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Molecular interference of fibrin's divalent polymerization mechanism enables modulation of multiscale material properties

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ABSTRACT

Protein based polymers provide an exciting and complex landscape for tunable natural biomaterials through modulation of molecular level interactions. Here we demonstrate the ability to modify protein polymer structural and mechanical properties at multiple length scales by molecular 'interference' of fibrin's native polymerization mechanism. We have previously reported that engagement of fibrin's polymerization '*hole b*', also known as 'b-pockets', through PEGylated complementary '*knob B*' mimics can increase fibrin network porosity but also, somewhat paradoxically, increase network stiffness. Here, we explore the possible mechanistic underpinning of this phenomenon through characterization of the effects of *knob B*-fibrin interaction at multiple length scales from molecular to bulk polymer. Despite its weak monovalent binding affinity for fibrin, addition of both *knob B* and PEGylated *knob B* at concentrations near the binding coefficient, K_d, increased fibrin network porosity, consistent with the reported role of *knob B-hole b* interactions in promoting lateral growth of fibrin fibers. Addition of PEGylated *knob B* decreases the extensibility of single fibrin fibers at concentrations near its K_d but increases extensibility of fibers at concentrations above its K_d. The data suggest this bimodal behavior is due to the individual contributions *knob B*, which decreases fiber extensibility, and PEG, which increase fiber extensibility. Taken together with laser trap-based microrheological and bulk rheological analyses of fibrin polymers, our data strongly suggests that *hole b* engagement increases in single fiber stiffness that translates to higher storage moduli of fibrin polymers despite their increased porosity. These data point to possible strategies for tuning fibrin polymer mechanical properties through modulation of single fiber mechanics.

1. Introduction

Protein-based polymers, such as those derived from extracellular matrix proteins, are widely utilized as biomaterials due to their inherent biocompatibility and utility in a range of medical and tissue engineering applications. Protein-based polymers provide a complex landscape for modulation at the molecular level, through either chemical modification or modulation of specific molecular interactions with the protein. Modification of protein-based polymers at the molecular level can affect material properties at the bulk scale; such modifications could provide a rich parameter space for rational design of biomaterial properties. However, modification of proteins at this length scale has been underutilized in the fields of biomaterials and tissue engineering. Here we demonstrate the ability to modify protein polymer structural and mechanical properties over multiple length scales by simple molecular 'interference' of the polymerization mechanism of the widely utilized protein polymer, fibrin.

Fibrin is a hydrogel formed from the naturally derived blood clotting protein fibrinogen. It is widely utilized for a number of

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biomedical applications due to its intrinsic ability to provide cell instructive cues to direct regenerative processes as well as its ability to be degraded by natural proteolytic processes [1,2]; it is the body's natural provisional wound-healing matrix. Fibrin monomer is derived from proteolytic cleavage of the soluble precursor molecule, fibrinogen, by the serine protease thrombin [3]. Upon activation fibrin monomers self-assemble through '*knob-hole*' interactions to form an insoluble network [4,5]. Fibrin network properties, both structural and mechanical, are influenced by the polymerization conditions including initial fibrinogen and thrombin concentrations, buffer ionic strength, pH, and calcium concentration [6–8]. Commercially available fibrin products utilize exceptionally high concentrations of fibrinogen and thrombin to achieve fast polymerization and impart mechanical integrity to the constructs [2]. However, typical fibrinogen concentrations of these products (30–100 mg/mL) are an order of magnitude higher than physiological circulating concentrations (~3 mg/mL). Despite their attractive physical properties, these high concentrations of fibrinogen and thrombin result in fibrin networks that lack the porosity necessary to facilitate optimal cellular infiltration [9]. There is interest, therefore, in modifying fibrin matrices to allow for increased network porosity while maintaining fast polymerization dynamics and mechanical integrity. To that end our group has previously created synthetic peptides variants that engage native fibrin polymerization mechanisms to altered network properties [10,11].

Fibrinogen is comprised of two identical subunits, which each contain three chains known as the Aα, Bβ and γ chains. The 'A' and 'B' designation refer to N-terminal 16- and 14-amino acid peptides, respectively, which are released by thrombin cleavage (activation), leading to exposure of peptide sequences at the N termini of the α and β chains, termed knobs *A* and *B*, respectively. *Knobs A* and *B* interact with complementary *holes a* and *b* located in the two distal 'D domains' of the γ and β chains on neighboring fibrinogen molecules. Because *holes a* and *b* do not require enzymatic cleavage to bind to their respective knob peptides, synthetic *knob* peptides have been utilized to modify fibrin network architecture for tissue engineering and/or drug delivery applications. Our group has created a variety of *knob* mimic constructs including *knob-A*-protein constructs [10], PEGylated *knobs A* and *B* [11,12] and *knob A* modified elastin like peptide micelles [13] and we have characterized their effect on fibrin properties such as polymerization, degradation, mechanical properties and network structure.

The N-terminal Gly–Pro–Arg (GPR) motif found on the α chain is the minimum *knob A* sequence required to facilitate binding to a complementary *hole a* located in the γ chains [14,15]. The human *knob B* motif is comprised of the N-terminal Gly–His–Arg–Pro (GHRP) motif and is complementary to *hole b* located in the β chains [16]. *A:a* interactions appear to be the primary contributor to fibrin polymerization; polymerization is inhibited in the presence of high concentrations of a synthetic *knob A* of the sequence Gly–Pro–Arg–Pro (GPRP) [15]. *B:b* interactions appear to be less crucial in primary fibrin formation and clots can be formed in the absence of *knob B* exposure [4,17]. There is some debate over the physiological relevance and specific functional role of *B:b* interactions, however our and others' studies suggest that *B:b* interactions promote lateral aggregation and play a role in determining clot stability and susceptibility to degradation [11,18].

Because *B:b* interactions are not essential to fibrin network formation, modification of fibrin network properties through synthetic *knob B* mimics is perhaps more attractive than through *knob A* mimics because one can alter properties without adversely affecting primary polymerization. Despite a few reports of *knob-hole* cross-reactivity, the *knob B* mimic derived from chicken (AHRP) has previously been demonstrated to only bind to *hole B* [19,20]. Further studies with the bovine *knob B* mimic (GHRPY) demonstrated that the Tyr5 residue contributes to an altered molecular packing of fibrinogen molecules that leads to altered network structure and delayed fibrinolysis [18,21]. We recently utilized a PEGylated *knob B* mimic, AHRPYAAC, to combine the *knob B* specificity of the AHRP sequence with the altered molecular packing of fibrinogen molecules of XHRPY sequences [11]. This PEGylated- AHRPYAAC construct enhanced the porosity of the fibrin network, decreased susceptibility to degradation and increased the complex modulus (G^*). These prior studies were performed at a 1:1 molar ratio of fibrinogen:knob mimic to allow for direct comparison with equimolar concentration of PEGylated *knob A* mimics, however the reaction kinetics of *A:a* and *B:b* interactions vary greatly. Furthermore, the initial release of *knob A* by thrombin cleavage is significantly faster than the exposure of *knob B*, but as polymerization proceeds, the rate of *knob B* exposure increases, a process thought to be driven by conformation changes [22]. Utilizing *knob B* mimics at concentrations which are close to its K_d would allow for more robust control over fibrin network properties mediated by pre-engagement of the *hole b*.

We hypothesized that the effect of AHRPYAAC-PEG on fibrin network properties would be more pronounced at concentrations near the K_d of *B:b* interactions. Here, we first characterize the binding kinetics of free and PEGylated *knob B* mimics to fibrinogen fragment D and then investigate the effect of these synthetic *knobs* on fibrin network properties at concentrations below, near and above the K_d. In these studies, we characterize the effect of PEGylated *knob B* on clot properties at multiple length scales by analyzing events at the molecular, nano- and micro-scale.

2. Materials and methods

2.1. Preparation and characterization of PEGylated knobs

Knob B and non-binding control cysteine-terminated peptides, AHRPYAAC and GPSPFPAC respectively, were custom-ordered from Genscript (Piscataway, NJ) in lyophilized form. Peptides were conjugated to 5 kDa maleimide-PEG (JenKem Technology, Allen, TX) by reacting components at a 10:1 peptide to PEG molar ratio in 100 mM phosphate buffer pH 7.2, 150 mM NaCl, 10 mM EDTA for four hours at room temperature. Excess unconjugated peptide was removed from the conjugated product through dialysis overnight into deionized water utilizing 2 kD molecular weight cutoff membranes (Slidalyzer, Thermo Fisher Scientific). The product was aliquoted, lyophilized and quantified. PEG and peptide concentrations were quantified through a barium chloride PEG assay and the CBQCA amine assay, respectively. Briefly, the barium chloride assay for PEG quantitation was based on the method of Sims and Snape and modified for a 96-well plate format [23]. 80 μL of sample was incubated with 20 μL of barium chloride solution (5% in 1 M HCL). 10 μL of 0.1 N iodine solution was then added to each well and absorbance was measured at 535 nm using a plate reader. PEG concentration was calculated through the use of a standard curve generated with unconjugated 5 kD PEG. Peptide concentration was then determined using the CBQCA assay kit (Invitrogen, Carlsbad, CA) according to manufacture specifications using unconjugated AHRPYAAC or GPSPFPAC peptides to generate standard curves.

2.2. Preparation of fibrinogen fragment D

Human fibrinogen (FIB 3, Enzyme Research Laboratories (ERL)) at 2 mg/mL was digested with 0.1 U/mL human plasmin (ERL) in HEPES (N-2-hydroxyethylpiperazine-N_-2-ethanesulfonic acid) $CaCl_2$ buffer (150 mM NaCl, 5 mM $CaCl_2$, 25 mM HEPES; pH 7.4) overnight at room temperature. Fragment D was isolated by incubating the plasmin-digested fibrinogen with GPRPAA beads at room temperature for 30 min, with occasional agitation [24,25]. The unbound proteins and protein fragments were removed with excessive washing with HEPES + $CaCl_2$ buffer. Fragment D was eluted with 1M sodium bromide and 50 mM sodium acetate (pH 5.3). Eluted samples were pooled together and exchanged back into HEPES + $CaCl_2$ buffer with a centrifugal filter (molecular weight cutoff, 10,000 Da). Fragment D was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stored at −80°C until use.

2.3. Surface plasmon resonance

The Biacore 2000 (Biacore Lifesciences, GE Healthcare) was used to investigate kinetic binding constants (k_a and k_d) of *knob* peptide variants for fibrinogen Fragment D, which contains the polymerization *holes*. Briefly, Fragment D was covalently immobilized to gold-coated SPR sensor chips via self-assembled monolayer surface chemistry to generate a nonfouling surface with a controlled density of reactive