Modulating Fibrin Matrices Using Knob$_x$-PEG Conjugates

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Statement of Purpose: Fibrin is a provisional protein matrix that forms in response to vascular injury, acting to stimulate and support cellular infiltration. The thrombin-catalyzed conversion of fibrinogen to fibrin involves the cleavage of fibrinopeptides, exposing N-terminal knobs that bind non-covalently to complementary C-terminal pockets on fibrin(ogen) to form fibrin networks. These networks are further stabilized by transglutaminase factor XIIIa that crosslink the alpha and gamma chains of fibrin(ogen). Fibrin network formation can be manipulated through variables such as substrate or enzyme concentration and buffer composition. Current surgical formulations of fibrin sealants use high concentrations of fibrinogen and enzymes, resulting in a tight meshwork that forms quickly but is not optimum for cellular infiltration. We propose a novel method of modifying fibrin network structure through the use of multiarm PEGs (polyethylene glycol) terminating in knob peptides that can form non-covalent networks with fibrinogen. We have previously shown that knob-protein fusions preserved their ability to bind fibrin(ogen) and hypothesize that knob$_x$-PEG conjugates will also retain their fibrin(ogen)-binding capacity. The resulting fibrinogen/PEG hybrid networks should manifest altered clotting kinetics upon the addition of thrombin and factor XIIIa, creating fibrin/PEG hydrogels with tunable mechanical properties.

Methods: *Production of knob$_x$-PEG conjugates*. Maleimide-activated PEGs of varying sizes and architectures were reacted with molar excess cysteine-terminated synthetic knob peptides. The dialyzed and lyophilized product was quantitated using Sims and Snape method for PEG and the CBQCA assay for amines.

Clotting assays. Fibrin clotting was followed kinetically using real-time turbidity measurements (A_{350}) of mixtures in 96-well plates. Following, the clottability of these mixtures was quantitated using the protein Quant-iT assay. The extent of factor XIIIa crosslinking was analyzed using protein band intensities on Coomassie-stained SDS-PAGE gels, normalized against the non-crosslinked beta chain. The biochemical makeup (i.e. α-α, γ-γ, or α-γ crosslinks) of higher molecular weight crosslinked products will be further identified via Western blots using antibodies specific against the alpha and gamma chains of fibrin(ogen).

Mechanical characterization. Viscoelastic measurements of the hydrogels will be obtained using the Bohlin CVO rheometer with plate-plate geometry. Fibrin clot permeability will be analyzed using the method of Sjoland. SEM images of fixed and critical-point-dried samples will be obtained using the Zeiss Ultra60.

Results: We have successfully produced a range of 1-arm, 2-arm and 4-arm knob$_x$-PEG conjugates of sizes 2 kDa to 20 kDa. As expected, fibrinogen/PEG mixture turbidity following enzyme addition is affected by conjugate dosage, and PEG size and architecture. Here, we present data using the 5 kDa knob-PEG, 5 kDa knob$_2$-PEG and 10 kDa knob$_4$-PEG conjugates. Mixture clottability upon enzyme addition was less impacted at conjugate:fibrinogen ratios near native knob:pocket ratios of unity (each fully activated fibrin molecule has 4 knobs and 4 pockets) or lower (Fig. 1A). The extent of crosslinking upon the addition of fXIIIa also shows an increase as compared to fibrinogen-only control mixtures (Fig. 1B), congruent with results reported by Lorand et al. using a 900 Da knob$_2$-PEG conjugate [1]. We are currently evaluating the effect of knob$_x$-PEG size and architecture on the mechanical and physiological chracteristics of such fibrin(ogen)/PEG hydrogels.

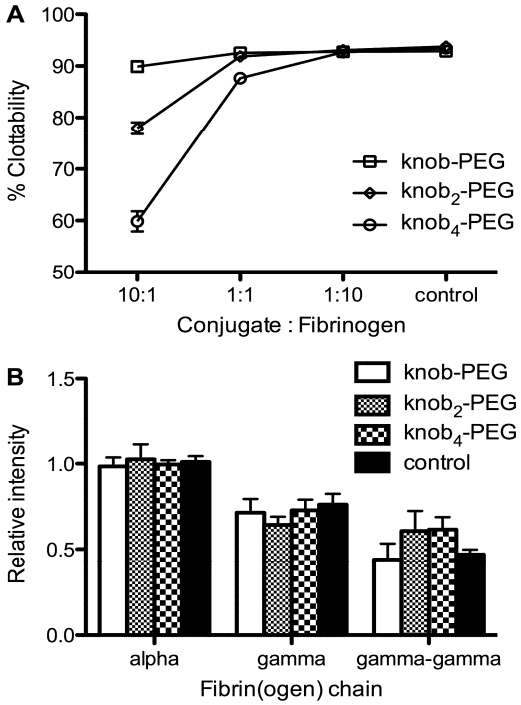

Fig. 1. (A) % clottability of fibrinogen mixtures in the presence of the indicated molar ratios of knob$_x$-PEG conjugates. (B) Relative band intensities of the alpha and gamma chains of fibrinogen/PEG mixtures at a 1:1 ratio upon the addition of factor XIIIa. Mean ± SEM.

Conclusions: We have employed the widely used sulfhydryl-maleimide conjugation chemistry for the creation of multiarm PEG structures terminating in knob peptides. We have shown that these multivalent knob$_x$-PEG conjugates can significantly modify fibrin clotting kinetics, suggesting that they will alter the global mechanical characteristics of the resulting hydrogel. The addition of knob$_x$-PEG conjugates to commercial formulations of fibrin sealants should allow us to generate mechanically strong but porous hydrogels that would facilitate wound repair and enhance tissue regeneration.

References: [1] Lorand L. et al. Proc Natl Acad Sci USA. 1998; 95:537-541.