

# Dynamic heterodimer-functionalized surfaces for endothelial cell adhesion

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## Abstract

The functionalization of hydrogels for receptor-mediated cell adhesion is one approach for targeted cell and tissue engineering applications. In this study, polyacrylamide gel surfaces were functionalized with specific cell adhesion ligands via the self-assembly of a peptide-based heterodimer. The system was comprised of a cysteine-terminated monomer, A (MW ~ 5400), grafted to the polyacrylamide gels and a complementary ligand presenting monomer, B<sub>X</sub> (MW ~ 5800) that was designed to heterodimerize with A. Two ligand presenting monomers were synthesized: one presenting the RGDS ligand, B<sub>D</sub>, for receptor-mediated cell adhesion, and the other, a control monomer presenting the nonadhesive RGEs ligand, B<sub>E</sub>. Assembly of the peptide pair A–B<sub>X</sub> by association of the monomers into a coiled coil was verified by circular dichroism in solution. Binding studies were conducted to determine the dissociation constant of the pair A–B<sub>X</sub>, which was found to be  $K_D \sim 10^{-8}$  M. Polyacrylamide gels functionalized with A–B<sub>X</sub> heterodimers were evaluated for cell adhesion using bovine aortic endothelial cells (BAECs). Endothelial cells cultured on the A–B<sub>D</sub> functionalized surfaces demonstrated typical cell morphologies and expected spreading behavior as a function of the density of RGDS ligand, calculated as the amount of B<sub>D</sub> associated with grafted A on the surface of the gels. In contrast, A–B<sub>E</sub> linked surfaces supported no cell adhesion. The adhesion of the substrate was dynamically altered through the reassembly of A–B<sub>X</sub> dimers as B<sub>D</sub> molecules in the solution replaced B<sub>E</sub> molecules at the substrate. The molecular constructs described here demonstrate the potential to design a broad family of switchable peptides that impart the dynamic control of biofunctionality at an interface, which would be useful for precise manipulation of cell physiology.

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## 1. Introduction

The development of surfaces and polymers functionalized with adhesion ligands has been critical for the elicitation of cell responses in materials for tissue engineering. For example, since the discovery of the functionally significant RGD binding domain of fibronectin for substrate-dependent cell adhesion [1], studies of RGD peptide grafted surfaces have shown that

peptide grafting density, ligand localization and the surrounding chemical environment can each affect the cellular processes of many types of cells on functionalized substrates [2–9]. The design of peptide-functionalized surfaces has been extended to micropatterned substrates that localize ligand presentation and control cell shape and function, elucidating the fact that spatial parameters imposed on a cell result in differences in the activation of a cell's physiology through the degree of mechanical constraint on the cell [10–15]. Therefore, the development of novel materials with increasing complexity for cell adhesion and tissue engineering will likely

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provide new insights into cell physiology as well as provide novel methods of controlling tissue morphogenesis.

In a natural extension of static surface patterning, several laboratories are pursuing methods for creating smart biomaterial interfaces in which the biochemical activity switches between states [16–21]. These materials have obvious applications for biosensing and tissue engineering (coordinating in-growth of cells into tissues). Mrksich and coworkers [17–20] have utilized patterned self-assembled monolayers with electrochemically conjugated binding ligand to produce substrates where the adhesive properties of the surface can be modified during cell culture. In these systems, cells are confined to an adhesive region defined by a pattern. The conjugation of a ligand in the region effectively turns the adhesion “on” across an interface. The success of this methodology suggests other designs, such as peptide-based assembly, in which the assembly of molecules can be controlled at an interface. Such a system might be used to promote endothelial cell adhesion and assembly at a surface as a means to control vascularization and angiogenesis in assembling tissues.

One promising alternative route for the development of dynamic ligand systems involves coupling that which is known about the requirements for the control of cell adhesion with the ability to design specific conformations into the molecule governing cell–substrate adhesion behavior. Advances in molecular biology techniques and genetic engineering have provided the means to synthesize precisely determined polypeptide sequences with potential for materials applications. In recent years, a novel class of self-assembling materials, composed of self-assembling oligopeptides, has emerged [22–25]. One of the most common dynamic folding motifs found in natural proteins is the coiled coil, and reversible hydrogels that dynamically respond to changes in near-neutral pH and mild temperatures have been made utilizing the dynamic folding of the coiled coil of leucine zipper derived domains [26,27]. However, few attempts have been made to utilize the assembly of coiled coils as a means to dynamically modulate the functionality of a biomaterial surface with adhesive ligands. Because peptides can ultimately be engineered to assemble in response to environmental cues, such as ion composition and temperature, this method for assembling peptides at interfaces might be useful for designing a spectrum of environmental controls for the assembly of different functionalities at interfaces. In this work, we grafted a designed heterodimerizing leucine zipper, based on human B-ZIP proteins [28], to a model polyacrylamide hydrogel system for the study of cell adhesion behavior. The assembling coil–coil domain of our system was designed by Vinson and coworkers for the nonimmunogenic, tunable delivery of radionucleotides to antigen-specific antibodies prelocalized to

cancer cells. The design of the molecules by Vinson et al. was optimized for enhanced stability of the heterodimer over the homodimer. Our adaptation of the design involves synthesis and functionalization of the two-component system with the cell adhesion binding domain, RGDS, on one peptide monomer, and a residue for specific surface grafting on the other peptide monomer (cysteine). The resulting functionalized hydrogel surfaces support endothelial cell adhesion and spreading, demonstrating that cellular adhesivity can be induced via the dimerization of a functionalized molecule with a complementary grafted molecule on a surface. Furthermore, the dimerization of the leucine zipper pair is dependent on concentration at physiological ionic strength and pH. Thus, the following report represents a basic demonstration of the ability to make self-assembling functionalized surfaces through peptide coiled-coil assembly, a necessary first step for the dynamic switching of interfacial interactions using peptide switching.

## 2. Experimental

### 2.1. Synthesis and purification of peptides

The peptides used for surface functionalization were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) amino acid chemistry on a PE Applied Biosystems model 433A solid phase peptide synthesizer (Applied Biosystems, Foster City, CA). Peptides were synthesized on a 0.25 mmol scale on standard amide resin. Cleavage from the resin was accomplished by incubating for 3 h with trifluoroacetic acid (TFA)/ethanedithiol (EDT)/water/triisopropylsilane (TIS) (95:2:2:1 by volume), and the cleaved peptide was precipitated from the TFA mixture with cold ether. All peptides were purified using reverse-phase HPLC on a preparative C4 column (Grace Vydac, Hesperia, CA) with a linear acetonitrile–water gradient (0.25% per minute increase of acetonitrile) in the presence of 0.1% TFA. Sample purity was measured with analytical reverse-phase HPLC, and the molecular weight of the peptides was determined by MALDI-TOF mass spectrometry using a Perspective Biosystems Voyager DE-RP BioSpectrometry Workstation (Applied Biosystems, Foster City, CA).

The sequences of the heterodimers produced are shown in Fig. 1. The modifications to the original dimer pairs designed by Vinson et al. [28] include the addition of a cysteine, C, at the amine terminus of peptide A, for grafting to the polyacrylamide gels, and the addition of either an adhesive binding moiety, RGDS, or a nonbinding moiety, RGEs, to the carboxy terminus of peptide B (B<sub>D</sub> and B<sub>E</sub>, respectively).

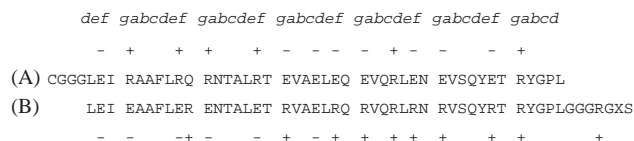


Fig. 1. Sequence information, residue positions in the helical structure, and charge distributions for the molecules synthesized. (A) The cysteine-terminated monomer designed for surface grafting. Expected MW = 5395 kDa; actual MW = 5462 kDa (by mass spectrometry, Experimental methods Section 2.1). (B) The complementary monomer designed for self-assembly and subsequent cellular adhesion ligand presentation on the surface ( $B_D$  when X = aspartic acid, D,  $B_E$  when X = glutamic acid, E). Expected molecular weights  $B_D$  = 5789 kDa,  $B_E$  = 5803 kDa; actual molecular weights  $B_D$  = 5789 kDa,  $B_E$  = 5803 kDa (by mass spectrometry).

## 2.2. Characterization of dimerization behavior

To verify the formation of heterodimer between A and B peptides in solution, circular dichroism (CD) spectra were acquired on an AVIV 62DS CD spectrometer (Proterion Corporation, Piscataway, NJ) using 1 mm pathlength quartz cuvettes. Each wavelength scan was taken from 200 to 260 nm as an average of three scans with an averaging time of 4 s at 37 °C (for physiological relevance). Peptide melting was observed by monitoring ellipticity at 222 nm from 0 to 98 °C with an averaging time of 20 s and a temperature equilibration time of 4 min. Scans were collected for peptide A, peptide  $B_D$ , and a 50:50 mixture of A and  $B_D$  at a concentration of 100  $\mu$ M in PBS, pH 7.0.

Formation of the heterodimer under an imposed surface constraint was observed in real time using surface plasmon resonance measurements on a Biacore X biosensor system (Biacore, Piscataway, NJ). To measure the binding of peptide  $B_{X(X=D \text{ or } E)}$  to surface-grafted A, peptide A was coupled to the sensor chip surface (CM5 chip) and the reaction of  $B_X$  from solution with the grafted A was monitored. To couple peptide A to the sensor chip, a continuous flow of HBS-EP buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) over the sensor chip was maintained at 5  $\mu$ L/min, while 35  $\mu$ L of a solution containing 0.2 M *N*-ethyl-*N'*-(3-diethylaminopropyl) carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) was injected to activate the carboxylated dextran surface of the CM5 chip. This was followed by a 35  $\mu$ L injection of 80 mM 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) in 0.1 M borate buffer, pH 8.5. All reagents for chip activation were obtained from Biacore (Biacore, Piscataway, NJ). The peptide for conjugation was introduced in a 25  $\mu$ L injection of 10  $\mu$ g/mL peptide A in 50 mM acetate, pH 4.0. The saturated response after conjugation of the peptide was  $\sim$ 1350 RU. Binding of peptide  $B_X$  to the surface was induced by injecting various concentrations of the peptide in HBS-EP buffer over the peptide A surface

at 5  $\mu$ L/min for 5 min. Dissociation was observed as pure HBS-EP buffer flowed across the surface causing detachment of peptide  $B_X$ . Surface regeneration (complete removal of bound peptide  $B_X$ ) between injections was accomplished by injecting 5  $\mu$ L of 10 mM NaOH.

The binding constants,  $k_a$  and  $k_d$ , were determined from the refractive index response signal as a function of time for association and dissociation of peptide  $B_X$  to immobilized peptide A using the kinetic expression given in Eq. (1), where B is the concentration of peptide  $B_X$

$$R = \left\{ \frac{k_a[B-1]R_{\max}}{k_a[B-1] + k_d} \right\} + \left\{ R_0 - \frac{k_a[B-1]R_{\max}}{k_a[B-1] + k_d} \right\} \times e^{-(k_a[B-1] + k_d)(t-t_0)} + \text{RI}, \quad (1)$$

where  $R_0$  is the refractive index before injection of peptide  $B_X$ ,  $R$  is the refractive index after injection of peptide  $B_X$  as a function of time, RI is the bulk refractive index contribution, and  $R_{\max}$  is the maximum value of  $R$  observed when all immobilized peptide A is bound with peptide B [29]. The equation was applied to all experimental results using the Biaevaluation data fitting software (Biacore, Piscataway, NJ). The fits were used to obtain estimates for  $k_a$ ,  $k_d$ , and  $R_{\max}$  for the heterodimerization of surface constrained A and mobile  $B_X$ .

## 2.3. Preparation of substrates

The protocol for producing polyacrylamide hydrogels for modification and subsequent cell culture in our laboratory has been thoroughly described by Reinhart-King et al. [9]. Briefly, the gels are prepared using a formulated monomer solution of acrylamide (40% w/v solution), *N,N*-methylene-*bis*-acrylamide (BIS, 2% w/v solution), *N*-tetramethylethylenediamine (TEMED), and ammonium persulfate (all from Bio-Rad Laboratories, Hercules, CA), in a ratio to produce 5% acrylamide/0.1% BIS gels. The formulation also contains 20  $\mu$ M/mL of a bifunctional linker, the *N*-succinimidyl ester of acrylamidohexanoic acid (N6). The acrylic N6 monomer copolymerizes with the acrylamide and provides an amine reactive *N*-succinimidyl ester throughout the gel for further functionalization. To produce each gel, a 25  $\mu$ L quantity of the formulated solution is dispensed onto glass coverslips and flattened using a no. 1, 22 mm diameter circular coverslip (Fisher Scientific, Pittsburgh, PA). After polymerization for 1 h, a gel of approximately 22 mm in diameter and 70  $\mu$ m thick, with a modulus of approximately 2500 Pa, is produced.

For the experiments described in this paper, the circular glass coverslip was removed, and the polymerized gels were then incubated for 1 h with 200  $\mu$ L of a 20 mM solution of 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA, from Biacore, Piscataway, NJ)

in 50 mM HEPES buffer. The amine of the PDEA reacts with the *N*-succinimidyl ester of the N6 in the gels to produce sites with sulfhydryl reactivity. The thiol reactive sites allow specific grafting of the cysteine-terminated peptide A with the release of the spectroscopically quantifiable 2-pyridinethione chromophore ( $\epsilon_{343\text{ nm}} \sim 8000\text{ cm}^{-1}\text{ M}^{-1}$ ). After reaction with the PDEA, the gels were then incubated with 200  $\mu\text{L}$  of a 1 mM solution of peptide A in  $1\times$  phosphate buffered saline (PBS) for at least 2 h—covered to reduce evaporation. After the reaction was complete, the solution was pipetted from the gel into a UV compatible cuvette, along with 1 mL of  $1\times$  PBS rinsed over the surface of the gel. The absorbance of the resultant 1.2 mL of solution was measured at 343 nm, and the absorption correlated to the amount of peptide grafted to the entire gel. To quantify the gel surface composition, the total amount of peptide grafted was adjusted to reflect the amount reacted in the topmost 10 nm of the polyacrylamide gel surface using a percentage of total volume [2,30].

After capping additional amine reactive sites in the gels with ethanolamine (500  $\mu\text{L}$  of 100  $\mu\text{L}$  EtOH in 10 mL HEPES, pH 8.0, on each gel) and additional thiol reactive sites with cysteine (500  $\mu\text{L}$  of an excess of cysteine in 10 mL of  $1\times$  PBS), the gels were prepared for incubation with peptide  $B_X$  in solution. Dimerization solutions were prepared using ratios of  $B_D$  to  $B_E$  in  $1\times$  PBS in order to access a full range of potential adhesion promoting surface compositions. The final peptide concentration for each solution was confirmed spectroscopically (based on tyrosine absorbance) and adjusted for the purity of the peptide samples (as determined by analytical HPLC). The final adjusted concentration for gel incubation in each case was 50  $\mu\text{M}$   $B_X$ . The amount of  $B_D$  adsorbed to the surface via dimerization was calculated by assuming a 1:1 binding of  $B_X$  with the peptide A grafted in the topmost 10 nm of the gel surface (see above), and adjusted for the ratio of  $B_D$  to  $B_E$  in the  $B_X$  solution. After dimerization, the gels were minimally rinsed ( $\sim 30$  s) to remove excess  $B_X$  but prevent dissociation of dimerized  $B_X$ .

#### 2.4. Cell culture

The stock cultures of bovine aortic endothelial cells (BAECs) for adhesion studies were maintained at 37 °C and 5%  $\text{CO}_2$  in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum, 0.5% penicillin–streptomycin, and 1% 200 mM L-glutamine. For experimental culture on the modified polyacrylamide gels, the BAECs were split using trypsin–EDTA (0.05% trypsin and 0.53 mM EDTA-4Na,) and resuspended in endothelial serum-free media. The serum-free media was supplemented with penicillin–streptomycin, basic fibroblast growth factor

(bFGF, 20 ng/mL), and epithelial growth factor (EGF, 10 ng/mL) (serum-free media and all supplements mentioned above, Invitrogen, Carlsbad, CA). The media was also supplemented with dimerization solution  $B_X$ , in the same ratio of  $B_D$  to  $B_E$  as the surface, for a total media concentration of  $B_X = 10^{-8}$  M. This media supplementation was necessary to offset dissociation of the dimer into the media when  $[B_X] = 0$ . The chambers used for cell culture on polyacrylamide gels are designed to accommodate 3 mL of media, and BAECs were plated for adhesion studies at a density of  $10^4$  cells/3 mL serum-free media (for information of chambers and chamber assembly, see Reinhart-King et al. [9]). Unless otherwise indicated, cells were maintained at 37 °C and 5%  $\text{CO}_2$  for at least 12 h before observation.

#### 2.5. Microscopy and measurement of cellular response

Images of the BAECs on the modified polyacrylamide gel surfaces were gathered using a Nikon Inverted Eclipse TE300 microscope with a Nikon 20 $\times$ , numerical aperture 0.75, phase objective (Nikon, Melville, NY). Photographs were taken using a Photometrics Cool Snap HQ camera (Roper Scientific, Trenton, NJ). For counting the number of adhesive and spread cells, a minimum of 100 cells was observed (10–20 images). Area measurements were manually taken on several typical cells of each population using Scion Image (National Institutes of Health, Bethesda, MD).

### 3. Results

#### 3.1. Dimerization of peptides produces surface functionality

Three peptides were synthesized for the functionalization of polyacrylamide gel surfaces: a cysteine-terminated monomer (A), an RGDS-functionalized monomer ( $B_D$ ), and an RGEs-functionalized monomer ( $B_E$ ) (Fig. 1). A and  $B_X$  (either  $B_D$  or  $B_E$ ) were designed to self-assemble. The RGEs, arginine–glycine–glutamic acid–serine, ligand was chosen as a control, since the single substitution of glutamic acid for the aspartic acid in RGDS disallows specific receptor-mediated cell adhesion [1]. After synthesis and purification, the heterodimerization of the molecules A and  $B_X$  was tested qualitatively and quantitatively, using circular dichroism and surface plasmon resonance measurements, respectively. Circular dichroism spectra indicated that each peptide monomer alone displays minimal structure formation (Fig. 2). However, the 50:50 mixture of peptide A and peptide  $B_D$  displayed a marked increase in helical structure and a corresponding increase in overall ellipticity. Analytical HPLC was used to determine purity of the molecules, which was on the

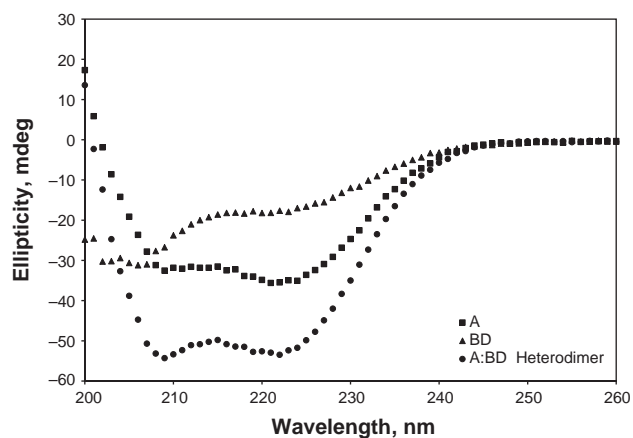


Fig. 2. CD wavelength scans recorded over the range of 200–260 nm at 37 °C. (circles) Heterodimer, A: $B_D$ ; (squares) monomer/homodimer, A; (triangles) ligand presenting monomer/homodimer,  $B_D$ .

order of 70–80% for each sample tested (data not shown). This lack of purity was not unexpected because of the length of the molecule and the method of synthesis. Iterative attempts at purifying the material further by HPLC resulted in substantial loss of peptide.

Kinetic rate constants for our modified dimer pair were determined by a nonlinear fitting of the time-dependent refractive index response measured by SPR (Fig. 3) to a standard rate equation for 1:1 binding. Fig. 4 shows the fits for each curve, estimated individually. Table 1 shows the values obtained through averaging the individual fits for each data set, as well as the values obtained from a global fit of all data sets for a given molecular pair. The range of  $k_a$  from global and local fits, respectively, is  $3.2\text{--}3.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ; the range of  $k_d$  was  $1.09\text{--}1.14 \times 10^{-3} \text{ s}^{-1}$ . Although the precise values for  $k_a$  and  $k_d$  do not match exactly for the individual and global fits, the values determined by each method are not statistically different. A measured  $K_D$  of  $10^{-8} \text{ M}$  was sufficient for the use of the peptides for surface grafting, as shown below.

Following characterization of the heterodimerizing molecular pair, surfaces for cell study were made by grafting monomer A to a polyacrylamide gel via two heterobifunctional linkers—the *N*-succinimidyl ester of acrylamidohexanoic acid (*N6*) to add a functional amine to the gel, and PDEA hydrochloride to link an ideal displacement group for the grafting of the cysteine-terminated peptide A. Fig. 5a shows that the polyacrylamide gel with monomer A grafted to the surface does not support nonspecific cell adhesion. However, addition of monomer  $B_X$  (at a ratio of  $B_D:B_E$ , 100:0) both to the surface (gels incubated before culture with  $5 \times 10^{-5} \text{ M}$  of  $B_X$ ) and in the culture medium (for a total  $B_X$  media concentration of  $10^{-8} \text{ M}$ ) results in adhesion of the bovine aortic endothelial cells to the surface of the gel (Fig. 5b). The observation of cell adhesion with characteristic morphology and spreading indicated that

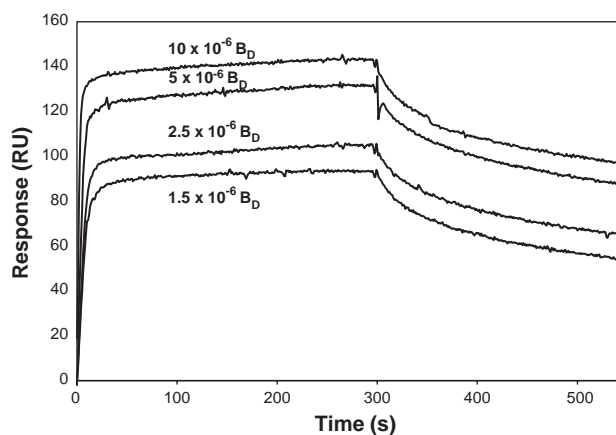


Fig. 3. Surface plasmon resonance (SPR) data obtained by flowing analyte,  $B_D$  (in HBS-EP buffer, pH 7.0, 25 °C), at various concentrations, over a gold, dextran-coated chip, functionalized with A. The graph shows the complete signal response, including association of the analyte during the programmed 5 min injection, and dissociation of the analyte with pure buffer flow (no analyte) for 5 min after injection.

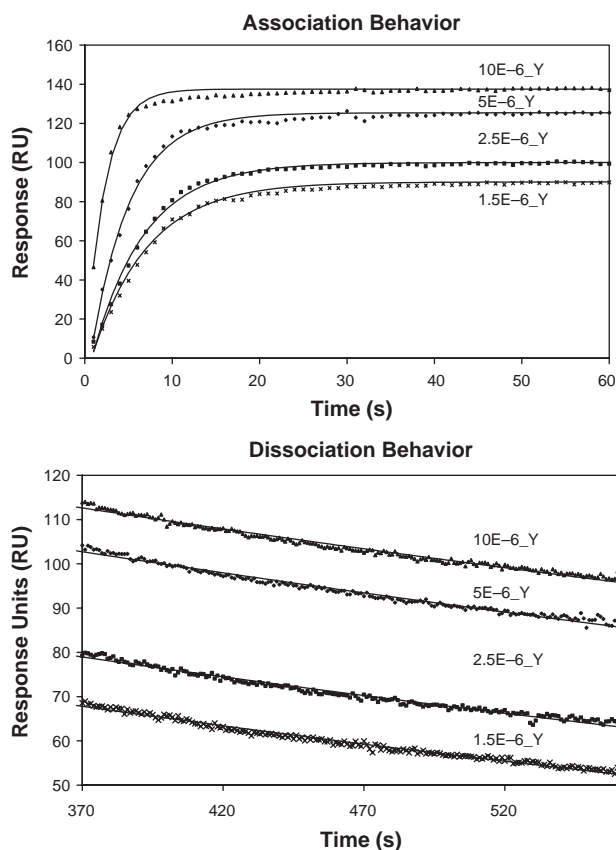


Fig. 4. Individually minimized fits to the association and dissociation portions of the refractive index response from SPR, as a function of time.

the surface of the nonadherent gel was rendered adherent with the ligand presenting molecule,  $B_D$ . To prove the specificity of the adhesive properties, cells were also cultured with monomer  $B_X$  ( $B_D:B_E$ , 0:100)

Table 1  
Kinetic parameters

	$k_a$ ( $s^{-1}$ )	$k_d$ ( $M^{-1}s^{-1}$ )	$K_A$ ( $M^{-1}$ )	$K_D$ (M)
A: $B_D$				
1:1 binding, average of individual fits	$3.21 \pm 1.03 \times 10^4$	$1.09 \pm 0.18 \times 10^{-3}$	$2.94 \pm 0.44 \times 10^7$	$3.40 \pm 0.55 \times 10^{-8}$
A: $B_D$				
1:1 binding, global fit	$3.81 \pm 1.91 \times 10^4$	$1.14 \pm 0.06 \times 10^{-3}$	$2.92 \pm 1.85 \times 10^7$	$3.89 \pm 1.79 \times 10^{-8}$
A: $B_E$				
1:1 binding, average of individual fits	$2.64 \pm 2.18 \times 10^4$	$2.61 \pm 0.61 \times 10^{-3}$	$1.01 \pm 4.20 \times 10^7$	$9.89 \pm 1.65 \times 10^{-8}$
A: $B_E$				
1:1 binding, global fit	$9.05 \pm 0.31 \times 10^3$	$2.18 \pm 0.09 \times 10^{-3}$	$4.15 \pm 0.03 \times 10^6$	$2.40 \pm 0.02 \times 10^{-7}$

Kinetic constants obtained by nonlinear fitting of the time-dependent refractive index response to a standard rate equation for 1:1 binding. A: $B_D$  heterodimer indicates the association of a grafted A monomer and a mobile  $B_D$  monomer (analyte). A: $B_E$  heterodimer indicates the association behavior of a grafted A monomer and a mobile  $B_E$  monomer. The same Dextran chip was used for both sets of experiments. The grafting density of the A monomer on the surface of the dextran layer was calculated to be approximately  $3 \times 10^{12}$  molecules/ $\mu m^2$ .

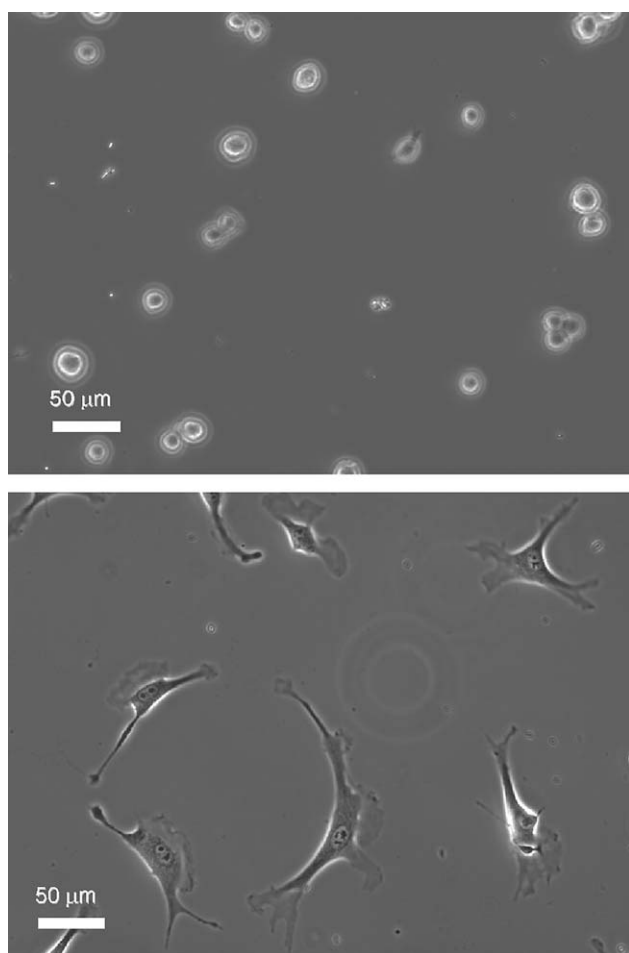


Fig. 5. Top: Cells incubated for 6 h on a polyacrylamide gel functionalized with A. The absence of adhesion and spreading confirms that the cysteine containing A monomer, although charged, does not support nonspecific adhesion when grafted to the surface of the gel. Bottom: An A-functionalized gel, incubated with excess ligand presenting  $B_D$  in PBS and rinsed ( $\sim 60$  s) to remove excess, undimerized  $B_D$ . The cells were plated with a supplement of  $B_D$  to the culture medium, resulting in  $[B_D] = 1 \times 10^{-8}$  M. The image was taken 12 h after cell plating ( $20 \times$  magnification).

added to the surface and to the medium. The system presenting only  $B_E$ , a nonadhesive ligand, did not result in cell adhesion (Fig. 6a), proving that the adhesion observed with monomer  $B_D$  was specific to integrin mediated, receptor-based adhesion to the substrate.

BAECs were also cultured on A- $B_D$  gels with media concentrations of  $B_D$  less than  $10^{-8}$  M. Over a 4 h time period, the amount of cell adhesion and spreading decreased, presumably as dissociation of the A- $B_D$  dimer pair occurred and the effective concentration of binding ligand  $B_D$  on the surface decreased. This is consistent with the value of  $k_d$  for dissociation of ligand from the receptor ( $10^{-3}$  s), where the B molecule could be expected to dissociate in about 15 min. Supplementation of the media to a concentration of  $B_D = 10^{-8}$  M, produced stable cell adhesion and spreading (data not shown). Therefore, the SPR result of a dimer  $K_D = 10^{-8}$  M was indirectly confirmed by our dynamic adhesion results.

The dynamic nature of the peptide dimerization was demonstrated by culture of cells on gels with  $B_X = B_D$ :  $B_E$ , 0:100 (nonadhesive ligand) that were supplemented in the culture medium with  $B_X = B_D$ :  $B_E$ , 100:0 (adhesive ligand) to a media concentration of  $10^{-8}$  M after 2 h of culture. A clear progressive increase in cell adhesion and spreading 4 h after the addition of  $B_D$  ligand was observed (Fig. 6b). The observed change in adhesivity indicated that the dynamic nature of the surface dimerization equilibrium ( $K_D \sim 10^{-8}$  M) allowed turn-over of the originally presented nonadhesive functionality ( $B_E$ ) on the surface to incorporation of adhesive functionality ( $B_D$ ) over a period of tens of minutes.

### 3.2. Quantification of adhesion behavior and cellular response

The quantitative relationship between adhesive ligand presentation on the surface of the dimer-functionalized

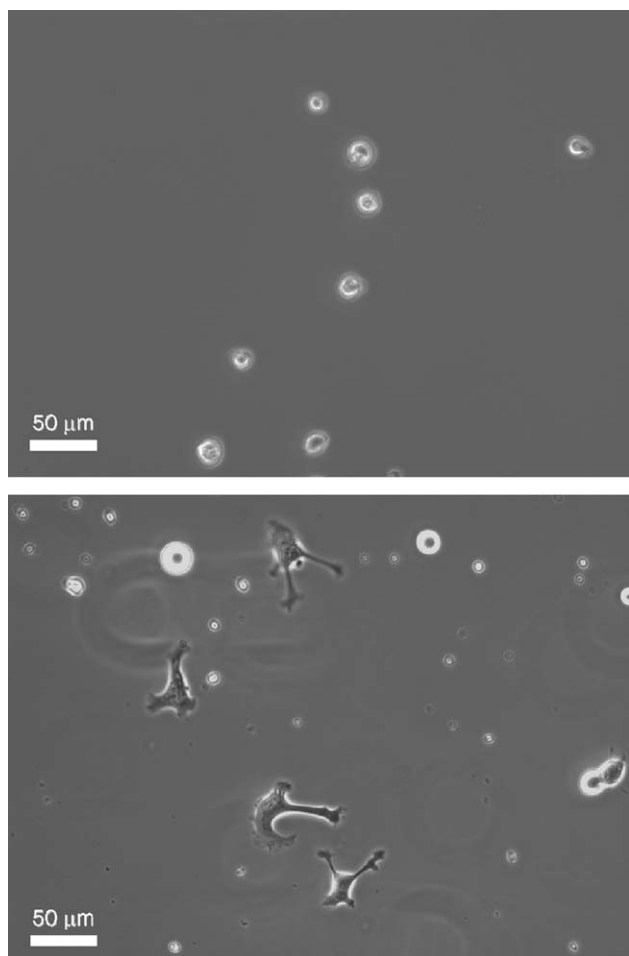


Fig. 6. Top: An A-functionalized gel, incubated with excess RGES ligand ( $B_E$ ) in PBS and rinsed ( $\sim 60$  s) to remove excess, undimerized  $B_E$ . The image exhibits a lack of adhesion and spreading 2 h after cell plating. Bottom: A supplement of RGDS ligand ( $B_D$ ) is added to the culture medium resulting in  $[B_D] = 1 \times 10^{-8}$  M. Adhesion, formation of lamellapodia and the initial stages of spreading are observed 4 h after addition of  $B_D$ , indicating displacement of  $B_E$  and dimerization of adhesive ligand with the surface.

gels with cellular adhesion and spreading is illustrated in Fig. 7. By using different ratios of  $B_D$  to  $B_E$ , for the same density of A ligand, the amount of  $B_D$  on the surface was varied, and thus the cell physiological response. The same mixture of  $B_D$  to  $B_E$  that was used on the surface was also used to supplement the media during these experiments, to ensure that desorbing  $B_D$  was replaced on the substrate. The total grafting density of  $B_X$ , and the subsequent amount of available  $B_D$ , was indirectly calculated from the measured grafting density of A (see methods Section 2.3) and the assumption of 1:1 dimerization of A: $B_X$ . A very clear increase in both the percentage of cells exhibiting adhesion (defined as the formation of clearly attached extensions of the cell membrane) and spreading (defined as the presence of multiple pseudopodia and a flattened cell center)

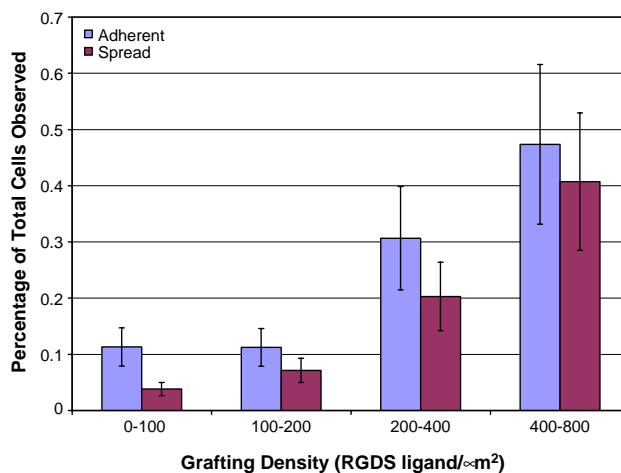


Fig. 7. Graph illustrating cellular response to increasing incorporation of adhesive ligand presenting  $B_D$ . As the grafting density of RGDS binding domain increases, the percentage of cells exhibiting adhesion and spreading morphologies increases. The increase in adhesion and spreading appears to accelerate after a grafting density of 200 molecules/ $\mu\text{m}^2$  is achieved.

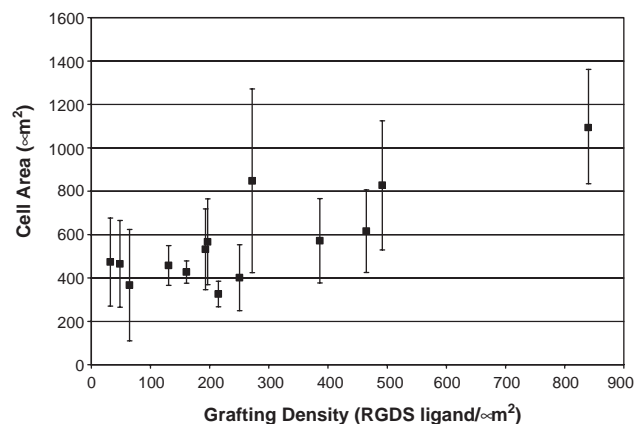


Fig. 8. Change in cell area as a function of grafting density of RGDS ligand ( $B_D$  associated with A on the surface).

increased as the availability of adhesive ligand  $B_D$  on the surface of the gel increased.

Correspondingly, cell area, as seen in Fig. 8, increases with the density of  $B_D$ . At  $B_D$  densities less than 200 RGDS molecules per square  $\mu\text{m}$ , the cell area does not change significantly. At  $B_D$  densities greater than 200 RGDS molecules/ $\mu\text{m}^2$ , the cell area begins to increase. An alternative way of considering the role of RGD density on cell spreading is to plot the adhesion as a function of interligand spacing, as first suggested by Massia and Hubbell [3]. Plotting the cell area as a function of the spacing between RGDS molecules, Fig. 9, illustrates a significant increase in cell area at a molecular spacing of 76 nm, equivalent to a grafting density of 200 molecules/ $\mu\text{m}^2$ .

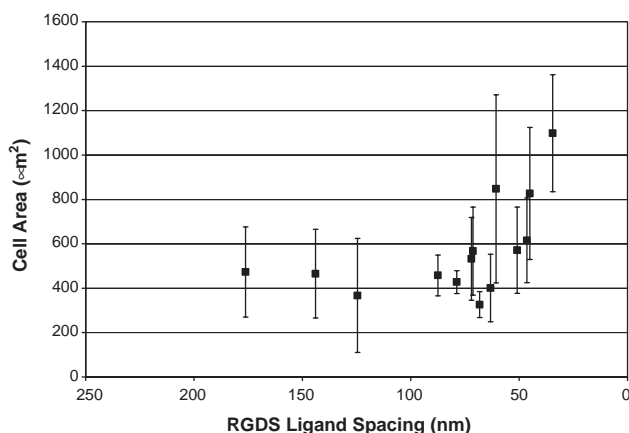


Fig. 9. Change in cell area as a function of molecular spacing, or the distance between RGDS ligands (an inverse measure of grafting density).

#### 4. Discussion

Grafting one monomer of a designed heterodimerizing peptide pair, A, to a polyacrylamide gel allows the introduction of cell binding ligand functionality (RGDS) to the surface via dimerization with complementary monomer, B<sub>D</sub>. The cell adhesion studies indicate that the surface of the modified gels, which are inherently nonadhesive to cells, are rendered adhesive after apparent A:B<sub>D</sub> dimerization. The use of a control peptide presenting the RGEs ligand, B<sub>E</sub>, which exhibits no ability to confer cell adhesivity, confirms that the adhesiveness of the B<sub>D</sub> functionalized gels is specific to receptor-mediated cell adhesion via the RGDS moiety.

The peptides used in this study were modified from the design of Vinson et al., who originally reported stabilities on the order of  $K_D \sim 10^{-11}$  M for the unmodified molecular pair [28]. The SPR results shown here give higher dissociation constants (lower affinities) for the modified pair  $K_{D(B_X)} \sim 10^{-8}$  M. The discrepancy in the stabilities of the modified peptides could be the result of end group modifications or an effect of sample impurity (70–80% purity by analytical HPLC). The response units obtained for binding of B<sub>X</sub> to A indicate that only a small fraction of the A on the surface is active. The purity of the samples may also account for the relatively low efficiency of binding by the A molecule as measured by SPR. For the SPR experiments, sample purity was not factored into the data analysis. However, for cell adhesion studies, the calculation of grafting density of B<sub>D</sub> to the polyacrylamide gels, via the measured presence of A, included adjustment of the activity of A based on the known purity for each peptide sample.

Another potential source of error in the refractive index signals of SPR for the different concentrations of peptide B<sub>X</sub> is the relatively small molecular weight of the

molecules and the sensitivity of the detector to immediate association behavior. However, surface adhesion studies on stably functionalized gels produced with media concentrations of peptide totaling  $10^{-8}$  M, indicate that the dissociation rates measured by SPR were the effective rates of the dimerization pair as they were used in surface functionalization. It is worth noting that the stability of the dimerization pair gave a  $K_D < 10^{-6}$  M—the dissociation constant for binding of ligand directly to an integrin receptor on the cell [31]. A dissociation constant greater than or equal to that of the cell receptor would have inhibited binding to the surface of the gels, as the cell receptors would have been occupied by B<sub>D</sub> bound directly from the media. To be effective, the  $K_D$  for surface association must be less than the  $K_D$  for ligand–receptor binding at the cell surface, such that the receptors are ligated only in the cell–substrate interface at a relatively high local concentration.

In a seminal paper by Massia and Hubbell [3], an RGDS grafting density of only 6 molecules/μm<sup>2</sup> was shown to be sufficient for fibroblast cell spreading on RGDS functionalized glass, dramatically lower than the grafting densities in our study. However, Massia and Hubbell reference the results of Brandley et al. [2] for cell spreading on RGDS grafted polyacrylamide, who calculated a grafting density of 200 RGDS molecules/μm<sup>2</sup> required for spreading. This number agrees with what we have found for polyacrylamide, and is likely a result of the assumptions made when calculating surface concentrations of ligand in the gels. The inherent roughness of the gels, compared to the smoothness of glass, would effectively reduce the surface concentration of ligand. However, our results for the density required for adhesion are consistent with that of Brandley and Schnaar, and suggests a ligand density above 200 molecules/μm<sup>2</sup> is needed for cell spreading.

#### 5. Conclusions

The results of this study show that heterodimerization of a coiled-coil zipper molecule is a viable means of functionalizing surfaces capable of ligand presentation. In this study, the dimerization molecules were “always on”—that is, under the solution conditions, the coil–coil domains were designed to self-associate, and no ion-inducible switch was built into the coil domains. The dynamic changes in adhesion seen in this paper are due to the repeated dissociation and reassociation of molecules between the bulk and the surface, which indicates a temporal control of surface activity. The incorporation of switchable domains, however, is easily envisioned. For example, the formation of the coiled coils could be designed as pH sensitive within a very narrow pH range. DeGrado and coworkers have

designed a novel four helix bundle that dimerizes via the binding of either metal ions or protons [32]. The dissociation of the helices in their system is very sensitive to pH in the range of 7–8. The sensitivity to pH for this particular four helix-bundle system is dependent on the complete absence of potential metal ions for binding, similar systems designed from considerations of structure and function could be produced. Other mechanisms for assembly and “switching” peptides are under development, and the Degrad laboratory has recently published a design for peptides that undergo a transition from unstructured monomer to a four-helix bundle upon phosphorylation by the enzyme cyclic AMP-dependent protein kinase [33].

Clearly, the paradigm described in this paper, and also suggested by the work of Mrksich [17–20], and Tirrell [26,27], represents an intriguing paradigm for the construction of smart interfaces, where the activity can be changed as a function of ion composition or concentration, temperature, or enzymatic activity. The availability of an array of different interfacial assembly molecules, with different control switches, would be a means of systematically building different activity for a variety of growth factors and ligands into interfaces [34,35]. Since cells often respond to a variety of different ligands—growth factors, chemokines, and often, many different adhesion ligands—the ability to make complex spatially and temporarily regulated interfaces would extend our ability to regulate cell physiology through biomaterial design in unprecedented ways. The peptide-based ligands presented here are one method for achieving this goal.

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