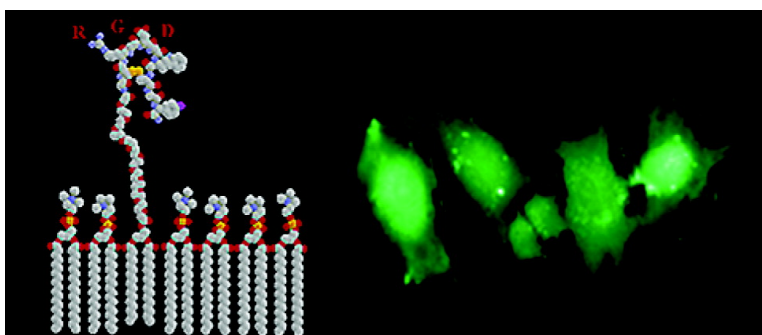


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Lipopeptides Incorporated into Supported Phospholipid Monolayers Have High Specific Activity at Low Incorporation Levels

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Abstract: The ability to present cell adhesion molecule (CAM) ligands in controlled amounts on a culture surface would greatly facilitate the control of cell growth and differentiation. Supported lipid monolayer/bilayer systems have previously been developed that allow for presentation of CAM ligands for cell interaction; however, these systems have employed peptide loadings much higher than those used in poly(ethylene glycol) (PEG)-based immobilization systems. We report the development of synthetic methods that can be used for the efficient and versatile creation of many linear and cyclic lipid-linked peptide moieties. Using RGD-based peptides for the $\alpha 5 \beta 1$ integrin as a model system, we have demonstrated that these lipopeptides support efficient cell binding and spreading at CAM ligand loadings as low as 0.1 mol %, which is well below that previously reported for supported lipid systems. Engineered lipopeptide-based surfaces offer unique presentation options not possible with other immobilization systems, and the high activity at low loadings we have shown here may be extremely useful in presenting multiple CAM ligands for studying cell growth, differentiation, and signaling.

Introduction

The growth and differentiation of many cell types are strongly influenced by adhesion to other cells and the extracellular matrix. The ability to present cell adhesion molecule (CAM) ligands in controlled densities and orientations on an otherwise non-adherent culture surface would enhance our understanding of the impact of engaging individual and multiple CAMs on cell growth and differentiation. Although both proteins and peptides have been used in this capacity, peptide-based mimetics of adhesion ligand epitopes have advantages that include control of presentation, ease of incorporating multiple ligands, and resistance to denaturation. The arg-gly-aspartic acid (RGD) peptide is a ubiquitous motif that is targeted by several members of the integrin class of CAMs.¹ The linear peptide sequence GRGDSP is found in the extracellular matrix protein fibronectin. High affinity cyclic RGD peptides, such as GC*RGDGWC* (C* denotes cyclization site), have been identified that specifically target $\alpha 5 \beta 1$ integrins.^{2,3} Linear and cyclic RGD (cRGD) peptides have been immobilized in a variety of ways to interact with RGD-specific integrins.

Poly(ethylene glycol) (PEG) tethers are commonly used to immobilize RGD and other peptides onto a surface. PEG-based

surfaces combine the advantage of low nonspecific binding with the ability to incorporate controlled levels of peptide-modified-PEG on the surface. Peptide-PEG conjugates have been immobilized via thiol-mediated attachment to gold,^{4,5} covalent attachment to PEG monoacrylate polymer networks,⁶ and conjugation to PEG comb and star polymers that are subsequently immobilized.^{7,8} These systems have enabled exploration of the effects of tether length on cell attachment, spreading, and migration at ligand concentrations ranging from 0.001 to 100 mol % for thiol/gold systems and ligand surface densities in the range of 160 to 30 000 fmol/cm² for other systems.

Peptide ligands can also be immobilized in the form of amphiphilic peptide-lipid conjugates (lipopeptides). Lipid-based systems have several unique advantages over other immobilization techniques. For example, lipid systems permit intimate mixing of various CAM ligands presented at the surface of vesicles and supported lipid monolayers. Through control of lipid chain length and saturation, lipid presentation systems allow for control of ligand surface properties, such as fluidity and domain formation, that are not accessible with polymer-tethered ligand systems.^{9–11} Both linear^{12–15} and cyclic^{16–18}

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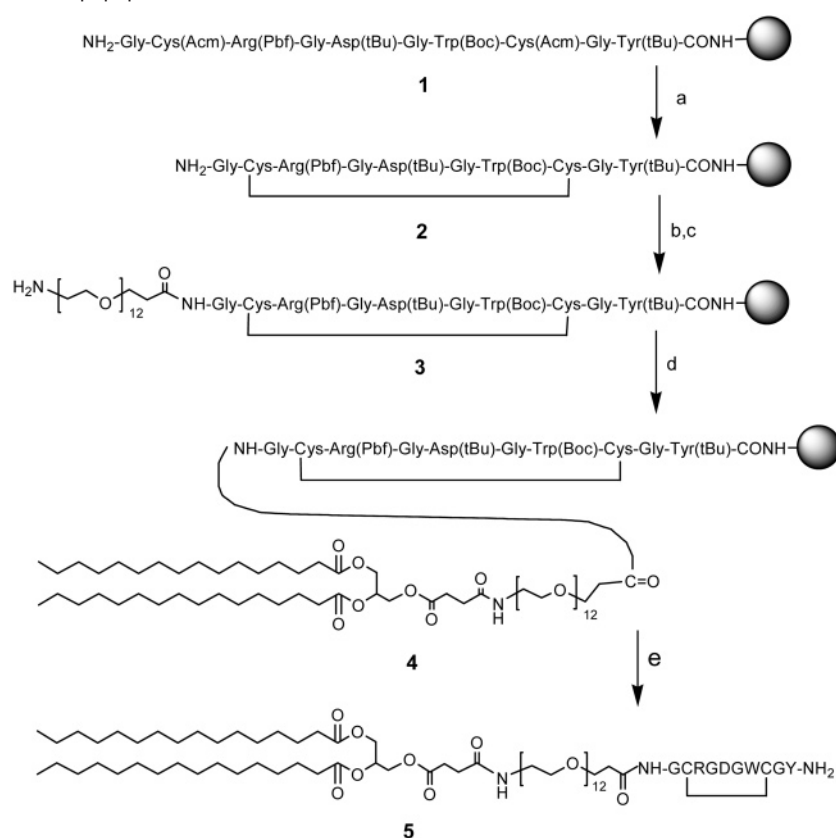
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Scheme 1. Synthesis of cRGD Lipopeptide^a

^a Reagents and conditions: (a) thallium(III) tris(trifluoroacetate) $[\text{Ti}(\text{CF}_3\text{COO})_3]$ in NMP -5°C , 80 min. (b) Fmoc-PEG600 acid/BOP/HOBt/DIEA in NMP, 4 days. (c) 25% piperidine in NMP, 20 min. (d) DPG-Su/BOP/HOBt/DIEA in NMP, 4 days. (e) 2.5% TIS/2.5% $\text{H}_2\text{O}/\text{TFA}$, 2 h.

peptides have been coupled to hydrophobic anchors and subsequently immobilized in supported lipid monolayers. It has been shown that a lipopeptide can be incorporated into a supported lipid monolayer at controlled densities^{12–14} and that mixtures of lipopeptides can be incorporated at controlled ratios.^{13,15} The resulting surfaces have been shown to bind to integrins in a cell-free system over a wide range of lipopeptide concentrations from 5 mol % to 100 mol % of total lipid on the surface.^{13,14,18} Cell spreading on lipopeptides in supported monolayers has also been shown for loadings of greater than 5 mol %, ^{12,17} which is much greater than those explored using nonlipid, PEG-tethered systems. We have attempted to bridge this gap in peptide density by developing a lipid-based system that demonstrates efficient cell binding and spreading at very low CAM ligand loading, using RGD-based peptides for the $\alpha 5\beta 1$ integrin as a model system.

In this paper, we report for the first time the solid-phase synthesis of glycerolipid-tethered cyclic and linear peptides and

their incorporation into a solid-supported lipid membrane for integrin-mediated cell attachment and spreading. We have developed custom cell culture cassettes that allow us to incubate cells on supported lipid monolayers and then directly measure both cell attachment, via a normal force adhesion assay, and cell spreading. Importantly, we demonstrate that the levels of immobilized peptide required for cell adhesion and spreading are at least an order of magnitude lower than those previously employed in lipid-based systems and are comparable to RGD loadings used in PEG-based immobilization systems.

Lipopeptide Synthesis. Our synthetic approach consisted of two steps: solid-phase peptide synthesis followed by coupling of a glycerolipid-PEG construct to the protected cyclic or linear peptide bound to the resin. This approach is illustrated for long tether cRGD lipopeptide **5** in Scheme 1. We first synthesized fully protected GCRGDGWCGY on Rink amide resin by standard Fmoc strategy.¹⁹ After coupling of the last amino acid, the N^α-Fmoc protection group was removed resulting in linear, protected peptide resin **1**. For the synthesis of cyclic peptide through a disulfide bridge, peptide cyclization was performed on solid phase while the peptide chain was fully protected. The peptide-resin **1** was treated with thallium(III) tris(trifluoroacetate) $[\text{Ti}(\text{CF}_3\text{COO})_3]$ by a modified disulfide bond formation method^{20,21} to form the cRGD peptide-resin **2**.

After peptide synthesis and cyclization, part of **2** was cleaved to generate free peptide for confirmation of the peptide sequence

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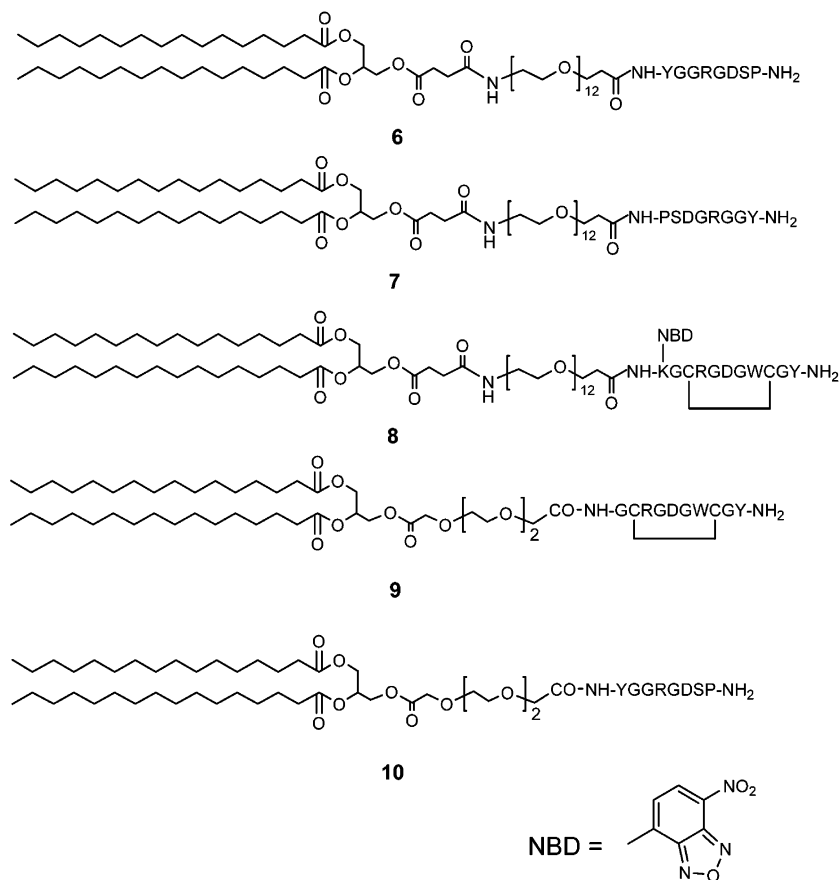


Figure 1. Structures of synthesized lipopeptides.

by ESI-MS and HPLC analyses before further coupling. Bifunctional *O*-(*N*-Fmoc-2-aminoethyl)-*O'*-(2-carboxyethyl)undecaethyleneglycol acid (FmocPEG600 acid; NovaBiochem) tether was reacted with the free N-terminus of **2** using a coupling mixture of benzotriazole-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP)/*N*-hydroxybenzotriazole (HOBt)/diisopropylethylamine (DIEA) in DCM with a 10 min preactivation step before coupling, and the N^{α} -Fmoc protection group was removed to form **3**. Separately, 1,2-dipalmitoyl-*sn*-glycerol (DPG; Fluka) was reacted with succinic anhydride in the presence of pyridine in DCM to form the acid terminal glycerolipid DPG-Su (see Supporting Information Scheme S.1). DPG-Su was subsequently reacted with the N-terminus of **3** using a mixture of BOP/HOBt/DIEA in DCM to produce lipid-tethered cRGD on the resin **4**. Simultaneous side chain deprotection and cleavage from the resin in the presence of scavenger afforded cRGD lipopeptide **5**.

Slight variations of this method were used to synthesize linear RGD lipopeptide **6**, linear RGD lipopeptide with a scrambled sequence **7**, a 7-nitrobenzofurazan (NBD)-labeled cRGD lipopeptide **8**, as well as cyclic and linear RGD lipopeptides with a short PEG tether **9**, **10** (Figure 1). The crude products were analyzed and purified by RP-HPLC, and their structures were confirmed by ESI-MS or MALDI-TOF MS.

Lipopeptide Deposition. Lipopeptide-containing surfaces for cell culture were created by fusion of small unilamellar lipid vesicles onto a hydrophobic surface formed by treatment of glass slides with octadecyltrichlorosilane (OTS). The vesicles contained both lipopeptide and a saturated carrier lipid, dipalmitoylphosphatidylcholine (DPPC), with the final ligand concen-

tration on the monolayer surface determined by the concentration of lipopeptide in the vesicle mixture. Lipopeptide deposition from vesicles was verified using fluorescence microscopy and radiolabeling. Surfaces resulting from vesicles incorporating either 1.0 mol % DPPE-NBD (Avanti Polar Lipids) in DPPC (Figure 2A) or 1.0 mol % **8** in DPPC (Figure 2B) were visually uniform across the surface of the well at all magnifications tested up to 400 \times . The surface loading of ^{125}I -labeled **5** was measured for vesicle lipopeptide content ranging from 0.1 mol % to 2.5 mol % in DPPC (Figure 2C). The resulting lipopeptide surface density in the culture wells was proportional to the level of lipopeptide incorporated into the vesicle suspension (8.5 ± 1.3 pmol cRGD/cm 2 /mol % incorporated in vesicles) across the measured range.

KG-1a Cell Adhesion. Cell adhesion to lipopeptide-containing monolayers was determined using the KG-1a cell line²² as a model for nonspreading, primitive, CD34 $^+$ hematopoietic stem and progenitor cells. The number of fluorescently stained KG-1a cells in each well after 2 h of incubation in serum-containing medium was quantified using automated image analysis of several defined areas in each well (Figure 3A and C). After nonadherent KG-1a cells were removed via normal force centrifugation, the number of adherent cells was quantified again by automated image analysis of the same defined areas in each well (Figure 3B and D). Cell adhesion for all values of adherence from 0 to 100% was homogeneous across the culture surface (Figure 3B and D and data not shown), indicating a uniform lipopeptide distribution.

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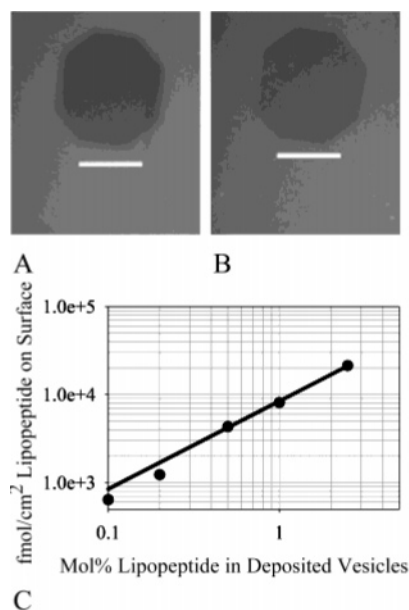


Figure 2. Deposition of lipopeptides into supported lipid monolayers was visualized (200 \times magnification) using DPPC vesicles containing 1.0 mol % DPPE-NBD (A) or 1.0 mol % **8** (B) and quantified using DPPC vesicles containing ¹²⁵I-labeled **5** (C). The octagonal spots in images A and B were photobleached to provide contrast with the coated surface. Scale bars in both images are 100 μ m.

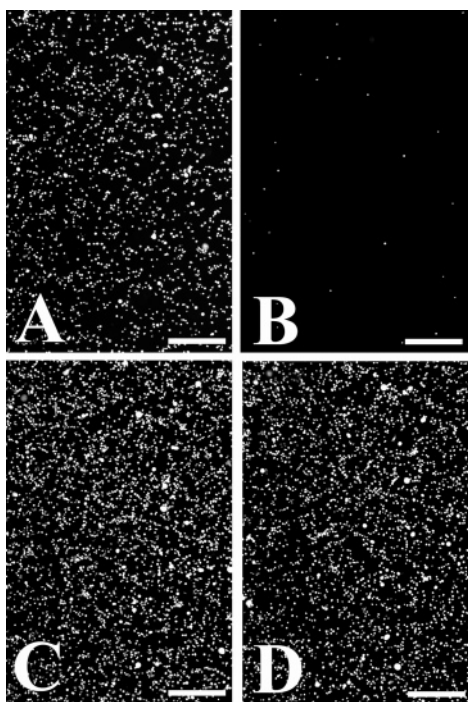


Figure 3. CFSE-stained KG-1a cells on control and lipopeptide-containing DPPC surfaces (all scale bars are 300 μ m) after 2 h of incubation on supported lipid monolayers before (A and C) and after centrifugation (B and D) on surfaces containing 0 mol % (A and B) and 1.0 mol % compound **5** (C and D). Image B shows 1.5% adhesion, and image D shows 90.3% adhesion.

When the high affinity cRGD **5** was incorporated into the surface, KG-1a cell adherence increased sharply for loadings greater than 0.05 mol %, with 40% adherent cells for 0.1 mol % and greater than 96% adherent cells for 1.0 mol % lipopeptide (Figure 4). Cell adhesion remained greater than 95% for loadings up to 5 mol % **5** (Figure 4).

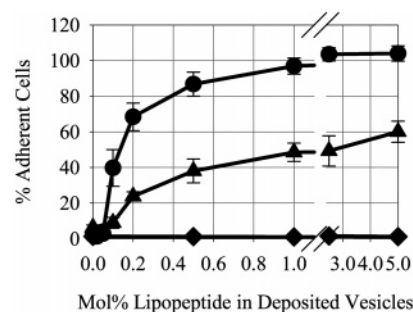


Figure 4. Adhesion of KG-1a cells to supported DPPC monolayers with various amounts of incorporated **5** (●), **6** (▲), or **7** (◆). Error bars indicate 99% confidence interval.

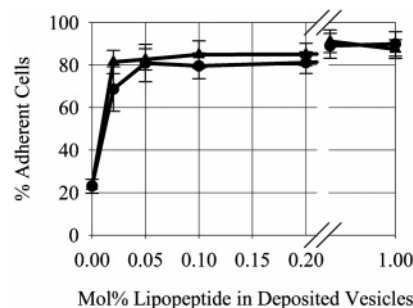


Figure 5. Fractional adhesion for CFSE-stained HUVECs to supported DPPC monolayers with various amounts of incorporated **5** (●) and **6** (▲). Error bars indicate 99% confidence interval.

For the lower affinity LinRGD **6**, KG-1a cell adhesion increased only slightly at 0.1 mol % lipopeptide incorporation and reached approximately 60% for a lipopeptide loading of 5.0 mol % (Figure 4). Above loadings of 5.0 mol %, cell adhesion increased only slightly to 65% at 10 mol % incorporated lipopeptide (data not shown). Besides supporting a lower maximal adhesion than cRGD **5**, LinRGD **6** required a higher lipopeptide loading to achieve half-maximal adhesion, 0.28 mol % versus 0.13 mol % for **5**. Incorporation of scrambled peptide **7** into the DPPC surface did not support adhesion above background at loadings up to 5.0 mol % (Figure 4). Furthermore, preincubation of the cells with soluble YGGRGDSP peptide (0.1 mM) decreased KG-1a cell adhesion to a level similar to that for the DPPC control on surfaces containing either **5** or **6** (data not shown).

When short tether cRGD **9** was incorporated into the DPPC surface, KG-1a cell adhesion increased to 9% for 0.5 mol % lipopeptide ($p < 0.01$) with no further increase up to 5.0 mol % (data not shown). When short tether LinRGD **10** was incorporated into the surface, no significant adhesion enhancement was measured for any loading up to 5.0 mol % (data not shown).

HUVEC Adhesion and Spreading. The adhesion of human umbilical vein endothelial cells (HUVECs) incubated for 4 h on lipopeptide-containing surfaces was measured in a manner analogous to that for KG-1a cells. For both LinRGD **6** and cRGD **5**, HUVEC adherence was between 70% and 80% for loadings as low as 0.02 mol %, with a maximal adhesion of 90% at 0.5 mol % and above (Figure 5). Thus, extensive HUVEC adhesion occurs at much lower lipopeptide loadings than for KG-1a cells.

HUVECs were also used to examine cell spreading on lipopeptide-containing surfaces. The extent of cell spreading was

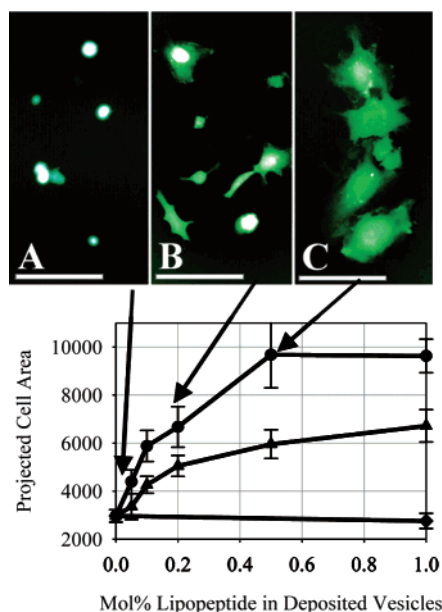


Figure 6. Spreading of CFSE-stained HUVECs on DPPC surfaces containing various amounts of incorporated **5** (●), **6** (▲), or **7** (◆). Error bars indicate 99% confidence interval. HUVECs after 4 h of incubation on surfaces containing (A) 0.0 mol %, (B) 0.1 mol %, and (C) 0.5 mol % compound **5** (all scale bars are 100 μm).

determined by calculating the average projected area of CFSE-dyed HUVECs incubated for either 4 (Figure 6) or 24 h (not shown). HUVEC spreading was detected at a cRGD **5** content as low as 0.05 mol %, with a maximal spreading of 3.3 times greater projected area than control obtained at 0.5 mol % with no further increase at 1.0 mol % (Figure 6). HUVEC spreading was less extensive on surfaces containing LinRGD **6**, with maximal spreading 2.3 times greater than that of control at 1.0 mol %. No further increase in cell spreading was seen for loadings of **6** as high as 2.5 mol % (data not shown). There was no increase in cell spreading relative to DPPC control when the scrambled lipopeptide **7** was incorporated at 1.0 mol % (Figure 6). After 24 h of incubation, the extent of cell spreading was essentially the same as that at 4 h, except that there was a 15–20% greater cell area on surfaces incorporating 1.0 mol % **5** or 0.5–1.0 mol % **6** (data not shown).

Discussion

We demonstrate here for the first time cell adhesion and spreading on supported lipid monolayers with very low levels of incorporated RGD-lipopeptides. The modular nature of the solid phase synthetic route facilitates the synthesis of a variety of lipopeptides consisting of linear or cyclized peptides, PEG tethers of variable length, and lipid anchors of variable chain length and saturation. The ability to cyclize peptides on the resin followed by both lipid attachment and fluorescent labeling on the solid phase simplifies the synthesis route from those previously published.^{16–18} In contrast to previous lipopeptides used for cell adhesion,^{12,17,23} our constructs utilize the naturally occurring glycerolipid dipalmitoyl glycerol as anchor, thus more closely mimicking the structure of naturally occurring lipids.

The development of unique cell culture cassettes allowed us to culture both spreading and nonspreading cell types in a

standard 96-well plate format, facilitating detailed quantitative studies of cell adhesion and spreading on ligand-presenting supported lipid monolayers. The results of our experiments show that near-maximal HUVEC adhesion occurs with as little as 170 fmol RGD/cm² (0.02 mol %). This very low RGD density is comparable to that observed previously for bovine capillary endothelial cells on thiol-immobilized PEG-RGD (0.001 mol %).⁴

In contrast to endothelial cells, fibroblasts, and other spreading cell types, little is known about the adhesion behavior of nonspreading hematopoietic progenitor cells on engineered ligand-presenting surfaces. In our experiments, nonspreading KG-1a cells required much higher levels of incorporated lipopeptide to achieve maximum adhesion (0.5 mol %; 4200 fmol/cm²) than did endothelial cells. This could be due in part to the rounded KG-1a cells having less extensive contact area with the supported monolayer. Although many hematopoietic cells do not spread during adhesion, their growth and differentiation are influenced by integrin engagement.^{24–26} In the future, normal force adhesion assays such as the one used here may aid in the further analysis of ligand densities necessary for adhesion of nonspreading cells.

Maximal spreading of HUVECs on the lipid monolayer system required about 10- to 20-fold higher levels of incorporated lipopeptide (0.5 mol %; 4200 fmol/cm²) than for maximal adhesion. This trend is consistent with spreading versus adhesion (10- to 100-fold higher) for endothelial cells⁴ and migration versus adhesion (5- to 10-fold higher) for fibroblasts⁷ with nonlipid PEG immobilization. Although there was some non-specific adhesion of HUVECs on control DPPC surfaces, the cells remained rounded and no spreading was observed. Lipid-based systems have been used previously to examine cell spreading,^{12,17,23} although at lipopeptide loadings of 5 to 100 mol %. This loading is much higher than required for our system, as well as for much of the previous work with nonlipid systems that support cell spreading at loadings as low as 0.1 mol % for thiol/gold systems and 1500 fmol/cm² for other systems.^{4,5,7}

As expected, both HUVEC spreading and KG-1a cell adhesion were greater for the same level of immobilized cRGD as for linear RGD. The greater affinity of cyclic versus linear RGD motifs for integrins has been shown previously^{2,3} and may be explained in part by differences in the ability of each peptide to mimic RGD epitopes found in naturally occurring integrin ligands.²⁷ However, the reported maximal response to cRGD may be equal to or greater than that to LinRGD. While we observed similar maximal HUVEC adhesion for linear and cyclic RGD (Figure 5), the maximal responses to linear RGD for HUVEC spreading and KG-1a cell adhesion did not reach those of cRGD for any of the linear RGD densities examined (Figures 4 and 6).

These results are consistent with other reports in the literature. At saturating concentrations, Verrier et al.²⁸ measured 15% less

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extensive cell spreading on linear RGD. Xiao and Truskey²⁹ found similar contact areas (spreading) on both linear and cRGD peptides over a 15 min incubation period, but a much lower shear stress was required to remove cells from saturating concentrations of linear RGD than that for saturating concentrations of cRGD. Finally, Kato and Mrksich³⁰ found that cell adhesion and focal plaque formation took substantially longer on linear RGD and that there were distinct differences in focal adhesion size and location for saturating concentrations of the linear and cRGD ligands.

In summary, we have developed a simple and versatile solid phase approach to synthesis of lipopeptides containing linear and cyclic cell adhesion ligands. Incorporation of these lipopeptides into supported lipid monolayers resulted in cell adhesion and spreading at ligand densities substantially lower than those previously reported for supported lipid systems. The low ligand density necessary to induce cell adhesion and spreading in this system may prove useful in future studies in which presentation of multiple cell adhesion ligands is desired. Immobilization of multiple ligands will likely be necessary to modulate the growth and differentiation of cells such as hematopoietic stem cells.^{31–33} Because of the unique properties of lipid monolayers, such as tunable monolayer fluidity and the ability to control phase segregation, this system may be used to explore unique ligand presentation configurations not possible with other immobilization strategies.

Experimental Section

Lipopeptide Synthesis. A. Peptide Synthesis. Reagents and solvents for peptide synthesis and protected Fmoc amino acids were purchased from Novabiochem and Advanced ChemTech. Peptides were synthesized manually on a Rink amide AM (GCRGDGWCGY; cRGD; 0.64 mmol/g) or Rink amide PEGA (YGGRGDSP; LinRGD; PSDGRGGY; LinDGR; 0.43 mmol/g) resin by the Fmoc solid phase peptide synthesis method with the following amino acid side chain protection: *t*-But (Ser, Asp, and Tyr), Pbf (Arg), Trt (His and Asn), Acm (Cys), and Boc (Trp). After coupling of the last amino acid in the sequence the N-terminus Fmoc was removed. For cRGD, the peptide resin was treated with Tl-(CF₃COO)₃ (2 equiv) in NMP at –5 °C for 80 min to cyclize the peptide on resin. After peptide synthesis, part of the peptide resin was cleaved, and ESI-MS or MALDI-TOF MS confirmed the sequence of the free peptide.

B. 1, 2-Dipalmitoyl-*sn*-glycerol-3-*O*-succinic Acid Monoester (DPG-Su; See Supporting Information). DPG (957 mg, 1.68 mmol) and succinic anhydride (337 mg, 3.36 mmol) were added to a solution containing 10 mL of pyridine and 5 mL of DCM. Reaction mixture was stirred and heated to 60 °C and refluxed overnight. The reaction was monitored by silica gel TLC (DCM–acetone = 20:1). After completion, the mixture was concentrated under reduced pressure and the residue was suspended in DCM. Insoluble solids were removed by filtration. The solution was diluted to a volume of 40 mL by addition of DCM and washed with water (20 mL × 4), 10% citric acid solution (20 mL), and water (20 mL). The DCM solution was then dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to a small volume (ca. 5 mL). Recrystallization by addition of hexane to the DCM solution afforded 1.08 g of crystal product (96%). ¹H NMR δ ppm (500 MHz, CDCl₃): 5.27 (1 H, m, sn-2 CH), 4.28–

4.34 (2 H, m, sn-1 CH₂), 4.13–4.20 (2 H, m, sn-3 CH₂), 2.68 (4 H, m, br, CH₂–CH₂ succinic acid), 2.31 (4 H, m, br, CH₂–C=O both acyl chains), 1.61 (4 H, s, br, CH₂–CH₂–C=O both acyl chains), 1.27 (48 H, s, br, (CH₂)₁₂ both acyl chains), 0.88 (6 H, t, *J* = 7.0 Hz, CH₃ both acyl chains). ¹³C NMR δ ppm (125 MHz, CDCl₃): 176.43 (COOH), 173.61 (COOR), 173.20 (COOR), 171.82 (COOR), 68.95, 62.90, 62.25 (glycerol carbons), 34.42 (CH₂–C=O), 34.29 (CH₂–C=O), 32.17 (CH₂–CH₂–CH₃ both acyl chains), 29.95, 29.91, 29.75, 29.62, 29.32, 28.92, 28.75 ((CH₂)₁₀ both acyl chains), 29.53, 39.37 (CH₂–CH₂ succinic acid), 25.12 (CH₂–CH₂–C=O both acyl chains), 22.94 (CH₂–CH₃ both acyl chains), 14.37 (CH₃ both acyl chains).

C. 1, 2-Dipalmitoyl-*sn*-glycerol-3-*O*-(3,6,9-trioxaundecanedioic acid) Monoester (DPG-PEG200 Acid; See Supporting Information). DCC (725 mg, 3.5 mmol) in 10 mL of DCM was added to a stirred solution containing 500 mg of DPG (0.88 mmol), 760 μL of PEG200 diacid (3.5 mmol), and 429 mg of DMAP (3.5 mmol) in 10 mL of DCM. The reaction mixture was stirred at room temperature, and the reaction was monitored by silica gel TLC (solvent system *n*-hexanes–ethyl acetate = 3:2). After overnight reaction, the byproduct precipitate was removed by filtration. The solution was concentrated, methanol was added to the residue to dissolve the impurities, and the insoluble product was collected by filtration. The solid was further purified by suspension in methanol and filtration. ¹H NMR δ ppm (500 MHz, CDCl₃): 5.28 (1 H, m, sn-2 CH), 4.38 (1 H, dd, *J* = 3.5 and 12.0 Hz), 4.30 (1 H, dd, *J* = 3.5 and 12.0 Hz), 4.13 (2 H, m), glycerol protons; 4.17 (2 H, s, O=C–CH₂–O–), 4.16 (2 H, s, O=C–CH₂–O–), 3.71–3.77 (8 H, m, –O–CH₂–CH₂–O–CH₂–CH₂–O–), 2.31 (4 H, m, br, CH₂–C=O both acyl chains), 1.60 (4 H, s, br, CH₂–CH₂–C=O both acyl chains), 1.27 (48 H, s, br, (CH₂)₁₂ both acyl chains), 0.87 (6 H, t, *J* = 6.0 Hz, CH₃ both acyl chains). ¹³C NMR δ ppm (125 MHz, CDCl₃): 173.38, 172.97, 172.60, 170.12, four C=O; 71.10, 70.81, 70.59, 70.34, 68.65, 68.31, –CH₂–O–; 69.16, 62.65, 61.97 (glycerol carbons), 34.17 (CH₂–C=O), 34.04 (CH₂–C=O), 31.93 (CH₂–CH₂–CH₃ both acyl chains), 24.87 (CH₂–CH₂–C=O), 24.85 (CH₂–CH₂–C=O), 22.70 (CH₂–CH₃ both acyl chains), 14.14 (CH₃ both acyl chains).

D. DPG-Su-PEG600-cRGD 5. *O*-(*N*-Fmoc-2-Aminoethyl)-*O'*-(2-carboxyethyl)undecaethyleneglycol (0.160 g, 0.192 mmol) (Fmoc-PEG600 acid; NovaBiochem), BOP (0.085 g, 0.192 mmol), HOBt (0.03 g, 0.192 mmol), and DIEA (50 μL, 0.287 mmol) were dissolved in 2.0 mL of DCM–NMP (1:1) and shaken for 10 min. The mixture was then added to 0.2 g of cRGD-resin **2** (0.128 mmol), and the mixture was rocked for 4 days. Then, the resin was washed with DCM × 3 and NMP × 3, followed by addition of a solution of 0.57 g of BOP (1.28 mmol), 0.196 g of HOBt (1.28 mmol), 73 μL of acetic acid (1.28 mmol), and 250 μL of DIEA (1.44 mmol) in 2 mL of DCM–NMP (1:1) for 2 h. The resin was washed with DCM × 3 and NMP × 4, followed by treatment with 25% piperidine in NMP for 20 min. The resin was washed with NMP × 3, DCM × 2, DCM–MeOH (1:1) × 2, IPA, and dried. DPG-Su (0.171 g, 0.256 mmol), BOP (0.113 g, 0.256 mmol), HOBt (0.04 g, 0.256 mmol), and DIEA (67 μL) were added to 1.0 mL of DCM and shaken for 10 min before adding to the PEG600-cRGD-resin. After the mixture was shaken for 4 days, 0.053 g of DCC (0.256 mmol) in 0.3 mL of DCM was added to the reaction vessel and shaken overnight. The resin was washed with DCM × 3, NMP × 4, DCM × 2, DCM–MeOH (1:1) × 2, and IPA and dried in a vacuum.

E. DPG-Su-PEG600-LinRGD 6 and DPG-Su-PEG600-LinDGR 7. 0.180 g of Fmoc-PEG600 acid (0.215 mmol), 0.095 g of BOP (0.215 mmol), 0.033 g of HOBt (0.215 mmol), and 70 μL of DIEA (0.43 mmol) were dissolved in 1.5 mL of DCM and shaken for 10 min. The mixture was then added to 0.25 g of LinRGD-resin (0.107 mmol) (or DPG-resin) and the mixture was shaken overnight. Resin was rinsed with DCM × 3 and NMP × 4 followed by the addition of 5 mL of 25% piperidine in NMP and shaken for 30 min. Resin was washed with NMP × 3, DCM × 3. DPG-Su (0.143 g, 0.215 mmol), BOP (0.095 g, 0.215 mmol), HOBt (0.033 g, 0.215 mmol), and DIEA (70 μL, 0.43

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mmol) were added to 1.5 mL of DCM and shaken for 10 min before being added to PEG600-LinRGD resin (or PEG600-DGR resin) and shaken overnight. The resin was washed with DCM \times 3, NMP \times 4, DCM \times 4, IPA \times 4 and dried.

F. DPG-Su-PEG600-cRGD-NBD 8. Fmoc-Lys(Mtt)-OH (0.240 g, 0.384 mmol; Novabiochem), BOP (0.170 g, 0.384 mmol), HOBt (0.059 g, 0.384 mmol), and DIEA (100 μ L, 0.575 mmol) were added to NMP and shaken 10 min before being added to c-RGD-resin **2** (0.2 g, 0.128 mmol) and rocked overnight. The resin was washed with NMP \times 4 followed by the addition of 5 mL of 25% piperidine in NMP. The reaction vessel was shaken for 30 min followed by rinsing the resin with NMP \times 3, DCM \times 3. Fmoc-PEG600 acid and DPG-Su were added as described for DPG-Su-PEG600-cRGD. Lys(Mtt) was deprotected by rinsing the resin with 30 mL of DCM-triisopropylsilane (TIS)-trifluoroacetic acid (TFA) (90:5:5) over a period of 10 min followed by rinsing in DCM \times 4 and NMP \times 3. NBD-Cl (0.153 g, 0.768 mmol) (Sigma) in 5 mL of NMP was added to the DPG-Su-PEG600-Lys-cRGD-resin and shaken for 90 min (the resins glowed green under UV light). Resin was rinsed in NMP \times 3. 4-Chloro-7-nitrobenzofurazan (NBD-Cl) (0.250 g, 1.28 mmol) in NMP was added to the resin and shaken for 48 h. This process was repeated for 0.250 g of NBD-Cl in DMF and then in THF. The resin was rinsed in THF \times 3, NMP \times 3, DCM \times 3, and IPA \times 3 and dried in a vacuum.

G. DPG-PEG200-cRGD 9. DPG-PEG200 acid (0.120 g, 0.120 mmol), BOP (0.052 g, 0.120 mmol), HOBt (0.028 g, 0.120 mmol), and DIEA (42 μ L, 0.240 mmol) were added to 1 mL of DCM and shaken for 10 min before being added to a reaction vessel containing 0.12 g of the cRGD-resin (0.043 mmol) and rocked overnight at room temperature. DCC (0.021 g, 0.100 mmol) in 0.3 mL of DCM was added to the reaction vessel and shaken overnight. The resin was washed with DCM \times 3, NMP \times 2, DCM-MeOH (1:1) \times 2, and IPA and dried in a vacuum.

H. DPG-PEG200-LinRGD 10. DPG-PEG200 acid (0.168 g, 0.215 mmol), BOP (0.095 g, 0.215 mmol), HOBt (0.033 g, 0.215 mmol), and DIEA (70 μ L, 0.43 mmol) were added to 1 mL of DCM and shaken for 10 min before being added to a reaction vessel containing 0.25 g of the peptide resin (0.107 mmol) and rocked overnight at room temperature. The resin was washed with DCM \times 3, NMP \times 2, DCM-MeOH (1:1) \times 2, and IPA and dried in a vacuum.

I. Lipopeptide Cleavage. Lipopeptide conjugates were cleaved from the resin by treatment with TFA-TIS (95:5) at room temperature for 2–3 h. The crude products were analyzed and purified by RP-HPLC with a C4 analytical column (10 μ m, 4.6 \times 250 mm²) and a C4 semipreparative column (10 μ m, 22 \times 250 mm²) (Vydac) by solvent gradients using solvent systems: (a) 10% acetonitrile (ACN) aqueous solution containing 0.1% TFA and (b) ACN-IPA (1:1) containing 0.1% TFA. The molecular weights of the purified products were confirmed by ESI-MS or MALDI-TOF MS.

J. ¹²⁵I Labeling. Labeling was based on a method of Winger et al.¹⁵ with some modification. Briefly, 0.3 mg of **5** was suspended in 0.15 mL of labeling buffer, 4:2:1 H₂O/ACN/IPA. Five iodobeads (Pierce) were rinsed with labeling buffer and placed in a reaction vessel. Labeling buffer (300 μ L) containing 2 mCi Na¹²⁵I (MP Biomedicals) was then added to the reaction vessel to incubate for 5 min. Lipopeptide suspension (150 μ L) was then added to the vessel. The vessel was shaken every 10 min for 1 h. The contents were then removed and placed into a desalting column (Amersham). The column was eluted with labeling buffer, and the collected fractions were measured for radioactivity. The first radioactive peak was then pooled (generally 3–4 fractions of approximately 0.3 mL each) and passed over a second desalting column. The first radioactive peak was again pooled. The labeling buffer was removed by bubbling nitrogen through the solution, and the lipopeptides were resuspended in chloroform. Lipopeptide concentration was determined by absorbance at 280 nm measured against a standard curve.

Cell Culture and Staining. KG-1a cells (ATCC) were maintained in IMDM medium (Sigma) supplemented with 5% fetal bovine serum (FBS) (Gibco) and 110 mg/L pyruvic acid. During the 2 h adhesion assay, cells were suspended in phenol red free DMEM supplemented with 5% FBS. HUVECs were maintained in phenol red free EGM medium (Clonetics) supplemented with Clonetics EGM Bullet Kit (bovine brain extract, hEGF, hydrocortisone, 2% FBS, Gentamicin and Amphotericin B) and passaged using trypsin according to the supplier's protocol. HUVECs were used between passages 3 and 10. Both cell types were cultured at 37 °C in a 5% CO₂ atmosphere and were used during the exponential growth phase for adhesion assays.

The 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining procedure for KG-1a cells is a modification of that by Glimm and Eaves.³⁴ Briefly, 20 h prior to use in adhesion experiments, 40 μ L of a 25 μ g/mL solution of CFSE in DMSO was added to 10 mL of PBS at 37 °C (100 ng/mL). KG-1a cells were resuspended at 7.5 \times 10⁶ cells/mL in CFSE-containing PBS and incubated at 37 °C for 30 min. Cells were washed twice in warm IMDM media and incubated overnight in supplemented IMDM at (5–8) \times 10⁶ cells/mL to allow excess CFSE to diffuse out of cells.

Adherent HUVECs, were stained with 50 ng/mL CFSE in Hanks balanced salt solution (HBSS) for 30 min followed by two rinses with supplemented EGM. HUVECs were then incubated in supplemented EGM for 4–6 h prior to use. When CFSE was allowed to diffuse out of HUVECs for 20 h, fully spread cells were difficult to image with the microscope.

Supported Monolayer Preparation. A. Hydrophobic Slides. Glass slides were cleaned based on the method of Cras et al.³⁵ Briefly, glass was immersed for 30 min in HCl/methanol (1:1), rinsed, and dried, followed by immersion in H₂SO₄, rinsing, and drying. Glass was silanized following the method of Silberzan et al.³⁶ with some modifications. A coating solution of hexadecane/carbon tetrachloride/chloroform (45:3:2 ratio; using water-saturated carbon tetrachloride and chloroform) containing 5 μ L/mL octadecyltrichlorosilane (OTS) was made 10–15 min prior to use. Clean glass slides were immersed in a vessel containing coating solution suspended in an ultrasonic bath. Slides were coated for 3–5 min at room temperature while sonicating until they readily shed excess solution. After silanization, glass slides were washed 3 times with chloroform while sonicating, followed by thorough rinsing with 18 M Ω water and drying in a convection oven at 55–60 °C for 20 to 30 min in a glass container. After drying, glass slides were kept in a sealed glass container until use.

B. Vesicle Preparation. Appropriate ratios of DPPC and lipopeptides in chloroform were added to a glass container and vortexed followed by removal of chloroform with nitrogen. Lipids were resuspended (to 0.5 mg/mL total lipid concentration) by adding water, heating to 50 °C, and vortexing until lipids detach from the glass. Lipid suspensions were then tip sonicated for 2–3 min until the suspension was clear, followed by filtering through a 0.22 μ m filter, and used immediately.

C. Culture Well Construction. Two cell culture well constructs were used in this work. First, wells were made by clamping silanized glass slides to a tissue-culture-treated polystyrene 96-well plate (Falcon), modified by cutting into 2 \times 8 well sections, removing the base plastic, and using a silicone rubber gasket to create a cell culture cassette. Alternatively, 16-well chamber slides (Lab-Tek) were removed from their base, and the injection gasket was removed from the well chamber. Well chambers were then clamped to silanized glass slides, and wells were sealed using injected silastic resin (Dow Corning).

D. Supported Monolayer Deposition. Lipid monolayers were created by adding 100 μ L of vesicle suspension to individual wells for 1.5 h at 55 °C. Excess vesicles were removed from the wells by

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performing dilution rinses (2.3-fold) 5 times with water followed by 3 dilution rinses with the appropriated cell culture media. Similar adhesion results were obtained when using up to 14 total dilution rinses (data not shown). Once coated, the lipid monolayer was never exposed to air.

Adhesion and Spreading Assays. A. Normal Force Adhesion Assay. CFSE-stained KG-1a cells were suspended in media at 6.0×10^5 cells/mL, and CFSE-stained HUVECs at 1×10^5 cells/mL. Mn^{2+} was added at 2 mM concentration, and 100 μ L of the cell suspension was added to each well. Cells were allowed to adhere for 2 h (KG-1a) or 4 h (HUVECs) in the incubator at 37 °C and 5% CO_2 . After incubation, images were taken at three distinct locations in each well using the 5 \times objective of a Leica DM IRB inverted fluorescence microscope with a Hg lamp. The cassette was then immersed in a heat sealable bag containing PBS at 37 °C and turned upside down, and nonadherent cells were removed by centrifugation at 30 rcf for 5 min in a Centra CL3 centrifuge plate spinner. Similar dose–response curves were obtained with compound **5** for centrifugation intensity ranging from 30 to 120 rcf (data not shown). After centrifugation, images were taken at locations matching the precentrifugation images. Cells in each pre- and post-spin image were counted using automated counting software (MetaMorph). Adhesion for each location in each well was calculated by dividing the post-spin image count by the pre-spin image count. Fractional adhesion values for a given lipopeptide density were calculated as the average of all values for a minimum of two independent experiments, with each experiment using a minimum of

two cell culture cassettes with 3–4 replicate wells per cassette (for a total of at least 36 images per loading density). Standard errors were calculated using the values calculated for each image.

B. Cell Spreading. Stained HUVECs were released from tissue culture plastic by rinsing once with warm HBSS followed by the addition of 0.25% trypsin solution for 10–15 min with subsequent addition of trypsin neutralizing solution. Cells were diluted in EGM, and 100 μ L of cell suspension were added to each well. After 4 h of incubation, 10 images were taken of each loading condition with a 20 \times objective. Cell spreading area was quantitated using ImageJ software (NIH freeware). The individual areas of all cells in each image (approximately 10–20 cells per image) were used to calculate the average. Standard errors were calculated using all the individual cell areas. Statistical significance was calculated using a two-tailed students t-test assuming equal variance.

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Supporting Information Available: Synthesis schemes for DPG-Su and DPG-PEG200 acid. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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