# Efficient Solid-Phase Synthesis of Peptide-Substituted Alkanethiols for the Preparation of Substrates That Support the Adhesion of Cells

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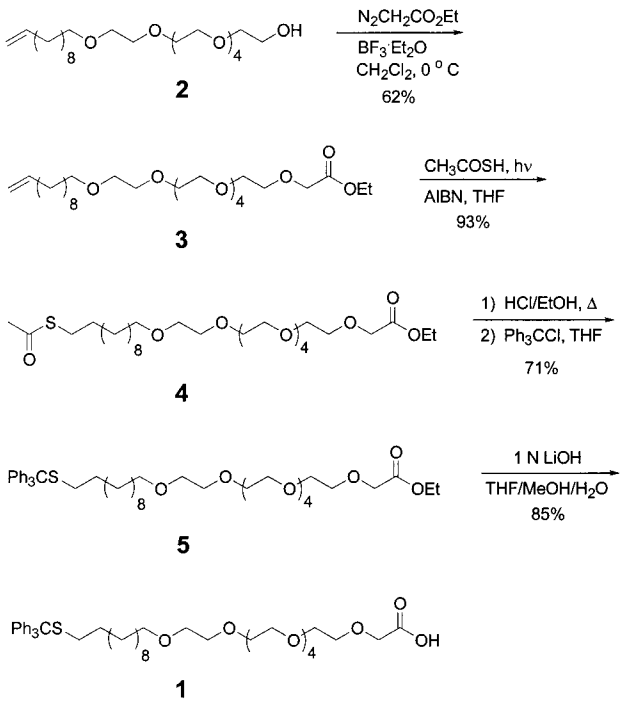
Self-assembled monolayers that present short peptide ligands are an important class of model substrates for studies of the interactions of mammalian cells with surfaces.[1] Substrates that present the Arg-Gly-Asp tripeptide, for example, have been important in understanding the adhesion and spreading of cells.[2−6] Although self-assembled monolayers of alkanethiolates on gold are a versatile class of model substrates for these studies, the effort required to synthesize peptide-terminated alkanethiols remains a disadvantage of this methodology.[2] In this note, we describe a rapid and efficient method, based on solid-phase peptide synthesis, for preparing alkanethiols terminated with peptide ligands. We utilize this methodology to synthesize a Gly-Arg-Gly-Asp-Ser alkanethiol conjugate and demonstrate that monolayers prepared from this compound support the adhesion and spreading of fibroblast cells.

Our strategy required a suitably protected alkanethiol that could be coupled to a peptide on solid support. Simultaneous deprotection and cleavage of the adduct from the resin would afford the final product. Scheme 1 shows the preparation of compound **1** from undec-1-en-11-ylhexa(ethylene glycol) (**2**).[7] Alkylation of **2** with ethyl diazoacetate in the presence of 10 mol % $BF_3{\cdot}Et_2O$ afforded ester **3**. Photochemical addition of thiolacetic acid to the terminal olefin of **3** gave thioacetate **4** in 93% yield. Acidic hydrolysis of **4**, followed by protection of the resultant thiol as a trityl thioether, provided **5**. This intermediate was saponified with aqueous lithium hydroxide in THF/MeOH to afford carboxylic acid **1** (35% yield over five steps).

Scheme 2 illustrates the preparation of peptide-substituted alkanethiol **6**, which contains the Gly-Arg-Gly-Asp-Ser sequence known to bind integrin receptors on the surface of mammalian fibroblast cells.[8] For this work, we chose Fmoc−Rink amide MHBA polystyrene as the solid support.[9] This resin was attractive for several reasons. The initial amino acid coupling step is accomplished in near quantitative yield. Deprotection

**Scheme 1**




and cleavage of the peptide from the resin can be performed simultaneously using aqueous trifluoroacetic acid. The product of cleavage contains an amide terminus that resembles the amide bond following the Arg-Gly-Asp sequence in extracellular matrix proteins.[8] We prepared the Gly-Arg-Gly-Asp-Ser pentapeptide moiety of **6** on this support using routine protocols.[10] Removal of the terminal glycinyl Fmoc-carbamate with 20% piperidine/DMF, followed by coupling with acid **1** in the presence of DCC and 1-hydroxybenzotriazole (HOBT), provided the fully protected conjugate. Cleavage of the peptide from the resin, followed by precipitation with ether and purification by gel permeation chromatography, afforded **6** in 51% overall yield based on initial loading of the resin. The chemical shift of the methylene protons adjacent to the sulfur (t, 2.4 ppm, $-CH_2S$) demonstrated the presence of a sulfhydryl group; no disulfide (t, 2.7 ppm) was observed. A thin layer chromatogram of the product (65:30:5 $CHCl_3$/MeOH/$H_2O$ v/v) turned bright yellow upon application of Ellman's reagent[11] (1 mg/mL in ethanol), confirming the presence of the sulfhydryl group. We have synthesized several other peptide-terminated alkanethiols with similar results.

We next examined the ability of self-assembled monolayers prepared from **6** to support cell adhesion. Glass coverslips coated with an optically transparent (12 nm) layer of gold were immersed for 5 h in an ethanolic solution containing (1-mercaptoundec-11-yl)tri(ethylene glycol)[7] and alkanethiol **6** (1 mM total thiol; 10 $\mu$M compound **6**). The tri(ethylene glycol) groups at the surface of the monolayer resist both nonspecific adsorption of protein and nonspecific attachment of cells.[12] The

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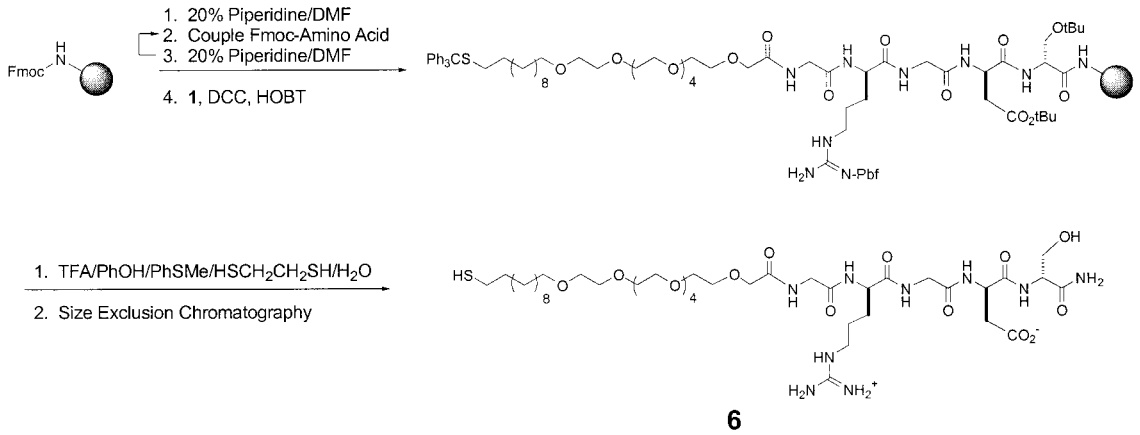
**Scheme 2**




**6**

monolayers were rinsed with ethanol and dried under a stream of nitrogen. 3T3 Swiss Albino fibroblast cells suspended in serum-free culture medium were allowed to attach to these substrates for 5 h at 37 °C. The substrates with attached cells were removed from the culture medium, fixed with a solution of 4% paraformaldehyde in phosphate-buffered saline, and photographed at 20× magnification (Figure 1). Adherent cells exhibited morphology similar to those attached to fibronectin or treated tissue culture dishes. Cells did not attach to monolayers that presented tri(ethylene glycol) groups alone.

We have demonstrated an efficient methodology for the preparation of peptide-terminated alkanethiols. These molecules are important precursors for the preparation of monolayers of alkanethiolates on gold that support the adhesion of cells. We believe that this methodology will be valuable for the preparation of a wide range of functionalized alkanethiols for studies of the adhesion, migration, and differentiation of cells.

## Experimental Section

**General Procedures.** $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker 500 MHz spectrometer with chemical shifts reported in parts per million relative to tetramethylsilane. All reagents for solution phase chemical synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI). Amino acids and the Fmoc−Rink amide MBHA resin were purchased from Anaspec, Inc. (La Jolla, CA). THF was distilled from sodium/benzyl ketyl, and dichloromethane was distilled from calcium hydride. Anhydrous DMF was purchased from Aldrich and used without further purification. The BCA protein assay reagent was purchased from Pierce Chemical Co. (Rockford, IL). Flash chromatography was carried out using EM Science Kiselgel 60 (230−400 mesh).

**Solid-Phase Peptide Synthesis.** Fmoc−Rink amide MBHA resin (312 mg, 0.15 mmol, substitution 0.48 mmol/g) was placed in a 10 mL polypropylene reaction vessel, washed with DMF (2 × 5 mL), and swollen for 30 min in DMF. The resin was rinsed with an additional portion of DMF before a solution of 20% piperidine in DMF (5 mL, 2 × 15 min) was added. The resin was washed with DMF (2 × 5 mL) before a solution of the Fmoc−amino acid (0.45 mmol), HOBT (68 mg, 0.5 mmol), and DCC (95 mg, 0.46 mmol) in DMF (5 mL) was added to the vessel. The mixture was agitated for 2 h, at which point the Kaiser ninhydrin test[13] was negative. The deprotection and coupling cycles were repeated until assembly of the Fmoc-protected Gly-Arg-Gly-Asp-Ser peptide was complete.

**Preparation of Self-Assembled Monolayers.** Substrates were prepared as described previously.[2,14] Briefly, titanium (1 nm) and then gold (12 nm) were evaporated onto glass slides. The slides were cut into pieces approximately 1 cm$^2$ in size and immersed in 0.3 mL of an ethanolic solution containing (1-mercaptoundec-11-yl)tri(ethylene glycol) and **6** (1 mM total thiol; 10 $\mu$M compound **6**). After 5 h, the substrates were removed from the solutions, rinsed with absolute ethanol, and dried under a stream of nitrogen.

**Cell Culture and Microscopy.** Swiss Albino 3T3 cells (ATCC, Rockville, MD) were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD). Gentamicin (Gibco BRL) was included in the culture medium to prevent bacterial growth. All cultures were maintained at 37 °C in a 10% $CO_2$ atmosphere. Near confluent monolayers of cells were detached by incubation in a solution of 0.05% trypsin/0.53 mM $Na_4EDTA$ (Gibco BRL) for 5 min. The trypsin was neutralized by the addition of culture medium containing 10% fetal bovine serum, and the cells were centrifugated and resuspended in serum-free culture medium. A fixed number of cells (15 000 cells in 1 cm$^3$ culture medium) were allowed to attach onto self-assembled monolayers presenting peptide and tri(ethylene glycol) groups. After 5 h at 37 °C, the substrates were gently rinsed with phosphate-buffered saline (pH 7.4, Gibco BRL) and fixed for 30 min in a solution of 4% paraformaldehyde in phosphate-buffered saline. Photographs were taken on Ilford PanF film (Malelo Camera, Chicago, IL) using a Zeiss Axiovert 135 microscope at 20× magnification.

**Hexa(ethylene glycol) Ethyl Ester 3.** To a solution of undec-1-en-11-ylhexas(ethylene glycol) (**2**)[7] (4 g, 9.22 mmol) in dry dichloromethane (20 mL) at 0 °C was added ethyl diazoacetate (1.4 mL, 11.06 mmol) and $BF_3{\cdot}Et_2O$ (97 $\mu$L, 0.92 mmol). After the mixture was stirred for 30 min at 0 °C, saturated aqueous ammonium chloride (10 mL) was added and the reaction mixture was placed in a separatory funnel. The organic phase was collected, and the aqueous phase was extracted with dichloromethane (5 × 50 mL). The combined organic phases were dried over magnesium sulfate and concentrated to a yellow oil. Silica gel chromatography using gradient elution (1:1 ethyl acetate/hexanes → ethyl acetate) afforded ester **3** (2.97 g, 5.69 mmol, 62%) as a clear oil: $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.26−1.36 (m, 16H), 1.53−1.58 (m, 2H), 2.00−2.04 (dd, 2H, $J$ = 7.02, 6.77 Hz), 3.43 (t, 2H, $J$ = 6.79 Hz), 3.49−3.76 (m, 24H), 4.13 (s, 2H), 4.20 (q, 2H, $J$ = 7.14 Hz), 4.88−4.96 (m, 2H), 5.74−5.84 (m, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$ 14.19, 26.06, 28.90, 29.09, 29.40, 29.44, 29.51, 29.61, 33.78, 60.76, 68.71, 70.04, 70.57, 70.61, 70.87, 71.52, 114.08, 139.20, 170.44; IR (thin film) 2922, 2855, 1744 cm$^{-1}$; HRMS (FAB) calculated for $C_{27}H_{53}O_9$ ($MH^+$) $m/e$ 521.3690, found $m/e$ 521.3678.

**Thioacetate 4.** A solution of olefin **3** (1.64 g, 3.14 mmol) in dry THF (32 mL) containing thiolacetic acid (0.55 mL, 7.85

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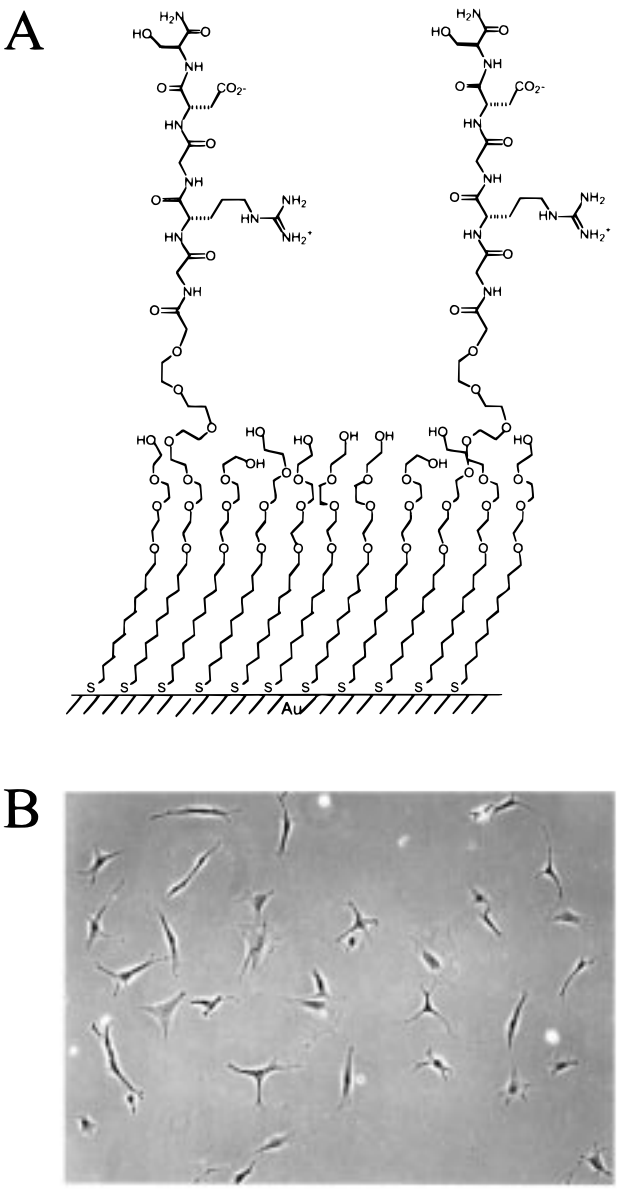


**Figure 1.** (A) Representation of the structure of a mixed self-assembled monolayer presenting Gly-Arg-Gly-Asp-Ser peptides and tri(ethylene glycol) groups. (B) Optical micrograph of 3T3 fibroblast cells attached to a self-assembled monolayer shown in (A) wherein 1% of the alkanethiolates present the peptide ligand. Cells were plated onto the substrate in serum-free culture medium and allowed to attach at 37 °C. This photograph was taken at 20× magnification after 5 h in culture.

mmol) and AIBN (51 mg, 0.31 mmol) was irradiated in a photochemical reactor (Rayonet reactor lamp, Southern New England Ultraviolet Co., model no. RPR-100) for 5 h under an atmosphere of nitrogen. Concentration of the reaction mixture, followed by flash chromatography (eluent 30:1 $CH_2Cl_2$/MeOH) gave compound **4** as a clear oil (1.75 g, 2.93 mmol, 93%): $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.25−1.34 (m, 18H), 1.52−1.59 (m, 4H), 2.31 (s, 3H), 2.85 (t, 2H, $J$ = 7.35 Hz), 3.43 (t, 2H, $J$ = 6.81 Hz), 3.56−3.74 (m, 24H), 4.14 (s, 2H), 4.21 (q, 2H, $J$ = 7.14 Hz); $^{13}$C NMR ($CDCl_3$) $\delta$ 14.16, 26.02, 28.74, 29.03, 29.07, 29.38, 29.40, 29.43, 29.48, 29.57, 30.58, 60.71, 68.66, 69.99, 70.53, 70.57, 70.82, 71.46, 170.39, 195.96; IR (thin film) 2921, 2854, 1750, 1691 cm$^{-1}$; HRMS (FAB) calculated for $C_{29}H_{57}O_{10}S$ ($MH^+$) $m/e$ 597.3672, found $m/e$ 597.3670.

**Trityl thioether 5.** A solution of **4** (1.75 g, 2.94 mmol) in absolute ethanol (35 mL) containing concentrated HCl (1.5 mL) was heated to reflux for 12 h. The reaction mixture was cooled to room temperature and adjusted to a pH of 7 with 5% methanolic ammonium hyroxide. The solution was concentrated in vacuo to a volume of 10 mL and extracted with dichloromethane (6 × 50 mL). The combined organic phases were dried over magnesium sulfate and concentrated to afford the free thiol as a pale yellow oil. To a solution of this compound in dry THF (8 mL) under nitrogen was added trityl chloride (1.23 g, 4.4 mmol). The reaction mixture was allowed to stir at room temperature for 24 h and reduced in vacuo to a yellow oil. Purification by silica gel chromatography (eluent 30:1 → 20:1 $CH_2Cl_2$/MeOH) provided **5** as a clear oil (1.67 g, 2.09 mmol, 71% over two steps): $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.13−1.23 (m, 18H), 1.25−1.36 (m, 2H), 1.55−1.69 (m, 2H), 2.12 (t, 2H, $J$ = 7.36 Hz), 3.43 (t, 2H, $J$ = 6.83 Hz), 3.56−3.74 (m, 24H), 4.14 (s, 2H), 4.20 (q, 2H, $J$ = 7.12 Hz), 7.20 (t, 3H, $J$ = 7.31 Hz), 7.27 (t, 6H, $J$ = 7.36 Hz), 7.40 (d, 6H, $J$ = 7.45 Hz); $^{13}$C NMR ($CDCl_3$) $\delta$ 14.20, 26.07, 28.57, 29.00, 29.16, 29.38, 29.46, 29.47, 29.54, 29.62, 32.01, 60.78, 66.33, 68.71, 70.04, 70.57, 70.52, 70.87, 71.53, 126.47, 127.76, 129.59, 145.07, 170.45; IR (thin film) 2921, 2855, 1750 cm$^{-1}$; HRMS (FAB) calculated for $C_{46}H_{67}O_9S$ ($MH^+$) $m/e$ 795.4506, found $m/e$ 795.4501.

**Acid 1.** To a solution of **5** (500 mg, 0.63 mmol) in 1:1 THF/MeOH (8 mL) was added 1 M aqueous lithium hydroxide (2 mL). The reaction mixture was stirred at room temperature for 3 h and cooled to 0 °C. The solution was acidified to a pH of 2 with 1 N HCl and extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with brine (10 mL), dried over magnesium sulfate, and concentrated to afford **1** (412 mg, 0.54 mmol, 85%) as a clear oil: $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.13−1.30 (m, 15H), 1.35−1.41 (m, 2H), 1.53−1.59 (m, 2H), 2.13 (t, 2H, $J$ = 7.36 Hz), 3.44 (t, 2H, $J$ = 6.84 Hz), 3.56−3.77 (m, 24H), 4.16 (s, 2H), 7.20 (t, 3H, $J$ = 7.31 Hz), 7.27 (t, 6H, $J$ = 7.36 Hz), 7.41 (d, 6H, $J$ = 7.45 Hz); $^{13}$C NMR ($CDCl_3$) $\delta$ 26.02, 28.56, 28.97, 29.13, 29.35, 29.42, 29.45, 29.50, 29.55, 31.99, 66.32, 68.92, 69.98, 70.34, 70.42, 70.45, 70.49, 70.52, 70.54, 70.61, 70.65, 71.32, 71.50, 126.44, 127.74, 129.57, 145.05, 171.95; IR (thin film) 3057, 2916, 2852, 1735 cm$^{-1}$; HRMS (FAB) calcd for $C_{44}H_{64}O_9SK$ ($M + K^+$) $m/e$ 807.3908, found $m/e$ 807.3909.

**Gly-Arg-Gly-Asp-Ser Alkanethiol 6.** To the resin containing the Fmoc-protected Gly-Arg-Gly-Asp-Ser peptide (0.15 mmol) was added a solution of 20% piperidine in DMF (5 mL, 2 × 15 min). The solid support was washed thoroughly with DMF to remove excess piperidine, and a solution of compound **1** (340 mg, 0.45 mmol), HOBT (68 mg, 0.5 mmol), and DCC (95 mg, 0.46 mmol) in THF (5 mL) was added. The mixture was agitated for 4 h before the resin was washed thoroughly with DMF and dichloromethane. The resin was placed in a 25 mL round-bottom flask, and a solution of 5 mL TFA containing 0.25 mL of $H_2O$, 0.375 g of phenol, 0.25 mL of ethanedithiol, and 0.25 mL of thioanisole was added. The yellow mixture was stirred at room temperature for 2 h and filtered to remove the polystyrene support. The beads were washed with TFA (2 × 2 mL), and the combined filtrates were concentrated in vacuo. Repeated precipitation from cold ether (4 × 40 mL) afforded a clear oil that was purified using gel permeation chromatography (Sephadex G-10, 20% aqueous MeOH/0.1% TFA). Fractions that stained positive with the BCA protein reagent were combined and lyophilized to afford **6** as a fluffy, white powder (75 mg, 51% based on loading of resin): $^1$H NMR (500 MHz, $CD_3OD$) $\delta$ 1.13−1.32 (m, 15H), 1.46−1.69 (m, 7H), 1.84−1.92 (m, 1H), 2.41 (t, 2H, $J$ = 7.13 Hz), 3.13 (t, 2H, $J$ = 6.93 Hz), 3.39 (t, 2H, $J$ = 6.63 Hz), 3.49−3.66 (m, 24H), 3.73−3.84 (m, 4H), 3.91 (s, 2H), 4.00 (s, 2H), 4.28−4.33 (m, 2H), 4.66 (t, 1H, $J$ = 6.52 Hz); $^{13}$C NMR ($CD_3OD$) $\delta$ 24.98, 26.09, 27.21, 29.41 29.66, 30.08, 30.22, 30.58, 30.65, 30.72, 31.36, 32.90, 35.23, 36.27, 41.91, 43.19, 43.98, 51.61, 54.46, 56.99, 57.08, 62.96, 71.12, 71.17, 71.32, 71.40, 71.44, 71.49, 72.05, 72.36, 158.56, 171.96, 172.01, 172.93, 173.85, 174.15, 174.81; HRMS (FAB) calculated for $C_{41}H_{80}O_{16}N_9S$ ($MH^+$) $m/e$ 998.5444, found $m/e$ 998.5447.

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**Supporting Information Available:** $^1$H NMR spectra of compounds **1** and **3**−**6** (5 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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