

## Design of a Novel Fibronectin-Mimetic Peptide–Amphiphile for Functionalized Biomaterials

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The interaction of the  $\alpha_5\beta_1$  integrin with its ligand, fibronectin, supports numerous adhesive functions and has an important role in health and disease. In recent years, there has been a considerable effort in designing fibronectin-mimetic peptides to target the integrin. However, to date, the therapeutic use of these peptides has been limited, as they cannot accurately mimic fibronectin's binding affinity for  $\alpha_5\beta_1$ . A peptide–amphiphile (PR\_b) was synthesized with a peptide headgroup composed of four building blocks: a spacer; RGDSP, the primary recognition site for  $\alpha_5\beta_1$ ; PHSRN, the synergy binding site; and a linker. The linker was designed to mimic two important criteria: the distance and the hydrophobicity/hydrophilicity between PHSRN and RGD in fibronectin. Human umbilical vein endothelial cells were seeded on different substrates and evaluated in terms of adhesion, spreading, specificity, cytoskeleton organization, focal adhesions, and secretion of extracellular fibronectin. This peptide was shown to perform comparably to fibronectin, indicating that a biomimetic approach can result in the design of novel peptides with therapeutic potential for biomaterial functionalization.

### Introduction

Materials employed in biomedical technology are increasingly being designed to have specific, desirable biological interactions with their surroundings, as opposed to the older, more common practice of trying to adapt traditional materials to biomedical applications. A common theme in engineering cell and tissue behavior at device surfaces is to modify the material's interface to interact selectively with a specific cell type through biomolecular recognition events. The cell surface has a variety of receptors that bind with other cells or specific proteins, which compose the environment (known as the extracellular matrix, ECM) surrounding the cells. A promising approach is the biomimetic modification of the material, in which peptides, or recombinant protein fragments containing the adhesion domains of the ECM proteins, are attached to the interface.<sup>1–10</sup>

The precise control of cell adhesion and migration in the body is crucial for biological processes such as embryogenesis, homeostasis, the immune response, and tissue remodeling and healing.<sup>11,12</sup> Central to this control is the integrin-mediated adhesion to proteins from the ECM. Although integrins and, in particular, the  $\alpha_5\beta_1$  integrin were originally characterized as a family of cell adhesion receptors that are responsible for anchoring

cells to the ECM, they have recently been shown to have a dramatic impact on dynamic processes such as mediating adenovirus infection, accelerating wound healing, providing a protection mechanism against Alzheimer's disease, and acting as a promising target for breast, colon, prostate, and rectal cancer.<sup>13–23</sup>

Many therapeutic strategies require the use of peptides, such as the short sequence arginine–glycine–aspartic acid (RGD), that mimic the cell adhesion domain of fibronectin in an attempt to target the  $\alpha_5\beta_1$  integrin and provide treatment. Even though surface modification with a biomimetic peptide remains one of the most promising strategies, the therapeutic use of RGD-containing peptides has been limited since they cannot accurately mimic the affinity of fibronectin for the  $\alpha_5\beta_1$  integrin.<sup>24–26</sup> This may be due to the fact that RGD peptides lack synergistic effects that come from the proline–histidine–serine–arginine–asparagine (PHSRN) site. Thus, the ability to design peptides

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**Table 1. Structure and Nomenclature of the Peptide–Amphiphiles Used in This Study**

peptide-amphiphile structure	abbreviation	linker between PHSRN and RGD	linker distance (3.7 Å/amino acid <sup>a</sup> )
(C <sub>16</sub> ) <sub>2</sub> –Glu–C <sub>2</sub> –GRGDSP	GRGDSP		
(C <sub>16</sub> ) <sub>2</sub> –Glu–C <sub>2</sub> –GRGESP	GRGESP		
(C <sub>16</sub> ) <sub>2</sub> –Glu–C <sub>2</sub> –PHSRN	PHSRN		
(C <sub>16</sub> ) <sub>2</sub> –Glu–C <sub>2</sub> –PHSRNSGSGSGSRGDSP	PR_a	(SG) <sub>4</sub>	29.6 Å
(C <sub>16</sub> ) <sub>2</sub> –Glu–C <sub>2</sub> –KSSPHSRNSGSGSGSGSRGDSP	PR_b	(SG) <sub>5</sub>	37 Å

<sup>a</sup> From refs 37 and 38.

that accurately mimic the fibronectin  $\alpha_5\beta_1$ -mediated adhesion has increased therapeutic potential and represents a significant undertaking.

The focus of this work is in engineering biomimetic peptide–amphiphiles that target and increase adhesion with the  $\alpha_5\beta_1$  integrin. The design features C<sub>16</sub> dialkyl ester tails, a glutamic acid (Glu) linker, a  $-(CH_2)_2-$  spacer, and the peptide headgroup. The headgroup contains two fibronectin-mimicking peptide sequences: fibronectin's primary binding ligand for the  $\alpha_5\beta_1$  integrin, RGDSP (natively found in the tenth type III module –FNIII10), and  $\alpha_5\beta_1$ 's synergy binding ligand, PHSRN. This synergy site in fibronectin type III repeat 9 (FNIII9) consists of about half a dozen amino acids on the side of the domain that is facing RGD.<sup>27–29</sup> In native fibronectin, PHSRN and RGD are separated by 30–40 Å.<sup>27</sup> Apparently, this distance is important for PHSRN to play a synergistic role in adhesion, as studies have shown that incremental extensions of the interdomain link between FNIII9 and FNIII10 reduced cell attachment and affected cell spreading and the phosphorylation of focal adhesion kinase.<sup>30</sup>

When RGD and PHSRN have been presented in a single peptide formulation in the past, results varied depending on the design. Motifs included no linker,<sup>31</sup> linkers of varying number of glycine (G) amino acids (G<sub>3</sub>–G<sub>13</sub>),<sup>32–34</sup> or a bivalent poly(ethylene glycol) hybrid linker.<sup>35</sup> However, for short periods of time before cells start secreting their own ECM, the sequences that were compared to fibronectin showed adhesion strengths that are smaller than fibronectin. Moreover, one study that examined ECM production demonstrated that ECM secretion was the lowest on surfaces functionalized with the colocalized peptide sequence (RGDG<sub>13</sub>–PHSRN) compared to surfaces with a scrambled peptide sequence (RDGG<sub>13</sub>HPRNS) or RGD, emphasizing the need to design peptides that are optimized to promote cell adhesion and encourage ECM production.<sup>34</sup>

A peptide–amphiphile that combines both PHSRN and RGDSP in one sequence, (C<sub>16</sub>)<sub>2</sub>–Glu–C<sub>2</sub>–PHSRN(SG)<sub>4</sub>RGDSP (referred to as PR\_a), was originally designed in our laboratory.<sup>36</sup> The goal in designing the linker was to mimic as closely as possible the distance and the hydrophobicity/hydrophilicity between the PHSRN and RGD sequences in the fibronectin molecule. A linker (SG)<sub>4</sub> was used to link these two bioactive

sequences in a specific orientation, that is, with the PHSRN sequence at the N-terminus, and the RGDSP sequence at the C-terminus. The combination of the hydrophilic serine (S), and the hydrophobic glycine (G) residues was chosen because analysis of the alignment of the amino acid sequences of repeats 7 through 10 in human fibronectin type III (FNIII7–10) indicated that the ratio of hydrophilic to hydrophobic residues between PHSRN and RGD is almost 1:1.<sup>36</sup> The PR\_a peptide–amphiphile was tested using atomic force microscopy (AFM) force measurements between immobilized pure  $\alpha_5\beta_1$  integrins on an AFM tip and Langmuir–Blodgett (LB) membranes of PR\_a on mica.<sup>36</sup> Results from this study demonstrated that the synergistic effect from PHSRN strongly depends on its accessibility to the receptor. Motivated by the findings of the AFM study, the second generation of the peptide–amphiphile ((C<sub>16</sub>)<sub>2</sub>–Glu–C<sub>2</sub>–KSSPHSRN(SG)<sub>5</sub>–RGDSP) was designed and referred to as PR\_b. When compared to the previous PR\_a design, the PR\_b structure contains an additional lysine–serine–serine (KSS) spacer at the N-terminus of the peptide headgroup, whose role is to extend the bioactive sequence further away from the interface and impart flexibility to the peptide to bind the receptor on the cell surface. Furthermore, an extra SG pair was added to the linker between PHSRN and RGDSP in PR\_b to increase the distance between the two to 37 Å (10 amino acids  $\times$  3.7 Å per amino acid residue<sup>37,38</sup>).

In the studies presented here, LB membranes on mica substrates constructed from PR\_a, PR\_b, GRGDSP, and an equimolar mixture of GRGDSP and PHSRN peptide–amphiphiles were used to investigate the effect of different substrates on cell adhesion, spreading, cytoskeletal organization, and fibronectin production. All experiments were conducted in the absence of fetal bovine serum (FBS) to investigate the effect of the bioactive peptide sequence on these phenomena. LB membranes of GRGESP peptide–amphiphiles were used as a negative control and fibronectin-coated glass slides (referred to as FN) were used as a positive control.

## Materials and Methods

**Surface Preparation.** The peptide–amphiphiles, listed in Table 1, were synthesized as described in the literature.<sup>39</sup> LB membranes constructed from peptide–amphiphiles were deposited onto 15 mm mica disks, as described elsewhere.<sup>38</sup> Shortly before cell assays, the LB films were transferred into 24-well plates containing 1X phosphate-buffered saline (PBS) solution (Mediatech), without exposing the surfaces to air, as they rearrange to form multilayers.<sup>40</sup> After equilibration for 5–10 min, PBS was exchanged with 1 mL of MCDB-131 basal media (Sigma) supplemented with 0.1% bovine serum albumin (BSA) (Sigma) and antibiotics (Sigma) (2.5  $\mu$ g/mL gentamicin, 2.5  $\mu$ g/mL amphotericin B, 50 U/mL penicillin, and 50

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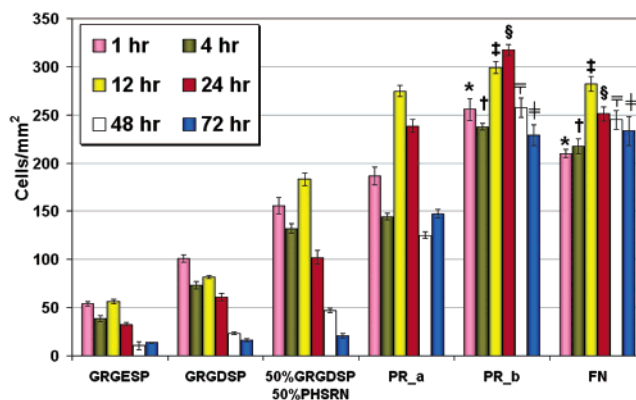
$\mu\text{g/mL}$  streptomycin), referred to as 0.1% BSA media. Surfaces were equilibrated at 37 °C, 5% CO<sub>2</sub>, for 30–60 min before adding the cells. Human fibronectin-coated round coverslips (FN), 22 mm in diameter (BD Biosciences), were put into 12-well plates, and 2 mL of 0.1% BSA media was added to each well. The surfaces were equilibrated for 30–60 min at 37 °C, 5% CO<sub>2</sub>.

**Cell Culture.** Human umbilical vein endothelial cells, HUVECs, (Cambrex Corporation) were cultured in MCDB-131 containing 20% FBS (Atlas Biologicals) at 37 °C, 5% CO<sub>2</sub>. Other nutrients and antibiotics supplementing the media were added on the basis of an optimal HUVEC proliferation study.<sup>41</sup> The media was changed in the culture flasks every other day. The cells used for adhesion and imaging studies were in passages 3 and 4.

**Cell Adhesion Experiments.** After cells became 90% confluent, they were washed with PBS, trypsinized with 0.25% Trypsin-EDTA (Cambrex Corporation), neutralized with MCDB-131 with 2% FBS, and spun at 1000 rpm for 10 min. The supernatant was removed, and the cell pellet was resuspended in 0.1% BSA media. Cells were counted with a hemocytometer and seeded onto LB and FN surfaces at a density of 497 cells/mm<sup>2</sup>. Surfaces were incubated with cells in 0.1% BSA media at 37 °C, 5% CO<sub>2</sub>, for 1, 4, 12, 24, 48, and 72 h. Cell adhesion was quantified using the CyQuant cell proliferation assay kit, with a dye that binds to cellular nucleic acids, following the manufacturer's protocol (Molecular Probes). A calibration standard curve was created for each experiment. The plates were read on a SpectraMAX GeminiXS (Molecular Devices).

**Adhesion Blocking Experiments.** Blocking experiments were done using mouse anti-human integrin  $\alpha_5$  (P1D6),  $\beta_1$  (P5D2),  $\alpha_5\beta_1$  (JBS5), and  $\alpha_v\beta_3$  (LM609) monoclonal antibodies (Chemicon Int.). All surfaces were transferred to 24- or 12-well plates and preincubated in 0.1% BSA media as described above. HUVECs were prepared and counted as described in the cell adhesion protocol. Cells were blocked by mixing the cell suspension with the appropriate antibody in 0.1% BSA media and incubating for 30 min at 37 °C, 5% CO<sub>2</sub>, with constant agitation. Blocked cells were then released onto LB and FN surfaces and allowed to adhere for 1 h at 37 °C, 5% CO<sub>2</sub>. For all blocking experiments, cells were seeded at 249 cells/mm<sup>2</sup> (lower seeding density was used here to reduce the amount of antibodies needed to block cell adhesion), and the final antibody concentration in each well was 20  $\mu\text{g/mL}$ . Following incubation, surfaces were washed, and adhered cells were quantified as described in the cell adhesion protocol.

**Cell Spreading and Fibronectin Production Experiments.** Surface transferring, cell seeding, and incubation were done in the same manner as that used in the adhesion experiment, with a cell density of 497 cells/mm<sup>2</sup> and incubation times of 1, 12, 24, 48, and 72 h. At the end of each incubation, the surfaces were removed, washed with 10 mL of PBS, and fixed in 1 mL of 4% paraformaldehyde (Sigma) in PBS for 15 min at 37 °C, 5% CO<sub>2</sub>. After washing with PBS, surfaces were first labeled for secreted fibronectin: surfaces were subjected to a blocking solution (0.1% BSA in PBS) for 30 min, incubated for 1 h with a primary antibody specific for secreted fibronectin (mouse anti-human fibronectin (PIH11) monoclonal antibody; Chemicon Int.) at a 1:500 dilution in blocking solution, and then incubated for 45 min with a secondary antibody (IgG fluorescein (FITC) conjugated antibody; Chemicon Int.) at a 1:200 dilution in PBS. As a final step, cell membranes and nuclei were stained using the Image-iT LIVE plasma membrane and nuclear labeling kit following the manufacturer's protocol for labeling fixed cells (Molecular Probes). After final washing, the surfaces were mounted onto glass slides and stored at 4 °C protected from light. An inverted fluorescent microscope (Nikon Eclipse TE200 with Pixcell II LCM) was used for imaging. Fibronectin production was assessed visually, by comparing 10–20 fluorescence images from two samples for each substrate (two independent experiments performed on different days). Cell spreading was quantified from two samples per substrate (two independent experiments performed on different days) by measuring cell area with NIH imaging software.



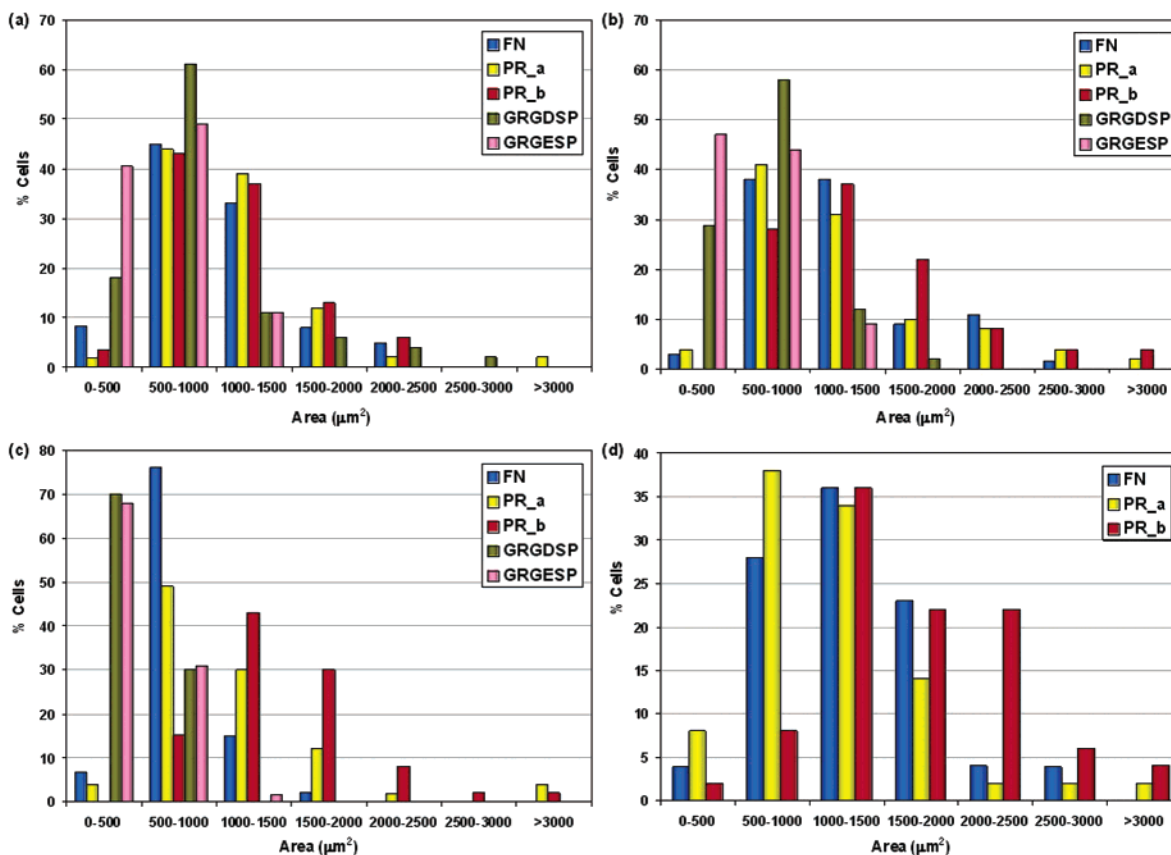
**Figure 1.** Effect of time and surface composition on HUVEC adhesion. Cell adhesion was evaluated on LB membranes of the following peptide—amphiphiles: GRGDSP, 50% GRGDSP—50% PHSRN, PR<sub>a</sub>, and PR<sub>b</sub>. The GRGESP peptide—amphiphile was used as a negative control, and the FN substrates were used as a positive control. HUVECs were incubated on these substrates for 1–72 h at 37 °C, 5% CO<sub>2</sub>, in the absence of FBS. The initial cell density was 497 cells/mm<sup>2</sup>. The PR<sub>b</sub> peptide—amphiphile outperforms all other peptide surfaces and, compared to the positive control FN, gives higher adhesion for 1–24 h (z-test analysis for \*, †, ‡, and §:  $p < 0.007$ ) and similar adhesion for 48–72 h (z-test analysis for †† and ‡‡:  $p < 0.1$ , signifying no statistical difference). Each histogram represents the mean  $\pm$  SD. For all substrates,  $n = 2$  (two independent experiments performed on different days).

**Actin Cytoskeleton and Focal Adhesion Staining.** Surface transferring, cell seeding, incubation, and fixing were done as described above. Staining for the actin cytoskeleton, focal adhesions, and nucleus of attached cells was done using the Actin Cytoskeleton and Focal Adhesion Staining Kit following the manufacturer's protocol (Chemicon Int.) and using 1:350 dilution for the anti-vinculin antibody, 1:200 for the secondary antibody, approximately 1:1000 dilution (equivalent to 1 unit/surface) for TRITC-conjugated phalloidin, and 1:1000 for DAPI. After being washed, surfaces were mounted onto glass slides and stored at 4 °C protected from light. A confocal microscope (BIORAD Multiphoton Confocal 1024) was used for imaging.

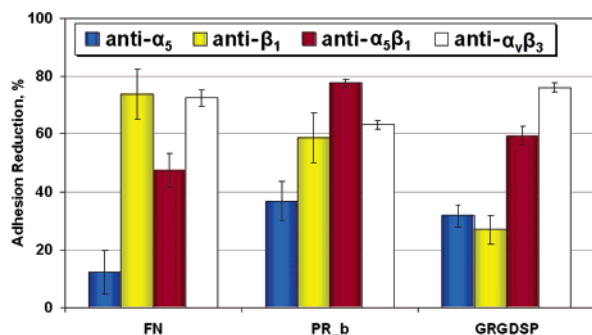
## Results and Discussion

HUVECs were seeded on different substrates (GRGESP, GRGDSP, 50% GRGDSP—50% PHSRN, PR<sub>a</sub>, PR<sub>b</sub>, and FN), and the cell density was examined after 1, 4, 12, 24, 48, and 72 h (Figure 1). For all LB surfaces, a small decrease in cell adhesion is observed after 1 h of incubation, which could be due to the absence of serum in culture media. Additionally, for all substrates, an increase in adhesion is observed going from 4 to 12 h that can be attributed to the onset of fibronectin production at 12 h, as shown in Figure 5. GRGDSP substrates failed to sustain HUVEC adhesion after 48 h, since, at this point, the cell density was similar to the one observed for the inactive GRGESP peptide. The absence of serum in the media and the minimal amount of fibronectin production on GRGDSP surfaces compared to PR<sub>a</sub>, PR<sub>b</sub>, and FN can be possible explanations for the failure of the GRGDSP film. Similarly, substrates functionalized with an equimolar mixture of GRGDSP and PHSRN did not sustain adhesion after 72 h. Cells effectively adhered to PR<sub>a</sub> and PR<sub>b</sub> for 72 h; however, the PR<sub>b</sub> peptide—amphiphile is the most promising sequence, comparing favorably to the natural protein ligand, as it gave higher adhesion than FN for 1–24 h. At 48 and 72 h, cell adhesion was similar on both PR<sub>b</sub> and FN surfaces. At this point in time, the areas under and around the cells on both PR<sub>b</sub> and FN were fully covered with ECM-secreted fibronectin (images of secreted fibronectin are shown at 72 h in Figure 5). Thus, the cells were attached to the produced protein, and the

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**Figure 2.** Effect of time and surface composition on HUVEC spreading. Cell spreading was evaluated on LB membranes of the following peptide–amphiphiles: GRGDSP, PR\_a, and PR\_b. The GRGESP peptide–amphiphile was used as a negative control, and the FN substrates were used as a positive control. HUVECs were incubated, in the absence of FBS, at 37 °C and 5% CO<sub>2</sub>, on these substrates for: (a) 1 h, (b) 12 h, (c) 24 h, and (d) 72 h. At 72 h, only PR\_a, PR\_b, and FN substrates were evaluated, as GRGDSP and GRGESP failed to sustain cell adhesion after 48 h. Results are reported as the percentage of cells spread within a cell area.



**Figure 3.** Integrin specificity. Inhibition assay using anti-integrin blocking antibodies against α<sub>5</sub> (P1D6), β<sub>1</sub> (P5D2), α<sub>5</sub>β<sub>1</sub> (JBS5), and α<sub>v</sub>β<sub>3</sub> (LM609) to determine the integrin engagement profile for HUVEC adhesion on FN, PR\_b, and GRGDSP substrates after 1 h of incubation at 37 °C and 5% CO<sub>2</sub>, in the absence of FBS. Results are reported as the percentage of reduction in cell adhesion by blocking antibodies compared to the control (nonblocked cells).

substrate that was below the secreted ECM fibronectin did not contribute to the cell attachment.

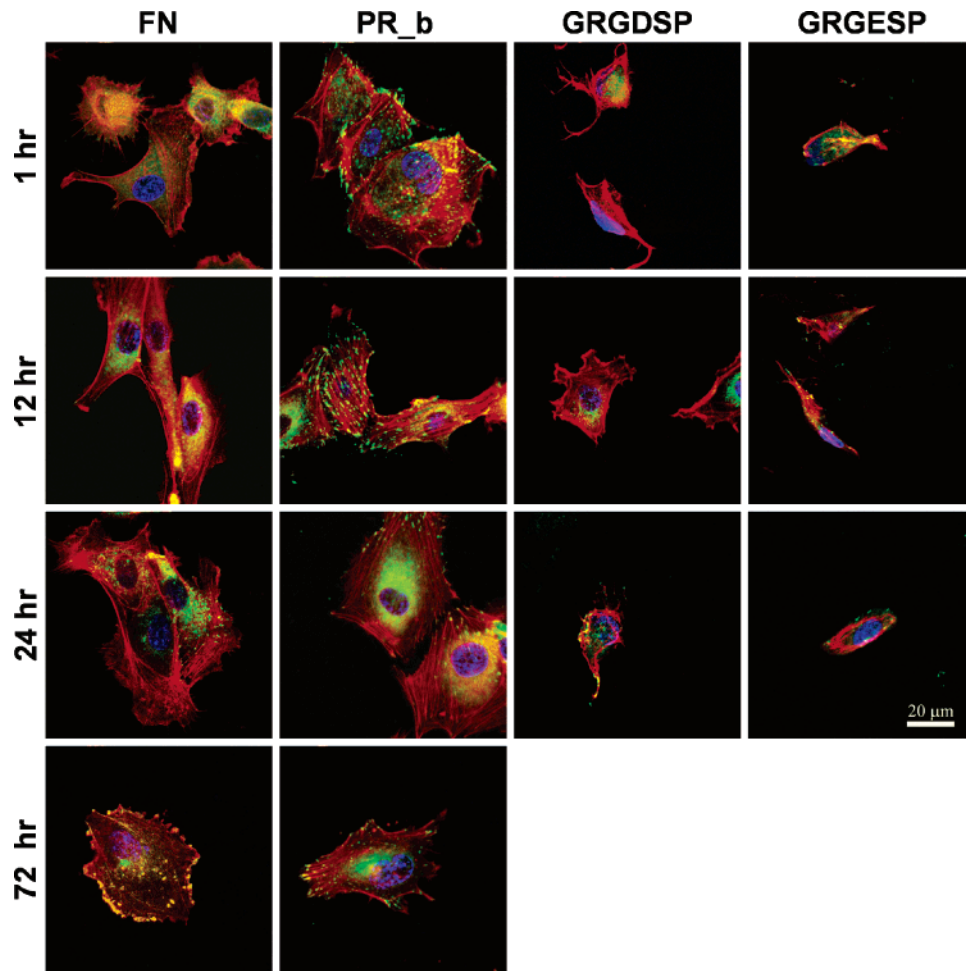
An increase in cell adhesion on PR\_b compared to FN for 1–24 h can be attributed to a combination of two factors. The first contribution comes from the amino acid sequence of PR\_b accurately mimicking the cell-binding domain of fibronectin. This is essential since, as discussed in the Introduction, none of the previous studies that used saturation levels of fibronectin-mimetic peptides showed such an improved performance compared to fully covered fibronectin surfaces. The second factor is a higher molar concentration of PR\_b versus FN, which is an

advantage one has in functionalizing an interface with a peptide versus a protein. The coating concentration of FN was 15–35 μg/mL and corresponded to a full monolayer of the protein (information provided by the manufacturer). Experimental studies show that, within this range of coating concentrations, the fibronectin surface density is 350–450 ng/cm<sup>2</sup>.<sup>42</sup> On the basis of the dimensions of the molecule (60 × 4 nm),<sup>43</sup> 350 ng/cm<sup>2</sup> (0.692 pmoles/cm<sup>2</sup>) represents the amount of fibronectin necessary to produce a monolayer coating. At the deposition pressure of the experiment, the LB isotherm of PR\_b shows an area of 0.51 nm<sup>2</sup>/molecule, which corresponds to 325 pmoles/cm<sup>2</sup>. Therefore, at saturation conditions, the ratio of PR\_b/FN molecules is 470:1.

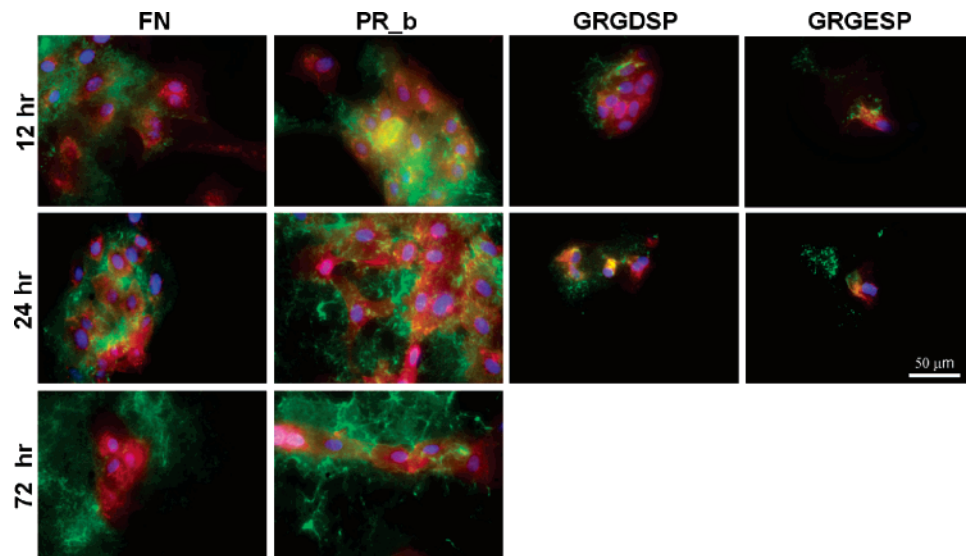
Cell spreading was evaluated for FN, PR\_a, PR\_b, GRGDSP, and GRGESP surfaces (Figure 2). The area of cells seeded on inactive GRGESP surfaces and incubated for up to 24 h was found to be the smallest, and the cells were mostly round (Figure 2a,b,c). The spreading of attached HUVECs on GRGDSP was higher compared to GRGESP, but much smaller than FN for 1–24 h (Figure 2a,b,c). In contrast, cells seeded on PR\_a, PR\_b, and FN surfaces were mostly spread to a different extent, however, depending on the bioactive sequence. It was also noticed that, initially, after 1 h of incubation, cells were evenly distributed throughout the PR\_a, PR\_b, and FN surfaces, whereas cell clusters were observed at 24 and 48 h. Adhered cells at 72 h were again more evenly distributed throughout the PR\_a, PR\_b, and FN

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**Figure 4.** Cytoskeletal organization and focal adhesions of HUVEC as a function of surface composition and time. HUVECs were seeded on FN and LB membranes of PR\_b, GRGDSP, and GRGESP for 1, 12, 24, and 72 h at 37 °C, 5% CO<sub>2</sub>, in the absence of FBS. At 72 h, only PR\_b and FN substrates were evaluated, as GRGDSP and GRGESP failed to sustain cell adhesion after 48 h. Cells were fixed and fluorescently stained for actin stress fibers (red), vinculin (green), and nucleus (blue) and were examined by confocal microscopy. The scale bar is 20 μm for all images.



**Figure 5.** Effect of substrate and time on HUVEC fibronectin secretion. HUVECs were seeded on FN and LB membranes of PR\_b, GRGDSP, and GRGESP for 12, 24, and 72 h at 37 °C, 5% CO<sub>2</sub>, in the absence of FBS. At 72 h, only PR\_b and FN substrates were evaluated, as GRGDSP and GRGESP failed to sustain cell adhesion after 48 h. Cells were fixed and fluorescently stained for secreted extracellular fibronectin (green), cell membrane (red), and nucleus (blue). The scale bar is 50 μm for all images.

surfaces. For all times examined, cells were found to spread the most on the PR\_b surfaces, with FN and PR\_a being second and

third, respectively (Figure 2). Figures 1 and 2 collectively demonstrate that, for all the peptide surfaces that include the

PHSRN sequence (50% GRGDSP–50% PHSRN, PR\_a, and PR\_b), PR\_b is the only fibronectin-mimetic sequence that compares favorably with FN, and remaining studies will be performed between FN, PR\_b, GRGDSP, and GRGESP surfaces.

Integrin specificity was examined by blocking HUVECs with anti- $\alpha_5$ , anti- $\beta_1$ , anti- $\alpha_5\beta_1$ , and anti- $\alpha_v\beta_3$  monoclonal antibodies, and then incubating blocked cells on PR\_b, GRGDSP, and FN surfaces for 1 h (results are shown in Figure 3). Cell adhesion on FN was equally reduced by anti- $\beta_1$  ( $73.84 \pm 8.69\%$  reduction in cell adhesion compared to adhesion of nonblocked cells) and anti- $\alpha_v\beta_3$  ( $72.39 \pm 2.87\%$ ). HUVEC adhesion on GRGDSP was reduced the most by anti- $\alpha_v\beta_3$  ( $75.99 \pm 1.51\%$ ), whereas cell adhesion on the PR\_b surface was reduced the most by the anti- $\alpha_5\beta_1$  antibody ( $77.55 \pm 1.50\%$ ). This indicates that HUVEC adhesion to PR\_b surfaces was  $\alpha_5\beta_1$ -mediated.

To investigate cytoskeletal organization and focal adhesion formation, HUVECs seeded on PR\_b, GRGDSP, and GRGESP membranes and FN surfaces were fixed and stained at 1, 12, 24, 48, and 72 h for actin stress fibers, nuclei, and vinculin, a major protein present in focal contacts.<sup>44</sup> Representative images are shown in Figure 4 for 1, 12, 24, and 72 h. At 12 h of incubation on the inactive GRGESP surface, HUVECs began to collapse into a spindle-shaped morphology with no distinct actin fibers and very few focal adhesion points. Cells seeded on GRGDSP surfaces showed some actin fiber formation at 12 h, although, at 24 h, actin organization became less pronounced. Cells did not form strong focal adhesion contacts on GRGDSP surfaces, and vinculin was present mainly in the perinuclear area of the cells. HUVECs seeded on both FN and PR\_b surfaces displayed actin stress fibers and focal contacts for 1–72 h (Figure 4). However, cells on PR\_b clearly showed a better-developed cytoskeletal structure compared to FN, suggesting a more firm adhesion on the fibronectin-mimetic peptide, PR\_b, (which is in agreement with the cell adhesion data in Figure 1). Adherent cells to PR\_b, for 1–72 h, assembled longer, thinner vinculin-positive focal adhesion complexes compared to FN, with sharp spikes of vinculin at the termination points and across the actin stress fibers. Strong actin cytoskeleton organization into elongated stress fibers was also more pronounced at all times on PR\_b compared to FN surfaces.

Extracellular fibronectin production was observed at 12 h of incubation on all surfaces (representative data are shown for FN, PR\_b, GRGDSP, and GRGESP surfaces in Figure 5), and may explain the increase in cell adhesion from 4 to 12 h observed in Figure 1. Secreted fibronectin was labeled with an anti-human fibronectin monoclonal antibody, specific for secreted fibronectin, and thus was distinguished from the FN coated glass slides. Therefore, fluorescent labeling of secreted fibronectin was only observed in the vicinity of the adhered cells, and not in the areas

without cells. Fibronectin production on GRGDSP and GRGESP surfaces was significantly less compared to that on FN and PR\_b at 12 and 24 h. After 48 h of incubation, there was minimal fibronectin secretion that was not sufficient to sustain cell adhesion on GRGDSP and GRGESP surfaces (data not shown). This result is also in agreement with the data from Figure 1, where cell density on these two substrates dramatically decreases after 48 h. The amount of fibronectin secretion was found to increase with incubation time on the two adhesive surfaces (FN and PR\_b), and was comparable for both substrates at all times examined (Figure 5). In addition, after 48 h for both PR\_b and FN, the areas around and under the cells were fully covered with secreted fibronectin. This finding is significant considering that extracellular protein production is of paramount importance for the viability and success of any functionalized biomaterial as a tissue-engineering construct.

## Conclusions

A new PR\_b peptide–amphiphile was designed in this study that mimics fibronectin's cell binding domain, and is specific for  $\alpha_5\beta_1$  integrins. Performance of PR\_b was evaluated in terms of cell adhesion, spreading, cytoskeletal organization, and extracellular fibronectin production. Results were compared to our earlier design of the fibronectin-mimetic peptide–amphiphile, PR\_a, as well as to a surface with equimolar amounts of GRGDSP and PHSRN, and to pure GRGDSP, GRGESP, and FN surfaces. PR\_b outperformed all other peptide substrates, and compared favorably to FN. This is the first study demonstrating that a peptide gives stronger cell adhesion than FN for 1–24 h. This result can be 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, an advantage peptides have over protein functionalized interfaces (the ratio of PR\_b/FN molecules was approximately 470:1). After 48 h, cell adhesion was equivalent on both PR\_b and FN because of high amounts of secreted ECM fibronectin that had covered areas under and around the cells. In addition, HUVECs were found to spread the most on the PR\_b surfaces for all times examined, from 1 to 72 h. Results from immunocytochemical studies showed that, compared to FN, the PR\_b sequence can effectively promote stronger cytoskeletal organization and focal adhesion formation.

In summary, the results presented here demonstrate the value of biomimetic surface science. The novel peptide sequence (PR\_b) can find applications in biomaterial functionalization, tissue engineering, and targeted drug delivery.

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