAMs composed of RGD between $\chi_{RGD} = 0.001$ and $\chi_{RGD} = 0.01$ promote complete cell adhesion and spreading, SPR established that they resist nonspecific protein adsorption (Figure 3). These results suggest that such surfaces allow cells to attach but resist the deposition of new extracellular matrix by cells. To examine directly whether cells can deposit ECM on surfaces of mixed SAMs, attached cells were cultured in the presence of radioactive amino acids, followed by direct measurement of *de novo* deposition of radioactive proteins onto the surfaces. Results show that mixed SAMs of RGD can reduce deposition of new proteins as compared to surfaces presenting fibronectin nonspecifically adsorbed onto hexadecanethiolate SAMs or RGD-containing

peptide nonspecifically adsorbed onto glass (Figure 5).³⁰ Surfaces containing χ_{RGD} of 0.001 significantly reduced protein deposition by severalfold ($p < 0.01$). We presume that deposition of radiolabeled matrix onto the fibronectin-coated SAM and the peptide-coated glass involved exchange of the radiolabeled protein with adsorbed protein. Because the mechanisms for exchange of soluble and adsorbed proteins are complicated and not well understood, we cannot provide an explanation for the greater amount of deposition on the peptide-coated glass substrate. While SAM substrates do not completely resist matrix deposition, the reduction of protein accumulation on SAM substrates may increase the window of time during which the effects of specific ligand–receptor interactions on cellular processes can be studied.

Cells Adhere Biospecifically to Surfaces Presenting RGD Ligand. The previous experiment demonstrates that a newly deposited layer of ECM forms on traditional surfaces within several hours after attachment of cells, possibly allowing cells to attach via mechanisms that do not involve binding to RGD. To test whether cells, over time, continued to attach to surfaces via RGD moieties, or if multiple interactions developed, cells were allowed to spread on substrates for up to 24 h and challenged with soluble GRGDSP peptide. Cells were first allowed to attach to the RGD-presenting substrates. By 2 h, they spread equally on the four different substrates—mixed SAMs containing either 0.1% or 1% RGD, fibronectin nonsp-

(30) SAMs of pure $(EG)_3OH$ alkanethiolate also resulted in similarly low (<500 cpm) nonspecific adsorption of radioactive amino acids in the absence of cells. Since cells presented with an $(EG)_3OH$ SAM do not attach, they rapidly die. The $(EG)_3OH$ surface is, therefore, not strictly a control substrate in the experiment.

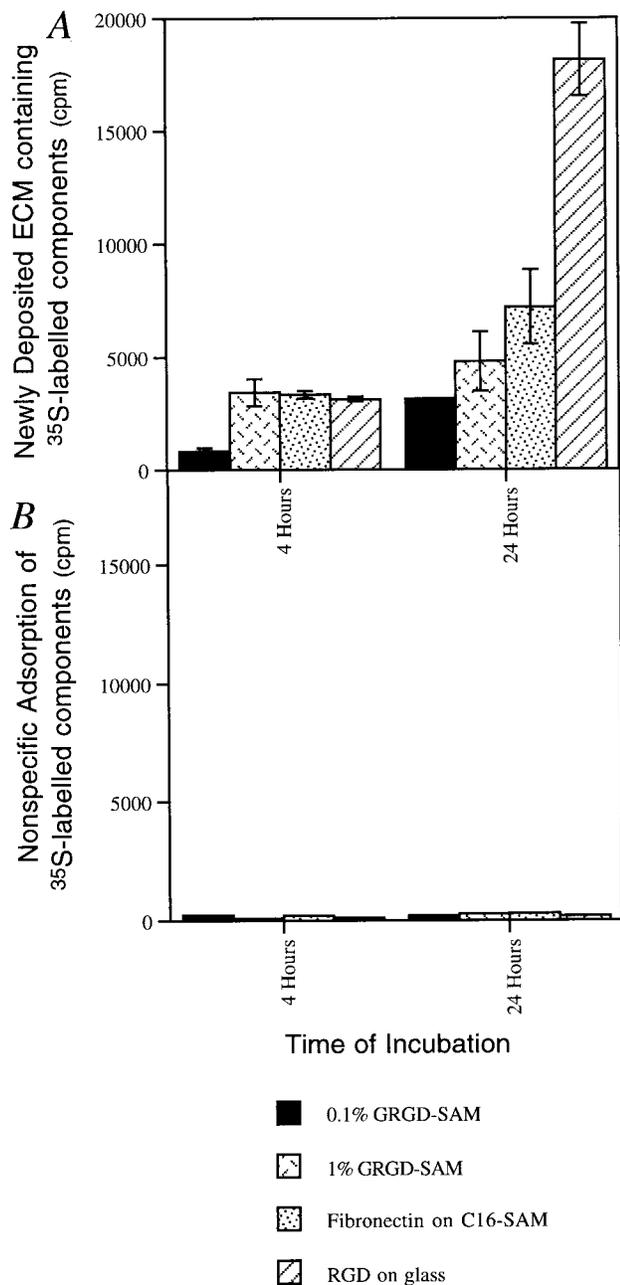


Figure 5. Direct measurement of *de novo* deposition of radioactive proteins onto mixed SAMs presenting RGD groups and tri(ethylene glycol) groups. Cells were cultured on mixed SAMs presenting RGD groups and tri(ethylene glycol) groups, fibronectin-coated SAMs of hexadecanethiolate (C16-SAM) or RGD-coated glass for 4 or 24 h in the presence of radioactive (³⁵S) amino acids. (A) Measurement of radioactivity (cpm) of substrate after removal of cells at 4 or 24 h, indicating amount of *de novo* deposition of proteins. (B) Measurement of radioactivity (cpm) of control substrates immersed in radioactive medium in the absence of cells for 4 or 24 h, indicating amount of passive adsorption of the labeled amino acids. Error bars indicated standard error of the mean.

cifically adsorbed to SAMs of hexadecanethiolate, and RGD-containing peptide nonspecifically adsorbed to glass. Addition of the soluble peptide caused the rapid retraction and subsequent release (2–30 min) of cells from all substrates (1%, 0.1% RGD, FN, and RGD coated directly on glass). These results support previous findings that cell attachment via RGD is reversible and demonstrates that it is only necessary to antagonize interactions with binding sites for RGD to cause release of an attached cell from a surface presenting this ligand.

When cells were attached for 4 h before challenging with soluble GRGDSP, the rate of cell retraction and subsequent detachment was already slower for cells attached to FN-coated SAMs and RGD-coated glass (Figure 6A; 6C top versus middle panel). In contrast, cells continued to rapidly detach from mixed SAMs upon addition of soluble GRGDSP (Figure 6A; 6C middle panel). By 24 h, all substrates show an increased ability to resist cell detachment by the soluble peptide (Figure 6B; 6C bottom panel). Cells on FN-coated substrates barely retracted and could not be detached even after 4 h of incubation with the soluble peptide. In contrast, cells on RGD mixed SAMs readily detached with soluble peptide, at rates equal to or greater than cells attached to FN-coated substrates for only 2 h. The increase in cellular retraction and detachment rate from RGD-coated glass between 4 and 24 h suggests that complex remodeling changes in the surface may be occurring, consistent with the markedly high rate of radioactive protein deposition found on this surface (Figure 5).

The significance of these results is to suggest that only the specific interactions of RGD with cellular receptors are involved in initial adhesion (i.e., less than 2 h) of cells to RGD-containing substrates. Events are more complex, however, with cells allowed to attach for 24 h to these systems. Within 4 h, cells were able to deposit a functional non-RGD matrix on FN-coated SAMs and RGD-coated glass substrates such that cell adhesion was partially mediated through binding of other cell-surface adhesive receptors to this matrix. By 24 h these undefined cell-matrix interactions altered the adhesion and spreading interactions of cells entirely, as evidenced by the changes in the ability of soluble GRGDSP to detach cells from these substrates. In contrast, adhesion of cells to the mixed SAMs presenting (EG)₃OH and (EG)₆OGRGD lost specificity at significantly slower rates such that even at 24 h, the detachment rate of cells attached to these surfaces remained equal or better than rates of cells attached to FN-coated surfaces for only 2 h. The cellular interactions with RGD remained important for much longer times.

The rapidity with which cells detached from the RGD mixed SAMs is remarkable and has not previously been reported. Past studies describe the average time of detachment of these cells to be between 20 and 60 min,²⁸ whereas most cells detached from SAM substrates in ≤2 min. These data suggest that substrates that are traditionally used to present RGD also contain additional unknown adhesive factors in the background of the substrate. These results cannot rule out, however, that the faster detachment of cells from the RGD-terminated SAMs is due to a lower affinity of the immobilized peptide for cellular integrin receptors. Nonetheless, substrates such as these mixed SAMs might be useful in experiments that require rapid release of attached cells without the damage to cell surfaces caused by treatment with proteases or by other, nondiscriminate methods of releasing cells.

These results define sharply the molecular interaction involved in adhesion between these cells and mixed SAMs presenting RGD and (EG)₃OH moieties and eliminate the ambiguity in interpretation that has obscured results obtained with surfaces that are less carefully tailored than these to limit adhesion to only one type of interaction.

Conclusions

Fundamental studies of mechanisms of cell adhesion have been limited in the past by the inability to design and generate surfaces with defined molecular structure. Using SAMs to address these technical limitations, we have demonstrated a

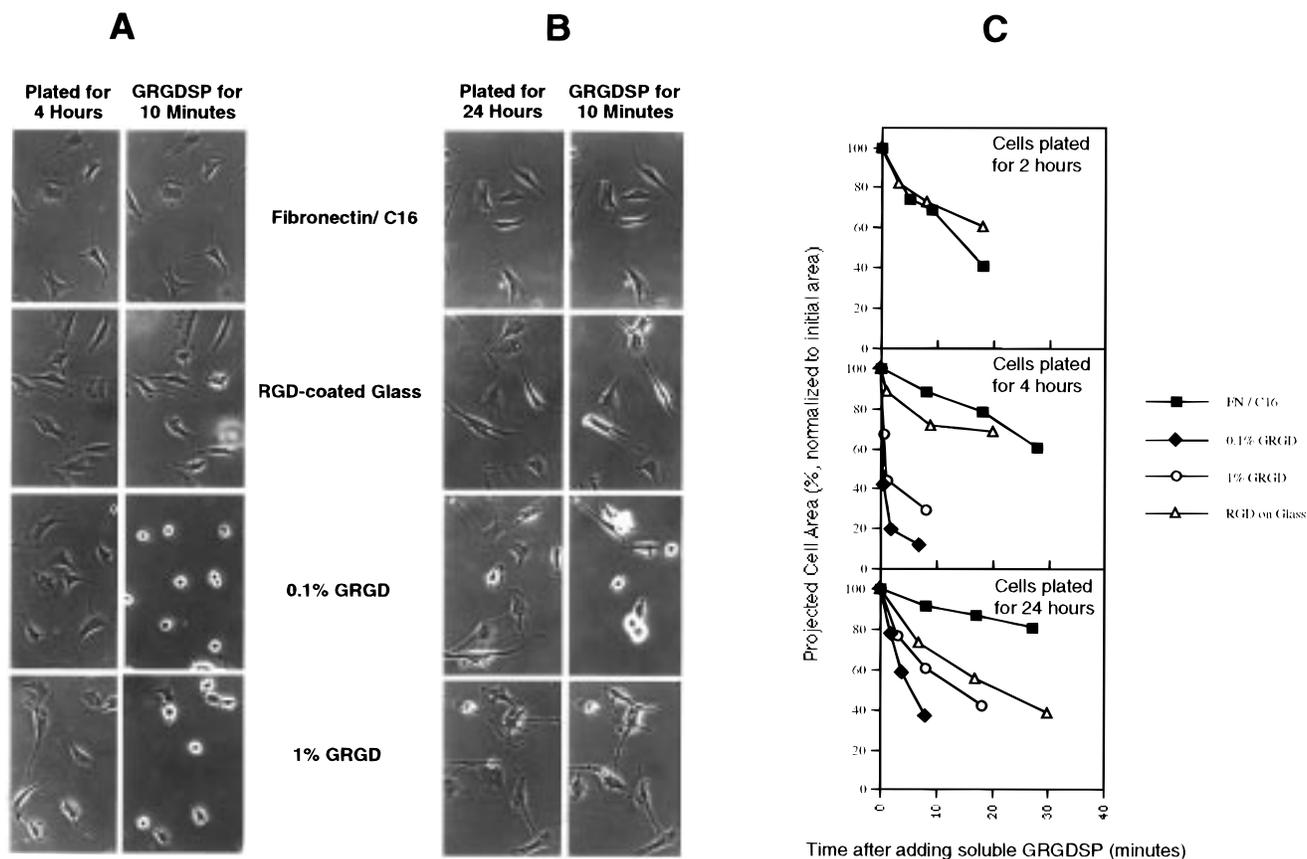


Figure 6. Retraction of cells caused by soluble GRGDSP peptide. Bovine capillary endothelial cells were allowed to attach to these substrates: mixed SAMs presenting RGD and tri(ethylene glycol) groups, fibronectin-coated SAMs of hexadecanethiolate, and RGD-coated glass. After allowing attachment to surfaces for 2, 4, or 24 h, soluble GRGDSP peptide to a concentration of 0.5 mg/mL. (A, B) Phase contrast micrographs of cells (attached 4 or 24 h, respectively) immediately before and 10 min after addition of peptide. (C) Plots of projected cell area over time after the addition of peptide. Between 25 and 50 cells were analyzed for each condition and time.

general approach to engineer surfaces with precise molecular and chemical compositions to study specific cell-substrate interactions.

Previous studies have shown that the binding of specific cell-surface integrins to the RGD peptide can mediate adhesion of cells to substrates.⁸⁻¹⁵ Interpretation of these studies was, however, limited because it was difficult to determine the initial density of RGD moieties required for efficient cell attachment and subsequent spreading and to study the changes of the surface over time that result from the active degradation and redeposition of extracellular matrix onto the substrate by the cells.

By engineering a surface presenting RGD peptide in an interface that is otherwise resistant to protein adsorption (because it comprises (EG)₃OH groups), we have created a substrate that allows biospecific adsorption of cells directly to the SAM. Furthermore, the synthetic strategy used to generate these SAMs presents a surface that is well-defined at a molecular scale. The use of an ethylene glycol in the SAM also reduces the deposition

of new matrix secreted by the attached cells onto the surface. As a result, the specificity of the cell-substrate interaction was maintained for at least 24 h. Thus, by using substrates that present RGD in an interface that is otherwise inert to adsorption and attachment of biological entities (proteins and cells), we demonstrate that integrin-RGD interactions alone are sufficient for long-term attachment and survival of cells.

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