

## ***Chapter VIII. Coordinated regulation of cell adhesion by cell-binding domain density and elastic modulus***

### **Abstract**

A family of artificial extracellular matrix (aECM) proteins that contain cell-binding domains derived from fibronectin and structural domains derived from elastin is described. These aECM proteins can be crosslinked into freestanding, three-dimensional films of tunable elastic modulus. Following crosslinking, the cell-binding domain sequences incorporated into the aECM films can still promote sequence-specific adhesion of endothelial cells. This cell adhesion can be tuned by altering the density of cell-binding domains within the film and by modifying the elastic modulus of the film from 0.1 - 1.0 MPa. The secretion and activation of matrix metalloproteinase-2 is also affected by elastic modulus. The ability to control both the cell-binding domain density and elastic modulus independently suggests that aECM films are ideal substrates to investigate the coordinated effects of biochemical and biomechanical cell-matrix interactions. Furthermore, the ability to tune the elastic modulus of aECM films to match that of native elastin while simultaneously promoting endothelial cell adhesion suggests that aECM proteins may be useful as small-diameter vascular grafts.

## 1. Introduction

Anchorage of cells to the underlying substrate plays a key role in determining cell structure and function [1]. One key criterion for small-diameter vascular grafts is the firm adhesion of endothelial cells to the graft material in the face of opposing hemodynamic forces [2, 3]. Our previous work has shown that endothelial cells can adhere strongly to films of adsorbed artificial extracellular matrix (aECM) proteins in a sequence-specific manner that resists physiologically relevant detachment forces [4]. This work validated the hypothesis that endothelial cell adhesion strength can be controlled by appropriate molecular engineering of aECM proteins. We now extend this hypothesis to suggest that the recognition of cellular domains and the observed effects on endothelial cell spreading and adhesion are retained upon crosslinking of aECM proteins into films suitable for construction of freestanding, implantable vascular grafts.

As reported previously, several crosslinking chemistries have emerged as providing tunable mechanical properties for aECM films [5, 6]. Recent studies suggest that in addition to recognizing ECM ligands, cells also respond to the mechanical properties of their surroundings by regulating adhesive interactions [7]. Rigid matrices, when compared to compliant matrices of identical composition, promote cell-surface assembly of fibronectin [8], decrease cell locomotion [9], display increased levels of protein phosphorylation at sites of cell-matrix contact [10], and strengthen integrin-cytoskeleton linkages [11]. Mounting evidence suggests that a balance of forces is maintained across the cell-matrix interface that is affected by many intracellular and extracellular components and provides a mechanical context for cellular response [12, 13]. Therefore, this work studies the effects of matrix rigidity as well as cell-binding domain density on endothelial cell spreading and adhesion on crosslinked aECM proteins.

Following arterial injury, which includes interventions for occlusive vascular disease, endothelial cells and smooth muscle cells play important roles in the hyperplastic response which includes cell migration into the intima along with secretion of ECM components and

matrix metalloproteinases (MMPs) [14]. Wound repair can be characterized by cell migration and consequent tissue remodeling by the secretion and activation of MMP molecules [15, 16]. Recent studies have been devoted to the regulation of MMPs by endothelial cells in order to understand the underlying mechanisms governing angiogenesis [17]. This work has shown that integrin recognition events as well as substrate mechanical properties may influence the secretion and activation of these important molecules [18-20]. Integrins of the  $\alpha_1$ -subfamily as well as the  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins are known to be involved in the signaling of matrix molecules during angiogenesis [21]. We believe that much of this work can be extended to aECM proteins to gain an understanding of how endothelial cells respond to local wounds inflicted during vascular grafting. Currently, the optimum endothelial cell response to encourage re-endothelialization and wound healing of a vascular graft is unknown. We hypothesize that the secretion and activation of various MMP molecules can be modulated through genetic engineering of the aECM protein and subsequent crosslinking to tune the mechanical properties.

The amino acid sequences of the aECM proteins used in this work are shown in Figure 1. Each includes elastin-like repeats comprising the pentapeptide VPGIG, interspersed with the CS5 or RGD cell-binding domains of fibronectin (termed **REVD** and **RGD**, respectively.) The **REVD** and **RDG** protein sequences are identical except the minimal recognition sites have been scrambled to provide negative control substrates for cell-binding studies. The elastin-like sequences give these materials elasticity and mechanical integrity [5, 6] while the cell-binding domains provide cell adhesion signals [4, 22]. Lysine residues were incorporated into each sequence to allow site-specific crosslinking without interruption of the cell-binding domains. A T7-tag leader sequence is included to increase expression levels and to aid in protein detection. A hexahistidine tag was incorporated as an alternate method of purification along with an enzymatic cleavage site to remove the fusion sequences.

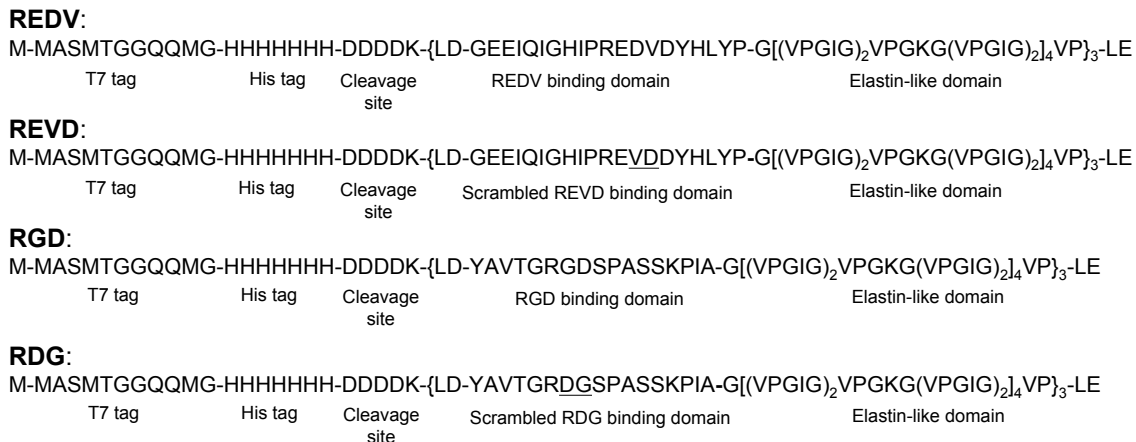


Figure 1. Amino acid sequences of the artificial extracellular matrix proteins. Each protein contains three cassettes of a cell-binding domain interspersed with elastin-like domains. The **REDV** and **RGD** proteins contain the CS5 and RGD cell-binding domains, respectively, while the **REVD** and **RDG** proteins are negative controls with scrambled cell-binding domains.

## 2. Experimental section

### 2.1. Protein expression and purification

Plasmids encoding sequences **REDV**, **REVD**, **RGD**, and **RDG** have been previously described [5, 22]. These sequences were expressed in *E. coli* and purified as previously described [4, 22]. Purity was assessed by SDS-PAGE, mass spectrometry, and Western blotting with anti-T7 tag-horseradish peroxidase conjugate antibody (Amersham).

### 2.2. Protein crosslinking

Protein films, 20% wt/vol in phosphate buffered saline (PBS), were crosslinked by addition of bis(sulfosuccinimidyl) suberate (BS3) pre-solubilized in 4°C water prior to injection in a 96-well plate (15  $\mu$ l/well) and centrifugation at 4°C for 15 min. Plates were covered with sealing tape and incubated 10 hours at 4°C prior to rinsing three times with PBS. Films were blocked with 0.2% heat-inactivated bovine serum albumin (BSA fraction V, Sigma) for 30 min and rinsed three times with PBS.

### 2.3. Cell culture

Human umbilical vein endothelial cells (HUVEC, Bio Whittaker) were maintained in a 37°C, 5% CO<sub>2</sub> humidified environmental chamber. The cells were grown in Endothelial Growth Medium-2 (5% serum, Bio Whittaker) and passaged non-enzymatically by treatment with 0.61 mM EDTA (Gibco). Passages 2-10 were used.

### 2.4. Cell resistance to detachment

Prior to each assay, a background fluorescence measurement of the crosslinked protein films was taken using a Perkin Elmer HTS 7000 Bio Assay Reader with excitation at 485 nm and emission at 538 nm. HUVEC in suspension were then labeled with a 5  $\mu$ M solution of calcein acetoxymethyl ester (Molecular Probes) in serum-free Endothelial Basal Medium (EBM, Cell Applications, San Diego, CA) at room temperature for 30 min. Cells were rinsed twice and resuspended in EBM at 2.67x10<sup>5</sup> cell/ml. Cells (150  $\mu$ l/well) were added to crosslinked films in 96-well plates in the presence or absence of competitive peptides. Competitive peptides (GRGDSP and GRDGSP) were provided at a concentration of 1.8 mM. After incubation at 37°C (for 30 min unless otherwise noted), the wells were completely filled with PBS and an initial fluorescence reading was measured prior to covering with sealing tape, inverting the plate, and centrifuging at 100 g for 10 min. After centrifugation, the sealing tape was removed and the fluorescence was again recorded. The detachment force applied was estimated to be 26 pN using Archimedes' theorem [22]. Three independent experiments with six replicates each were performed. Cell retention is calculated as

$$\left( \frac{\text{Final fluorescence} - \text{Background fluorescence}}{\text{Initial fluorescence} - \text{Background fluorescence}} \right) \times 100\%.$$

### 2.5. Zymography

Samples of 16-hour conditioned medium were collected from HUVEC monolayers grown on crosslinked protein films. The samples were analyzed by gelatin zymography prior to densitometry analysis using Image J software (public domain image analysis

software provided by NIH). Briefly, samples were run at 200 V at 4°C on a 10% SDS-PAGE impregnated with 1 mg/ml gelatin. The gel was washed twice with 2.5% TritonX-100 and incubated overnight at 37°C in an activation buffer (50 mM TrisHCl, 5mM CaCl<sub>2</sub>, 1mM ZnCl<sub>2</sub>, 1% TritonX-100, 0.02% NaN<sub>3</sub>). After staining, the majority of the gel is tinted, representing the impregnated gelatin, while clear bands represent gelatin degradation caused by MMPs in the conditioned medium. Three independent experiments were performed.

### 3. Results and discussion

#### 3.1. Sequence-specific cell adhesion

Prior studies have shown that human umbilical vein endothelial cells (HUVEC) adhere to aECM proteins primarily through interactions with the cell-binding domains when the proteins are adsorbed to tissue culture polystyrene [4, 22]. Interestingly, HUVEC adhere more strongly to the **RGD** protein after it has been crosslinked into a coherent film, Figure 2. This increase in cell retention is probably not the result of an increase in cell-binding domain concentration, because the adsorbed **RGD** substrate already presents over two-orders of magnitude more cell-binding domains as potential cell-surface receptors.

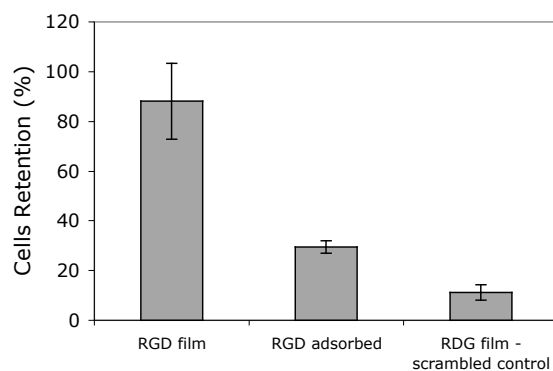


Figure 2. HUVEC retention on **RGD** presented as a crosslinked, coherent film or as an adsorbed protein substrate after 30 min of adhesion time and 10 min of exposure to a 26 pN detachment force. Three independent experiments were performed. Error bars represent one standard deviation.

To verify that cell adhesion was primarily a result of interactions with the RGD minimal binding sequence, the adhesion assay was repeated in the presence and absence of competitive peptides. As expected, the GRGDSP peptide almost completely inhibited HUVEC binding to crosslinked films of **RGD** while the scrambled sequence GRDGSP peptide was found to have no effect on cell retention, Figure 3.

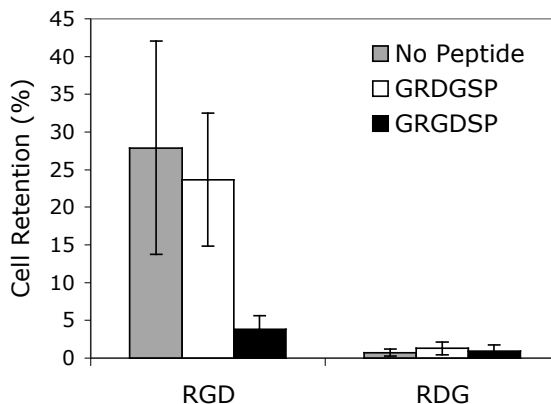


Figure 3. HUVEC retention following 30 min of adhesion time and 10 min exposure to a 26 pN detachment force on crosslinked **RGD** and **RDG** films in the presence and absence of competitive peptides. Three independent experiments were performed. Error bars represent one standard deviation.

Previous studies of cell spreading on aECM proteins found that HUVEC were fully spread on adsorbed **RGD** substrates after 30 min while HUVEC on adsorbed **REDV** substrates required much longer times for cell spreading to occur [22]. This contrast in cell spreading times was also found in comparisons of crosslinked **RGD** and **REDV** films, Figures 3 and 4. While 30 min of incubation was sufficient to retain about 30% of HUVEC on crosslinked **RGD** throughout 10 min of exposure to a 29 pN detachment force (Figure 3), fewer than 5% of HUVEC remained adherent during a similar force after 30 min of incubation on **REDV** (data not shown). HUVEC were required to incubate on **REDV** films for about 2 hours before this level of cell adhesion was demonstrated, Figure 4. However, at even longer incubation times, cell adhesion was observed to decrease. This is perhaps attributable to interactions between the CS5 cell-binding domain and the  $\alpha_4\beta_1$

integrin, which has been shown to promote weak adhesion and increase cell migration [23]. At 2 hours, cell adhesion to the negative control **REVD** film was significantly less than adhesion to **REDV**. Collectively, these results demonstrate that HUVEC are able to recognize the cell-binding domain sequences incorporated into the engineered **REDV** and **RGD** proteins even after crosslinking into coherent films.

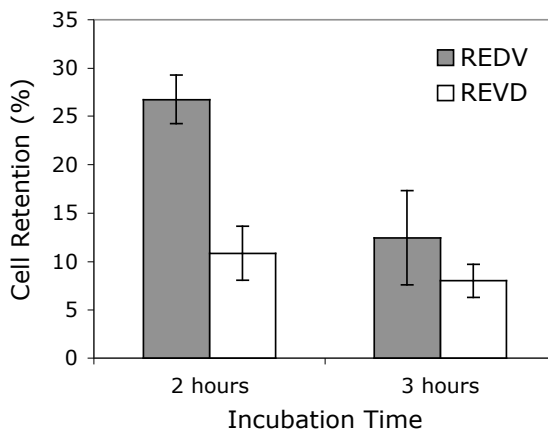


Figure 4. HUVEC retention on crosslinked **REDV** and **REVD** films following two or three hours of incubation prior to 10 min exposure to a 26 pN detachment force. These are preliminary results that have been repeated twice. Error bars represent one standard deviation.

### 3.2. Tuning cell adhesion through material properties

Two material properties were evaluated for their ability to tune cell adhesion: cell-binding domain density and elastic modulus. The cell-binding domain density was modified by mixing together **RGD** and **RDG** proteins at various concentrations prior to crosslinking. Decreasing the concentration of **RGD** in the film from 100 - 90% resulted in a decrease in cell retention of over 50%, Figure 5. No significant changes in cell retention were observed for films from 90 - 50% **RGD**. These changes in cell adhesion simply could be a result of the decrease in density of the cell-binding domain or could be affected by the local spatial arrangement of the RGD domains. These crosslinked aECM films are



assumed to be homogenous; therefore, a decrease in cell-binding domain density will also result in larger spacing between adjacent binding motifs.

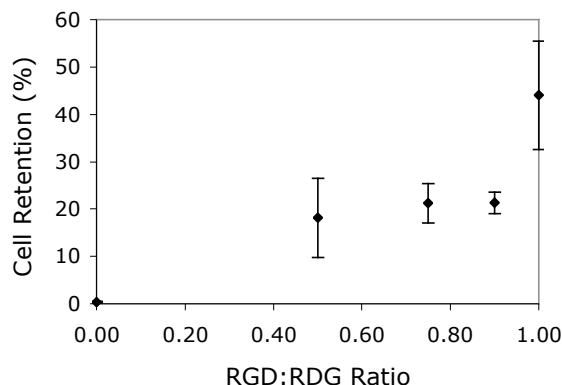


Figure 5. HUVEC retention on crosslinked films made of mixtures of **RGD** and **RDG** after 30 min incubation and 10 min exposure to a 26 pN detachment force. These are preliminary results that have been repeated twice. Error bars represent one standard deviation.

To vary the elastic modulus of the materials, the proteins were crosslinked with various stoichiometric amounts of BS3 crosslinker. Previous reports have shown that elastic modulus is approximately linearly dependent on crosslinker stoichiometry up to one full stoichiometric ratio [5]. Films prepared with 1X stoichiometric amount of BS3 results in a film with an elastic modulus about 0.7 MPa, similar to that of native elastin. HUVEC were found to adhere more firmly after a 30 min incubation to films prepared with higher stoichiometric ratios of BS3, Figure 6. This result is somewhat similar to reports by others that cells display larger spread areas and increased adhesion and intracellular signalling on stiffer substrates; however, these reports were generally concerning materials with elastic moduli 10-100X less than those tested here [7-11].

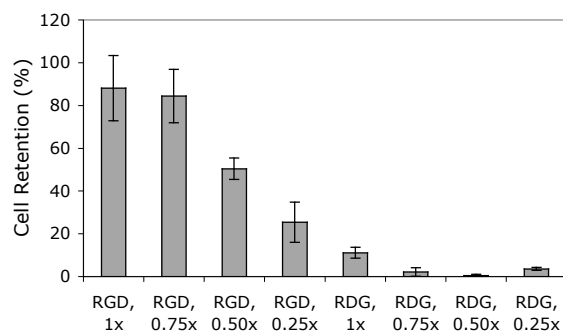


Figure 6. HUVEC retention on films of **RGD** and **RDG** prepared with 1 - 0.25 stoichiometric amount of BS3 crosslinker. These are preliminary results that have been repeated twice. Error bars represent one standard deviation.

### 3.3. Matrix metalloproteinase-2 activation

Matrix metalloproteinase-2 (MMP-2) is a gelatinase secreted and activated by endothelial cells. Medium samples were taken from HUVEC monolayers grown on crosslinked aECM films for 16 hours and analyzed by gelatin zymography, Figure 7. Crosslinked aECM films of **REDV** and **REVD** were formed using 0.5 or 1.0 stoichiometric amount of BS3 crosslinker, resulting in films with elastic modulus of about 0.1 and 0.3 MPa, respectively [5, 24]. The clear bands represent degradation of gelatin, which was impregnated in the acrylamide gel, by proteases. In lanes one and three (films with 0.5X BS3), three bands are present at 72, 62, and 59 kDa, corresponding to the inactive, partially active, and fully active forms of MMP-2, respectively [18, 19, 25]. In contrast, lanes two and four (films with 1.0X BS3) only have bands at 72 kDa, suggesting that all secreted MMP-2 on these substrates is in the inactive form. MMP-2 secretion and activation were increased on films with lower elastic moduli, irrespective of whether the authentic or scrambled CS5 cell-binding domain sequence was presented. These results were quantified using densitometry and are reported in Table 1. The intensity values in Table 1 are reported relative to the intensity of the brightest band (inactive MMP-2 secretion on a **REVD** film with 0.5X crosslinker). These results corroborate earlier studies that

showed endothelial cells increase MMP-2 activation and undergo less cytoskeleton remodeling when grown on flexible substrates [18, 19]. Taken together with the results in Figure 6, these data suggest that HUVEC grown on more compliant films undergo less cytoskeleton remodeling which results in weaker resistance to detachment forces and increased secretion and activation of MMP-2.

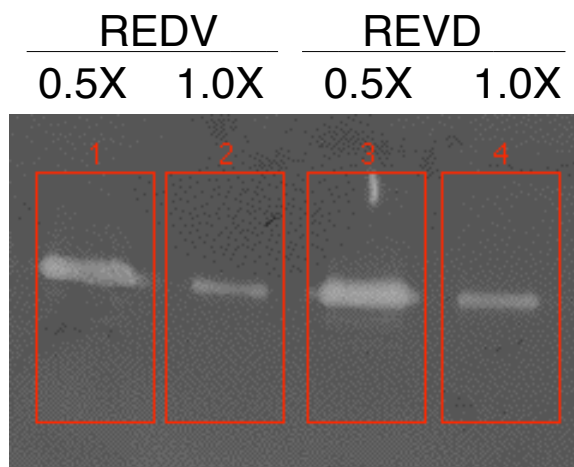


Figure 7. Gelatin zymogram of conditioned medium samples taken from HUVEC grown on various aECM films for 16 hours. Films were prepared from **REDV** and **REVD** proteins with either 0.5 or 1.0 stoichiometric amount of BS3.

Table 1. MMP-2 secretion and activation by HUVEC monolayers grown on various aECM films for 16 hours as measured by individual band intensity relative to the highest intensity band.

Molecular weight	MMP-2 form	<b>REDV</b>	<b>REDV</b>	<b>REVD</b>	<b>REVD</b>
		0.5X	1.0X	0.5X	1.0X
72,000	inactive	75.4	24.0	100.0	31.0
62,000	partially active	2.3	0.0	5.0	0.0
59,000	fully active	2.2	0.0	1.6	0.0

#### 4. Conclusion

Towards the goal of designing a new protein-based material for small-diameter vascular grafts, we have previously shown that genetically engineered artificial extracellular matrix proteins can adhere endothelial cells in a sequence-specific manner while physically adsorbed to tissue culture polystyrene. These proteins can also be crosslinked into freestanding, three-dimensional structures with elastic moduli similar to that of native elastin. The work presented here demonstrates that this crosslinking procedure does not inhibit sequence-specific endothelial cell adhesion. HUVEC adhesion to these crosslinked films can be tuned by controlling the density of cell-binding domains presented or by modifying the elastic modulus. Furthermore, secretion and activation of MMP-2 was influenced by elastic modulus and not cell-binding domain authenticity. These results suggest that crosslinked aECM films may be useful as small-diameter vascular grafts and also have potential as substrates for well-characterized studies of cell-matrix interactions.

#### 5. References

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