Effect of Linker and Spacer on the Design of a Fibronectin-Mimetic Peptide Evaluated via Cell Studies and AFM Adhesion Forces

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The design of a fibronectin-mimetic peptide that specifically binds to the \(\alpha_5\beta_1\) integrin has been widely studied because of this integrin’s participation in many physiological and pathological processes. A promising design for such a peptide includes both the primary binding site RGD and the synergy site PHSRN connected by a linker and extended off of a surface by a spacer. Our original hypothesis was that the degree of hydrophobicity/hydrophilicity between the two sequences (RGD and PHSRN) in fibronectin is an important parameter in designing a fibronectin-mimetic peptide (Mardilovich, A.; Kokkoli, E. Biomacromolecules 2004, 5, 950–957). A peptide-amphiphile, PR_b, that was previously designed in our laboratory employed a hydrophobic tail connected to the N terminus of a peptide headgroup that was composed of a spacer, the synergy site sequence, a linker mimicking both the distance and hydrophobicity/hydrophilicity present in the native protein fibronectin (thus presenting an overall “neutral” linker), and finally the primary binding sequence. Even though our previous work (Mardilovich, A.; Craig, J. A.; McCammon, M. Q.; Garg, A.; Kokkoli, E. Langmuir 2006, 22, 3259–3264) demonstrated that PR_b is a promising sequence compared to fibronectin, this is the first study that tests our hypothesis by comparing PR_b to other peptides with hydrophobic or hydrophilic linkers. Furthermore, different peptide-amphiphiles were designed that could be used to study the effect of building blocks systematically, such as the peptide headgroup linker length and hydrophobicity/hydrophilicity as well as the headgroup spacer length on integrin adhesion. Circular dichroism spectroscopy was first employed, and the collected spectra demonstrated that only one peptide-amphiphile exhibited a secondary structure. Their surface topography was evaluated by taking atomic force microscopy (AFM) images of Langmuir–Blodgett peptide-amphiphile membranes supported on mica. Their adhesion was first evaluated with AFM force measurements between the different sequences and an AFM tip functionalized with purified integrins. The amphiphiles were further characterized via 1–12 h cell studies that examined human umbilical vein endothelial cell adhesion and extracellular matrix fibronectin production. The AFM studies were in good agreement with the cell studies. Overall, the adhesion studies validated our hypothesis and demonstrated for the first time that a “neutral” linker, which more closely mimics the cell adhesion domain of fibronectin, supports higher levels of adhesion compared to other peptide designs with a hydrophobic or hydrophilic linker or even fibronectin. Neutral linker lengths that were within the distance found between PHSRN and RGD in fibronectin performed equally well. However, the 10 amino acid neutral linker gave slightly better cell adhesion than did the control fibronectin at all times. Also, a short spacer was shown to give higher adhesion than other sequences with no spacer or a longer spacer, suggesting that a short spacer is necessary to extend the sequence further away from the interface. In conclusion, this work outlines a logical approach that can be applied for the rational design of any protein-mimetic peptide with two binding sites.

Introduction

An important field of study in biological surface modification is the attachment of cell-signaling ligands to provide biofunctionality to synthetic materials. By mimicking specific portions of extracellular matrix (ECM) proteins, individual receptors can be targeted and activated to induce particular responses such as cell adhesion, migration, proliferation, and even death. One highly prevalent ECM protein, fibronectin, binds to integrins found on numerous cell types such as fibroblasts, hepatocytes, and endothelial, stimulating cell adhesion and spreading. One particular tripeptide arginine-glycine-aspartic acid (RGD), located in the 10th type III repeat of the fibronectin molecule, is the minimal binding ligand for multiple integrins including \(\alpha_5\beta_1\). The use of RGD alone as a fibronectin-mimetic peptide, however, has not provided the same level of adhesion as with the native protein. To increase binding affinity, a synergistic proline-histidine-serine-arginine-asparagine (PHSRN) sequence, located in the ninth type III repeat of fibronectin, has been found that enables a stronger and more specific interaction with the \(\alpha_5\beta_1\) integrin. For example, different fibronectin constructs that contained the PHSRN sequence exhibited relative activities that were more than 100 times higher than for a standard RGD-containing peptide, and antibody inhibition studies demonstrated the use of the fibronectin receptor (integrin \(\alpha_5\beta_1\)) for cell adhesion to PHSRN-containing constructs. This particular integrin has

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been shown to be involved in both essential biological processes such as wound healing as well as harmful pathologies such as adenovirus infection, Alzheimer’s disease, breast, colon, prostate, and rectal cancers. Thus, the ability to specifically target and activate its signaling cascades is a significant field of study at this time.

Using an oligopeptide sequence to target the \( \alpha_5\beta_1 \) integrin, rather than an entire fibronectin molecule, has many advantages including structural stability, control over the orientation of the peptide, cost efficiency, and a higher ligand concentration per area. To this end, many single peptides have been designed that incorporate RGD and PHSRN with varying linkers attaching the two recognition sequences. These designs feature either no linker, polyglycine (G) linkers of between 3 and 13 amino acids long, or a bivalent poly(ethylene glycol) hybrid. Unfortunately, these designs were unsuccessful in achieving cell adhesion densities as high as for the positive control fibronectin during short incubation times. It is during this initial time period that the cells’ receptors are most affected by the presented ligands because they have not yet begun to produce their own ECM. At later times, cells should be producing their own ECM. One study testing a peptide with a G\(_{13}\) linker, however, showed higher ECM production when only RGD or a scrambled peptide was presented than with RGD\(_{a}\)PHSRN (results were not compared to fibronectin), despite its higher cell density and focal contact formation.

In an attempt to design a peptide that promotes both cell adhesion and ECM production at or above the level that the native fibronectin protein supports, our group designed a first-generation peptide combining the sequence RGDSP with the C terminus with its synergy site PHSRN oriented at the N terminus. The linker used was designed to mimic both the distance and the hydrobobicity/hydrophilicity between the two active sites in the fibronectin molecule itself. This was done with four pairs of alternating hydrophilic serines and hydrophobic glycines (–(SG)\(_{5}\)–). The smallest hydrophilic amino acid serine (S), and the smallest hydrophobic or nonpolar amino acid glycine (G) were chosen because the analysis of the amino acid sequence between the RGD and PHSRN in human fibronectin type III also showed an equal number of hydrophilic and hydrophobic residues. Larger amino acids with more hydrophobic and hydrophilic side groups were avoided because they would contribute to nonspecific hydrophobic and hydrophilic interactions and to a bulkier headgroup that could change the packing shape of the peptide-amphiphiles and thus their final self-assembled structure. The peptide headgroup with the (SG)\(_{1}\) linker was attached to a hydrophobic tail that features C\(_{16}\) dialkyl ester tails, a glutamic acid (Glu) tail connector, and a (CH\(_{2}\))\(_{5}\) tail spacer to form a peptide-amphiphile called PR\(_{a}\). The Langmuir–Blodgett (LB) technique was used to form a membrane on a solid substrate with the peptide headgroup exposed at the interface. Using atomic force microscopy (AFM), force measurements were made between the peptide and pure \( \alpha_5\beta_1 \) integrins immobilized on an AFM tip. The results of this study indicated that the PHSRN was not fully accessible to the integrins for optimal binding because of its proximity to the membrane surface. This led to the design of a second-generation peptide-amphiphile called PR\(_{b}\). PR\(_{b}\)’s peptide features two changes to PR\(_{a}\)’s design. A three amino acid spacer consisting of a lysine followed by two serines (KSS) was added to extend the active sites further off of the surface, and an extra serine-glycine pair was added to the linker. The addition to the linker lengthened the distance between the RGD and PHSRN to 37 Å (10 amino acids at 3.7 Å per amino acid) at the longer end of the 30–40 Å distance in the native protein. Our previous AFM single-molecule work with peptide-amphiphiles that have no secondary structure demonstrated that the persistence length (the length of the statistical segment) of an amino acid is 0.37 ± 0.02 nm and this length, even though it can vary from one sequence to another, was originally used as a rough guideline in designing the peptide sequences.

Both PR\(_{a}\) and PR\(_{b}\) were tested with human umbilical vein endothelial cells (HUVECs) to look for cell adhesion density, cell spreading, cytoskeletal organization, and fibronectin production, and PR\(_{b}\) was tested for integrin specificity. Results were compared to the cells’ performance on native fibronectin as a positive control, to GRGESP as a negative control, and other peptide surfaces (GRGDSP and a random mixture of 50% GRGDSP and 50% PHSRN). All interfaces were fully covered with adhesive ligands, either peptide or fibronectin. PR\(_{b}\) was shown to be \( \alpha_5\beta_1 \)-specific and gave superior results to all peptides tested at all times, as well as outperforming the fibronectin (FN) protein in cell adhesion levels from 1–24 h and giving equivalent levels at 48 and 72 h. Cells spread more at all times and gave stronger cytoskeletal formation on the PR\(_{b}\) surfaces and similar ECM production compared to fibronectin. This result was attributed first to the PR\(_{b}\) peptide sequence accurately mimicking the cell binding domain of fibronectin and second, to a higher molar concentration of PR\(_{b}\) compared to FN, which is an advantage that peptides have over protein-functionalized interfaces. (The ratio of PR\(_{b}\)/FN molecules was approximately 470: 1.) These positive results motivated the current work in which the two building blocks of the peptide headgroup—the spacer and the linker—were systematically altered.

In addition to PR\(_{a}\) and PR\(_{b}\), four new peptide headgroups were designed in order to test the effects of the linker’s hydrobobicity/hydrophilicity (this is the first study that tests our...
hypothesis by comparing PR_b with the neutral linker to other peptides with hydrophobic or hydrophilic linkers), linker length (within the recommended 30–40 Å distance\(^3\)), and spacer length on their performance as a fibronectin-mimetic peptides. The sequences and nomenclature are listed in Table 1. To compare linker length, first-generation peptide PR_a was directly compared to a new peptide named PR_e. PR_e has no spacer and a neutral linker like PR_a, but the linker is two amino acids longer (an extra serine-glycine pair) than PR_a’s linker. To compare linker hydrophobicity/hydrophilicity, PR_c and PR_d were designed with the same spacer and the same linker length (10 amino acids) as for PR_b, but PR_c’s linker has 10 hydrophobic glycines whereas PR_d’s linker has 10 hydrophilic serines. Finally, to compare the effect of spacer length, PR_b, PR_c, and the final new peptide PR_f, were compared. All of these three peptides have the same \(\sim(SG)\sim\) linker, but PR_e has no spacer, PR_b has a KSS spacer, and PR_f has the longest spacer, KSSSSS. All six peptides were attached to C16 dialkyl ester tails to form peptide-amphiphiles, and the LB technique was used to prepare membranes on mica substrates.

The secondary structure of the peptide-amphiphiles was characterized with circular dichroism (CD) spectroscopy, and their surface topography was evaluated by taking AFM images of LB peptide-amphiphile membranes supported on mica. The peptide-amphiphiles (PR_a to PR_f) were further evaluated by comparing AFM adhesion forces in a liquid environment between LB membranes of the peptide-amphiphiles and 20-nm-diameter silicon nitride AFM tips functionalized with pure \(\alpha_\beta_1\) integrins according to a protocol that we developed previously,\(^3\) as well as comparing HUVEC performance in terms of cell adhesion and fibronecton production. Human fibronectin-coated coverslips were used as a positive control for both the AFM and HUVEC studies.

### Materials and Methods

#### Isotherm Preparation

All six peptide-amphiphiles, as shown in Table 1, were synthesized as described elsewhere.\(^3\) To obtain a surface pressure—area isotherm for each peptide-amphiphile, the LB technique was used on a KSV 5000 LB system (KSV Instruments, Helsinki, Finland) as in our previous work,\(^1,2\) except that for the HUVEC studies both the water and 15 mm mica disks used were treated in an autoclave for sterility. All depositions were done at a surface pressure of between 41 and 47 mN/m, well below the collapse pressure and in the liquid-condensed or solid phase. The deposition speed for both the up and down strokes was 1 mm/min. Freshly cleaved mica disks of radius 7.5 mm were used as substrates for the supported bilayer membranes. The DSPE layer was deposited first at the upstroke to make the mica surfaces hydrophobic. The second layer with peptide-amphiphiles was deposited in the down stroke. Transfer ratios for each layer were calculated to be in the range of 0.8–1, indicating that monolayers were deposited on mica surfaces with minimal disruption. The transfer ratio of PR_d, however, was consistently low (0.4–0.6), which might be due to possible hydrogen bonding between the serine residues of the linker. Therefore, instead of the monolayer coating the hydrophobic mica substrate, the PR_d molecules preferred to interact with each other and allow the disk to slip through the monolayer. The resulting supported bilayer membranes were transferred into glass vials under water. Care was taken to avoid exposing the surface to air because they rearrange to form multilayers.

Approximately 90 min before cells were seeded on the LB films to begin cell adhesion experiments, mica surfaces were transferred into 1X phosphate-buffered saline (PBS), with particular care taken to ensure that the films were not exposed to air. After 5–10 min of equilibration, surfaces were transferred into 24-well plates containing a supplemented MCDB-131 basal media (Sigma), as described in our earlier work.\(^2\) Following surface transfer, 24-well plates were moved to a 37 °C, 5% CO\(_2\) incubator for 45–60 min. As a control, 22-mm-diameter human fibronectin-coated coverslips (BD Biosciences), referred to as FN, were put into 12-well plates, covered with 2 mL of the supplemented media, and also incubated for 45–60 min.

#### \(\alpha_\beta_1\) Integrin Immobilization on AFM Tips

Purified human \(\alpha_\beta_1\) integrins were purchased from Chemicon International (Temecula, CA) and immobilized on the AFM tip with the use of the activating TS2/16 antibody. TS2/16 has been shown to enhance cell adhesion by inducing a conformational change in the extracellular domain of the integrin. This high affinity state of the receptor facilitates ligand binding without requiring receptor cross-linking or intracellular signaling pathways.\(^3\) Most importantly, this effect was observed both with intact cells and on detergent-solubilized \(\alpha_\beta_1\) receptors.\(^35\) In addition to activating the integrins, TS2/16 (anti-\(\beta_1\) antibody) was also used in our study to orient the integrin at the interface. An IgG Fc antibody was used to interact specifically with the Fc chain of the TS2/16 and orient the TS2/16 at the interface. An IgG Fc antibody was used to interact specifically with the Fc chain of the TS2/16 and orient the TS2/16 at the interface. An IgG Fc antibody was used to interact specifically with the Fc chain of the TS2/16 and orient the TS2/16 at the interface.

<table>
<thead>
<tr>
<th>Peptide-Amphiphile</th>
<th>Approximate Spacer Length for an Unordered Peptide (Å/Amoeba)</th>
<th>Approximate Linker Length for an Unordered Peptide (Å/Amoeba)</th>
</tr>
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<tbody>
<tr>
<td>((\text{C}_{16})_2\text{-Glu-C}_2\text{-PSHRN(SG)}_3\text{RGDSP})</td>
<td>PR_a</td>
<td>29.6</td>
</tr>
<tr>
<td>((\text{C}_{16})_2\text{-Glu-C}_2\text{-K(S)}_2\text{-PSHRN(SG)}_3\text{RGDSP})</td>
<td>PR_b</td>
<td>11.1</td>
</tr>
<tr>
<td>((\text{C}_{16})_2\text{-Glu-C}_2\text{-K(S)}_5\text{-PSHRN(SG)}_5\text{RGDSP})</td>
<td>PR_c</td>
<td>11.1</td>
</tr>
<tr>
<td>((\text{C}_{16})_2\text{-Glu-C}_2\text{-K(S)}_2\text{-PSHRN} \text{(S)}_10\text{RGDSP})</td>
<td>PR_d</td>
<td>37</td>
</tr>
<tr>
<td>((\text{C}_{16})_2\text{-Glu-C}_2\text{-PSHRN(SG)}_2\text{RGDSP})</td>
<td>PR_e</td>
<td>37</td>
</tr>
<tr>
<td>((\text{C}_{16})_2\text{-Glu-C}_2\text{-K(S)}_5\text{-PSHRN(SG)}_2\text{RGDSP})</td>
<td>PR_f</td>
<td>22.2</td>
</tr>
</tbody>
</table>

*From refs 30 and 31.*
above solution was adjusted to 7.3 by adding 0.1 M NaOH solution. Standard V-shaped silicon nitride AFM cantilevers were washed with ethanol and then placed under a low-wavelength UV light in the presence of water vapor for 10 min. After being cleaned, cantilevers were washed with PBS. Goat antiserum to mouse IgG (Cappel, Aurora, OH) was physically adsorbed on cantilevers by incubating them overnight at 37 °C and 5% CO₂ with IgG at a final antibody concentration of 8 µg/mL in PBS. Cantilevers were then washed with PBS and incubated with purified monoclonal mouse antihuman TS2/16 antibody (Endogen, Woburn, MA), 20 µg/mL. After being washed with PBS, cantilevers were incubated with purified human α5β1 integrins at 37 °C and 5% CO₂ for 2.5 h and washed with PBS before use. The activity of the integrins was verified via an ELISA test. Force measurements were made immediately after the cantilevers were prepared.

AFM Images and Force Measurements. AFM characterization of the LB films was done with a Nanoscope III Multimode (Veeco Instruments, Woodbury, NY) equipped with a fluid cell for tapping mode. All images were obtained in tapping mode in DI water using standard 100 µm V-shaped silicon nitride AFM cantilevers with pyramidal tips (Veeco Probes, Camarillo, CA) of nominal radius 5–40 nm and nominal spring constant 0.58 N/m. Reproducible images were acquired within 2 days of deposition, on several different surfaces of the same composition and on different areas within the same sample.

AFM force measurements were performed on a Nanoscope III Multimode (Veeco Instruments, Woodbury, NY). Forces were obtained with standard silicon nitride (Si₃N₄) tips on a 200 µm V-shaped cantilever (Veeco Probes, Camarillo, CA) with a nominal tip diameter of 20 nm and a nominal spring constant of 0.06 N/m. AFM collective force measurements (forces between multiple ligand–receptor pairs) were performed at room temperature in a fluid cell filled with 1 mM MnCl₂ solution. All forces for the Si₃N₄-functionalized AFM tips were collected using a loading rate, defined as the spring constant of the cantilever times the velocity of the piezo, of 59.8 nN/s. Data reported reflect the total adhesion (pull-off force) measured between the functionalized tips and different surfaces.

To minimize drift effects, the AFM was warmed for at least 0.5 h before the experiment.

The biomimetic system used here to measure forces with pure integrins immobilized on an AFM silicon nitride tip through the use of IgG and TS2/16 has been previously validated by measuring the effect of the loading rate on the single-molecule interaction of the α5β1 with GRGDSP peptide-ampiphiles; therefore, it allows for the characterization of integrin-specific interactions.

**Cell Adhesion Experiments.** HUVECs (Cambrex Corporation) were cultured as described previously. Only cells from passage 3 were used in the cell studies. Flasks of HUVECs with at least 90% confluency were washed with 1X PBS, trypsinized with 0.25% Trypsin-EDTA (Fisher Scientific), and neutralized with 2% fetal bovine serum in MCDB-131 basal media. The suspended cells from all flasks were placed in one 50 mL centrifuge tube and centrifuged at 1000 rpm for 5 min. The medium was removed, and the cell pellet was resuspended in the supplemented medium described above. A hemocytometer was used to count the cells, and then 497 cells/mm² were seeded onto both LB and FN surfaces. Surfaces were incubated with cells at 37 °C, 5% CO₂ for 1, 4, or 12 h. Cell adhesion following these times was determined using the CyQuant cell proliferation assay kit (Molecular Probes), which measures the fluorescence of a cellular nucleic acid-binding dye using the SpectraMAX GeminiXS plate reader (Molecular Devices). Standard calibration curves were created within each experiment for both 12- and 24-well plates.

**Statistical Analysis.** Cell adhesion data were analyzed using z-test analysis. Results were considered to be statistically significant for p < 0.03.

**Fibronectin Production Staining.** Surface preparation, transfer, and cellular work were all done as described in the previous methods. Only surfaces incubated with cells for 12 h were stained. The staining protocol, involving a primary antibody for secreted fibronectin (Chemicon), an FITC-conjugated secondary antibody (Chemicon), and the Image-iT LIVE plasma membrane and nuclear labeling kit (Molecular Probes), was carried out as described in our previous work. A single-photon confocal microscope (Olympus Fluoview FV1000) was used for imaging. Two slides per substrate were prepared during two separate experiments, and each slide was imaged two to four times. The final images chosen were all taken from one.
Results and Discussion

Surface pressure—area compression isotherms obtained from the six peptide-amphiphiles studied are shown in Figure 1. All peptide-amphiphiles show a phase transition at a pressure somewhere between 18 and 45 mN/m except for PR_d. Also, all six peptide-amphiphiles show a collapse pressure above the deposition pressure range of 41 to 47 mN/m. This collapse pressure ranges from 61 to 71 mN/m.

The surface pressure—area compression isotherms shown in Figure 1 were recorded in order to determine the phase behavior of each peptide-amphiphile at the air—water interface. All peptide-amphiphile monolayers collapse into 3D structures between 61 and 71 mN/m. All depositions onto mica are done at surface pressures between 41 and 47 mN/m, always after the secondary phase transition, when the monolayer is in the liquid-condensed or solid phase, but well before the ultimate collapse. The four peptide-amphiphiles PR_a, PR_b, PR_c, and PR_e all show similar behavior, with secondary phase transitions in the peptide-headgroup, indicated by the hump in the isotherm, occurring at pressures between 35 and 45 mN/m. PR_f also shows a similar phase transition, although at the much lower surface pressure of 18.5 mN/m. PR_f’s headgroup is the longest of all of the peptide-amphiphiles (26 amino acids) with a large number of serines in the linker and spacer (a total of 10 serines in the linker and spacer). Each of these serines contains an aliphatic hydroxyl group, which is available to form hydrogen bonds with other serines. Therefore, interactions among the peptide-amphiphile molecules at the interface may contribute to the formation of an extended liquid phase that appears at smaller pressures. In fact, as the number of serines decreases, the phase transition appears at higher pressures, with PR_c (which has only two serines in its linker and spacer) showing the highest surface pressure phase transition. Peptide-amphiphile PR_d behaves much differently from those previously discussed because no secondary phase transition is present in its isotherm. This peptide headgroup contains the highest number of serines in its headgroup. A network of hydrogen-bonded headgroups then may form that does not undergo any straightening phase transition until the monolayer collapses at 60 mN/m. This hydrogen-bonding network also resulted in low transfer ratios between 0.4 and 0.6 when PR_d was deposited onto the hydrophobized mica surfaces. All other peptide-amphiphiles had transfer ratios between 0.8 and 1, indicating little disturbance in the transferred monolayer.

Circular dichroism (CD) spectroscopy in aqueous solutions was performed to evaluate the peptide-amphiphile secondary structure (Figure 2). This method reveals different secondary structures found in proteins that show a characteristic absorption in the far-UV region (240 nm and below) due to peptide bonds that are located in a regular folded environment. The $\alpha$-helix CD spectrum has a negative minimum at about 208 nm and a second, more pronounced minimum at 222 nm, a positive peak in the region of 190–195 nm, and a crossover from positive to negative below 172 nm. All-$\beta$ proteins exhibit a minimum in the 210–220 nm range, a second minimum in the 170–180 nm range, a crossover from positive to negative above 185 nm, and positive ellipticity around 200 nm. The $\alpha+\beta$ and $\alpha/\beta$ proteins have CD spectra that are dominated by the $\alpha$-helix portion and exhibit two minima at about 208 and 222 nm, a positive band in the range of 190–195 nm, and the crossover from positive to negative above 172 nm, which serves to distinguish the $\alpha$-$\beta$ proteins from the all-$\alpha$ proteins. In the case of the $\alpha+\beta$ proteins, the 208 nm minimum is larger in magnitude and more prominent than at 222 nm, which often is a very shallow minimum. Figure 2 shows that only PR_d exhibits a secondary

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structure. PR_d has a total of 12 serine residues in its linker and spacer, 2 at the N-terminus of the headgroup, and a 10-serine linker connecting the synergy (PHSRN) site and primary binding (RGD) site. Polyserine domains naturally exist as flexible linkers in several proteins and exhibit different secondary structures, which frequently appear in turns and loops rather than helices. PR_d’s spectrum has two minima at approximately 208 and 221 nm and also a maximum at 194 nm. Because the band at 208 nm is larger in magnitude than the 221 nm band, its CD spectrum exhibits the characteristics of the R+/beta two protein CD spectrum. Therefore, it is likely that PR_d is a mixture of mostly all-R and some all-beta regions. No other peptide-amphiphiles exhibited any secondary structure. This can be explained if one considers that the glycine amino acids are typically found to break the helix formation and Monte Carlo studies of a polyglycine peptide, (G)20, did not show any stable secondary structure. For example, even though PR_f has a total of 10 serines in its linker and spacer, a 5-serine spacer at the N terminus of the headgroup, and 5 serine residues alternating with 5 glycines in the linker, the presence of the glycine amino acids disrupts any tendency to form helices. As a result, PR_f’s CD spectrum does not display any structure.

AFM was used to characterize the surface topography of the supported bioartificial membranes constructed from PR_b, PR_d (the peptide with the lowest transfer ratio that exhibits secondary structure), and PR_f (the longest peptide sequence). Figure 3 presents the AFM topography of the peptide-amphiphiles acquired at room temperature in tapping mode in DI water. The images shown are representative of the images taken over different areas on the same surface. The presence of height differences of 3.10 \pm 0.37 nm on the PR_b surface (Figure 3A) and 3.33 \pm 0.67 nm on the PR_f surface (Figure 3C) suggests that some of the headgroups of the peptide-amphiphiles are bent (the fully extended headgroup lengths of PR_b and PR_f are calculated to be 8.51 nm and 9.62 nm, respectively, assuming the amino acid length of an unordered sequence to be 0.37 nm31), which is in agreement with AFM images of other peptide-amphiphiles. However, on the PR_d surface (Figure 3B) two different height differences were measured, 3.23 \pm 0.57 nm and 7.09 \pm 1.13 nm, consistent with the idea that some headgroups are bent and some peptide-amphiphiles are missing (in agreement with the observed low transfer ratios). It is not straightforward to calculate the theoretical length of the peptide headgroup because the CD spectrum (Figure 2) showed that PR_d is a mixture of mostly all-alpha and some all-beta regions. The length of an amino acid in the PR_d sequence can vary between 0.15 nm, for an alpha-helix, to 0.35 nm, for a beta-sheet. Therefore, the length of the PR_d headgroup will be between 3.45 nm (if it was an all-alpha helix headgroup) and 8.05 nm (for an all-beta sheet headgroup).
The peptide-amphiphiles were next evaluated with AFM adhesion forces. For that, AFM tips with a 20 nm nominal diameter were functionalized with purified integrins and were used to collect force profiles. Figure 4 shows AFM adhesion forces between an $\alpha_5\beta_1$-functionalized tip and LB membranes of three peptide-amphiphiles with similar spacer lengths and linker lengths but with varying degrees of hydrophobicity/hydrophilicity (from PR_b to PR_d). An FN surface was used as a positive control. The AFM force curves were collected in 1 mM MnCl$_2$ solution. $\alpha_5\beta_1$ integrins can be activated by direct stimuli, such as anti-$\beta_1$ chain monoclonal antibodies (e.g., TS2/16 that was used to immobilize the integrin on the AFM tip as well as activate the integrin$^{35,43}$ and divalent ions (Mg$^{2+}$ and Mn$^{2+}$).$^{1,44,45}$ The AFM adhesion data in Figure 4 show that the peptide-amphiphile PR_b, which has a neutral linker, gives the highest level of adhesion compared to the other two peptide sequences with a hydrophobic or hydrophilic linker and fibronectin. Our original hypothesis was that the degree of hydrophobicity/hydrophilicity between the two sequences (RGD and PHSRN) in fibronectin has to play a role when it comes to designing a fibronectin-mimetic peptide.$^{1}$

Even though our previous work$^4$ demonstrated that PR_b is a promising sequence compared to fibronectin and other peptide surfaces that had only RGD or had RGD and PHSRN in fibronectin has to play a role when it comes to designing a fibronectin-mimetic peptide.$^4$ Even though our previous work$^4$ demonstrated that PR_b is a promising sequence compared to fibronectin and other peptide surfaces that had only RGD or had RGD and PHSRN, this is the first time that we tested our hypothesis by comparing PR_b to other peptides with only hydrophobic or hydrophilic linkers. This graph provides the first validation of our hypothesis and demonstrates that a neutral linker, which more closely mimics fibronectin’s cell-adhesion domain, supports higher levels of adhesion compared to other designs.

Figure 5 compares peptides with neutral linkers. PR_a and PR_e have no spacer and examine the effect of the linker length within the recommended 30–40 Å distance.$^{32}$ The two peptides give similar adhesion levels within the standard deviation of the force measurements. Next we question the effect of the spacer length for peptides with the same linker. PR_e has no spacer; PR_b has a three amino acid spacer, and PR_f has a six amino acid spacer. Results show that PR_b gives higher adhesion; therefore, a short spacer is necessary to extend the sequence further away from the interface. A longer spacer may give more flexibility to the design but imparts no added benefit when it comes to AFM adhesion forces.

Figures 6–8 display the results of the cellular adhesion timed experiments. In all three Figures, cellular adhesions to FN are also given for comparison. The timed experiment results in Figure 6 compare adhesion when no spacer is present in the peptide-amphiphile. Both linkers are within the recommended 30–40 Å distance$^{32}$ and are neutral. Thus, the only difference between the two peptide-amphiphiles compared is the length of the linker, with PR_a containing only four serine-glycine pairs versus PR_e’s five pairs. This gives PR_e an approximately 7.4 Å longer linker between the PHSRN and RGDSP amino acid sequences. Within the 1 h time period, PR_e gives higher HUVEC adhesion than both PR_a ($p < 0.03$) and FN ($p < 0.03$), whereas PR_a and FN show no statistical difference in their adhesion after 1 h. At 4 h, PR_a’s adhesion increases, whereas in our previous work cell adhesion on the PR_a surfaces decreased from 1 to 4 h and increased at 12 h (probably as a result of the production of the extracellular matrix).$^2$ Figure 6 shows that for the 4 and 12 h time periods there is no statistical difference at the level of adhesion between PR_e and PR_a. However, PR_e shows higher adhesion than FN ($p < 0.03$) at all times (1–12 h). Overall, both PR_a and PR_e performed equally well because both sequences have linkers that are neutral and within the 30–40 Å distance found between PHSRN and RGD in fibronectin.$^{32}$ However, if one has to choose a linker, the recommendation would be a headgroup with the 10 amino acid neutral linker because PR_e gives slightly better cell adhesion performance as compared to the control FN at all times ($p < 0.03$).

In Figure 7, the importance of the hydrophobicity/hydrophilicity of the linker between the PHSRN and the RGDSP sequences is tested. All three peptide-amphiphiles contain the same spacer (KSS) and the same number of amino acids in the linker. PR_b’s linker is composed of five hydrophilic serines alternating with five hydrophobic glycines and is neutral overall, whereas PR_c’s linker has 10 hydrophobic glycines, and PR_d’s linker has 10 hydrophobic serines. Figure 7 shows that PR_b performs significantly better than PR_c at all times except 1 h, better than
**Figure 6.** Effect of peptide linker length on HUVEC adhesion. Cell adhesion was evaluated on LB membranes of PR_a, PR_e, and positive control FN at 1, 4, and 12 h. HUVECs were incubated at a seeding density of 497 cells/mm² on substrates at 37 °C, 5% CO₂ in the absence of serum. Results are given as the cell density value for each particular surface and time period divided by the cell density value of PR_b at 1 h. The values represent the mean ± SD of three separate experiments performed in triplicate. * indicates significant differences (p < 0.03) compared with the PR_a group. † indicates significant differences (p < 0.03) compared with the PR_e group.

**Figure 7.** Effect of peptide linker hydrophobicity/hydrophilicity on HUVEC adhesion. Cell adhesion was evaluated on LB membranes of PR_b, PR_c, and PR_d and positive control FN at 1, 4, and 12 h. HUVECs were incubated at a seeding density of 497 cells/mm² on substrates at 37 °C, 5% CO₂ in the absence of serum. Results are given as the cell density value for each particular surface and time period divided by the cell density value of PR_b at 1 h. The values represent the mean ± SD of three separate experiments performed in triplicate. * indicates significant differences (p < 0.03) compared with the PR_b group.

PR_d at all times except 4 h, and better than FN at all times. Overall, PR_b’s neutral linker supports the highest adhesion in the absence of serum, compared with a hydrophobic or hydrophilic linker, and this is even more evident for longer times (12 h). This finding is in agreement with the AFM adhesion data shown in Figure 4. Thus, both AFM data and cell adhesion results validate...
our original hypothesis that a neutral linker, which more accurately mimics the cell adhesion domain of fibronectin, is better than a hydrophobic or a hydrophilic linker. In addition, adhesion to RGD-PHSCRN peptides with polyglycine linkers, such as PR_c, has been shown to be mediated by the Rv/betatwo integrin.21

The final factor to consider when designing a fibronectin-mimetic peptide is the length of the spacer that extends the active sites away from the surface of the membrane. Figure 8 compares the length of the spacer with all three peptide-amphiphiles containing the exact same linker sequence (SG)_5. PR_e contains no spacer at all, PR_b contains the three amino acid sequence of a lysine followed by two serines, and PR_f’s spacer adds another three serines, increasing its length by approximately 11 Å as compared to PR_b. The AFM adhesion results (Figure 5) show that PR_e with no spacer and PR_f with a six amino acid spacer KSSSSS give comparable levels of force, whereas PR_b containing a three amino acid spacer KSS gives the highest force. The AFM data are in general agreement with the cell adhesion results. Figure 8 demonstrates that for all time periods PR_b performs statistically better than both PR_f and FN. When comparing PR_b and PR_e, there is no statistical difference between the averages for 1 and 4 h. At 12 h, PR_b outperforms PR_e at a significance level of p < 0.03. Also, PR_b’s adhesion level shows the least drop off between 4 and 12 h. This is particularly significant because our past work has shown that adhesion is maintained between 12 and 24 h as a result of the start of fibronectin production by the cells.2 Thus, the higher 12 h adhesion equates to higher long-term adhesion as well. Overall, PR_b’s three amino acid spacer seems to allow the best adhesion, extending the active sequences the optimal distance off the surface of the bilayer membrane to better expose them to the α5β1 integrins.

Fluorescent images stained to show the cell nucleus and membrane and the fibronectin produced by the cells are depicted in Figure 9. Each image is on one of the six peptide-amphiphile LB surfaces or the fibronectin-coated coverslip and was fixed after 12 h of incubation with the HUVECs. Extracellular matrix protein production by cells adhered to a surface indicates that the cells are content, allowing them to maintain long-term adhesion. Once cells cover the surface with their own matrix, the original surface, in this case the bilayer peptide-amphiphile membrane, is no longer seen by cell integrins and becomes irrelevant to their continued adhesion. One of these excreted ECM proteins is fibronectin. Fibronectin begins to be produced by adhered cells sometime between the 4 and 12 h time periods,2 so fibronectin staining was done only on 12 h surfaces. Fibronectin was labeled using an antihuman fibronectin monoclonal antibody specific to secreted fibronectin. The goal of the fibronectin staining was to ensure that adhered cells on all surfaces were producing ECM, thus supporting each surface’s respective level of adhesion. In Figure 9, the secreted fibronectin is shown in green, with the cell membranes shown in red and the nuclei in blue. All surfaces, including both the six peptide-amphiphile surfaces and the control FN, allow both cell adhesion and ECM production as seen from the presence of secreted fibronectin on all. This important finding demonstrates that all six versions of the fibronectin-mimetic peptide engineered here present the primary and secondary active binding sites, RGD and PHSRN, respectively, to HUVEC α5β1 integrins, allowing some level of cell adhesion. The fibronectin’s presence at 12 h also agrees with the experimental parameter of

![Figure 8](image-url) Effect of peptide spacer length on HUVEC adhesion. Cell adhesion was evaluated on LB membranes of PR_b, PR_e, and PR_f and positive control FN at 1, 4, and 12 h. HUVECs were incubated at a seeding density of 497 cells/mm² on substrates at 37 °C, 5% CO₂ in the absence of serum. Results are given as the cell density value for each particular surface and time period divided by the cell density value of PR_b at 1 h. Symbols above a particular column represent the p value given the null hypothesis that that column’s adhesion density and the PR_b adhesion density for that same time period (i.e., 1, 4, or 12 h) are the same. The values represent the mean ± SD of three separate experiments performed in triplicate. * indicates significant differences (p < 0.03) compared with the PR_b group.
ending the timed experiments at 12 h because by that time produced ECM surrounds the cells and blocks interaction with any remaining peptide.

**Conclusions**

Six peptide-amphiphile designs containing the primary binding sequence RGD and the $\alpha_5\beta_1$ integrin-specific synergy site PHSRN were characterized with CD and AFM surface images. Their ability to support adhesion was examined via AFM adhesion forces with purified $\alpha_5\beta_1$ integrin receptors immobilized on AFM tips and HUVEC adhesion from 1–12 h. The production of ECM fibronectin was also examined. The peptide-amphiphiles were systematically constructed in order to test the effect of the length and hydrophobic/hydrophilic linker connecting the PHSRN to the RGD sequence as well as the effect of the length of the spacer used between the amphiphile’s hydrophobic tails and the headgroup’s active sites.

The CD experiment revealed that only PR_d (which has a total of 12 serine residues in its linker and spacer) exhibits a secondary structure that is probably a mixture of mostly all-$\alpha$ and some all-$\beta$ regions. No other peptide-amphiphiles exhibited any secondary structure.

The AFM images of LB membranes of peptide-amphiphiles PR_b and PR_f showed height differences that suggest that some of the headgroups are bent at the interface. However, on the PR_d surface two different height differences were measured, consistent with the idea that some headgroups are bent and some peptide-amphiphiles are missing (in agreement with the low transfer ratios for this surface).

The AFM adhesion data, with the purified integrins, were in general in good agreement with the HUVEC adhesion studies. When the linker length was compared, both PR_a and PR_e gave similar levels of adhesion with AFM forces and longer cell adhesion experiments (4 and 12 h). Overall, both PR_a and PR_e performed equally well because both sequences have linkers that are neutral and within the 30–40 Å distance found between PHSRN and RGD in fibronectin. However, because PR_e gives slightly better cell adhesion performance as compared to the control FN at all times ($p < 0.03$), the recommendation would be a headgroup with the 10 amino acid neutral linker. Linker hydrophobicity/hydrophilicity was compared using PR_b, PR_c, and PR_d. PR_b’s neutral linker allowed the highest level of adhesion, evident from both the AFM and cell studies, with the least amount of decay in cell adhesion over the 12 h period. Hence, both AFM forces and in vitro studies validated our original hypothesis that the degree of hydrophobicity/hydrophilicity between the two sequences (RGD and PHSRN) in fibronectin has to play a role when it comes to designing a fibronectin-
mimetic peptide. Finally, when the effect of spacer length was compared, PR_b's three amino acid spacer performs much better than PR_f's six amino acid spacer at all times and better than PR_e's no spacer at the important 12 h time, when ECM production is observed. AFM measurements also showed that PR_b gave higher forces than PR_e and PR_f. Therefore, a short spacer is necessary to extend the sequence further away from the interface.

It was seen from secreted fibronectin staining that all six peptide-amphiphile surfaces, as well as the positive control FN, allowed attached cells to produce fibronectin. On the basis of the adhesion experiments, however, the current best choice for use in biomaterial or targeted drug delivery applications is PR_b.

The strategy employed in this work for designing a fibronectin-mimetic peptide can be used in the future anytime two active sites are being combined in one peptide to mimic a protein's cell adhesion domain. Using a spacer to expose the active sites and a linker to mimic the protein's natural distance and hydrophobicity/hydrophilicity between the active sites provides a methodical approach to rational peptide design.

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