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Surface Presentation of Bioactive Ligands in a Non-Adhesive Background using DOPA-Tethered Biotinylated Poly(Ethylene Glycol)

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Abstract

We have developed surfaces for the selective presentation of biotinylated peptides and proteins in a background that resists nonspecific protein adsorption; controlled amounts of biotinylated polyethylene glycol (MW 3400; PEG3400) anchored to titanium-dioxide-coated surfaces via an adhesive tri-peptide sequence of L-3,4-dihydroxyphenylalanine (DOPA₃-PEG3400-biotin; DPB) were incorporated within a DOPA₃-PEG2000 background. Using optical waveguide lightmode spectroscopy, we found that the amounts of sequentially adsorbed NeutrAvidin and singly-biotinylated molecules increased proportionally with the amount of DPB in the surface. Biotinylated peptides (MW ca. 2000) were able to fill all three of the remaining avidin-binding sites, while only one molecule of biotinylated PEG5000 or stem cell factor bound to each avidin. The resulting biotin-avidin-biotin linkages were stable for prolonged periods under continuous perfusion, even in the presence of excess free biotin. Hematopoietic M07e cells bound to immobilized peptide ligands for $\alpha 5\beta 1$ (cyclic RGD) and $\alpha 4\beta 1$ (cyclic LDV) integrins in a DPB-dose-dependent manner, with near-maximal binding to cyclic LDV for surfaces containing 1% DPB. Multiple ligands were adsorbed in a controlled manner by incubating NeutrAvidin with the respective ligands in the desired molar ratio and then adding the resulting complexes to DPB-containing surfaces. Cell adhesion to surfaces containing both cyclic LDV and cyclic RGD increased in an additive manner compared to that for the individual ligands. The bioactivity of adsorbed biotinylated stem cell factor was retained, as demonstrated by DPB-dose-dependent M07e cell adhesion and ERK1/2 activation.

1. Introduction

Improved methods for functionalizing surfaces with bioactive peptides and proteins would accelerate the development of immunoassays¹, diagnostics², and tissue engineering applications^{3,4}. The main obstacles that inhibit this development are inactivation of biomolecules due to uncontrolled immobilization and nonspecific adsorption of serum proteins. Strategies employing conjugation chemistries such as histidine tag⁵, chemical cross-linkers⁶, and biotin-avidin interaction⁷ have been developed to attach biomolecules to surfaces in a controlled manner. Meanwhile, nonspecific adsorption has been minimized by coating surfaces with hydrophilic polymers such as poly(ethylene glycol) (PEG)^{8,9}. The non-fouling properties of PEG have been attributed to both increased steric hindrance and osmotic repulsion^{10,11}.

Peptides have been conjugated directly to PEG to improve both the activity and compatibility of biomaterials¹². However, mimicking complex *in vivo* cell microenvironments with multiple ligands would require several steps of PEG and peptide conjugation. A more convenient strategy is to combine the non-fouling properties of PEG with a conjugation step that allows for incorporation of multiple peptides. Biotin-avidin interactions are known to be the strongest non-covalent bond¹³. Once formed, these interactions can withstand a wide range of pH and temperature. Previous studies with biotinylated PEG have focused on the binding of one biotinylated macromolecule, typically to biosensor surfaces^{7,13,14}. For example, Huang et al.⁷ attached biotin-derivatized copolymers of positively-charged poly(L-lysine)-*g*-PEG (PEG2000 or PEG3400) to negatively-charged Nb₂O₅ surfaces. The resulting surfaces supported avidin-mediated adsorption of biotinylated lactamase⁷, but their application was limited by the need to use a negatively-charged surface to physically adsorb poly(L-lysine). Other chemistries, such as silane^{14–16} and photograft¹⁷, have been utilized to covalently attach PEG2000 and PEG3400 to surfaces. However, the bioactivity of such surfaces may be decreased by the harsh conditions associated with the surface preparation.

We have developed a method for attaching biotin-derivatized PEG3400 to TiO₂-coated surfaces under mild conditions within a background of PEG2000 via the modified amino acid L-3,4-dihydroxyphenylalanine (DOPA), which is largely responsible for the adhesive properties of mussel adhesive peptides^{18,19}. We have previously shown that DOPA-PEG5000 surfaces greatly reduce the nonspecific adhesion of proteins and cells^{8,20}. The non-fouling properties of PEG5000 improved with increasing DOPA content, due to the corresponding increase in the PEG5000 surface density. Although the interaction of DOPA with metal oxides was observed to be reversible with AFM²¹, the DOPA-oxide bond is strong and DOPA-PEG5000 surfaces are stable under cell culture conditions and suitable for long-term application^{20,21}.

Here, we demonstrate a versatile system in which biotinylated immunoglobulin, PEG5000, fibronectin-inspired peptides, and cytokines can be presented via biotin-avidin interactions in surfaces containing varying amounts of DOPA-PEG3400-biotin within a non-adhesive DOPA-PEG2000 background. In particular, we show dose-dependent adhesion of the human hematopoietic M07e cell line to surfaces containing peptide mimics of fibronectin-binding domains for either $\alpha 4\beta 1$ (cyclic LDV) or $\alpha 5\beta 1$ (cyclic RGD) integrins. Incorporation of both cyclic RGD and cyclic LDV peptides increased M07e cell adhesion in an additive manner. Immobilization of stem cell factor – an important cytokine for the maintenance and expansion of hematopoietic stem cells – in an active conformation was demonstrated by dose-dependent adhesion of M07e cells, as well as activation of the ERK1/2 mitogen-activated protein kinases. Studies using optical waveguide lightmode spectroscopy showed that the modified surfaces were stable for extended periods of time under continuous buffer flow and in the presence of excess free biotin.

2. Materials and Methods

2.1 DOPA₃-PEG2000 Synthesis

675 mg of methoxy-PEG2000-NH₂ (0.338 mmol, 2000 MW PEG, Nektar, Huntsville, AL) was dissolved in 13 mL anhydrous tetrahydrofuran and purged with argon in a round-bottom flask. 518 mg diacetyl-DOPA *N*-carboxyanhydride (DOPA(Ac₂)-NCA, 1.69 mmol) prepared using published procedures²² was added and the mixture was stirred at room temperature (RT) for 5 days with a drying tube attached to the flask outlet to minimize the moisture content. The polymer solution was added to diethyl ether and the precipitate was vacuum-filtered and dried in a desiccator to yield 700 mg PEG-Ac₂DOPA₃. The peptide-coupled PEG was dissolved in 7 mL anhydrous dimethylformamide and bubbled with argon for 10 min. 332 μ L piperidine was added to remove the acetyl groups and the solution was stirred for 15 min with argon

bubbling. The mixture was rotary-evaporated to remove excess piperidine and precipitated in diethyl ether. After drying in a desiccator, 480 mg of a pale yellow solid was obtained. Using UV-vis as described²³, the number of DOPA molecules bound to each PEG chain was determined to be 3.0 ± 1.0 . The molar yield based on input PEG2000-NH₂ was 56%.

2.2 DOPA₃-PEG3400-Biotin Synthesis

DOPA₃-PEG3400-biotin was synthesized from biotin-PEG3400-NHS (Nektar) through first converting the terminal NHS group to a primary amine, which was then used as a polymer-bound initiator for DOPA(Ac₂)-NCA polymerization. The primary amine conversion was achieved by conjugating NHS with Boc-protected ethylene diamine (Boc-ED) that was synthesized as follows: 15 mL ethylene diamine (224 mmol, Sigma, St. Louis, MO) was added to 50 mL dichloromethane and stirred in a 0°C water bath. 5 g *t*-butyl-dicarbonate (22.4 mmol, Sigma) in 20 mL tetrahydrofuran was added drop-wise over 20 min. The reaction mixture was stirred at RT for 1 hr. After adding 100 mL dichloromethane, the mixture was successively washed with 1 M HCl and saturated NaCl solution. The organic layer was dried over MgSO₄ and the solvent was evaporated to yield Boc-ED, a viscous oil. Boc-ED was used without further purification and contained a fraction of doubly protected Bis-Boc-ED, which did not affect the outcome of the next reaction step.

217 μ L Boc-ED (1.37 mmol) and 239 μ L N,N'-diisopropylethylamine (1.37 mmol) were added to 10 mL chloroform. 500 mg biotin-PEG3400-NHS (0.137 mmol) was added and the mixture was stirred at RT for 2 hr. The polymer solution was added to 100 mL diethyl ether. The precipitate was vacuum-filtered and dried to yield 510 mg biotin-PEG3400-Boc-ED. Based on ¹H NMR, nearly 100% of the PEG terminal group was converted to Boc-ED. 510 mg biotin-PEG3400-Boc-ED was dissolved in 2 mL chloroform, and 3 mL 4 M HCl in dioxane was added. The reaction mixture was stirred at RT for 15 min and HCl was removed through rotary evaporation. The polymer solution was added to 50 mL diethyl ether and the precipitate was vacuum-filtered and dried to yield 488 mg biotin-PEG3400-NH₂. ¹H NMR revealed that Boc was completely removed.

488 mg biotin-PEG3400-NH₂ was dissolved in 10 mL anhydrous tetrahydrofuran. 23 μ L N,N'-diisopropylethylamine (0.132 mmol) and 322 mg DOPA(Ac₂)-NCA (1.05 mmol) were added and the reaction mixture was stirred at RT with dry tube outlet for 3 days. The polymer solution was added to 100 mL diethyl ether and the precipitate was vacuum-filtered and dried. The DOPA-coupled PEG3400 was dissolved in 2.5 mL anhydrous dimethylformamide and bubbled with argon for 10 min; 115 μ L pyridine was added and the solution was stirred for 15 min with argon bubbling. The mixture was rotary-evaporated to remove excess pyridine and precipitated in diethyl ether. After drying in a desiccator, 221 mg of a pale yellow solid was obtained. Using UV-vis as described²³, the number of DOPA molecules bound to PEG3400 was determined to be 2.9 ± 1.0 . The molar yield based on input biotin-PEG3400-NHS was 40%. DOPA₃-PEG2000 and DOPA₃-PEG3400-biotin are referred to as DP and DPB, respectively, throughout the text. For mixtures of DP and DPB, an abbreviation such as DPB10 has been used to indicate a mixture of 10 mol% DPB and 90% DP.

2.3. Synthesis of biotin-PEG5000

Biotin-PEG5000 was synthesized by conjugating 5-(biotinamide)pentylamine (biotinamide-NH₂, Pierce, Rockford, IL) to the carboxylic acid group of PEG5000 terminated with propionic acid (PEG5000-SPA, 0.05 mmol, Nektar). 21.4 mg biotinamide-NH₂ (0.065 mmol) was dissolved in a mixture of 0.7 mL dimethylformamide, 1.5 mL methanol, and 2 mL dichloromethane. The solution was added to an amber vial containing 250 mg PEG5000-SPA and agitated overnight at RT using a flask shaker (St. John Associates, Inc., Beltsville, MD). The mixture was purged with argon to remove dichloromethane and dried through rotary

evaporation to remove remaining dimethylformamide and methanol. The oily film was added to diethyl ether and precipitated overnight at 4°C. The white precipitate was centrifuged at 4500×g for 10 min and re-washed with diethyl ether. After centrifugation, the presence of free biotinamide-NH₂ was still detected on a TLC plate via the ninhydrin test. Biotin-PEG5000 was purified with a Sephadex® LH-20 gel column (Sigma) to remove free biotinamide-NH₂ as follows: Dried crude biotin-PEG5000 was dissolved in 0.5 mL methanol and 0.5 mL dichloromethane. After soaking and rinsing the gel with methanol, the crude solution was loaded onto the gel interface. The column was eluted with methanol. Fractions that contained biotin-PEG5000, which was detected by ninhydrin and phosphomolybdic acid tests on TLC plates, were collected, evaporated, and dried in a desiccator. These fractions (total yield = 50 mg) also contained PEG5000. The fraction of biotinylated PEG5000 was found to be 0.6 using the HABA/Avidin kit (Pierce), and this factor was used when adding biotin-PEG5000 to surfaces.

2.4. Synthesis of Biotinylated Peptides

Peptides were synthesized manually on Rink Amide AM (GCRGDGWCGY [cyclic RGD] and YGGRGDSP [linear RGD], 0.7 mmol/g, Novabiochem, San Diego, CA) or on Rink Amide MBHA (GCWLDVCGY [cyclic LDV] and SEPLIGRKKTY [heparin-binding domain; HBD], 0.6 mmol/g, Novabiochem) resin using Fmoc solid-phase synthesis. Peptide synthesis and the cyclization of the cyclic RGD and cyclic LDV peptides were as previously described²⁴ for cyclic RGD. The cyclic RGD, cyclic LDV, and HBD peptides were conjugated with Fmoc-PEG600-acid (Novabiochem) as described²⁴ except that N-hydroxybenzotriazole was replaced with 2-(1-H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU, Novabiochem). After Fmoc deprotection, the free amine group of PEG600 was reacted with derivatized biotin. Succinimidyl-6-(biotinamido) hexanoate (200 mg, 0.284 mmol) (EZ-link NHS-LC-biotin; Pierce) and N,N'-diisopropylethylamine (0.825 mmol) were dissolved in 6 mL dimethylformamide and added to the resin overnight at RT. The peptide-PEG600-biotin construct was cleaved from the resin with a mixture of trifluoroacetic acid (TFA, Sigma, St. Louis, MO), triisopropylsilane (Sigma), and water (95:2.5:2.5); precipitated in diethyl ether; and dried under vacuum. The crude products were purified with RP-HPLC using a C18 semipreparative column (Vydac, Deerfield, IL) with solvent systems of (A) 10% acetonitrile in aqueous solution containing 0.1% TFA and (B) acetonitrile-isopropyl alcohol (1:1) containing 0.1% TFA. The linear solvent gradient ranged from 20% A to 50% A over 90 min. The molecular weights of the purified products (~20% of the crude peptides) were confirmed using matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) on a Voyager DE (delayed extraction)-Pro Mass Spectrometer (PerSeptive Biosystems, Framingham, MA) in positive-ion reflection mode. The accelerating voltage was 20 kV with 100-ns extraction delay time. α -Cyano-4-hydroxycinnamic acid (Sigma) was used as the matrix at 10 mg/mL in 50% (v/v) water/acetonitrile containing 0.1% TFA. The molecular weights of the peptides are 1070 for cyclic RGD, 807 for linear RGD, 1012 for cyclic LDV, and 1292 for HBD. The molecular weights of the peptides after conjugation with biotin-PEG600 are 2010 for cyclic RGD, 1952 for cyclic LDV, and 2231 for HBD.

2.5 Biotinylation of Stem Cell Factor

Stem cell factor (SCF, MW 18.5 kDa; Amgen, Thousand Oaks, CA) was conjugated to sulfosuccinimidyl-6-(biotinamido) hexanoate (EZ-link sulfo-NHS-LC-biotin; Pierce) following the manufacturer's protocols. Briefly, 200 μ g SCF was diluted to 0.5 mg/mL in phosphate-buffered saline (PBS, pH 7.4) and biotinylated at a biotin-to-protein molar ratio of 2:1 at RT for 90 min while vortexing, followed by dialysis against PBS. Samples of the biotinylated material were dialyzed against ultrapure water (Millipore, Billerica, MA) for analysis by MALDI-TOF MS (using the conditions described in Section 2.4), which indicated

that SCF molecules contained either zero or one biotin molecule (MW 18.9 kDa). The HABA assay indicated that an average of 0.25 ± 0.10 moles of biotin were added per mole of SCF. This factor was used when preparing biotin-SCF solutions.

2.6. Surface Preparation and Modification

Glass microscope slides (3"×1", Fisher Scientific, Waltham, MA) were cleaned in a piranha solution (7:3 18 M H₂SO₄:H₂O) overnight. The slides were rinsed with deionized water and dried inside a 55°C oven. Optical waveguide chips (Microvacuum, Ltd., Budapest, Hungary) were sonicated in isopropyl alcohol and dried under nitrogen. Both the glass slides and the waveguide chips were coated with a 10-nm-thick TiO₂ layer inside an EB3 e-beam evaporation unit (BOC Edwards, Wilmington, MA). Before polymer modification, TiO₂-coated glass slides and waveguide chips were sonicated in isopropyl alcohol, dried with nitrogen, and cleaned in an oxygen plasma chamber (Harrick Scientific, Ossining, NY) for 3 min. Surfaces were modified with DP at a polymer concentration of 1 mg/ml in cloud-point buffer (0.1 M MOPS with 0.6 M K₂SO₄, pH 6.0) overnight at 40°C. For mixed DPB and DP surfaces, solutions of DPB and DP were prepared in cloud-point buffer, as described above for DP, with a molar ratio of DPB to DP ranging from 0.01 to 0.15. After polymer modification, surfaces were rinsed with ultrapure water and dried with nitrogen.

For cell adhesion, cell signaling assays, and ELISA experiments, 16-well chambers (Lab-Tek™, Nalge Nunc International, Rochester, NY) were removed from their glass base and bound to TiO₂-coated glass microscope slides using injected silastic resin (T-2, Dow Corning, Midland, MI). The TiO₂ surface in each well was modified with 0.1 ml DP or DP/DPB in cloud-point buffer at 40°C overnight and rinsed 5 times with PBS.

2.7 Optical Waveguide Lightmode Spectroscopy

Optical waveguide lightmode spectroscopy (OWLS) was used as described²⁰. A TiO₂ waveguide chip coated with DP or DP/DPB was mounted onto the flow cell of an OWLS110 (Microvacuum Ltd.) and stabilized by flowing PBS for 3 hr at 37°C. Adsorption of streptavidin, NeutrAvidin (Pierce), and biotinylated molecules was performed by injecting the protein solution (0.1 mg/mL in PBS) in a stop-flow mode for 1 hr. Unbound molecules were removed by rinsing with PBS for 15 min. For analysis of surface stability, PBS was injected through the flow cell at 0.3 mL/hr for 10 hr. Alternatively, PBS containing 0.01 mg/mL free biotin was injected at 0.1 mL/hr for 7 hr. The change in the incoupling angles, α_{TM} and α_{TE} , was recorded during each adsorption step. The refractive indices were calculated from the incoupling angles using software supplied by the manufacturer. The refractive index increments, dn/dc , were calculated by linear interpolation, and were close to 0.18 cm³/g for all of the molecules evaluated. In order to remove adsorbed material, used waveguide chips were sonicated in cleaning solution (200 mM HCl, 1% sodium dodecyl sulfate), rinsed with deionized water, dried with nitrogen, and regenerated in the oxygen plasma chamber for 3 min prior to reuse^{20,25}. OWLS results are given either as a function of mol% DPB or the surface density of DPB, which was determined from the *in situ* adsorption of mixed DP/DPB solutions onto TiO₂-coated waveguides using OWLS.

2.8 Surface Characterization

Ellipsometry and X-ray photoelectron spectroscopy (XPS) analysis were performed as described²⁶. Briefly, ellipsometry was performed using a variable-angle spectroscopic ellipsometer (ESM-300, J. A. Woodlam, Inc., Lincoln, NE) at 65°, 70°, and 75° incident angles using wavelengths from 370 to 1000 nm. Ellipsometric thickness was derived from fitting the Ψ and Δ data with fixed A_n (1.46) and B_n (0.01) values using the Cauchy model and WVASE software (J. A. Woodlam, Inc.). The reported thickness, before and after the adsorption of streptavidin and biotinylated IgG₁ (5–8 biotin molecules per IgG₁; biotin_(5–8)-IgG₁; BD

Biosciences, San Jose, CA), was the average from 3 surfaces with 3 different spots measured on each surface. XPS spectra were collected on an Omicron ESCALAB (Omicron, Taunusstein, Germany) with a monochromatic Al K α (1486.6 eV) 300-W X-ray source. All binding energies were calibrated using the C 1s peak (284.6 eV). Surface composition was calculated from the spectral area ratios corrected with individual elemental sensitivity factors. Curve fitting was performed using CasaXPS (Casa Software Ltd., www.casaxps.com).

2.9 Culture Cassette Preparation

Well chambers coated with DP or DPB/DP were treated with 0.5% bovine serum albumin (BSA, Sigma, St. Louis, MO) in PBS (0.5% BSA) for 30 min to block any defects on the microscope slides and to minimize nonspecific adsorption to the polystyrene wells. For sequential adsorption, NeutrAvidin (0.1 mg/mL in PBS, Pierce) was incubated inside the wells for 1 hr with gentle mixing at 37°C. The wells were rinsed (via replacement of 200 μ l from total well volume of 350 μ l) 5 times with PBS, followed by addition of biotin-SCF (0.01 mg/mL in 0.5% BSA to minimize nonspecific adsorption of SCF to the polystyrene wells), biotin-PEG600-cyclic LDV (0.1 mg/mL in PBS), or biotin-PEG600-cyclic RGD (0.1 mg/mL in PBS) for 1 hr at 37°C. For adsorption of a peptide-NeutrAvidin complex, NeutrAvidin was mixed at various molar ratios with biotin-PEG600-cyclic LDV and/or biotin-PEG600-cyclic RGD in PBS for 1 hr, and then added to DPB/DP-coated wells for 1 hr. Wells were rinsed 8 times with PBS after sequential or complex adsorption of biotinylated peptides, and 15 times with 0.5% BSA after adsorption of biotin-SCF. Finally, wells were rinsed 3 times with culture media and incubated for 30 min at 37°C prior to cell loading. The DPB/DP-coated well surfaces were never exposed to air after NeutrAvidin and biotinylated molecule adsorption.

2.10 Cell Culture and Staining

Hematopoietic M07e cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) adapted to growth with low serum were maintained in Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with 2.5% fetal bovine serum (FBS, HyClone, Logan, UT) and cultured under 5% CO₂ in a humidified incubator at 37°C. Cells were used during the exponential growth phase for adhesion and signaling assays. Cell-staining solution for use in adhesion assays was prepared by adding 100 μ L Calcein AM (Invitrogen, Carlsbad, CA) in dimethylsulfoxide (Sigma) to 10 mL PBS at 37°C. M07e cells were pelleted at 50 $\times$ g for 10 min, resuspended in staining solution, vortexed, and maintained at 37°C for 15 min with periodic vortexing. Stained cells were washed in phenol-red-free Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 2.5% FBS and resuspended at 7×10^5 cells/mL.

2.11 Cell Adhesion

The cell adhesion assay and data analysis were performed as described²⁴. Briefly, cells in phenol-red-free DMEM (0.1 ml) were allowed to adhere for 2 hr in a humidified incubator at 37°C and 5% CO₂. Calcein-AM-stained cells were supplemented with 2 mM Mn²⁺ for experiments with biotin-PEG600-cyclic LDV or biotin-PEG600-cyclic RGD. For competition experiments, cells were incubated with unbiotinylated cyclic LDV, linear RGD, or SCF for 30 min. Fractional adhesion is reported as the mean $\pm$ standard error of the mean (SEM) for 3–4 independent experiments. The fractional adhesion for surfaces containing mixed peptides (FA_{P1+P2}) was compared to the sum of the fractional adhesion values for surfaces containing the same amounts of the individual peptides (FA_{P1} + FA_{P2}) by testing the null hypothesis, H₀: 0 = FA_{P1} + FA_{P2} – FA_{P1+P2}. A probability of less than 0.05 was considered statistically significant.

2.12 ELISA

Well chambers with a TiO₂-coated microscope slide base were coated overnight with DP and DPB mixtures at cloud-point conditions. The wells were rinsed 5 times with 0.5% BSA and blocked with 0.5% BSA for 30 min at 37°C. Streptavidin-horseradish peroxidase (streptavidin-HRP, R&D Systems, Minneapolis, MN) was diluted 1:2000 in 0.5% BSA and incubated in the wells for 20 min at RT, followed by 5 rinses with 0.5% BSA. Tetramethylbenzene substrate (Sigma) was developed in streptavidin-HRP-coated surfaces for 20 min. The absorbance was read with a Lambda plate reader (Perkin-Elmer, Waltham, MA) at 450 nm immediately after the reaction was stopped by adding 50 μ l of 2 M H₂SO₄ (Fisher Scientific) to each well. A calibration curve for the mass of streptavidin-HRP bound to DPB on the surface was prepared from a serial dilution of streptavidin-HRP in 0.5% BSA (data not shown).

2.13 Cell Signaling Studies

A flow cytometric method²⁷ was adapted for measuring phosphorylated ERK1/2. Briefly, growth-factor-starved M07e cells were stimulated with unmodified or biotinylated SCF (in soluble or surface-immobilized form) for 20 min. Cells were harvested, fixed with 2% formaldehyde at 4°C for 10 min, permeabilized with 0.5 mL ice-cold 90% methanol, and placed on ice for 20 min. Samples were rinsed with 0.5 mL 1% BSA followed by blocking for 30 min at 4°C with 1% BSA. Samples were stained with phospho-specific anti-ERK1/2 rabbit antibody (Cell Signaling Technologies, Danvers, MA) for 30 min at RT, rinsed twice with 1% BSA, and incubated for 30 min at 4°C in 1% BSA. Samples were then stained with goat anti-rabbit secondary antibody conjugated to phycoerythrin (PE, Jackson ImmunoResearch, West Grove, PA) for 20 min at RT and washed twice with 1% BSA. Data were acquired using an LSR II flow cytometer and analyzed with FACS DIVA software (BD Immunocytometry Systems, San Jose, CA). The fold-over-isotype value – calculated by dividing the mean fluorescence intensity of a sample by that of the isotype-matched control – was used to normalize for experiment-to-experiment variation. The significance of differences in signaling between different conditions was evaluated using a two-tailed Student's t-test.

3. Results and Discussion

3.1. Characterization of DOPA-PEG3400-Biotin Surfaces using Streptavidin and Biotinylated IgG₁

We examined sequential adsorption of streptavidin and biotin₍₅₋₈₎-IgG₁ to characterize the binding activity of DPB/DP surfaces. Nonspecific adsorption of streptavidin and biotin₍₅₋₈₎-IgG₁ was very low for surfaces coated with 100% DP (DPB0) (Fig. 1A). Streptavidin binding to surfaces coated with DPB10 was 40-fold greater than for DPB0, and biotin₍₅₋₈₎-IgG₁ specifically adsorbed to unfilled streptavidin binding pockets. Binding of streptavidin and biotin₍₅₋₈₎-IgG₁ to DPB10 surfaces was evaluated using ellipsometry. The thickness of the DPB10 layer was 22 ± 2 Å. Sequential adsorption of streptavidin and biotin₍₅₋₈₎-IgG₁ increased the thickness by a total of 18 ± 1 Å. The binding of streptavidin and biotin₍₅₋₈₎-IgG₁ to DPB10 was confirmed using X-ray photoelectron spectroscopy (XPS; Fig. 1B). Before protein adsorption, the main peak in the XPS C(1s) spectra at 286.3 eV corresponded to C–O bonds in the PEG chains. The small shoulder at 288.1 eV indicates the presence of the C–N bond from the DOPA anchor group, while the peak at 284.6 eV was attributed to C–C bonds. After sequential binding of streptavidin and biotin₍₅₋₈₎-IgG₁, substantial increases in the peak heights were observed for C–C and C–N bonds. The stepwise increase in total C (from 53.3% to 53.2% to 60.0%) and total N (from 4.2% to 5.4% to 6.3%) content and the decrease in Ti content (from 6.3% to 6.2% to 4.0%) (data not shown) confirmed the sequential binding of streptavidin and biotin₍₅₋₈₎-IgG₁.

The surface concentration of streptavidin increased linearly with that of DPB (Fig. 1C). However, only about 0.1 mole of streptavidin bound per mole of DPB on the surface. This suggests that most of the biotin moieties were buried in the PEG layer and therefore not accessible for binding streptavidin. The low level of streptavidin binding could also be due to preferential adsorption of DP (vs. DPB) during surface preparation. However, DPB, DP, and their mixtures exhibited very similar lower-critical-solution temperatures in the deposition buffer (data not shown), so we would not expect closer packing or preferential adsorption of DP on the surface. The linear increase of streptavidin binding to surfaces with increasing DPB content was confirmed with ELISA using HRP-conjugated streptavidin (Fig. 1D). The ratio of 0.14 ± 0.02 moles of streptavidin-HRP per mole of DPB obtained from ELISA was similar to the molar ratio of 0.08 ± 0.05 obtained for streptavidin via OWLS. Comparable molar ratios of streptavidin to immobilized biotin have been reported by Zhen et al.²⁸ and Huang et al.⁷ for biotinylated poly(L-lysine)-g-PEG3400. Solvated PEG adopts a flexible extended coil conformation¹¹. Biotin has been shown to be present not only at the aqueous interface, but also buried within the PEG layer, even though the PEG3400 chain attached to biotin was substantially longer than the PEG2000 background^{7,28}.

In contrast to linear streptavidin adsorption, the surface concentration of biotin₍₅₋₈₎-IgG₁ increased much more slowly for DPB concentrations above 2.5 pmole/cm² (Fig. 1C). At low levels of DPB, the molar ratio of biotin₍₅₋₈₎-IgG₁ to streptavidin was close to one, but the ratio steadily decreased at higher DPB levels. This trend may be expected because each IgG₁ molecule is conjugated with 5–8 biotin molecules, and these can bind to more than one streptavidin molecule at higher DPB (and streptavidin) concentrations. Moreover, the IgG₁ molecule (MW ~160 kDa) is bulky in comparison to streptavidin (~60 kDa), so there would be additional steric hindrance for IgG₁ molecules to pack at higher concentrations of DPB. For comparison, Huang et al.⁷ observed that the surface concentration of biotinylated goat anti-rabbit-IgG antibody reached a maximum of ca. 0.4 pmole/cm² at a biotin surface concentration of ca. 11 pmole/cm² before the biotinylated antibody surface concentration dropped to 0.2 pmole/cm² at a biotin surface concentration of 19 pmole/cm².

BSA was used to evaluate nonspecific adsorption to the surfaces. The BSA surface concentration on DP was 0.022 ± 0.001 pmol/cm². BSA adsorption tended to increase with the DPB content, but was very low (ca. 1–2 ng/cm²) for all DPB levels (Fig. 1C). Moreover, adding BSA prior to incubation with NeutrAvidin, which is a neutral version of avidin, did not alter the specificity or extent of NeutrAvidin binding to DPB on the surface (data not shown).

Several other groups have attached biotin-derivatized PEG^{7,14,29} to surfaces using different chemistries. Huang et al.⁷ exploited electrostatic interactions to attach biotin-derivatized poly(L-lysine)-g-PEG3400 to metal oxide surfaces. Although such surfaces can be readily prepared under ambient conditions, the poly(L-lysine)-g-PEG coating can exhibit stability problems in media with high ionic strength, at pH lower than pI of the substrate, or above pH 10³⁰. Nam et al.³¹ used streptavidin and lipids tethered with biotin-PEG600 to capture biotinylated epidermal growth factor (EGF). Although lipid bilayers are well suited for short-term live-cell assays,³¹ they are not covalently linked to the surface and may exhibit poor stability during extended use. Metzger et al.¹⁴ utilized silane chemistry to stably attach biotinylated PEG3400 to surfaces. However, harsh conditions are required for synthesis and the surface homogeneity depends on combinations of factors such as temperature, humidity, and solvents used for silane deposition³². In contrast, the DOPA anchor group of DPB strongly binds to organic and inorganic surfaces under mild conditions and is stable over a wide range of pH and osmolality^{21,33}.

3.2. Controlled Adsorption of Singly-Biotinylated PEG5000, Fibronectin-Inspired Peptides and Stem Cell Factor

To evaluate the versatility of DPB/DP surfaces, we adsorbed PEG5000, bioactive peptides, and a cytokine conjugated with a single biotin molecule to DPB via NeutrAvidin (Fig. 2A). The dose-dependence of NeutrAvidin adsorption was similar to that for streptavidin, which indicates that electrostatic interactions with TiO_2 do not greatly affect streptavidin binding to DPB. In contrast to biotin₍₅₋₈₎-IgG₁, the surface concentrations for all of the singly-biotinylated molecules increased linearly with increasing DPB surface concentration. The ratio of biotinylated molecules bound per NeutrAvidin was ca. 3 for biotin-PEG600-cyclic LDV, 1.2 for biotin-PEG5000, and 0.8 for biotin-SCF. Thus, large molecules that are comparable in size to NeutrAvidin, such as biotinylated SCF ($8.7 \text{ nm} \times 3.2 \text{ nm} \times 2.5 \text{ nm}$)³⁴ and PEG5000 ($R_H \sim 6 \text{ nm}$), bind to ca. one of the biotin sites on NeutrAvidin, whereas the much smaller biotinylated peptide (MW ca. 2000) was able to access all of the available binding sites.

The biotin-NeutrAvidin-biotin linkages were very stable, as demonstrated by the observation that there was no decrease in surface density for any of the singly-biotinylated molecules after 10 hours of continuous perfusion in the OWLS chamber (Fig. 2B and data not shown). Furthermore, subsequent incubation for up to 7 hours in perfusion mode with free biotin at a concentration of 0.01 mg/mL, which is 1000-fold greater than the concentration of biotin in cell culture medium, did not release the adsorbed molecules from the surface (data not shown). This is consistent with previous studies by our group demonstrating that DOPA-PEG5000 surfaces remain non-fouling for at least 2 weeks^{8,20}.

Although sequential adsorption worked well for the biotinylated molecules discussed above, we observed nonspecific adsorption of biotinylated-peptides with isoelectric point (pI) of ca. 6 or more. For example, biotin-PEG600-cyclic RGD (pI 5.9; net charge of -0.1 at pH 7.0; <http://www.innovagen.se/index.asp>) adsorbed nonspecifically to surfaces modified with DPB10 (Fig. 3A) and DPB0 ($20 \pm 3 \text{ ng/cm}^2$). Nonspecific adsorption was even greater for biotin-PEG-HBD (pI 10.6; net charge of $+2.0$) on DPB10 ($44 \pm 8 \text{ ng/cm}^2$) and DPB0 ($51 \pm 9 \text{ ng/cm}^2$) surfaces. Although PEG reduces nonspecific protein adsorption, the non-fouling properties of PEG depend on the size of the protein^{10,35}. Small proteins can overcome the steric hindrance of PEG chains and bind to the underlying surface. This suggests that the biotinylated peptides we employed could readily penetrate the PEG2000 network. It is likely that the positively-charged biotin-PEG600-HBD diffused through the PEG2000 layer and adsorbed to the negatively-charged TiO_2 surface (pI ~ 5.2 ^{36,37}). This is consistent with substantial adsorption (ca. 40 ng/cm^2) of biotin-PEG600-HBD to bare TiO_2 surfaces. Biotin-PEG600-cyclic LDV (pI 0; net charge of -1.1) did not adsorb to either DPB0 surfaces or bare TiO_2 (data not shown). Although biotin-PEG600-cyclic RGD extensively adsorbed to DPB/DP surfaces, it did not adsorb to bare TiO_2 (data not shown). This suggests that nonspecific adsorption of biotin-PEG600-cyclic RGD was due to PEG-PEG interactions and/or interactions between the cyclic RGD peptide with PEG chains and/or DOPA groups. Similar hydrophobic interactions have been reported between the ethylene group of ethylene oxide and amino acids with hydrophobic side chains larger than alanine³⁸, as well as between PEG5000 brushes and self-assembled alkane thiol monolayers³⁹.

Nonspecific adsorption of biotin-PEG600-cyclic RGD to DPB/DP surfaces could be essentially eliminated by forming a complex between NeutrAvidin and the biotinylated peptide in solution prior to adsorption. For example, $53 \pm 3 \text{ ng/cm}^2$ of a NeutrAvidin-biotin-PEG600-cyclic RGD complex, which was formed by incubating biotin-PEG600-cyclic RGD and NeutrAvidin at a 2:1 molar ratio, adsorbed to a DPB10 surface (Fig. 3B). This can be compared with $50 \pm 2 \text{ ng/cm}^2$ of NeutrAvidin alone that adsorbed to DPB10. The ca. 3 ng/cm^2 difference in surface density between NeutrAvidin-biotin-PEG600-cyclic RGD complexes and NeutrAvidin alone is consistent with the binding of two molecules of biotin-PEG600-cyclic

RGD to each NeutrAvidin molecule. In contrast, forming a complex with NeutrAvidin prior to adsorption did not substantially decrease nonspecific adsorption of biotin-PEG600-HBD (data not shown). This difference is consistent with the observation that biotin-PEG600-HBD, but not biotin-PEG600-cyclic RGD, adsorbed to bare TiO₂ surfaces.

3.3 Surfaces Containing Biotin-PEG600-Cyclic LDV and/or Biotin-PEG600-Cyclic RGD Support Dose-Dependent Adhesion of M07e Cells

The bioactivity of adsorbed biotin-PEG600-cyclic LDV and biotin-PEG600-cyclic RGD was evaluated using M07e cell adhesion. M07e cells adhered to surfaces prepared via sequential adsorption of NeutrAvidin and biotin-PEG600-cyclic LDV in a DPB-dose-dependent manner, and the fractional adhesion approached a maximum of 0.95–1.0 between 1 and 2.5% DPB (Fig. 4A, open circles). In contrast, M07e cells only poorly adhered to biotin-PEG600-cyclic RGD on surfaces prepared via sequential adsorption (Fig. 4B, open circles). Since biotin-PEG600-cyclic RGD nonspecifically adsorbs to DPB surfaces (Fig. 3A), poor cell adhesion to biotin-PEG600-cyclic RGD can be attributed to suboptimal presentation of cyclic RGD at the surface. This is consistent with DPB-dose-dependent cell adhesion on biotin-PEG600-cyclic RGD surfaces prepared via complex adsorption (Fig. 4B, solid circles), although M07e cell binding was still much less extensive than that to biotin-PEG600-cyclic LDV surfaces containing the same amount of DPB. Prior complexing with NeutrAvidin did not substantially alter M07e cell adhesion to biotin-PEG600-cyclic LDV (Fig. 4A, solid circles). Adhesion to biotin-PEG600-cyclic LDV and biotin-PEG600-cyclic RGD was specifically mediated by the peptides. Pre-blocking cells with unbiotinylated cyclic LDV decreased the fractional adhesion to biotin-PEG600-cyclic LDV-containing surfaces prepared via sequential adsorption on DPB2.5 from ca. 0.9 to 0.3. In a similar manner, pre-blocking cells with unbiotinylated linear RGD decreased the fractional adhesion on biotin-PEG600-cyclic RGD-containing surfaces prepared via complex adsorption on DPB5 from ca. 0.6 to 0.1. It has previously been shown that avidin-mediated incorporation of biotinylated RGD peptides supports cell adhesion and spreading on surfaces containing biotinylated PEG linked to poly(lactic acid)⁴⁰. Here, we show dose-dependent M07e cell adhesion to immobilized biotin-PEG600-cyclic LDV, with nearly 100% of cells adhering to surfaces containing as little as 1% DPB.

Since biotinylated peptides are able to bind to all three of the available NeutrAvidin binding pockets (Fig. 2A), DPB/DP surfaces are well suited for the simultaneous presentation of multiple peptides. Incorporating both biotin-PEG600-cyclic RGD and biotin-PEG600-cyclic LDV increased M07e cell adhesion at least additively compared to adhesion to the individual peptides (Fig. 4C). For example, the fractional adhesion to surfaces prepared via complex adsorption at a NeutrAvidin:biotin-PEG600-cyclic RGD:biotin-PEG600-cyclic LDV molar ratio of 1:1:1 was equal to the sum of the fractional adhesion values for the two single-peptide surfaces prepared at a NeutrAvidin:biotinylated-peptide ratio of 1:1. For surfaces prepared using NeutrAvidin:biotin-PEG600-cyclic RGD:biotin-PEG600-cyclic LDV molar ratios of 1:2:1 or 1:1:2, the fractional cell adhesion was slightly (ca. 10–20%), but not significantly ($p = 0.31$ for 1:2:1; $p = 0.09$ for 1:1:2), greater than the sum of the fractional adhesion values for the single-peptide surfaces prepared at the respective NeutrAvidin:biotinylated-peptide ratios. Additive binding to LDV from the CS1 domain and REDV from the CS5 domain of fibronectin has been reported previously for B16-F10 murine melanoma cells⁴¹. However, we are not aware of any previous reports on the additive effect of peptides from the CS1 and FN-III(10) fibronectin domains. This approach can be readily extended for the presentation of other biotinylated peptides from fibronectin and other extracellular matrix proteins.

3.4. Biotinylated Stem Cell Factor Supports M07e Cell Adhesion and ERK Activation

The bioactivity of immobilized SCF was demonstrated via M07e cell adhesion and activation of the ERK signaling pathway. Cells adhered to immobilized biotin-SCF in a DPB-dose-

dependent manner, with a plateau in fractional adhesion at ca. 0.8 between 2.5% and 5% DPB (Fig. 5A). Fractional adhesion to biotin-SCF on surfaces containing 2.5% DPB was decreased to 0.1 by pre-blocking the cells with unbiotinylated SCF. M07e cells stimulated for 20 min with biotin-SCF exhibited a DPB-dose-dependent increase in the level of phosphorylated ERK1/2 (pERK1/2) as the surface DPB content was increased from 0% to 1% (Fig. 5B). However, pERK1/2 levels did not increase further when the DPB content was increased to 2.5%. Thus, ERK1/2 activation plateaued at a much lower DPB content than cell binding. Stimulation of M07e cells with unbiotinylated SCF in control tissue culture polystyrene (TCPS) well plates produced a similar level of ERK1/2 activation for an equivalent amount of total SCF up to 0.5 ng/well (Fig. 5C). ERK1/2 activation with soluble SCF plateaued at a slightly greater, although not significantly different ($p = 0.11$), value compared to that for immobilized SCF. Background ERK1/2 activation of cells in the absence of SCF was similar on TCPS or untreated DPB0 surfaces ($p = 0.43$, Fig. 5C). However, nonspecific adsorption of (NeutrAvidin or) biotin-SCF to DPB0 surfaces or the TCPS wells of the cassette resulted in pERK1/2 levels that were significantly greater than those for cells incubated with DPB0 surfaces in the absence of NeutrAvidin and biotin-SCF ($p < 0.001$, Fig. 5B). In contrast, there was no difference in M07e cell adhesion between blank DPB0 surfaces and those incubated with NeutrAvidin and biotin-SCF, which indicates a lower sensitivity to nonspecific SCF adsorption for the cell adhesion assay.

Maximal ERK1/2 activation by immobilized SCF was slightly lower than that by soluble SCF (Fig. 5C). Several studies have addressed the activity loss of proteins upon binding, which is primarily due to steric effects from random attachment sites^{42–44}. Even with a controlled position of attachment, the activity of immobilized proteins is typically lower than the soluble forms. For example, Zhen et al.²⁸ showed that the activity of β -lactamase immobilized to biotin-functionalized poly(L-lysine)-g-PEG via NeutrAvidin decreased by 7-fold. Several studies have evaluated the effects of immobilizing SCF. Doheny et al. used a fusion protein combining the cellulose-binding domain of cellulase Cex to immobilize SCF (SCF-CBD) on cellulose surfaces.⁴⁵ Immobilized SCF-CBD fusion proteins were found to be 7-fold more potent at stimulating the proliferation of M07e and TF-1 cells than the soluble form or native SCF.⁴⁵ Additional characterization of SCF-CBD surfaces using mouse B6SutA cells showed extended phosphorylation of the SCF receptor, ckit⁴⁶. In contrast, an immobilized fusion protein of IgG₁ and rat SCF exhibited lower maximal M07e cell growth potential than soluble rat SCF.⁴⁷

4. Conclusions

By conjugating biotin to the end group of DOPA₃-PEG3400, we have developed surfaces that are conducive for the stable presentation of bioactive peptides and proteins in a background that resists nonspecific protein adsorption. Various biotinylated ligands can be incorporated into surfaces via avidin linkers and their surface density is readily controlled. This approach is applicable to a wide variety of substrates due to the DOPA anchor group of the PEG chains³². Furthermore, this chemistry can be used for attaching multiple ligands, which will be necessary for mimicking complex cell environments, such as the hematopoietic stem cell niche.

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Figure 1.

Streptavidin and biotin₍₅₋₈₎-IgG₁ adsorption onto DOPA₃-PEG3400-biotin/DOPA₃-PEG2000 (DPB/DP) surfaces. (A) OWLS was used to monitor specific (solid line) and nonspecific (dashed line) sequential adsorption of streptavidin and biotin₍₅₋₈₎-IgG₁ onto surfaces with 10 mol% DPB (DPB10) and 0 mol% DPB (DPB0), respectively. (B) XPS measurements of TiO₂-coated slides modified with DPB10 (solid line). The surface was then modified via sequential adsorption of streptavidin and biotin₍₅₋₈₎-IgG₁ without exposing the substrate to air prior to XPS measurements (dashed line). (C) OWLS was used to measure the increase in streptavidin (solid circles), biotin₍₅₋₈₎-IgG₁ (open triangles), and BSA (open squares) surface concentrations with increasing surface concentrations of DPB. (D) The surface concentration of HRP-conjugated streptavidin, as measured with ELISA, increased linearly with the surface concentration of DPB. For (C) and (D) the data are presented as the mean $\pm$ standard deviation of at least three experiments. 1 mol% DPB corresponds to a surface concentration of 0.9 pmole DPB/cm².

Figure 2.

Sequential adsorption of NeutrAvidin and various singly-biotinylated molecules to DPB/DP-coated surfaces was monitored using OWLS. (A) The surface concentrations of NeutrAvidin (solid circles), biotin-PEG600-cyclic LDV (cLDV; open circles), biotin-PEG5000 (open squares), and biotin-SCF (open triangles) increased linearly with the DPB surface concentration. Data are presented as the mean $\pm$ standard deviation of three experiments. The schematic diagram illustrates molecular arrangements consistent with the adsorption data. (B) The final baseline after NeutrAvidin and biotin-PEG600-cyclic LDV adsorption to a DPB15 surface was essentially constant during more than 10 hours of continuous flow through the OWLS chamber; this illustrates the stability of the biotin-NeutrAvidin-biotin linkage.

Figure 3.

Adsorption of biotin-PEG600-cyclic RGD, alone and as a complex with NeutrAvidin, onto surfaces treated with DPB10. (A) Biotin-PEG600-cyclic RGD (cRGD) nonspecifically adsorbs to surfaces modified with DPB10 in the absence of NeutrAvidin. (B) In order to minimize nonspecific adsorption, biotin-PEG600-cyclic RGD and NeutrAvidin were incubated at a 2:1 molar ratio prior to adsorption to surfaces modified with DPB10 (solid curve). The adsorption of NeutrAvidin alone to surfaces modified with DPB10 (dotted curve) is shown for comparison.

Figure 4.

M07e cell adhesion to biotinylated peptides. M07e cell adhesion onto DPB/DP surfaces prepared via sequential (open circles) and complex (solid circles) adsorption of NeutrAvidin and (A) biotin-PEG600-cyclic LDV or (B) biotin-PEG600-cyclic RGD. Complex adsorption was performed using a peptide-to-NeutrAvidin molar ratio of 2:1. (C) M07e cell adhesion onto DPB0.5 surfaces prepared via complex adsorption of biotin-PEG600-cyclic RGD and/or biotin-PEG600-cyclic LDV using various peptide-to-NeutrAvidin molar ratios. The numbers along the abscissa represent the moles of biotin-PEG600-cyclic RGD (cRGD) and/or biotin-PEG600-cyclic LDV (cLDV) added per mole of NeutrAvidin during complex formation for each condition. Data are presented as the mean $\pm$ SEM of at least three adhesion experiments.

Figure 5.

M07e cell interactions with immobilized biotin-SCF. (A) M07e cells adhered to surfaces prepared via sequential adsorption of NeutrAvidin and biotin-SCF in a DPB-dose-dependent manner. (B) Growth-factor-starved M07e cells incubated for 20 min on surfaces prepared via sequential adsorption of NeutrAvidin and biotin-SCF exhibited DPB-dose-dependent ERK1/2 phosphorylation (solid circles). Control DP surfaces not treated with NeutrAvidin and biotin-SCF are indicated by an open circle. (C) Cells were also stimulated with soluble SCF on control surfaces made of tissue culture polystyrene (TCPS; solid squares). The ng/well values for DPB/DP surfaces were calculated using OWLS data (Figure 2A) and those for TCPS surfaces were

calculated using the soluble SCF concentration and the medium volume within the well. Data are presented as the mean $\pm$ SEM of six activation experiments.