Increased Stability of Glycol-Terminated Self-Assembled Monolayers for Long-Term Patterned Cell Culture

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Materials and Instrumentation

All reagents were obtained from commercial sources and used without further purification. Reactions were carried out in an argon atmosphere with dry solvents unless otherwise noted. ^{1}H NMR and ^{13}C NMR spectra were obtained on a 300 MHz Varian Innova instrument. Electrospray ionization mass spectrometry were obtained on either a Bruker Maxis Q-TOF or a Thermo LCQ Deca XP+, samples were dissolved in acetonitrile. Electron beam deposition was achieved using a PVD 75 electron beam evaporator (Kurt J. Lesker, Clairton, PA). Plasma oxidation was carried out in a Femto standard low-pressure plasma system (Diener electronic GmbH+Co. KG, Nagold). Live-cell phase-contrast images were obtained using a Nikon TE2000-PFS microscope running NIS-Elements imaging software and equipped with a Prior XY stage, Photometrics CoolSNAP monochrome camera, and In Vivo Scientific incubation system.

Synthesis of Ester-linked glycol thiol (2)

12-thioacetatedodecanoic acid (**4**) 12-Bromohexadecanoic acid (1.00 g, 3.6 mmol) was dissolved in dimethylformamide (DMF) (20 ml) at 0 °C. Potassium thioacetate (1.00 g, 9 mmol) was added as a solid, turning the solution a deep red color, and the reaction was allowed to proceed for 30 minutes. The reaction was diluted with methylene chloride (50 ml) and washed three times with water (30 ml). The organic layer was dried over anhydrous magnesium sulfate. Followed by coevaporation of the DMF with toluene. The resulting material was purified by silica chromatography (toluene/ethyl acetate 20:1) to afford the product as a white solid. Yield: 0.604g (61.2%). Mp 64 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.3-1.33 ppm (14 H, s), 1.6 (4 H, m), 2.36 (3 H, s), 2.38 (2 H, t), 2.90 (2 H, t). ^{13}C NMR (300 MHz, $CDCl_3$): δ 24.95, 29.09, 29.33, 29.40, 29.45, 29.73, 30.96, 34.26. MS (ESI+) m/z calculated for $C_{14}H_{26}O_3$ +Na 297.1495, found 297.1509.

*Dodecanoic acid disulfide (***5***)* 12-Thioacetatedodecanoic acid, **4**,(0.4211 g, 1.5 mmol) was dissolved in methanol (15 ml) and 25% sodium methoxide in methanol (3 ml). Air was bubbled through the reaction for 24 hours; the reaction was neutralized by addition of HCl, and diluted with methylene chloride (50 ml). The organic layer was washed 3 times with deionized water (60 ml) and dried over sodium sulfate. Concentration by rotary evaporation afforded the product as a white solid. Yield: 0.306 g (86.2%). Mp 83 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25-1.32 ppm (28 H, m), 1.64-1.7 ppm (8 H, m), 2.38 ppm (4 H, t), 2.72 ppm (4 H, t). ^{13}C NMR (300 MHz, $CDCl_3$) δ 24.88, 28.59, 28.74, 29.21, 29.27, 29.41, 29.43, 29.55, 29.60, 29.66, 34.21, 180.15.

Tetraethylene glycol dodecanoate disulfide (**6**) Dodecanoic acid disulfide, **5**, (0.132 g, 0.28 mmol) was dissolved in methylene chloride (15 ml). Dicyclohexylcarbodiimide (DCC) (0.8248 g, 3.9 mmol) and dimethylaminopyridine (DMAP) (0.065 g, 0.53 mmol) were added to the reaction mixture as solids. Tetraethylene glycol (2.7473 g, 14.14 mmol) was then added to the reaction and the reaction was allowed to proceed for 3.5 h. A white precipitate formed and was removed by filtering through celite. The solvent was removed by rotary evaporation, and the reaction taken up in ethyl acetate (50 ml) and washed with water (60 ml). The organic layer was dried with sodium sulfate and the solvent was removed *in vacuo*. The product was purified by silica chromatography (ethyl acetate:methanol 90:10) to afford a white wax. Yield: 0.352g (22.8%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25-1.32 ppm (28 H, m), 1.64-1.7 ppm (8 H, m), 2.38 ppm (4 H, t), 2.72 ppm (4 H, t), 3.6-3.7 (28 H, m), 4.2 (2 H, t). 13C NMR (300 MHz, $CDCl_3$): δ 25.11, 28.73, 29.12, 29.34, 29.43, 29.47, 29.64, 29.69, 29.73, 34.41, 39.38, 61.94, 63.53, 69.46, 70.55, 70.74, 70.76, 70.86, 72.73, 174.09. MS (ESI+) m/z calculated for $C_{40}H_{78}O_{12}S_2$ +Na 837.49, found 837.4912.

Mercaptododecaote tetraethylene glycol (**2**) Tetraethylene glycol dodecanoate disulfide (0.052g, 0.063 mmol) was purged with argon gas and diluted in methylene chloride (5 ml). A 0.4 mmol/ml solution of tributyl phosphine (600 μl, 0.24 mmol) was added and the reaction was allowed to stir for 1.5 h. The solvent was removed by rotary evaporation and the product was purified by silica chromatography (ethyl acetate) to give the product as a white wax. Yield: 16.4 mg (31.5%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.24-1.32 ppm (14 H, m), 1.70 (4 H, m), 2.2 (2 H, t), 1.9 (2 H, t), 2.36 (2 H, t), 3.44 (2 H, t), 3.6-3.71 (12

H, m), 4.15 (2 H, t). ^{13}C NMR (300 MHz, $CDCl_3$): δ 25.15, 28.43, 29.02, 29.38, 29.52, 29.71, 33.11, 34.45, 62.02, 63.59, 69.53, 70.60, 70.82, 70.91, 72.75. MS (ESI+) m/z calculated for $C_{20}H_{40}O_6S$ +Na 431.2438, found 431.2429.

Synthesis of Amide-linked glycol thiol (3)

Tosyltetraethylene glycol (**7**) Tetraethylene glycol (10.49 g, 54.01 mmol) was dissolved in dry tetrahydrofuran (THF) (20 ml). Pyridine (4.5 ml, 55.18 mmol) was added and the reaction was allowed to proceed for 5 minutes at 0 °C. Recrystallized tosyl chloride (6.79 g, 35.62 mmol) dissolved in dry THF (10 ml) was added to the reaction dropwise and allowed to proceed for 2 hours at room temperature. The reaction was evaporated to dryness, diluted with chloroform (40 ml), and washed with 1M HCl (40 ml), 1M NaOH (40 ml), and water (60 ml). The organic layer was dried over sodium sulfate and the solvent was removed by rotary evaporation. The resulting oil was purified by silica chromatography (ethyl acetate/ hexanes 80:20) to afford the product as a colorless oil. Yield: 4.215 g (34%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.45 ppm (3 H, s), 3.6-3.65 (14 H, m), 7.37 (2 H, d), 7.81 (2 H, d). ^{13}C NMR (300 MHz, $CDCl_3$): δ 24.3, 61.4, 67.5, 69.1, 70.5, 72.7, 129.6, 130.6, 138.2, 144.3. MS (ESI+) m/z calculated for $C_{15}H_{24}O_7S$ $+H_1$ 349.1316, found 349.1313.

Azidotetraethylene glycol (**8**) Tosyltetraethylene glycol, **7,** (3.26 g, 9.36 mmol) was refluxed with 95% ethanol (50 ml) and sodium azide (1.4g, 21.6 mmol) for 19 hours. The solvent was removed by rotary evaporation followed by addition of chloroform (50 ml). This was rinsed with water (75 ml) and the organic layer was dried *in vacuo* to

afford the product as a colorless oil. Yield: 1.26 g (61.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.4 (2 H, t) 3.6-3.65 (14 H, m). ^{13}C NMR (300 MHz, $CDCl_3$): δ 50.0, 61.4, 70.0, 70.2, 70.5, 72.7. MS (ESI+) m/z calculated for $C_8H_{17}N_3O_4$ +H_1 220.1325, found 220.1289.

Aminotetraethylene glycol (**9**) Azidotetraethylene glycol, **8**, (0.656 g, 3.04 mmol) was dissolved in THF (10 ml) followed by addition of triphenyl phosphine (0.9224 g, 3.52 mmol) as a solid. The reaction was allowed to proceed for 10 h. Deionized water (120 μL, 6.72 mmol) was added to the reaction which proceeded for an additional 13 h. The reaction was diluted by water (40 ml) and rinsed with toluene (50 ml). The water was removed by rotary evaporation to afford the product as a colorless oil. Yield: 0.539 g (91.8%). ^{1}H NMR (300 MHz; $CDCl_3$): δ 2.92 (2 H, t) 3.6-3.65 (14 H, m). ^{13}C NMR (300 MHz; $CDCl_3$): δ 41.39, 61.50, 70.23, 70.38, 70.66, 70.76, 72.59, 73.15. MS (ESI+) m/z calculated for $C_8H_{19}NO_4$ +H_1 194.1387, found 194.1382.

12-thioacetatedodecanimidotetraethylene glycol (**10**) 12-Thioacetatedodecanoic acid, **4**, (0.679 g, 2.48 mmol) was dissolved in DMF (20 ml) followed by addition of diisopropylethylamine (875 μl, 5.02 mmol). *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) (1.437 g, 3.79 mmol) was added to the reaction as a solid followed by aminotetraethylene glycol, **9**, (0.6453 g, 3.33 mmol). The reaction was allowed to proceed for 6 h. The reaction was diluted with dichloromethane (75 ml) and rinsed with water (100 ml). The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed by rotary evaporation. The product was

further purified by silica column (ethyl acetate) to afford the product as a colorless oil. Yield: 0.503 g (45.1%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.3 ppm (14 H, s), 1.60 (4 H, m), 2.18 (2 H, t), 2.3 (3 H, s), 2.86 (2 H, t) 3.43-3.73 (15 H, m). ^{13}C NMR (300 MHz, $CDCl_3$): δ 26.02, 28.99, 29.06, 29.36, 29.60, 29.65, 29.71, 30.88, 36.76, 36.74, 39.29, 61.56, 70.06, 70.46, 70.50, 70.64, 72.65, 72.63, 174.23, 196.30. MS (ESI+) m/z calculated for $C_{22}H_{43}O_6NS$+Na 472.2703, found 472.2695.

12-mercaptododecanimide tetraethylene glycol (**3**) 12-Thioacetatedodecanimidotetraethylene glycol, **10**, (0.18 g, 0.356 mmol) was diluted in methanol (10 ml) followed by addition of acetyl chloride (50 µl, 0.857 mmol). The reaction was allowed to reflux for 4 h. The reaction was dried by rotary evaporation, dissolved in chloroform (30 ml), and rinsed with water (30 ml). The solvent was removed *in vacuo* and the product was further purified by silica chromatography (ethyl acetate) to afford a white wax. Yield: 0.153 g (92.6 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.3 ppm (15 H, s), 1.6 (4 H, m), 2.2 (2 H, t), 2.56 (2 H, t) 3.5-3.78 (16 H, m). ^{13}C NMR (300 MHz, $CDCl_3$): δ 24.90, 26.10, 28.62, 29.34, 29.761, 34.30, 36.88, 39.30, 61.81, 70.25, 70.69, 70.91, 72.85. MS (ESI+) m/z calculated for $C_{20}H_{41}NO_5S$ +Na 430.2603, found 430.2594.

PDMS Stamp Preparation.

Master Formation. A virgin silicon wafer (50 mm, Montco Silicon) was cleaned with acetone. AZ 9245 (1-2 mL, Mays Chemical Company, Indianapolis, IN) was applied to the wafer using a Cee 200CB spin/bake system (Brewer Science, Rolla, MO) and an even

coating of resist (nominally 4.5 µm) was achieved using a two-cycle spin-coater program (1000 rpm/500 rpm/s/5 s, 3800 rpm/3800 rpm/s/30 s). The wafer was soft baked at 110 °C for 2 min. Photolithography was carried out using a LaserWriter system equipped with a 325 nm laser (Microtech s.r.l., Palermo, Italy). The wafer was developed in 1:2 400K developer (Mays Chemical Company, Indianapolis, IN):deionized water for 2 min. The resulting master was used for stamp formation.

PDMS Stamp Formation. Sylgard 182 (Dow Corning, Midland, MI) was mixed 10:1 (resin:hardener) and poured over the patterned silicon master. The polymer was de-gassed using a vacuum dessicator and cured at 70 °C for 2 h. The final stamp was separated from the master and cut to size.

Patterned Cell Growth.

Patterning SAMs. Glass coverslips (25 mm, No. 1, VWR, Batavia, IL) were cleaned by oxygen plasma oxidation for 20 min at 100% power. Coverslips were then twice rinsed with water and ethanol, and dried under nitrogen. Deposition of 50 Å titanium followed by 50 Å, 100Å, 150 Å, 200 Å, or 250 Å gold onto the glass coverslips was carried out with a PVD 75 electron beam evaporator under vacuum (1 E^{-6}-1 E^{-7} Torr).
The stamp was coated with hexadecanethiol (Alfa Aesar, Ward Hill, MA) (10 mM in ethanol) by dropping the solution onto the stamp (5-6 drops) and drying with nitrogen. Slides were then stamped for 10 s. The bare regions of gold were allowed to react with 1 mM (1-mercaptoundec-11-yl)tetra(ethyleneglycol) (**1**), ester-linked glycol thiol (**2**), or

amide-linked glycol thiol (**3**) in ethanol for 12-14 h. After soaking, coverslips were twice rinsed with ethanol and dried under nitrogen.

Cell Culture. A patterned coverslip (stamped with 10 mM hexadecanethiol and incubated in 1 mM **1**, **2**, or **3** for 12-14 h) was coated with fibronectin at 20 μg/mL in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco) at 37 °C for 1 h. Excess protein was removed by rinsing with DPBS (3x) and the coverslip was covered with fresh DPBS. CHO-K1 cells (ATCC, Manassas, VA) were detached using TrypLE Express (Invitrogen), followed by resuspension in Dulbecco's Modified Eagle Medium (DMEM, low glucose 1X, glutamax, 1 g/L D-glucose, 110 mg/L sodium pyruvate, 10% FBS, 1% penicillin/streptomycin (10,000 units/mL Penicillin G Sodium and 10,000 μg/mL Streptomycin Sulfate in 0.85% saline), Invitrogen), and counted using a hemacytometer (Bright-Line, Hausser Scientific). After rinsing the patterned coverslip with DPBS, approximately 100,000 cells were applied in 1 mL of DMEM. Plated cells were grown at 37 °C and 5% CO_2). Cultures were visualized using live-cell inverted microscopy.

Analysis of Cells Growing Outside the Pattern. Cells growing outside the 95 μm circle pattern were counted as either round or spread cells. A cell possessing any projections or appearing elongated was considered spread, while cells having a round morphology were considered round. Cell density was determined by dividing the number of cells outside the pattern by the background area. Outliers were eliminated using the Grubbs' test with a critical value of 0.05.

Scanning probe microscopy (SPM)

All SPM images were obtained on a Multimode VIII with Peak Force Quantitative Nanomechanical property mapping (Bruker, Santa Barbra, CA) using a silicon tip on a silicon nitride cantilever with a nominal spring constant of 0.4 N/m (Scanasyst-Air, Bruker Probes, Camarillo, CA). Images of patterned gold substrates were obtained with 512 points per line and 512 lines per image with a frequency of 0.97 Hz. Bare gold substrates were acquired with 1024 points per line and 1024 lines per image with a frequency of 0.488 Hz.