In Situ Modulation of Cell Behavior via Smart Dual-Ligand Surfaces
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ABSTRACT: Due to the highly complex nature of the extracellular matrix (ECM), the design and implementation of dynamic, stimuli-responsive surfaces that present well-defined ligands and serve as model ECM substrates have been of tremendous interest to biomaterials, biosensor, and cell biology communities. Such tools provide strategies for identifying specific ligand–receptor interactions that induce vital biological consequences. Herein, we report a novel dual-ligand-presenting surface methodology that modulates dynamic ECM properties to investigate various cell behaviors. Peptides PHSRN, cRGD, and KKKTTK, which mimic the cell- and heparan sulfate-binding domains of fibronectin, and carbohydrates Gal and Man were combined with cell adhesive RGD to survey possible synergistic or antagonist ligand effects on cell adhesion, spreading, growth, and migration. Soluble molecule and enzymatic inhibition assays were also performed, and the levels of focal adhesion kinase in cells subjected to different ligand combinations were quantified. A redox-responsive trigger was incorporated into this surface strategy to spontaneously release ligands in the presence of adhered cells, and cell spreading, growth, and migration responses were measured and compared. The identity and nature of the dual-ligand combination directly influenced cell behavior.

INTRODUCTION

The extracellular matrix (ECM) is a highly dynamic, insoluble aggregate of collagens, proteoglycans, structural glycoproteins, and elastin that provides structural support for the adhesion, growth, differentiation, migration, and survival of mammalian cells.1–3 Improper cell attachment and migration have been implicated in cancer cell metastasis and other diseased states, including fibrosis.4–7 For a cell to undergo migration, it must first adhere to another cell or the ECM through cell surface receptor-ligand interactions.8 Integrins and syndecans, which are transmembrane proteins, represent the most common cell surface receptor families that facilitate cell adhesion to the ECM and transduce extra- and intracellular signals.9–11

Fibronectin (FN) is a predominant ECM glycoprotein that contains three homologous globular domains—types I, II, and III—and possesses a number of interaction sites for both integrins and syndecans.12 As such, FN plays an important role in cell adhesion, growth, migration, and differentiation and is critical to cellular processes, including embryogenesis and tissue repair.13 A number of cell types bind to FN regions that span the 9th to 10th type III (FNIII9–10) cell-binding domain. Arg-Gly-Asp (RGD), found in FNIII9–10, was identified as the minimal cell attachment sequence of α5β1 and αVβ3 integrin recognition.14 A synergy site that presents Phe-His-Ser-Arg-Asn (PHSRN) was then identified in FNIII9 and shown to enhance FN’s association with α5β1 integrins, mediating cell adhesion and migration.15–18 RGD and PHSRN are presented on the same plane of FN, connected by a flexible 30-40 Å linker.19 Spatial orientation and positioning of these signals are crucial for inducing synergistic effects on cell adhesion and migration. Furthermore, conflicting reports of whether PHSRN alone is capable of supporting cell adhesion have been a topic of debate over the past decade.15–18

Although α5β1 and αVβ3 integrins serve as the primary cell surface receptors that mediate adhesion, syndecan-4, a transmembrane heparan sulfate proteoglycan (HSPG), is a coreceptor for FN.20,21 A heparan sulfate (HS) binding domain spans FNIII12–14. Simultaneous interactions of syndecan-4 and α5β1 integrin with FNIII12–14 and FNIII9–10, respectively, induces downstream signaling events, leading to the activation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) with subsequent complete cell attachment and enhanced spreading via focal adhesion complex (FAC) formation.12,20,21 A few HS binding domain mimics have been tested; however, similar to the synergistic effect of RGD and PHSRN on cell adhesion, these small molecules or sequences are less efficient in promoting cell attachment alone. Such mimics contain the sequence B-B-B-X-X-B, where B is a basic amino acid (e.g., Arg or Lys) and X is a hydropathic sequences are less e

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infection and trafficking into the nucleus. Two separate works demonstrated that different hAd types, which lacked the KKTK motif, were able to attach and infect hepatic cells in vivo. However, little is known concerning the role of HS binding sequences on cell adhesion and migration and its possible synergistic effects, if any, with RGD.

Due to the complex nature of the ECM, identifying all the diverse small molecules and ligand−cell surface receptor combinations that induce specific biochemical processes remains challenging. Rather than performing in vitro studies with large native FN (~440 kDa), which is purified from blood plasma and tends to denature or adsorb in unnatural orientations and conformations on surfaces, researchers have sought to discover alternative approaches. As such, tremendous effort has been extended to creating model substrates that mimic the ECM using structurally well-defined, decoupled biomolecules, including RGD and PHSRN. Such surfaces enable the spatial and temporal presentation of well-defined ligands for the interrogation of biospecific ligand−cell surface receptor interactions, providing great tools for applications in cell biology, biotechnology, and tissue engineering.

Over the past decade, self-assembled monolayers (SAMs) of alkanethiolates on gold have proven to be smart, dynamic, and stimuli-responsive model surfaces for a number of cell biological investigations. "Dynamic" refers to the in situ control of cell behavior in response to an applied external stimulus. Liu et al. demonstrated that cells attach to the E isomer of RGD-conjugated azobenzene SAMs. However, when irradiated with UV light, azobenzene adopted the Z conformation, masking RGD, and the cells detached. Using a photodeprotection strategy, Lee and colleagues selectively exposed and conjugated SAM regions with RGD to probe cell adhesion, polarization, and migration behaviors. Moreover, Lamb and Yousaf reported a dynamic and switchable strategy based on electrochemically controlled hydroquinone (HQ) SAMs, in which the affinity of RGD ligands was altered. Upon the application of a specific oxidative (Ox) or reductive (Red) electrochemical potential, the HQ could be turned “off” and “on” to reveal or hide RGD ligands for cell attachment studies. Furthermore, in a mild, reducing environment, HQ has been shown to release its covalently bound ligand in the presence of living cells for subsequent immobilization and release cycles with negligible effects on cell behavior and viability. In vivo, ECM proteins and cell surfaces are constantly being remodeled and modified, where ligands are subjected to different compositions and orientations for biomolecular recognition. Therefore, model substrates that can mimic and modulate the highly evolving ECM would serve as great analytical tools, providing insight into the mechanisms of cell adhesion and migration in real time.

Herein, we report a dynamic, redox-responsive strategy to immobilize and release ligands in the presence of cells for cell adhesion, spreading, morphology, and migration studies (Figure 1). Electrochemistry enables the complete quantitative control over ligand density and provides a dynamic molecular switch for the combinatorial discovery of ligand effects. Two bioorthogonal coupling methodologies, click and oxime chemistry, were incorporated, and an HQ- and azide (N₃)-functionalized RGD peptide (HQ-RGD) was synthesized to modulate the ECM. Commercially available lysine-N₃ and derivatized glycine-HQ were incorporated into Ser-Ser-Asp-Gly-Arg-Gly-C₆ linker via solid-phase peptide synthesis to generate K(N₃)₃-C₆ linker-G(HQ)GRGDSS, where the N₃ moiety is conjugated to alkylamine-terminated SAMs via click chemistry and the HQ serves as a conjugation site for a variety of oxyamine-containing ligands. Synergy peptide PHSRN, high affinity cyclic RGD (cRGD), putative HS-binding sequence KKKTTK, and monosaccharides galactose (Gal) and mannose (Man) were functionalized with oxyamine groups and surveyed for potential synergistic or antagonistic effects with cell-adhesive RGD. Fibroblasts (Fs) were seeded to the different ECM mimics substrates, with or without HQ-RGD, and the number of attached cells, spreading area, morphologies, and migration rates were tabulated. Inhibition and competitive binding studies were also performed in which soluble FN, cRGD, and HS were added. Chondroitinase ABC and heparinase I and II were also delivered to Fs in culture to determine whether HS-binding KKKTTK exhibited a synergistic effect on cell adhesion and spreading. FAK protein levels were also detected and quantified. Furthermore, the ligands were released in the presence of cells, providing the dynamic component to our system, and cell adhesion, morphology, and migration rates were again examined. To our knowledge, this is...
the first report that uses a density-controlled, bioorthogonal, and stimuli-responsive model ECM to probe ligand–cell surface integrin and syndecan interactions in situ. The ability to switch ligands for the combinatorial screening of synergistic antagonistic ligand effects provides a platform that would be of tremendous significance to the biosensor and biomaterials research communities.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** All chemicals and reagents were of analytical grade and used without further purification. Common chemicals were obtained from Fischer Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO) unless specified. Rink amide 4-methylbenzhydrylamine (MBHA) resin, amino acids [Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Ser(But)-OH, Fmoc-Thr(But)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Lys(N2)-OH], Boc-aminooxyacetic acid, Fmoc-c-Abz-OH, and HBTU were purchased from Anaspec (San Jose, CA). Antibodies antiphospho and antivinculin and Cy-2 goat anti-mouse IgG were purchased from BD Biosciences (San Jose, CA) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), respectively. Fluorescent dyes DAPI and phalloidin and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fluorescence mounting medium was purchased from Dako (Carpinteria, CA). Swiss albino 3T3 mouse fibroblasts were obtained from the UNC-CH Tissue Culture Facility (Chapel Hill, NC). Heparin sulfate, heparinase I and II, and chondroitinase ABC were obtained from Sigma-Aldrich (St. Louis, MO).

**Syntheses.** Alkyne-terminated tetra(ethylene glycol) alkanethiol (alkyne-EGSH, 7) was synthesized as previously reported.40 Tetra-(ethylene glycol)-terminated alkanethiol (EG4SH, 8) was prepared as previously described.41 Rhodamine-oxime (Rhod-OA) was synthesized as previously demonstrated.42 Galactose- and mannose-oxime (A and B, respectively) were also synthesized as previously reported.43 Synthetic routes and a list of molecules are respectively shown in Schemes 1 and 2.

1,4-Bis((tetrahydro-2H-pyran-2-yl)oxy)benzene (1,6-dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.32H-pyran) (C) was then added, and the reaction was stirred then warmed to room temperature for 3 h.

**Scheme 1. Synthetic Scheme of HQ-RGD: (A) Solution Synthesis of Fmoc-glycine-HQ2 and (B) Solid-Phase Peptide Synthesis of HQ-RGD Using MBHA Resin**

**Reagents and conditions:** (i) DHP (4.5 equiv), HCl (cat.), THF, 16 h, 67.4%; (ii) tert-butylthiol (2 equiv), 1,6-dibromohexane (2 equiv), dry THF, 0–25 °C, 20 h, 50.6%; (iii) Na2N (1 equiv), DMF, 20 h, 80 °C; and (iv) Fmoc-propargylglycine (0.5 equiv), CuSO4·5H2O (1.5 equiv), NaAsc (1 equiv), DMF/H2O/EtOH (2:1:1), 12 h, 61.1%.**Syntheses:** Alkyne-terminated tetra(ethylene glycol) alkanethiol (alkyne-EGSH, 7) was synthesized as previously reported.40 Tetra-(ethylene glycol)-terminated alkanethiol (EG4SH, 8) was prepared as previously described.41 Rhodamine-oxime (Rhod-OA) was synthesized as previously demonstrated.42 Galactose- and mannose-oxime (A and B, respectively) were also synthesized as previously reported.43 Synthetic routes and a list of molecules are respectively shown in Schemes 1 and 2. 1,4-Bis((tetrahydro-2H-pyran-2-yl)oxy)benzene (1).

To a solution of hydroquinone (6.0 g, 54.5 mmol) in tetrahydrofuran (THF, 40 mL) were added 3,4-dihydropyran (20.8 mL, 245.3 mmol, 4.5 equiv) and three drops of concentrated HCl (cat.). The mixture was stirred for 16 h at room temperature. The reaction contents were then diluted with ethyl acetate (EtOAc, 20 mL) and extracted with 1 M sodium bicarbonate (3 × 25 mL) and brine (1 × 25 mL) and concentrated to afford a white solid, A. The solid product was dried under vacuum for an additional 3 h (10.23 g, 67.4%).1H NMR (400 MHz, CDCl3, δ): 1.58–1.67 (m, 6H, J = 36 Hz; −CH2−), 1.85–1.88 (m, 4H, J = 12 Hz; −CH2−), 2.00–2.03 (m, 2H, J = 8 Hz; −CH2−), 3.60–3.62 (m, 2H, J = 8 Hz; −CH2−), 3.96–3.98 (m, 2H, J = 8 Hz; −CH2−), 5.32–5.34 (t, 2H, J = 7 Hz; −CH−), 7.00 (s, 4H, Ar-H).

2,2′-((2-(2-Azidoethyl)-1,4-phenylene)bis(oxy))bis(tetrahydro-2H-pyran) (C). To a stirring solution of A (3.0 g, 10.8 mmol) in dry THF (100 mL) at 0 °C was added tert-butylthiol (1.5 M in pentane, 10.1 mL, 13.0 mmol, 1.2 equiv) dropwise. After the base addition, a white precipitate formed. The mixture was stirred for 2 h at 0 °C and then warmed to room temperature for 3 h. 1,6-Dibromohexane (3.3 mL, 21.6 mmol, 2 equiv) was then added, and the reaction was stirred for 16 h to afford a pale yellow liquid. The mixture was then diluted with EtOAc (20 mL), extracted with NH4Cl (2 × 25 mL), H2O (1 × 50 mL), and brine (1 × 50 mL); dried over MgSO4; and concentrated to a pale yellow oil. Silica flash column chromatography (Hex/EtOAc/DCM, 8:1:1) was employed to purify B (2.4 g, 50.6%).1H NMR (400 MHz, CDCl3, δ): 1.40–1.42, 1.46–1.47 (d × 4, 4H, J = 8 Hz; −CH2−), 1.49 (m, 4H, −CH2−), 1.86–1.88 (m, 6H, J = 8 Hz; −CH2−), 2.02 (d, 2H, J = 8 Hz; −CH2−), 2.60–2.62 (t, 2H, J = 8 Hz; −CH2−), 3.41–3.44 (m, 2H, J = 12 Hz; −CH2−), 3.61–3.62 (m, 2H, J = 4 Hz; −CH2−), 3.96–3.99 (m, 2H, J = 12 Hz; −CH2−), 5.31 (s, 2H, −CH2−), 6.85–6.87 (m, 2H, J = 8 Hz; Ar-H), 7.02–7.04 (d, 1H, J = 8 Hz; Ar-H).

2-((9H-Fluoren-9-yl)ethoxy)carbonyl)amino)-3-(1-(6-(2,5-bis(tetrahydro-2H-pyran-2-yl)oxy)phenyl)hexyl)-1H-1,2,3-triazol-4-yl)propionic Acid (Fmoc-Gly-HQ), D. To a solution of C (1.64 g, 4.51 mmol, 2 equiv) and Fmoc-propargylglycine (0.76 g, 2.26 mmol) in DMF (20 mL) and EtOH (5 mL) was added a solution of CuSO4·5H2O (0.85 g, 3.38 mmol, 1.5 equiv) and sodium ascorbate (0.67 g,
Scheme 2. List of Molecules and Surface Groups Used in This Study

“The following molecules are depicted: (1) hydroquinone- and azide-functionalized RGD, HQ-RGD; (2) cyclic RGD-functionalized oxyamine, cRGD; (3) PHSRN-functionalized oxyamine, PHSRN; (4) KKKTTK-functionalized oxyamine, KKKTTK; (5) galactose-functionalized oxyamine, Gal; (6) mannos-functionalized oxyamine, Man; (7) alkyne-terminated tetra(ethylene glycol) alkanethiol, alkyne-EG4SH; and (8) tetra(ethylene glycol)-terminatated alkanethiol, EG4SH.

The following sequences, separately, over the course of 12 h: resin-S-S-4, resin-K-T-T-K-K-K-C6(linker)-ONH2 (C6(linker)-ONH2 (min, 0.1% TFA and 5 D-G-R-G-G(HQ)-C 6(linker)-K(N 3)-amide (the following sequences, separately, over the course of 12 h: resin-S-S-

Preparation of Gold-Coated Substrates and Monolayers. Glass coverslips (75 × 25 mm²) were immersed into a piranha solution (1:3 (v:v) concentrated H2SO4:30% H2O2; use with caution) for 4 h, followed by rinsing with deionized H2O and EtOH. Gold substrates were prepared by electron-beam deposition (Model VE-100, Thermionics Laboratory, Inc., Port Townsend, WA) of titanium (5 nm) and then gold (12 nm for cell work and 50 nm for electrochemical measurements). The gold-coated slides were cut into 1 × 2 cm² pieces. To form SAMs on gold, the slides were immersed in an ethanolic solution containing the alkanethiols (1 mM of 5% alkyne-EG4SH for cell studies and 100% alkyne-EG4SH for electrochemical characterization) for at least 16 h. Once removed from solution, the surfaces were rinsed with EtOH and dried with an air stream before use.

HQ-RGD Immobilization. After monolayer formation with 1 mM of 1% alkyne-EG4SH/EG4SH, a solution containing 10 mmol HQ-RGD, 15 mmol CuSO4·5H2O, and 15 mmol NaAsc (1:1:1) in H2O and EtOH (3:1) was added to the substrates and allowed to react for 90 min. Substrates were then rinsed with EtOH and dried with a stream of air, and HQ-RGD immobilization was confirmed by cyclic voltammetry (CV).

Electrochemical Activation. Electrochemical experiments were performed using a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, IN) in a 1 M HClO4 electrolyte solution with an Ag/AgCl electrode serving as the reference, the gold monolayer as the working electrode, and a Pt wire as the counter electrode. Surfaces were scanned at a rate of 100 mV/s from −100 to 850 mV to activate “on” (quinnime form).

Ligand Immobilization and Release. Peptides and sugars were added to RGD-Q-presenting surfaces (20 mM in H2O) and allowed to react for 90 min. Immobilized ligands were confirmed using the same electrochemical parameters listed above. The ligands were released by applying potential for 12 cyclic scans (−100 to 850 mV) in PBS buffer (pH 7) and were characterized using similar conditions.

Cell Culture and Surface Seeding. Swiss albino 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle medium containing 10% calf bovine serum and 1% penicillin/streptomycin. When passaging or seeding, a 1 mL solution of 0.05% trypsin in 0.53 mM EDTA was employed to remove cells from the tissue culture flask containing fresh media and placed in the incubator (37 °C, 5% CO2) to grow and divide. For surface seeding, cells were resuspended in serum-free medium (106 cells/mL) and diluted to 105 cells/mL with serum-free medium. The cells were then seeded to surfaces for 2 h, and after 2 h, serum-containing media was added to promote cell growth.

Cell Staining. After 2 h of cell growth, the cells were fixed with formaldehyde (3.2% in PBS) and then permeated (PBS containing 0.1% Triton X-100) at 37 °C, pH 7 and were characterized using similar conditions.

Fluorescent images were obtained using a Nikon Eclipse TE2000-E inverted microscope (Nikon USA, Inc., Melville, NY) and a Plan Fluor 40X oil immersion objective (1.30 NA, Nikon USA). Immersion oil was purchased from Carl Zeiss MicroImaging, Inc. (Thornwood, NY) and the lens paper was used with caution.
obtained from Fisher. Image analysis was performed using MetaMorph software (Molecular Devices, Downingtown, PA).

**Cell Adhesion Assay.** The number of adhered cells were measured 2 h after seeding on the following substrates at 2% ligand density: ±HQ-RGD and Gal, Man, PHSRN, KKKTTK, and cRGD. Two hundred microliters of cells (∼10⁵ cells/mL) was added to each substrate (1 cm²), which was subsequently incubated at 37 °C and 5% CO₂ for 2 h. Two random regions of four different substrates for each ligand were imaged at a 4× resolution using a Nikon Eclipse TS100 (Nikon USA, Inc., Melville, NY). The attached cells were then counted. The data are expressed as the mean ± SEM of eight replicates.

**Cell Area Determination Assay.** Cell areas (μm²) were measured 2 h after seeding on the following substrates at 2% ligand density: ±HQ-RGD and Gal, Man, PHSRN, KKKTTK, and cRGD. Two hundred microliters of cells (∼10⁵ cells/mL) was added to each substrate (1 cm²), which was subsequently incubated at 37 °C and 5% CO₂ for 2 h. The cells were then fixed as previously reported, and two random cell areas from four different substrates for each ligand were imaged at a 40× resolution using a Nikon Eclipse TE2000-E inverted microscope; Nikon USA, Inc., Melville, NY), was carried out with MetaMorph software (Molecular Devices, Downingtown, PA). The data are expressed as the mean ± SEM of eight replicates.

**Cell Migration Rate Determination.** Cells were cultured and seeded at concentrations of approximately 10⁵ cells/mL onto the following substrates at 2% ligand density, as described: HQ-RGD and HQ-RGD + Gal, Man, PHSRN, KKKTTK, or cRGD. Migration rates were determined after recording the movement of cells for 18 h in bright-field mode (Nikon Eclipse TE2000-E inverted microscope; Nikon USA, Inc., Melville, NY). Eight randomly selected cells (four cells per substrate condition, two substrates per condition) were imaged each hour for 18 h, and the displacements from their original adherent position were tracked and calculated using MetaMorph software (Molecular Devices, Downingtown, PA). The data are expressed as the mean ± SEM of eight replicates.

**Electrochemical Characterization.** HQ-RGD was immobilized to alkyne-terminated SAMs (100%, 1 mM in EtOH, 16 h), as previously described. The immobilization was confirmed using a BAS 100B/W electrochemical analyzer (Bioanalytical Systems, Inc., West Lafayette, IN) in a 1 M HClO₄ electrolyte solution with an Ag/AgCl electrode serving as the reference, the gold monolayer as the working electrode, and a Pt wire as the counter electrode. Samples were scanned at 100 mV/s ranging from −100 to 850 mV. To immobilize oxime-containing ligands, the substrates were activated and turned on after performing a linear scan from −100 to 850 mV at 100 mV/s. After ligand immobilization, the redox reversible oxime signal was confirmed using CV with the parameters just mentioned. The ligands were then released, as described, and confirmed by CV.

**Enzymatic Adhesion Assay.** Fbs (∼10⁵ cells/mL) were suspended in serum-free media and treated separately with heparinase (104 cells/mL) were seeded in a suspension of serum-free media on the Scheme 2) monosaccharides due to a few published works. Du et al. reported the enhancement of hepatocyte adhesion using a hybrid Gal/RGD monolayer via hepatic asialoglycoprotein receptor interactions.66 Fbs that express the mannose receptor, which contains a NFII domain, have been shown to exhibit specificity in binding to type I, III, and IV collagens to facilitate cell–ECM adhesion.47 Thus, we hypothesized to observe an

**RESULTS AND DISCUSSION**

**Dynamic Surface Design and Ligand Selection Rationale.** The development and use of smart materials that have switchable or stimuliresponsive properties have proven to be important for a number of biological studies ranging from fundamental basic cell biology research to biomedical implants and tissue engineering scaffolds.31−34 Therefore, we aimed to modulate the dynamic ECM with redox-responsive surfaces to survey ligand effects on cell behavior. FN is an abundant glycoprotein that promotes cell adhesion to the ECM via integrin (i.e., transmembrane, cell surface receptors) interactions (Figure 1A). Two key peptide sequences, Arg-Gly-Asp (RGD, Scheme 2) and Phe-His-Ser-Arg-Asn (PHSRN, Scheme 2), located in FNIII₁₀ and FNIII₁₆, respectively, were identified as cell-binding ligands. Polymers, nanoparticles, and other biomaterials are routinely functionalized with both linear (K₉−μM) and cyclic (2, cRGD; K₉−nM, Scheme 2) forms of RGD to promote integrin recognition and subsequent cell attachment.44,45 PHSRN is described as a synergy ligand with RGD and interacts simultaneously with α₅β₁ integrins to mediate cell adhesion and migration.15−18 Furthermore, an HS binding domain, located within FNIII₁₂−₁₆, was identified and promotes cell surface syndecan and integrin corecognition and interaction. With the FN structure in mind, we hoped to reproduce the combined ligand effects of coupling PHSRN and cRGD with RGD on cell adhesion, spreading, morphology, and migration. As a new ligand, we chose to survey the combined effects of RGD and Lys-Lys-Lys-Thr-Thr-Lys (4, KKKTTK, Scheme 2), which bears four positive charges, on cell behavior. We assumed that KKKTTK would mediate electrostatic interactions with negatively charged cell surface HSPGs, mimicking the HS binding domain on FN and producing a synergistic effect on cell adhesion, growth, and migration. Detailed peptide structures are displayed in Scheme S2 in the Supporting Information.

We also chose to investigate the dual-ligand effects of RGD with galactose (5, Gal, Scheme 2) and mannose (6, Man, Scheme 2) monosaccharides due to a few published works. Du et al. reported the enhancement of hepatocyte adhesion using a hybrid Gal/RGD monolayer via hepatic asialoglycoprotein receptor interactions.66 Fbs that express the mannose receptor, which contains a NFII domain, have been shown to exhibit specificity in binding to type I, III, and IV collagens to facilitate cell–ECM adhesion.47 Thus, we hypothesized to observe an
increase in cell adhesion and spreading and a decrease in migration when presented with RGD.

The general dynamic redox-responsive surface strategy to present two ECM ligands is represented in Figure 1B,C, and a more detailed schematic is provided in Scheme S1 (Supporting Information (SI)). Mixed EG₄SH/alkyne-EG₄SH SAMs (Scheme 2) were formed on gold substrates in a ratio of 99:1 (1 mM total in EtOH, 16 h). Both alkanethiols are resistant to nonspecific protein adsorption and cell adhesion, which is extremely important when conducting biospecific ligand–receptor interaction studies. Here, EG₄SH (99% density) serves as the inert background and alkyne-EG₄SH (1% density) provides an alkyn terminal group for ligand immobilization via click chemistry with an azide-containing RGD ligand. The molecule density was maintained at 1% to ensure that only the specific interactions between the ligands in question with Fb cell surface receptors occur.

The redox-responsive trigger, in the form of a hydroquinone/quinone (HQ/Q) couple, was built into an Fmoc-protected glycine residue (E, Scheme 1A) and was compatible with routine solid-phase peptide synthesis (Figure S1, SI). E was incorporated into an RGD-containing peptide (1, HQ-RGD, Schemes 1 and 2) that was capped with an azide-functionalized lysine residue for coupling to the 1% alkyne-EG₄SH SAMs via click chemistry (20 mM in H₂O/EtOH (3:1), cat. CuSO₄·5H₂O and NaAsc, 90 min). We have previously shown and extensively characterized the immobilization and release of oxime (OA)-functionalized ligands (i.e., peptides, small molecules, and carbohydrates) to and from HQ/Q_SAMs on gold substrates for a number of biotechnological applications and cell behavioral studies.

As shown in Figure 1B, after HQ-RGD-N₃ immobilization to alkyne-EG₄SH SAMs, the substrates are oxidized [Ox] using linear sweep voltammetry to the corresponding Q (1 M HClO₄, −100 to 850 mV, 100 mV/s), which then reacts rapidly and chemoselectively at room temperature and under physiological conditions (20 mM in PBS, pH of 7, 2 h) to form an oxime conjugate. The oxime bond is stable in all pH ranges until application of a reducing potential at a pH of 7, at which the ligands are spontaneously cleaved, regenerating the original HQ-RGD-tethered SAM. Ligand immobilization and release to and from HQ-RGD-presenting SAMs were characterized by electrochemistry and fluorescence microscopy (Figures S2 and S3, respectively, SI).

A key feature of this system is that both HQ-RGD and the corresponding oxime conjugate exhibit signature redox signals that can be monitored and quantified by cyclic voltammetry (CV) in terms of ligand immobilization, release, and density, characteristics that are significant when designing a platform for biological investigation. Thus, this dual-ligand ECM and combinatorial screening strategy possesses the following features: a dynamic, molecular redox-responsive trigger for immobilizing and releasing structurally well-defined ligands in the presence of cells; density control over ligands; and rapid, chemoselective, and bioorthogonal coupling reactions (i.e., Huisgen cycloaddition and oxime chemistry).

**Cell Adhesion Assays.** Before testing our dual-ligand ECM system, we first verified whether Fbs could adhere and healthily spread on substrates presenting HQ-RGD and each decoupled ligand (Figure 2). As such, HQ-RGD and cRGD, Man, Gal, PHSRN, and KKKTTK ligands were immobilized to alkyne-EG₄SH (20 mM in H₂O/EtOH (3:1), cat CuSO₄·5H₂O and NaAsc, 90 min) and HQ-terminated (20 mM in PBS, 2 h) SAMs, respectively, at a 2% ligand density. Fbs cells were then seeded on surfaces (~10⁵ cells/mL, 2 h), fixed, imaged, and counted after 2 h. As shown in Figure 2, all ligands were able to support cell adhesion to varying degrees. The data are represented as the average number of attached cells per 4× frame (eight random regions). HQ-RGD and cRGD demonstrated similar affinity for attracting Fbs when compared to the surfaces presenting Man, Gal, KKKTTK, and PHSRN, which exhibited almost a 2-fold reduction in the amount of attached Fbs. Additionally, the Fbs were more loosely adhered and adopted a rounder morphology on substrates presenting Man, Gal, KKKTTK, and PHSRN. However, when these ligands were combined with and immobilized to SAMs with 1% Q-RGD (2% ligand total), the amount of cells increased significantly, approximately 2-fold, resembling the results observed with 2% HQ-RGD. When Man and Gal were coupled to HQ-RGD, Fbs behaved similarly to when in the presence of cell adhesive RGD; similar numbers of attached cells and morphologies were observed. When PHSRN was coupled to HQ-RGD, a synergistic effect on the amount of cells adhered and overall spreading and morphology was observed, although, at surface value, the actual number of attached cells did not increase significantly from the substrates with 2% HQ-RGD. Surprisingly, KKKTTK coupled to HQ-RGD showed a dramatic increase in the amount of attached Fbs when compared to HQ-RGD- and KKKTTK-presenting substrates, indicating a profound synergistic effect between KKKTTK and RGD on adhesion. A marked 0.5-fold increase from HQ-RGD substrates was observed on KKKTTK + HQ-RGD.

**Cell Spreading, Morphology, and Migration Assays.** Upon adhering to the ECM or another cell, a cell undergoes the following sequential events: spreading, actin cytoskeleton organization, and focal adhesion (FA) formation (Figure 1A). The cell then waits in anticipation of receiving and processing extracellular signals and migrates from various epithelial layers to target locations, where it differentiates to form a specialized cell that comprises different tissues and organs.
Similar immobilization and fixing conditions were employed to survey the possible synergistic or antagonistic effects of coupling cell adhesive RGD with a number of peptides and carbohydrates on cell spreading, morphology, and migration rate. Cell areas were measured, and FAs and actin were visualized by staining for vinculin (antivinculin and Cy-2, green) and F-actin (phalloidin, red) after 2 h of culture on all surfaces (Figure 3A,B). Cells were then observed to migrate on substrates during an 18-h period via live-cell recordings, and migration rates were calculated (Figure 3C). Notably, the focus of this work was to observe the possible synergistic or antagonist effects of an artificial, dual-ligand ECM. Our cell attachment and spreading results in Figures 2 and 3A, respectively, demonstrate significant increases in the size and number of cells adhered to dual-ligand-presenting SAMs when compared to substrates displaying single HQ-RGD, cRGD, PHSRN, KKKTTK, Gal, and Man ligands. Thus, cell migration behavior was only examined on dual-ligand surfaces.

As shown in Figure 3AB, when compared to HQ-RGD, Fbs were extremely well spread on both cRGD and HQ-RGD + cRGD presenting surfaces. These results are not surprising, because the RGD sequence in native FN is located on a β-turn, and thus, the cyclic form of RGD has a nanomolar affinity for integrin binding, compared to linear RGD, which has micromolar cell-binding affinity. Cells exhibited a 30% increase (HQ-RGD, 630 μm²; cRGD, 850 μm²; HQ-RGD + cRGD, 850 μm²) in area from HQ-RGD with a more pronounced and intricate network of actin striations and FAs on the periphery and along the actin extensions of the main cell body (right panel, Figure 3B). These morphological characteristics are indicative of well-adhered, healthy cells. Moreover, when comparing Fb migration on HQ-RGD (7.2 μm/h), cells exhibited a 2-fold decreased rate (3.1 μm/h) on HQ-RGD + cRGD, corroborating the adhesion, spreading, and morphology data (Figure 3C).

Synergistic effects of coupling HQ-RGD + PHSRN on Fb spreading and morphology were observed, verifying the results from the cell adhesion assay. An approximate 4-fold increase in average cell area from PHSRN (180 μm²) to HQ-RGD + PHSRN (700 μm²) was recorded, indicating that PHSRN, which resembles FNIII₉, does not alone sustain adequate attachment and growth. This result was expected due to the lower binding affinity of integrins for PHSRN. Moreover, cells were larger on average than those on HQ-RGD alone (630 μm²). The cell morphologies were drastically different upon observing Figure 3A,B. Cells on PHSRN alone remained small with rounded ruffling features at the cell periphery and little to no FA formations, as compared to cells on HQ-RGD. However, when coupled to HQ-RGD, cells spread but appeared to be in a migratory state; the actin cytoskeleton was not fully extended and striated and FAs were not strongly pronounced. These cellular characteristics were further verified with the migration data represented in Figure 3C. Cells on HQ-RGD + PHSRN (11.9 μm/h), demonstrated a 66% increase in migration rate when compared to cells on HQ-RGD (7.2 μm/h). These results demonstrate a relationship between increased migration rate and cell ruffling dynamics, suggesting that our cell findings on RGD/PHSRN substrates were induced by ligand effects. These data corroborate previous reports showing that PHSRN causes ECM invasion of Fbs and keratinocytes and stimulates corneal epithelial cell migration, accelerating wound healing in mice and rabbits, respectively.53,54

Figure 3. Representative cell areas (A), morphologies (B), and migration rates (C) after 2 h of culture on the following substrates presenting single ligands (green) cRGD, Man, KKKTTK, Gal, or PHSRN and dual ligands HQ-RGD (blue) + cRGD, Man, KKKTTK, Gal, or PHSRN. Cells in part B were stained for actin (red, phalloidin), nucleus (blue, DAPI), focal adhesions (FAs green, antivinculin/Cy 2). Migration rates were calculated from an 18-h period using live-cell recording and imaging software. Each bar represents the mean ± SEM (n = 8). Statistical analyses were performed with respect to HQ-RGD: *p < 0.001 and **p < 0.01.
Although marked differences were observed in the Fb spreading, areas, and morphologies on Gal and HQ-RGD + Gal (420–600 μm²) and Man and HQ-RGD + Man (380–610 μm²), no overall synergistic effects were observed when compared to HQ-RGD alone (630 μm²) (Figure 3A). This conclusion was also apparent from the images in Figure 3B. Similar to PHSRN, Fbs on Gal and Man adopted small and round shapes with no noticeable FA formations and organized actin cytoskeleton. Ruffling characteristics are typical of cells that are feeling and sensing their environment. The increase in cell area after Man and Gal immobilization to Q-RGD is most likely due to the presence of cell adhesive RGD. The cells appear more spread with FA formations at the tips of spikey actin extensions. However, the cell bodies remain rounded, and the actin cytoskeleton is not well structured; it appears that these monosaccharide ligands may be partially masking the adhesive properties of RGD. When observing the migration data, the rate differences were minimal when comparing cells on HQ-RGD and HQ-RGD + Man or Gal substrates (7.2, 7.4, and 7.5 μm/h, respectively). Interestingly, Fbs migrated at approximately the same rate on both RGD and carbohydrate-presenting SAMs (Figure 3C).

As previously mentioned, KKKTTK weakly supported the attachment of Fbs. However, when compared to the average area of cells on HQ-RGD (630 μm²), cells on KKKTTK alone exhibited a comparable average area of 560 μm². Although no defined FAs were formed on the cell peripheries (left panel, Figure 3B), the actin cytoskeleton appeared to be partially organized, with striated patterns and small extensions. This area may be due to the electrostatic interactions, generated from the positive lysine residues at physiological conditions and highly negatively charged cell surface. When coupled to HQ-RGD, the average cell area on KKKTTK + HQ-RGD increased by approximately 50% that of cells on HQ-RGD alone, indicating a synergistic effect of spreading and growth. When imaged, cells maintained a rounded, spread, dense cell body, as seen with cells on KKKTTK alone; however, the appearance of strongly pronounced FAs at the cell periphery and increased actin organization was observed (left panel, Figure 3B). When considering the difference in cell migration rates, Fbs on HQ-RGD + KKKTTK demonstrated a 20% decrease from that of cells on HQ-RGD alone. The data was statistically significant (p < 0.0001), indicating that the presence of KKKTTK does exhibit a combined ligand effect. These significant morphological distinctions and decreased migration rate further support the synergistic effects of RGD (FNIII10) and KKKTTK (FNIII12-14) in promoting cell surface integrin and syndecan interactions.

**Ligand Inhibition Studies on Cell Adhesion.** Because the ligand combinations HQ-RGD + cRGD, HQ-RGD + KKKTTK, and HQ-RGD + PHSRN demonstrated an effect on Fb adhesion, several soluble molecule and enzyme inhibition studies were performed. We first surveyed the number of attached cells on HQ-RGD + cRGD, HQ-RGD + KKKTTK, and HQ-RGD + PHSRN substrates (2%) before and after the addition of FN, cRGD, and HS (0.1 mmol in PBS). FN is a natural adhesion protein in the ECM, and we chose cRGD because integrins have a high binding (nM) affinity for the ligand. HS is a repeating disaccharide that possesses a carboxylate and varying sulfate groups. Thus, at physiological conditions, the overall net charge of HS is highly negative. We assumed that addition of soluble FN, cRGD, and HS would interfere with the binding interactions of Fbs and our dual-ligand ECM substrates. In Figure 4A, the addition of soluble FN had significant effects on the cells that were adhered to HQ-RGD and HQ-RGD + KKKTTK; an approximate 75% reduction of attached cells was observed. Thirty percent of the Fbs on HQ-RGD + cRGD detached from the surface after FN addition, and only 10% lifted from HQ-RGD + PHSRN, as expected. Therefore, RGD alone and the combination of RGD with KKKTTK or cRGD do not exhibit as strong of a synergistic effect on promoting strong cell attachment as originally anticipated. For all substrates, the addition of soluble cRGD and HS had minimal effects on Fb detachment. The high binding affinity small peptide and negatively charge oligosaccharide were not strong enough to detach the cells from the dual-ligand ECM mimics, most likely due to already established cell surface integrin– and syndecan–ligand interactions. On the basis of the other evidence in this section, RGD + KKKTTK remains a good dual-ligand ECM platform, but is not as ideal as natural ligand FN.

![Figure 4. Cell adhesion inhibition assays. (A) Soluble competitive inhibition studies with (orange) FN, (blue) cRGD, and (light purple) HS and (B) enzymatic treatment of cells with (olive green) chondroitinase ABC and (dark purple) heparinase I/II on the following substrates: HQ-RGD and HQ-RGD + cRGD, KKKTTK, or PHSRN. Each bar (mean ± SEM) represents an average of five to eight trials (~10⁴ cells/mL).](image-url)

We also investigated the enzymatic effects of heparinase I and II (Hep I/II) and chondroitinase ABC (chon ABC) on cleaving the HS and CS chains of PGs that are found on Fb membranes. These enzymes were incubated with Fbs at 0.025 units/mg in serum-free media for 1 h, after which the cells were added to HQ-RGD and HQ-RGD + KKKTTK (2%) for 2 h. The Fbs were then fixed, counted, and compared to the
controls (−Hep I/II or −Chon ABC). Figure 4B presents the number of adhered cells after enzyme treatment. As shown, HQ-RGD showed little change in attracting cells to the surface with the following results: no treatment, 59; +Hep I/II, 57; and +chon ABC, 55. This could be due to the fact that only a net charge of +1 is present in the same ligand area (arginine in HQ-RGD). Therefore, cleaving the HS and CS groups from cell surfaces did not have an effect on integrin-mediated cell attachment. However, when compared to HQ-RGD + KKKTTK, Fbs experienced a 50% decrease in adhesion after enzymatic treatment of Hep I/II and chon ABC with the following results: no treatment, 83; +Hep I/II, 41; +chon ABC, 53. Furthermore, the results corroborated the previous adhesion results; more cells adhered to surfaces due to the synergistic effects of HQ-RGD and KKKTTK. The inhibition in cell adhesion after Hep I/II and chon ABC treatment is most likely due to the decreased electrostatic interactions of the negatively charged cell surface with positively charged dual-ligand ECM (+5 net charge in one concentrated area), as well as less interaction with syndecan-4 surface receptors.

**Focal Adhesion Kinase Assay.** To confirm the results from inhibition and morphological assays, we detected and quantified the FAK levels in cells subject to HQ-RGD + cRGD, HQ-RGD + KKKTTK, and HQ-RGD + PHSRN substrates. FAK serves a major role in cell adhesion, spreading, differentiation, migration, division, and apoptosis. Evidence of enhanced FAC was observed in morphological data of the dual-ligand ECM systems listed above. Thus, Fbs were incubated on these substrates for 4 h, and an ELISA kit and spectrophotometry, the FAK levels were quantified. The lysates of each cell population were diluted to measure the FAK level range and generate a linear relationship between the optical density and FAK concentration. The total FAK concentration was then determined according to each substrate. As shown in Figure 5, higher FAK concentrations were observed in Fbs after 4 h adjusted time, the cells more or less adopted similar phenotypes (Figure 6C), motility (Figure 6B), and spreading areas (Figure 6A) to those of substrates that presented HQ-RGD. The Fbs that were subject to Man, Gal, and PHSRN from HQ-RGD. Fbs were cultured on the dual ECM ligand-presenting surfaces for 2 h, after which the ECM ligands were electrochemically released (PBS, pH 7, 12 cyclic scans: −100 to 850 mV, 100 mV/s) and incubated for an additional 2 h. Migration rates were calculated from 18-h period using live-cell recording and imaging software after ligand release. Each bar (mean ± S.E.M.) represents an average of eight trials (10^3 cells/mL).

**Figure 6.** Cell behavioral responses to the dynamic release of ECM ligands. Cell (A) areas, (B) migration rates, and (C) morphologies after releasing (blue) cRGD, Man, KKKTTK, Gal, or PHSRN from HQ-RGD. Fbs were cultured on the dual ECM ligand-presenting surfaces for 2 h, after which the ECM ligands were electrochemically released (PBS, pH 7, 12 cyclic scans: −100 to 850 mV, 100 mV/s) and incubated for an additional 2 h. Migration rates were calculated from an 18-h period using live-cell recording and imaging software after ligand release. Each bar (mean ± S.E.M.) represents an average of eight trials (10^3 cells/mL).

ligand immobilization to HQ-RGD SAMs when compared to substrates only baring HQ-RGD (35 ng/mL, black), with +cRGD (59 ng/mL, red) and +KKKTTK (58 ng/mL, blue) surfaces possessing similar FAK levels, followed by +PHSRN (50 ng/mL, green) functionalized substrates. Therefore, the FAK assay confirmed the morphological data; more FAs were formed in the cells on +KKKTTK and +cRGD and less in the cells on +PHSRN when compared to HQ-RGD. The combined ligand effects of RGD with KKKTTK on enhancing cell adhesion and FA formation, as determined by the increased FAK concentration, were again verified. Thus, RGD + KKKTTK is a good dual-ligand ECM mimic.

**Modulation of Cell Behavior via a Dynamic ECM Model Surface.** The final study in this work concerned the dynamic release of immobilized ligands in the presence of cells. Fb spreading areas, migration rates, and morphologies were investigated after releasing PHSRN, Gal, Man, KKKTTK, and cRGD from separate HQ-RGD-presenting SAMs. The data are presented in Figure 6A–C. Remarkably, after ligand release and...
CONCLUSIONS

In summary, we developed a model substrate for in situ cell biological studies that dynamically modulates the ECM. A small library of biomolecules, Gal, Man, PHSRN, cRGD, and KKKTTK, was synthesized to bear an oxime group that reacts rapidly and chemoselectively with carbonyl moieties. This reaction is also bioorthogonal and can be performed in the presence of cells without inducing any side reactions with proteins and lipids. A cell adhesive HQ-containing RGD was also synthesized and immobilized to biointerface alkyl-EG₅SH SAMs to provide the ketone (in the form of electrochemically oxidized quinone) for dual-ligand display, and cell adhesion, spreading and growth, migration, and adhesion inhibition were measured. An added benefit of this platform included the redox-responsive trigger that turns the system on and off. Such a feature allows modulation of the dynamic ECM environment, where ligands, proteins, and small molecules are constantly being hidden and revealed to cells. In surveying the synergistic or antagonistic effects of the immobilized and released ligands to and from HQ-RGD, many key results were observed. When comparing the ligands, the number of attached cells increased for all ligands when immobilized to HQ-RGD. However, HQ-RGD + KKKTTK showed a dramatic increase of 50%, indicating a possible synergistic effect of simultaneous cell surface integrin and syndecan-4 interactions with the cell- and HS-binding domain mimics of FN. Furthermore, the formation of more FA in the morphological data was observed in the images and confirmed by the increased FAK levels from control substrates (HQ-RGD). The decreased migration rates and soluble and enzymatic inhibition assays also demonstrated that KKKTTK serves as a synergistic ECM ligand with RGD in promoting cell attachment, spreading, and division. When cRGD and PHSRN were investigated, results similar to those reported previously in the literature were observed, and Man and Gal had no effect on cell adhesion, spreading, and migration. Thus, not only does this dynamic dual-ligand ECM enable the immobilization and release of ligands in the presence of cells, but it also provides a platform for the combinatorial screening of ligands to further probe the synergistic or antagonistic effects of cell adhesive RGD with other molecules. This surface strategy can be applied to nanoparticles for the redox-state-dependent delivery and release of therapeutics and imaging probes in vitro. Finally, the ability to generate defined dynamic and gradient surfaces will allow for many fundamental studies of cell behavior and for their use as biotechnology tools to assay various cell functions.

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ABBREVIATIONS USED

Alkyl-EG₅SH, alkyl-terminated tetra(ethylene glycol) alkanethiol; ECM, extracellular matrix; EG₅SH, tetra(ethylene glycol) alkanethiol; FA, focal adhesion; Fbs, fibroblasts; FN, fibronectin; cRGD, cyclic Arg-Gly-Asp; Gal, galactose; HQ, hydroquinone; HQ-RGD, hydroquinone Arg-Gly-Asp; K₅, dissociation constant; KKKTTK, Lys-Lys-Lys-Thr-Thr-Lys; Man, mannose; OA, oxime; Q, quinone; PHSRN, Phe-His-Ser-Arg-Asn; SAMs, self-assembled monolayers.

REFERENCES

(1) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. Molecular Biology of the Cell; Garland Science: New York, 2002.

(2) Rhodes, J.; Simons, M. The extracellular matrix and blood vessel formation: Not just a scaffold. J. Cell Mol. Med. 2007, 11, 176–205.

(3) Danilov, Y.; Juliano, R. (Arg-Gly-Asp)n–albumin conjugates as a model substratum for integrin-mediated cell adhesion. Exp. Cell Res. 1989, 182, 186–196.

(4) Williams, C. M.; Engler, A. J.; Slone, R. D.; Galante, L. L.; Schwarzbaumer, J. E. Fibrinectin expression modulates mammary epithelial cell proliferation during acinar differentiation. Cancer Res. 2008, 68, 3185–3192.

(5) Hood, J.; Cheresh, D. A. Role of integrins in cell invasion and migration. Nat. Rev. Cancer 2002, 2, 91–100.

(6) Bogenrieder, T.; Herlyn, M. Axis of evil: Molecular mechanisms of cancer metastasis. Oncogene 2003, 22, 6524–6536.

(7) Liotta, L.; Kohn, E. The microenvironment of the tumour-host interface. Nature 2001, 411, 375–379.

(8) Hynes, R. Integrins: A family of cell surface receptors. Cell 1987, 48, 549–554.

(9) Tamkun, J.; DeSimone, D.; Fonda, D.; Patel, R.; Buck, C.; Horwitz, A.; Hynes, R. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 1986, 46, 271–282.

(10) Juliano, R.; Reddig, P.; Alahari, S.; Edin, M.; Howe, A.; Aplin, A. Integrin regulation of cell signalling and motility. Biochem. Soc. Trans. 2004, 32, 443–446.

(11) Ginsberg, M. H.; Partridge, A.; Chattil, S. J. Integrin regulation. Curr. Opin. Cell Biol. 2005, 17, 509–516.

(12) Hynes, R. Molecular biology of fibronectin. Annu. Rev. Cell Biol. 1985, 1, 67–90.

(13) Pankov, R.; Yamada, K. M. Fibronectin at a glance. J. Cell Sci. 2002, 115, 3861–3863.

(14) Pierschbacher, M. D.; Ruoslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984, 309, 30–33.

(15) Aota, S.; Nomizu, M.; Yamada, K. M. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell- adhesive function. J. Biol. Chem. 1994, 269, 47536–274761.

(16) Bowditch, R. D.; Harharan, M.; Tomim, E. F.; Smith, J. W.; Yamada, K. M.; Getzoff, E. D.; Ginsberg, M. H. Identification of a novel integrin binding site in fibronectin. Differential utilization by beta 3 integrins. J. Biol. Chem. 1994, 269, 10856–10863.

(17) Garcia, A. J.; Schwarzbauer, J. E.; Boettiger, D. Distinct activation states of alpha 5 beta 1 integrin show differential binding to...
RGD and synergy domains of fibronectin. *Biochemistry* 2002, 41, 9063–9069.

(18) Feng, Y.; Mrksich, M. The synergy peptide PHSRN and the adhesion peptide RGD mediate cell adhesion through a common mechanism. *Biochemistry* 2004, 43, 15811–15821.

(19) Leahy, D. J.; Aukhil, I.; Erickson, H. P. 2.0 Angstrom crystal structure of a four-domain segment of human fibronectin encompassing the RGD loop and synergy region. *Cell* 1996, 84, 155–164.

(20) Ingham, K. C.; Brew, S. A.; Atha, D. H. Interaction of heparin with fibronectin and isolated fibronectin domains. *Biochem. J.* 1990, 272, 605–611.

(21) Kim, J. H.; Park, S. O.; Jang, H. J.; Jang, J. H. Importance of the heparin-binding domain of fibronectin for enhancing cell adhesion activity of the recombinant fibronectin. *Biotechnol. Lett.* 2006, 28, 1409–1413.

(22) Lebaron, R. G.; Athanasiou, K. A. Extracellular matrix cell adhesion peptides: Functional applications in orthopedic materials. *Tissue Eng.* 2000, 6, 85–103.

(23) Dee, K. C.; Anderson, T. T.; Bizios, R. Design and function of novel osteoblast-adhesive peptides for chemical modification of biomaterials. *J. Biomed. Mater. Res.* 1998, 40, 371–377.

(24) McCarthy, J. B.; Skubitz, A. P.; Qi, Z.; Yi, X. Y.; Mickelson, D. J.; Klein, D. J.; Furcht, L. T. RGD-independent cell adhesion to the carboxy-terminal heparin-binding fragment of fibronectin involves heparin-dependent and -independent activities. *J. Cell Biol.* 1990, 110, 777–787.

(25) Kritz, A. B.; et al. Adenovirus 5 fibers mutated at the putative HSIPG-binding site show restricted retargeting with targeting peptides in the HI loop. *Mol. Ther.* 2007, 15, 741–749.

(26) Darr, S.; Madisch, I.; Hofmayer, S.; Rehren, F.; Heim, A. HSPG-binding site show restricted retargeting with targeting peptides in the HI loop. *J. Gen. Virol.* 2007, 88, 2529–2539.

(27) Di Paolo, N. C.; Kalyuzhnii, O.; Shayakhmetov, D. M. Fiber shaft-chimeric adenovirus vectors lacking the KKKTK motif efficiently infect liver cells in vivo. *J. Virol.* 2007, 81, 12249–12259.

(28) Buck, C. A.; Horwitz, A. F. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* 1987, 3, 179–205.

(29) Humphries, J. D.; Byron, A.; Humphries, M. J. Integrin ligands at a glance. *J. Cell Sci.* 2006, 119, 3901–3903.

(30) Eisenberg, J. L.; Piper, J. L.; Mrksich, M. Using self-assembled monolayers to model cell adhesion to the 9th and 10th type III oxidation and reduction.

(31) Nakanishi, J. Switchable substrates for analyzing and engineering the RGD loop and synergy region. *Langmuir* 2009, 25, 12512–12529.

(32) Wischerhoff, E.; Badi, N.; Lutz, J.-F.; Laschewsky, A. Smart adhesion peptides: Functional applications in orthopedic materials. *Langmuir* 2007, 23, 11826–11835.

(33) Mendes, P. M. Stimuli-responsive surfaces for bio-applications. *J. Am. Chem. Soc.* 2009, 131, 10780–10783.

(34) Luo, W.; Chan, E. W. L.; Yousaf, M. N. Synthetic chemoselective rewiring of cell surfaces: Generation of three-dimensional tissue structures. *J. Am. Chem. Soc.* 2011, 133, 8704–8713.

(35) Ostuni, E.; Chapman, R. C.; Liang, M. N.; Meluleni, G.; Pier, G.; Inger, D. E.; Whitesides, G. M. Self-assembled monolayers that resist the adsorption of proteins and the adhesion of bacterial and mammalian cells. *Langmuir* 2001, 17, 6336–6343.

(36) Xiao, Y.; Truskey, G. A. Effect of receptor-ligand affinity on the strength of endothelial cell adhesion. *J. Biophys.* 1996, 71, 2869–2884.

(37) Livant, D. L.; Brabec, R. K.; Kurachi, K.; Allen, D. L.; Wu, Y.; Haaseth, R.; Andrews, P.; Ethier, S. P.; Markwart, S. The PHSRN sequence induces extracellular Matrix Invasion and Accelerates Wound Healing in Obese Diabetic Mice. *J. Clin. Invest.* 2000, 105, 1537–1545.

(38) Kimura, K.; Hattori, A.; Uusi, Y.; Kitazawa, K.; Naganuma, M.; Kawamoto, K.; Teranishi, K.; Nomizu, M.; Nishida, T. Stimulation of cell surface receptors.

(39) Palerosdemanze, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. Formation of self-assembled monolayers by chemisorption of derivatives of oligo(ethylene glycol) of structure HS-(CH2)-11(OCH2CH2)Meta-OH on gold.

(40) Li, J.; Thiara, P. S.; Mrksich, M. Rapid evaluation and screening of interfacial reactions on self-assembled monolayers. *Langmuir* 2007, 23, 11826–11835.

(41) Palerosdemanze, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. Formation of self-assembled monolayers by chemisorption of derivatives of oligo(ethylene glycol) of structure HS-(CH2)-11(OCH2CH2)Meta-OH on gold. *J. Am. Chem. Soc.* 1991, 113, 12–20.

(42) Chen, E. W. L.; Yousaf, M. N. Immobilization of ligands with precise control of density to electroactive surfaces. *J. Am. Chem. Soc.* 2006, 128, 15542–15546.

(43) Nakanishi, J. Switchable substrates for analyzing and engineering the RGD loop and synergy region. *Langmuir* 2007, 25, 12512–12529.

(44) Lu, J.; Shi, M.; Shoichet, M. S. Click chemistry functionalized polymeric nanoparticles target corneal epithelial cells through RGD-cell surface receptors. *Bioconjugate Chem.* 2009, 20, 87–94.

(45) Durr, D.; Pulsipher, A.; Luo, W.; Mak, H.; Yousaf, M. N. Engineering cell surfaces via liposome fusion. *Bioconjugate Chem.* 2011, 22, 2423–2433.

(46) Durr, D.; Pulsipher, A.; Luo, W.; Yousaf, M. N. Synthetic chemoselective rewiring of cell surfaces: Generation of three-dimensional tissue structures.