FINAL REPORT
R21EB008463
Barker, TH (PI)

A. Specific Aims

Our long-term research goal is to understand and exploit the natural biochemical properties of the fibrinogen/fibrin system for greater control of fibrin polymerization and degradation. In R21EB008463, we hypothesized that a fibrin knob fusion with thermo-responsive elastin-like peptide repeats will bind to fibrinogen polymerization pockets and prevent polymerization in a temperature-dependent manner. We proposed 2 specific aims to address this hypothesis; SA1) *design and express recombinant proteins displaying N-terminal fibrin polymerization knobs, and SA2) create recombinant knob-ELP fusion proteins and test their fibrinogen-binding/fibrin blocking activity.*

B. Studies and Results

Overview: This project consists of two aims, with Year 1 being primary devoted to the completion of Aim 1 and Year 2 focused on the completion of Aim 2. Specific Aim 1 focuses on the core technology and establishing the capacity of native and mimetic fibrin knob peptide sequences to retain their fibrinogen/fibrin binding activity when presented in the context of a non-fibrin element. We initially proposed and tested a model protein based on fibronectin type-III repeats that is biotechnologically easy to produce and would facilitate straight-forward analysis and interpretation in directing binding studies. Our Aim 1 studies confirm the hypothesis that fibrin knob-type III repeat fusion proteins exposed to fibrinogen retain high affinity for fibrinogen. This is demonstrated through numerous direct binding studies and competition analysis with free fibrin knob peptides. The binding of fibrin knob peptides to polymerization pockets should confer inhibitory activity in polymerization assays. Initial experimentation confirms this, although the inhibition was determined to be time sensitive and thus, upon activation, fibrinogen is still capable of forming fibrin polymer in the presence of fibrin knob fusion proteins, albeit at a much slower rate. We have extended our study to include the use of PEG. We believe this alternative approach may serve two roles. First, PEG is known to significantly inhibit protein-protein interaction and thus may confer additional activity to the fibrin knob peptides. Second, PEGylation is a more readily scalable process than protein production, thus the technology may be more readily applied in future animal and human studies. We are currently completing our proposed studies for Aim 1 and anticipate a manuscript submission in the next 4 months. Once complete we begin experimentation for Aim 2.

***Specific Aim 1**: Design and express recombinant proteins displaying N-terminal fibrin polymerization knobs.*

The NOVEL fibrin knob mimic GPRPFPAAC displays far superior fibrinogen binding characteristics.

Using molecular dynamics simulations (MDS) we explored the structural landscape of fibrin knob mimics and correlated those data with quantitative peptide-protein binding studies performed with surface

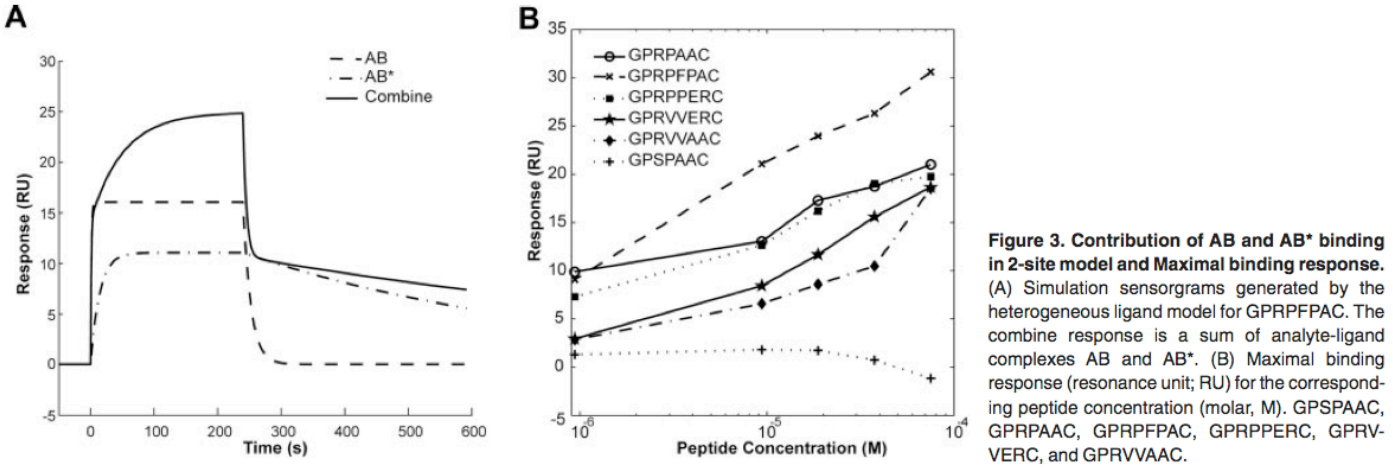
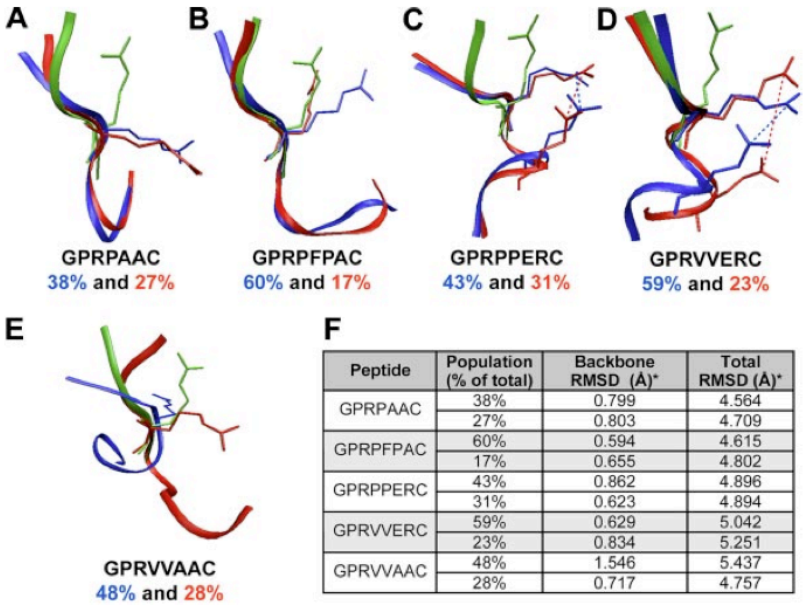


Figure 3. Contribution of AB and AB* binding in 2-site model and Maximal binding response. (A) Simulation sensorgrams generated by the heterogeneous ligand model for GPRPFPAC. The combine response is a sum of analyte-ligand complexes AB and AB*. (B) Maximal binding response (resonance unit; RU) for the corresponding peptide concentration (molar, M). GPSPAAC, GPRPAAC, GPRPFPAC, GPRPPERC, GPRVVERC, and GPRVVAAC.

Figure 1 from Stabenfeldt S, *Blood* 2010, 116 1352-9

plasmon resonance (SPR; **Figure 1**). The findings, reported in *Blood*, indicate that the binding affinity of fibrin knob peptides to fibrinogen are dominated by the structure of the peptides in solution. The backbone stability and the position of the Arg3 side chain (trans, gauche +/-) apprear critical to predicting the binding affinity (**Figure 2**). Peptides with high structural stability and whose Arg3 side chain is predominately in a gauche + conformation display the highest binding affinities to fibrinogen and fibrin fragment D. This study is significant since it is the first demonstration of knob peptide structure in solution and the first that explores the determinants of peptide "docking" to the fibrinogen molecule. It also defines the design criteria for regulating knob peptide binding affinity.

Figure 4. Structural analysis. Representative trajectories of the top 2 (blue and red) most populated groups from hierarchical cluster analysis superimposed on the active GPRP or GPRV conformation (green); GPRPxxx peptides were compared with active GPRP and GPRVxxx peptide were compared with active GPRV. (A) GPRPAAC, (B) GPRPFPAC, (C) GPRPPERC, (D) GPRVVERC, and (E) GPRVVAAC. (F) The population percentage represents the percentage of total number trajectories in 1 conformational cluster; the top 2 populated clusters at the fourth level of the hierarchical cluster are reported. RMSD calculations for the first 3 residues were in reference to the active conformation (ie, GPRP or GPRV); both backbone and total RMSDs were calculated after optimal superposition along the backbone.



F

Peptide	Population (% of total)	Backbone RMSD (Å)*	Total RMSD (Å)*
GPRPAAC	38%	0.799	4.564
	27%	0.803	4.709
GPRPFPAC	60%	0.594	4.615
	17%	0.655	4.802
GPRPPERC	43%	0.862	4.896
	31%	0.623	4.894
GPRVVERC	59%	0.629	5.042
	23%	0.834	5.251
GPRVVAAC	48%	1.546	5.437
	28%	0.717	4.757

Figure 2 from Stabenfeldt S, *Blood* 2010, 116 1352-9

Fibrin knob-FN fusions bind specifically and efficiently to fibrinogen and are retained within fibrin polymers.

Using the fibronectin 9-10 Type III repeats (FN) as our model protein, we have shown that non-fibrin proteins displaying knobs are capable of binding fibrinogen in SPR (**Figure 3**). Further demonstrating specificity of the binding, short synthetic fibrin knob peptides were added to block the knob-binding pockets on fibrinogen. While high concentrations of these recombinant proteins were capable of inhibiting fibrin polymerization, we observed that they are more useful in targeting and retention within fibrin polymers (**Figure 4**). This study is significant in that it presents a novel mechanism for fibrin drug delivery that is significantly simpler than previously published methods that rely on enzymatic crosslinking, etc.

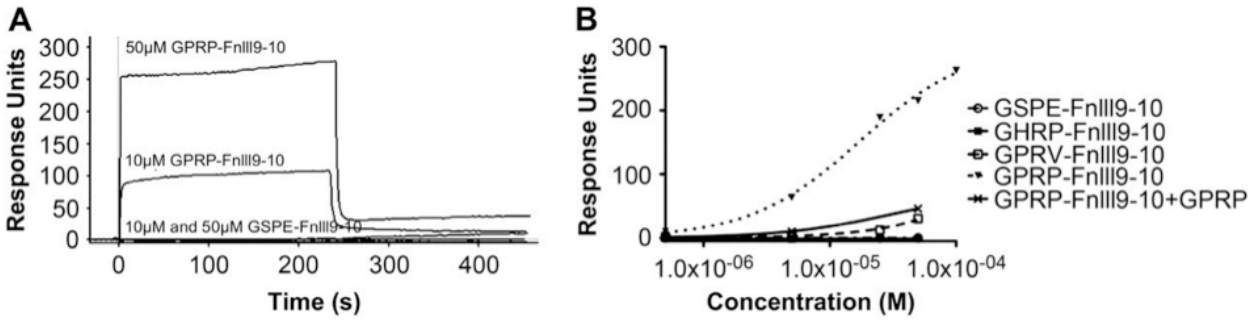
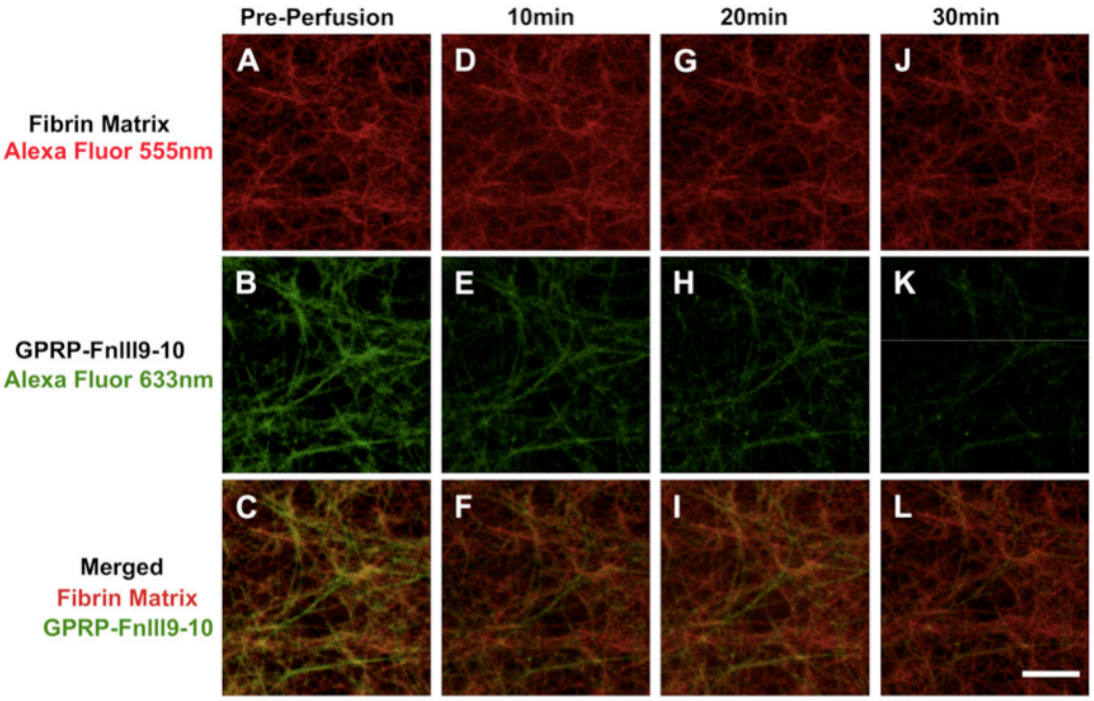


Fig. 3. SPR studies demonstrating Gxxx-FnIII9–10 affinity for fibrinogen fragment D. (A) Representative sensorgrams showing protein-protein interaction between immobilized fragment D and GPRP-FnIII9–10 but not the non-binding control GSPE-FnIII9–10. Binding interactions were observed as an increase in response units (1 RU = 1 pg/mm^2). (B) At equilibrium, the sensorgram responses for each analyte were plotted against the injection concentration and fit to a single-site equilibrium model. Global fits were performed to determine K_D. The highest affinity was observed with GPRP-FnIII9–10.

Figure 3 from Soon ASC, *Biomaterials* 2010, 31 1944-54



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Time	Mean Intensity		Relative Area of Colocalization	Colocalization Coefficient		Correlation Coefficient (R)
	Fibrin	GPRP-Fn		Fibrin	GPRP-Fn	
0 min	100±33	100±33	94.2%	0.981	0.965	0.47
10 min	105±22	55±14	28.5%	0.325	0.956	0.19
20min	105±22	52±13	19.1%	0.219	0.948	0.14
30min	99±19	51±12	12.8%	0.159	0.926	0.11

Fig. 8. Incorporation and retention of GPRP-FnIII9–10. A 6 μM fibrinogen and 0.6 μM GPRP-FnIII9–10 solution was incubated for 30 min. Upon adding 1 U/mL thrombin and 1 U/mL factor XIIIa, the solution was injected into the microfluidic channel and allowed to clot for 1 h. Confocal micrographs were taken before perfusion (A–C), 10 min (D–F), 20 min (G–I), and 30 min (J–L) after perfusing Tris + Ca buffer at a rate of 10 μL/min. Colocalization of the fibrin and GPRP-FnIII9–10 (A–L) was immediately observed after polymerization. Subsequent perfusion of buffer through the matrix diminished the GPRP-FnIII9–10 signal over a 30 min period. (M) Quantitation of signal intensity and colocalization at each time point. Red = Fibrin conjugated to Alexa Fluor-555; Green = GPRP-FnIII9–10 conjugated to Alexa Fluor-633. Scale bar = 10 μm.

Figure 4 from Soon ASC, *Biomaterials* 2010, 31 1944-54

Multivalent PEGylated fibrin knob peptides can inhibit thrombin-mediated polymerization, but are also capable of altering the dynamics of polymerization and end polymer structure.

PEGylation of knob peptides was explored to enhance their ability to inhibit fibrin polymerization, this being a major goal of the study. We explored the use of mono-, bi-, and multi-functional PEG moieties, specifically moPEG (2.5-30 kD), biPEG (2.5-30 kDa), and mPEGx (5-40 kDa). Monofunctional PEG inhibited polymerization at concentrations 100-fold less than the unmodified peptides (**Figure 5**) however, multifunctional PEGs modified with the knob peptide support multimerization of fibrinogen, even prior to the addition of thrombin. We chose to explore this phenomena further and found that multifunctional knob-PEG structures initially support the formation of an amorphous polymer with fibrinogen and integrate within thrombin-catalyzed fibrin polymers. The result is a fibrin polymer with different polymerization dynamics and resulting structure (**Figure 6**).

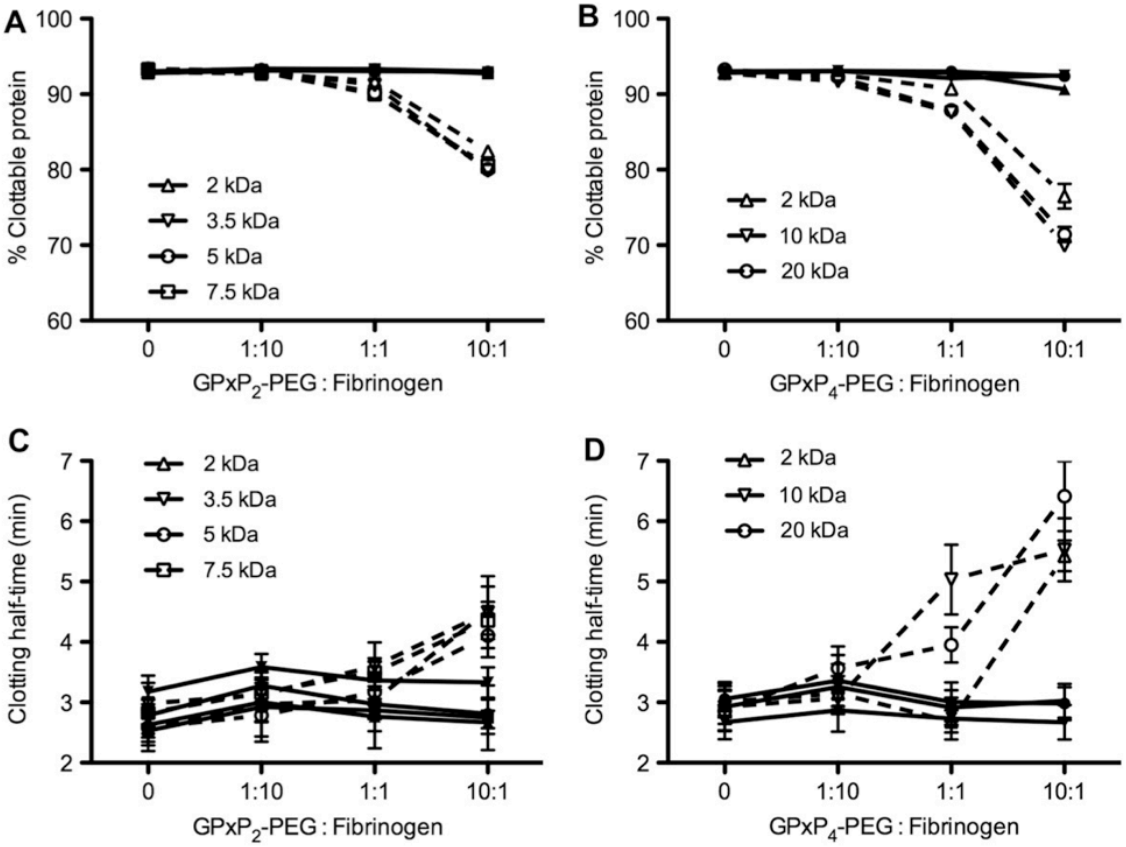

Fig. 3. Fibrin polymerization parameters in the presence of peptide-PEG conjugates. % Clottable protein of hydrogels formed in the presence of $GPxP_2$-PEG (A) and $GPxP_4$-PEG conjugates (B) at 1:10, 1:1 and 10:1 conjugate-to-fibrinogen molar ratios. Clotting half-times in the presence of $GPxP_2$-PEG (C) and $GPxP_4$-PEG conjugates (D). Solid symbols and lines correspond to data for the $GPSP_n$-PEG control conjugates; empty symbols and dashed lines correspond to data for the $GPRP_n$-PEG conjugates.

Figure 5 from Soon ASC, *Biomaterials* 2011, 32 4406-4414

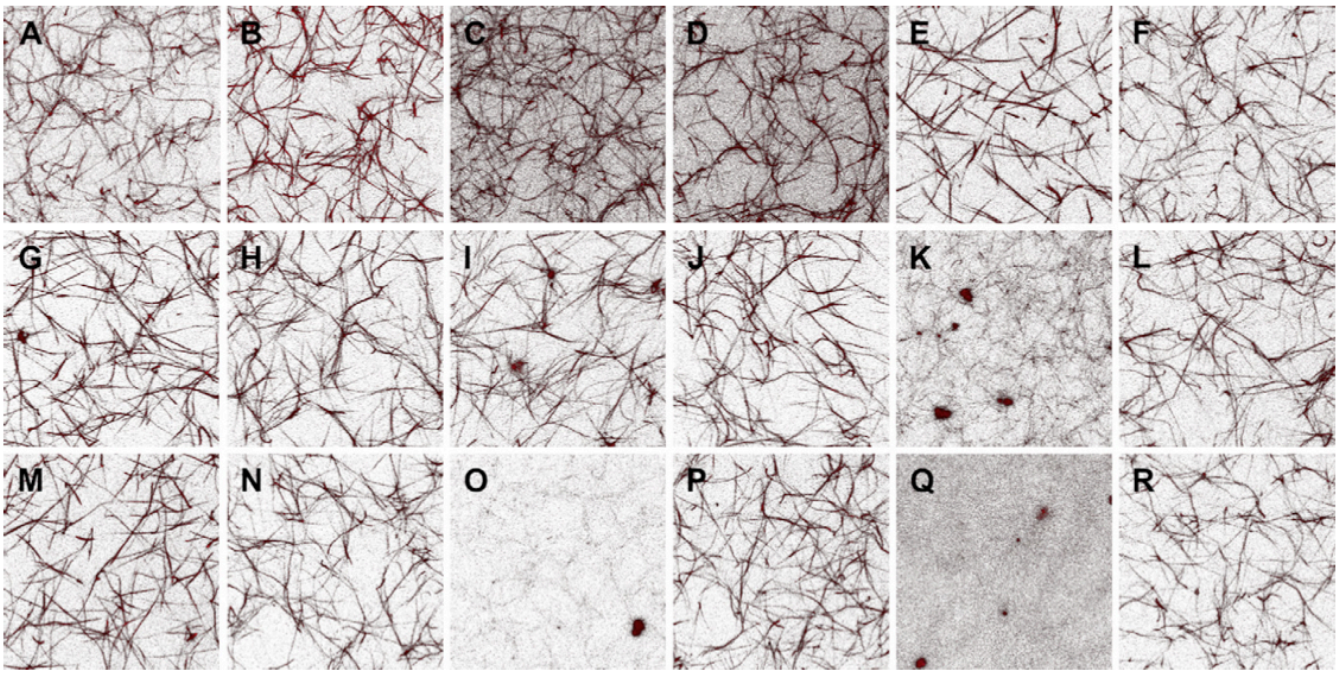

Fig. 6. Confocal images of hydrogels. Hydrogels were formed within sealed chambers on glass slides and imaged using laser scanning confocal microscopy. Images were rendered from 45 × 45 × 10 μm slices. (A) Control clot (1 mg/mL fibrinogen, 0.25 NIH U/mL thrombin, 5 Loewy units/mL factor XIIIa). (B) Representative network structure formed in the presence of 100:1 molar ratio of PEG-to-fibrinogen. (C) Network structure in the presence of 10:1 molar ratio of GPRP-to-fibrinogen. (D) Network structure in the presence of 10:1 molar ratio of GPSP-to-fibrinogen. Subsequent paired images are of hydrogels formed in the presence of $GPRP_n$-PEG or $GPSP_n$-PEG conjugates at a 1:1 conjugate:fibrinogen molar ratio as follows: 2 kDa $GPxP_2$-PEG (E,F), 3.5 kDa $GPxP_2$-PEG (G,H), 5 kDa $GPxP_2$-PEG (I,J), 7.5 kDa $GPxP_2$-PEG (K,L), 2 kDa $GPxP_4$-PEG (M,N), 10 kDa $GPxP_4$-PEG (O,P), 20 kDa $GPxP_4$-PEG (Q,R).

Figure 6 from Soon ASC, *Biomaterials* 2011, 32 4406-4414

C. Significance

The goal of R21EB008463 was the use of fibrin knob peptide-modified proteins to 1) bind fibrinogen and 2) inhibit polymerization and to regulate this activity through the use of thermally sensitive proteins like elastin-like polypeptides. We successfully created a 'plug-and-play' protein expression system that enable the creation of recombinant proteins that properly display the fibrin knob motif at the N-terminus. We successfully demonstrated that proteins made in this system bind fibrinogen and are retained in fibrin polymers. As an alternative approach we used synthetic polymers in conjugation to knob motifs and demonstrated the ability of these constructs to inhibit polymerization of fibrinogen upon exposure to thrombin and at subcritical doses to significantly alter fibrin structure. Lacking for our success has been the realization of thermally sensitive knob-displaying proteins for thermal control of fibrin polymerization. The construction of such proteins proved difficult and the initial design of a single polypeptide chain (ELP-knob) was critically flawed since the thermal transition of the ELP protein results in massive precipitation of the ELP and fibrinogen. In future studies we will make attempts at designing ELP nanoparticles that may circumvent these initial design flaws and some will also work on ELP-synthetic hybrid nanoparticles.

The significance of the work preformed under R21EB008463 are 1) the first report of knob peptide structures in solution and the first quantitative study on structure-function relationships of these unique peptides, 2) the first demonstration of using fibrin knob peptides as a fibrin targeting/delivery method, 3) the demonstration that multifunction PEG-knob conjugates have the capacity to integrate with the polymerizing fibrin and significantly alter the end structure. The studies conducted with R21EB8463 lead to our successful R01 application (R01EB011566).

D. Deliverables *(See Attachments)*

1. 13 oral and poster presentations over the 3 year grant period (03/01/08 – 02/28/11)
2. Soon ASC, *Biomaterials* 2010, 31 1944-54
3. Soon ASC, *Biomaterials* 2011, 32 4406-4414
4. Stabenfeldt S, *Blood* 2010, 116 1352-9
5. Barker, T.H. Peptides for Binding Fibrinogen and Fibrin. GTRC 4021, *U.S. Patent* 61/089,180; 2010.