Use of novel fibrin-binding peptides as a delivery vehicle for proteins into fibrin matrices

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Statement of Purpose:

As the field of tissue engineering develops and we understand how cells respond to their physical and chemical environment, the impetus has been to move away from passive matrices and towards functionalized matrices that will be a better substitute for the tissue that is being replaced. While several synthetic polymer systems have shown promise, fibrin, the natural blood clotting protein, still represents the most useful, biologically relevant polymer system for regenerative medicine applications. Fibrin, as the matrix of choice produced in the body in response to injury, is currently used worldwide in surgeries as a sealant and adhesive. However, its role may be expanded for the delivery of protein factors at the site of application. There have been many approaches in the functionalization of fibrin scaffolds but until recently, few have attempted to take advantage of the natural binding sites and activities of fibrin for protein attachment. The best-known case is the use of Factor XIIIa crosslinking sites for the covalent incorporation of recombinant proteins into fibrin. However, since Factor XIIIa displays extremely slow enzyme kinetics, this technology is significantly limited in *in situ* polymerization applications. We have developed a system using fibrin-binding peptides (FBPs) that display inherent fibrin binding capacity, thus eliminating the need for additional factors for their incorporation. Despite their non-covalent nature, these interactions are extremely stable. Moreover, proteins fused with these FBPs may have a targeting capacity to fibrin clots, allowing the delivery of factors to sites of injury. In this study, fibronectin type III repeats ($FNIII_{9-10}$) were used as a model protein displaying the FBPs.

Methods:

Production of the fusion protein. Variants of the FBP sequence were created in the E. coli expression plasmid pGEX4T using the Stratagene QuikChange® II-E site-directed mutagenesis kit. The open-reading frame for $FNIII_{9-10}$ was inserted in-frame using standard molecular biology techniques. All plasmids were sequence-verified. Proteins were expressed in the E. coli expression strain, BL21, and purified using FPLC. Protein purity, concentration and identity were verified via Western blots and ELISA.

Binding kinetics. Data for the binding kinetics were obtained using Surface Plasmon Resonance (SPR) on Biacore CM5 gold sensor chips (coated with carboxymethylated dextran). Fusion proteins were flowed over (and passively attached to) the chips, followed by purified fibrinogen.

Polymer characterization. Hydrogels were obtained by pipetting thrombin into mixtures of fibrinogen and the fusion protein. SEM images of fixed and critical-point-dried samples were obtained from the Zeiss Ultra60.

Biochemical characterization. Polymerization kinetics were observed via absorbance readings at 350nm. Briefly, a solution of 4 mg/ml fibrinogen containing various doses of the recombinant fusion protein in TBS with 10mM $CaCl_2$ was place in the well of a 96-well plate. An equal volume of thrombin (2 U/ml) was added, the solution titurated and the absorbance at 350 nm measured kinetically.

Results:

Sequencing and western blots demonstrated the fidelity of the synthesized fusion proteins (FBP- $FNIII_{9-10}$). SPR studies showed that these fusion proteins were capable of binding to fibrinogen (Figure 1). Further dose response and competitive binding assays will be conducted.

Tethered protein	Tethered mass(ng/cm²)	Bound FGN (ng/cm2)
Var A	26.5	79.0
Var B	55.4	5.8

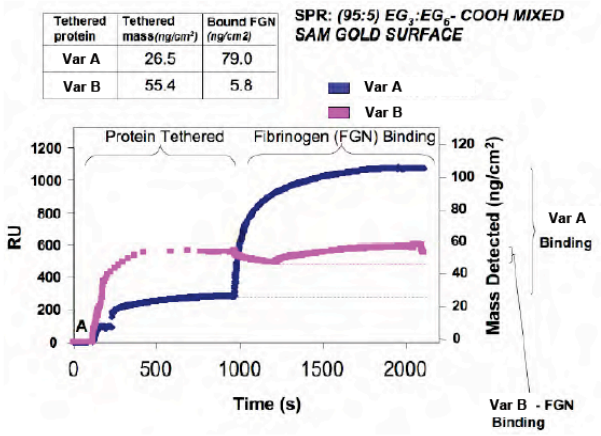

Figure 1. Binding kinetics of fibrinogen to fusion proteins attached to the SPR chip.

Absorbance readings of the polymerizing hydrogels indicated that the fusion protein inhibits the formation of the fibrin network at competitive stoichiometries, but not at lower doses. Based on preliminary SEM results we do not believe that the structure of the fibrin network is significantly affected at the lower dosages of protein incorporation used but further studies are necessary.

Conclusions/Future Studies:

We have successfully produced FBP fusion proteins and these have been shown to be capable of binding to fibrinogen. We are confident that supporting assays, such as viscoeleasticity, release kinetics, and degradation kinetics assays will support our hypothesis that the incorporation of these fusion proteins will not significantly affect the polymer properties and that release profiles can be tuned based on the peptide sequence and its non-covalent binding affinity to fibrin polymer.

References:

[1] Litvinov RI, et al. Blood. 2005;106(9): 2944-2951
[2] Ehrbar M, et al. Biomaterials. 2008;29(11):1720-1729