FVII Dependent Coagulation Activation in Citrated Plasma by Polymer Hydrogels

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Polymer hydrogels containing positively charged functional groups were used to investigate the critical material and biological components of FVII activation and subsequent fibrin formation in citrated plasma. A FVIIa ELISA confirmed the ability of the polymer to induce FVII activation and provided insight into the material parameters which were influential in this activation. Experiments utilizing coagulation factor depleted and inhibited plasmas indicated that FVII, FX, FII, and FI are all vital to the process outlining the general mechanism of fibrin formation from the onset of FVII activation. Dynamic mechanical analysis and swelling experiments were used to establish a critical correlation between polymer microstructure and FVII activation.

Introduction

Blood coagulation is a complex process involving a series or "cascade" of enzyme activation reactions. At each stage a precursor protein (zymogen) is converted to an active protease by cleavage of one or more peptide bonds in the precursor molecule. Activation of the coagulation cascade is believed to occur through two main pathways: the contact activation pathway and/or the tissue factor pathway. These two pathways converge in the activation of a final common pathway, which in turn results in the formation of a cross-linked fibrin network.[1,2] The activation and sustainment of the tissue factor pathway involves the coordination of a multitude of enzymes and cofactors including factor VII (FVII), calcium, tissue factor pathway inhibitor (TFPI), tissue factor (TF), and a phospholipid surface.[3] Human coagulation FVII is a single chain, glycoprotein which circulates in normal human blood, and is the main activator of the tissue factor pathway.[3,4] Cleavage of a single peptide bond results in a structural change that activates the zymogen, transforming it into a potent vitamin K-dependent serine protease.[5] This structural change also allows for the protein to effectively bind to its cofactor, TF, in the presence of calcium.[6,7] The distribution of TF is carefully coordinated in a hemostatic layer surrounding the vascular endothelium.[8−12] Upon disruption of the endothelium TF forms a stable complex with activated FVII (FVIIa) within blood, and this complex (TF-FVIIa) is capable of directly initiating the common pathway via the activation of FX or indirectly through the activation of FIX, which in turn is able to activate FX.[13] TF is most effective as a procoagulant when incorporated into a phospholipid membrane, a surface which is typically provided by platelets.[14−16] TFPI is the main inhibitor of the tissue factor pathway, exerting its inhibitory action on the FVIIa-TF-FX complex.[17]

There has been considerable research into the effects of charged surfaces on the coagulation cascade. The ability of negatively charged surfaces, such as glass or the mineral kaolin, to initiate the contact activation pathway in blood or plasma via activation of FXII has been known for over 50 years.[18−20] Recently, research has shown that negatively charged polyphosphate species are capable of enhancing fibrin clot structure in buffer solutions.[21] It had previously been discovered by Bjourn et al.,[22] that highly positively charged materials have the ability to activate FVII. Later research by Pederson et al.[23] explained the ability of these positively charged surfaces to activate FVII via an autoactivation theory. Other research groups have shown that amine containing polymers such as poly(lysine) are capable of enhancing the activation of various coagulation proteins, including FVII, FX, and FII in various buffers.[23−29]

Although the ability of charged surfaces to induce specific coagulation factor activation is well-known, the roles that specific material properties have in the activation is not well understood. Our research helps to establish a better understanding of the effects that material properties, specifically the interplay of electrostatic charge and microstructure, have on FVII dependent coagulation activation. A better understanding of the way in which specific material properties affect the body's blood clotting response is vital to the development of next-generation blood contacting biomaterials.

Materials and Methods

Materials. Acrylamide, *N*,*N*′-methylenebisacrylamide (BIS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), ammonium persulfate (APS), and human fibrinogen (FI) were purchased from Sigma-Aldrich (Milwaukee, WI). *N*-(3-Aminopropyl)methacrylamide hydrochloride (APM) was purchased from Polysciences (Warrington, PA). *N*-(2-Hydroxyethyl)-methacrylamide (HEM) was purchased from Monomer-Polymer (Trevose, PA). Sodium hydroxide (NaOH), sodium phosphate monobasic, sodium chloride (NaCl), potassium chloride (KCl), and hydrochloric acid (HCl) were purchased from Mallinckrodt Baker (Phillipsburg, NJ).

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Dextrose (anhydrous, ACS grade) was purchased from EMD Chemicals (Madison, WI). Deionized water (DI water) was obtained using a Millipore Super-Q water system (Billerica, MA). Plastic-capped glass vials were purchased from VWR Scientific (West Chester, PA). 24-Well cell culture plates were purchased from Corning Life Science (Corning, NY).

Normal, human source plasma (pooled, sterile filtered, 4% (w/v) sodium citrate), and FI deficient plasma (pooled, sterile filtered, 4% (w/v) sodium citrate) were purchased from Vital Products (Boynton Beach, FL). Human FI (fibrinogen) was purchased from Sigma-Aldrich (Milwaukee, WI). Human coagulation factors II (FII), FV, FVII, FIX, and FX were purchased from Haematologic Technologies (Essex Junction, VT) and were buffered in 50% (v/v) glycerol/water. FII-, FV-, FVII-, FVIII-, FIX-, FX-, FXI-, and FXII-deficient plasma along with the chemical inhibitors corn trypsin inhibitor (CTI), D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), and L-glutamyl-L-glycyl-L-arginine chloromethyl ketone (GGACK) were all purchased from Haematologic Technologies (Essex Junction, VT). All factor-deficient plasmas were prepared from citrated (4% (w/v) sodium citrate), normal human plasma (pooled, sterile filtered). Goat polyclonal primary fibrinogen antibody was purchased from Abcam (Cambridge, MA). The immunogen of this antibody was fibrinogen of human plasma (Abcam, ab6666). Alexa Fluor 488 labeled donkey antigoat IgG antibody was purchased from Invitrogen (Carlsbad, CA).

Hydrogel Synthesis. A specific amount of either acrylamide, BIS, HEM, or APM was dissolved in DI water and mixed thoroughly until all components were completely solvated. The solution was then titrated to a pH between 6.8 and 7.2 using NaOH and HCl. After pH adjustment, the monomer solution was initiated by adding 20 μL/mL of a 7.5% (v/v) TEMED solution, followed by adding 20 μL/mL of a 15% (w/v) solution of APS, for polymerization. After polymerizing overnight, all polymer hydrogels were dried in a vacuum (85 kPa) oven at 85 °C and then crushed using a mortar and pestle. All hydrogels were washed in approximately 325 mL of DI water for 24 h and the dried, crushed particles were sieved between 75 and 250 μm.

For dynamic mechanical analysis (DMA) and swelling experimentation 500 μL of monomer solution, immediately following initiation, was added to a well of a 24-well cell culture plate and allowed to polymerize overnight.

Characterization. A total of 3 mL of ovine plasma (3.8% (w/v) acid citrate dextrose) were added to a vial containing 100 mg of dried hydrogel (1.5 M APM, 1.5 M acrylamide, 0.3 M BIS). The vial was then rotated for 18 h on a Barnstead International (Dubuque, IA) Labquake.

For hematoxylin and eosin (H&E) staining, sample slides were exposed to each stain for 90 s. For immunohistochemical (IHC) staining slides were incubated with a primary fibrinogen antibody (200 μL, 62.5 μg/mL) for 60 min, followed by incubation with an Alexa Fluor 488-labeled donkey antigoat IgG antibody (200 μL, 5 μg/mL) for 60 min. Micrographs were taken with a Zeiss, Hitech Instruments (Broomall, PA), Axiovert 40 CFL microscope, and Diagnostic Instruments (Sterling Heights, MI) digital camera with corresponding software. For environmental scanning electron microscopy (ESEM) the coagulate complex was imaged using a FEI (Hillsboro, OR) Quanta environmental scanning electron microscope.

Dynamic FVII Activation Experiment. A set of six acrylamide-*co*-APM-*co*-BIS hydrogel compositions was tested in this experiment. The total concentration of APM and acrylamide was kept constant at 3 M, and only the comonomer ratio (acrylamide/APM/BIS) was varied. The HEM control (HEM CTRL) was composed of 1.5 M of HEM, 1.5 M of acrylamide and 0.3 M of BIS. The acrylamide control (Acrylamide CTRL) was composed of 3 M acrylamide and 0.3 M of BIS. 3.5 mL of human plasma (4% (w/v) sodium citrate) were added to each vial containing 100 mg of dried, sieved hydrogel, which was immediately rotated on a Labquake. Plasma samples (250 μL aliquot) were taken at 30, 90, and 180 min. All aliquoted samples were filtered with Whatman (Piscataway, N.J.) 0.22 μm Puradisc polyethersulfone membrane filters. The aliquoted samples were analyzed to determine FVIIa concentration using an American Diagnostica (Stamford, CT) IMUBIND Factor VIIa ELISA.

Factor Deficient and Factor Inhibition Experiment. The hydrogel composition of 1.5 M APM, 1.5 M acrylamide, and 0.3 M acrylamide (Composition C in Dynamic FVII Activation Experiment) was tested with various factor deficient and factor inhibited plasmas (18 h cycle). After the 18 h cycle, the coagulate complex formed was drained of plasma and visually scored for fibrin formation on a scale of 0 (no fibrin formation) to 10 (substantial fibrin formation).

Factor deficient plasmas were prepared via the immunodepletion of citrated, normal human plasma (Haemtech Inc.). Factor inhibited plasmas were prepared by adding the necessary amount of chemical inhibitor to normal, human plasma (4% (w/v) sodium citrate) to obtain the appropriate concentration for factor inhibition. CTI at a concentration of 7 μM was used to inhibit FXIIa.[30] GGACK at a concentration of 500 μM was used to inhibit FXa and PPACK at a concentration of 200 μM was used to inhibit FIIa.[31] Although PPACK and GGACK have been shown to have secondary targets, their primary targets are FIIa and FXa respectively. A total of 1.11 mL of factor deficient or factor inhibited human plasma were added to a vial containing 35 mg of dried hydrogel, which was then rotated for 18 h. All rotations occurred on a Barnstead International Labquake.

TFPI Activity Experiment. The same protocol used for the Dynamic FVII Activation Experiment was used for the TFPI Activity Experiment. The aliquoted samples were analyzed to determine TFPI activity using an American Diagnostica ACTICHROME TFPI Activity Assay.

Artificial Plasma Experiment. The hydrogel composition of 1.5 M APM, 1.5 M acrylamide, and 0.3 M acrylamide (Composition C in Dynamic FVII Activation Experiment) along with a control hydrogel (HEM Control in Dynamic FVII Activation Experiment) were both tested in a synthetically produced plasma explicitly lacking calcium, TF, and platelets. The artificial plasma was prepared based on a platelet-suspension buffer used by Motlagh et al.,[32] composed of 137 mM NaCl, 2.7 mM KCl, 0.4 mM sodium phosphate monobasic, 5.5 mM dextrose, 10 mM HEPES, 0.5 μg/mL FVII,[33] 10 μg/mL FX,[34] 100 μg/mL FII,[35] 7 μg/mL FV,[36] 5 μg/mL FIX,[37] 2.6 mg/mL FI,[34] and 30 μg/mL FXIII.[38] A total of 1 mL of this artificial plasma was added to each vial containing 35 mg of dried hydrogel, which was then rotated for 18 h. The resulting coagulate complex was stained for fibrin using the IHC protocol described above.

Dynamic Mechanical Analysis. All hydrogel compositions listed in the Dynamic FVII Activation Experiment were tested with a TA Instruments (New Castle, DE) Q800 Dynamic Mechanical Analyzer under submersion compression mode. Hydrogel disks were immersed in an excess (at least 100 mL) of DI water (multifrequency) or citrated (4% (w/v) sodium citrate) human plasma (stress−strain) for at least 18 h. All samples were submerged in 1.5 mL of either DI water (multifrequency) or human plasma (stress−strain) during testing.

For the moduli comparison experiment, BIS concentration was varied from 0.3 to 0.175 M by 0.025 M increments at four different monomer charge ratios, that is, APM/acrylamide, including 1:2 (1 M APM/2 M acrylamide), 1:1 (1.5 M APM/1.5 M acrylamide), 2:1 (2 M APM/1 M acrylamide), and 2:0 (2 M APM/0 M acrylamide). Each sample was swollen in plasma for at least 18 h and then subjected to stress−strain compression testing (0−5% strain). All moduli were determined by fitting linear curves in the selected regions of strain. A small amount of each hydrogel was also prepared, dried in a vacuum (85 kPa) oven at 85 °C overnight, then crushed using a mortar and pestle, and sieved to obtain particles between 75 and 250 μm. A total of 35 mg of the dried particles were added to 1 mL of citrated (4% (w/v) sodium citrate) human plasma and rotated for 18 h. After the 18 h cycle the presence of fibrin formation was visually confirmed.

Swelling Experiment. BIS concentration was varied from 0.3 to 0.175 M by 0.025 M increments at three different monomer charge ratios, that is, APM/acrylamide, including 1:2 (1 M APM/2 M

acrylamide), 1:1 (1.5 M APM/1.5 M acrylamide), and 2:1 (2 M APM/1 M acrylamide). All hydrogels were prepared in disk form according to the DMA protocol mentioned above. The hydrogel discs were swollen in an excess of plasma for 18 h (equilibrium), drained of plasma, and weighed accordingly. The sample discs were then dried in a vacuum (85 kPa) oven at 85 °C overnight to remove all water and weighed again. The swelling percent of gel was calculated using the formula below.

$$\mathrm{SP} = 100 \times \frac{W_{\mathrm{swollen}} - W_{\mathrm{dry}}}{W_{\mathrm{dry}}} \quad (1)$$

where SP = swelling percent, W_{swollen} = weight of swollen gel, and W_{dry} = weight of dry gel.

To determine the ability of the hydrogel to induce fibrin formation, the hydrogel was dried according to above protocols, crushed using a mortar and pestle, and separated with sieves to obtain particles between 75−250 μm. A total of 35 mg of the dried particles was added to 1 mL of citrated (4% (w/v) sodium citrate) human plasma and rotated for 18 h. After the 18 h cycle, the presence of fibrin formation was visually confirmed.

Results and Discussion

We synthesized various polymer hydrogel compositions with a primary amine containing functional monomer, APM, to help isolate the specific effect that positive electrostatic charge and polymer microstructure has on FVII activation and corresponding fibrin formation in citrated plasma. The pK_a of the polymer hyrdogel, as determined through potentiometric acid−base titration, is approximately 8.3. Therefore, the majority of the functional APM amine groups on the polymer are expected to be protonated at a pH of 7.4 (pH of plasma). Histological and microscopic characterization techniques were used to investigate the formed coagulate complex. A dynamic FVII activation experiment confirmed the ability of several multicomponent charged hydrogel compositions to induce FVII activation and offered insight into the dependence on electrostatic charge and mechanical structure. Experiments utilizing artificial plasma, coagulation factor depleted plasmas, coagulation factor inhibited plasmas, and an assay capable of determining TFPI activity, were conducted to elucidate the biological mechanism by which the hydrogel was inducing fibrin formation. DMA and swelling experiments were used to help establish a correlation between the microstructure of the polymer and its ability to induce FVII activation and fibrin formation in citrated plasma.

It should be noted that all experiments, unless otherwise noted, were conducted in plasma where calcium was artificially depleted by the use of citrate. Due to the lack of free calcium in citrated plasma, the kinetics of fibrin formation were considerably delayed, and because of this, a longer incubation time was chosen for most experiments. This time period allowed for the qualitative assessment of the material's ability to activate the cascade, based on the amount of fibrin formed. It should be noted that no fibrinolysis was observed during the experiments and the formed clots were stable for several days after the 18 h incubation period. Also, no further fibrin formation occurred after this time period.

Characterization. Histological and microscopic techniques were used to investigate the microstructure of the clot formed in the presence of the charged hydrogel. An example of a clot induced by such a charged hydrogel is shown in Figure 1. Initial IHC staining, using a primary fibrinogen antibody, was completed on the coagulate complex to confirm the presence of fibrin

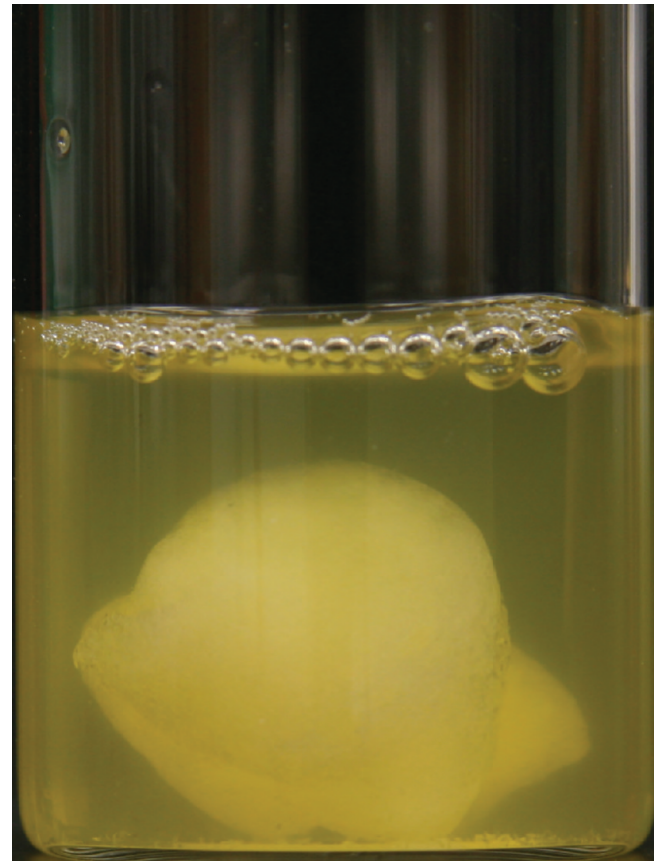

Figure 1. Typical coagulate complex (fibrin−hydrogel complex) formed after rotating a charged polymer hydrogel (1.5 M APM, 1.5 M acrylamide, 0.3 M BIS) in citrated (4% (w/v) sodium citrate) human plasma for 18 h. A total of 300 mg dried hydrogel was placed in 9 mL of plasma.

(Figure 2A). The fluorescence on the image indicates the presence of fibrin within the complex and suggests that the polymer is indeed activating the coagulation cascade in citrated plasma. H&E staining, Figure 2B, shows the presence of two distinct materials. The polymer hydrogel appears as the smooth, lighter colored material on the right side of the micrograph surrounded by a second fibrous material, located on the left side of the micrograph. The darker, fibrous appearance of this material is consistent with fibrin staining.

The ESEM image of the surface shows the presence of a continuous fibrin layer coating the exterior of the complex (Figure 2C). Cross-sectional images showed little to no fibrin presence within the interior of the complex suggesting activation of fibrin is occurring via interaction with the hydrogel matrix, eventually leading to the aggregation and envelopment of the particles resulting in a fibrin-covered composite.

Dynamic FVII Activation Experiment. A dynamic FVII activation experiment was completed in order to confirm the ability of such charged polymer materials to induce FVII, and also to investigate the critical material parameters necessary for this activation. All compositions capable of inducing fibrin formation (compositions B, C, E) showed elevated levels of FVIIa compared to the controls (Figure 3). All compositions incapable of inducing fibrin formation (compositions A, D, F) showed FVIIa levels similar to the controls, consistent with normal physiologic concentrations (~5 ng/mL). Composition C induces substantial activation of FVII resulting in FVIIa concentrations approximately 8 times the resting levels (5 ng/mL) after 30 min and almost 15 times the resting concentration

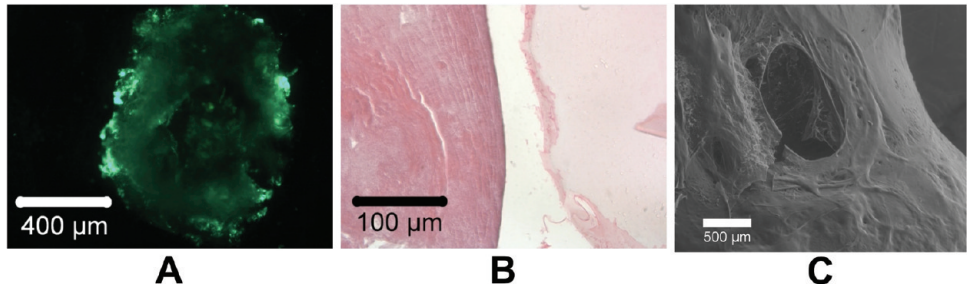

Figure 2. (A) IHC stained micrograph image of coagulate complex. (B) H&E stained micrograph image of coagulate complex. Polymer hydrogel appears as lighter, smoother material on right side of the micrograph while fibrin appears as the darker, rougher material on the left side of the micrograph. (C) ESEM surface image of the coagulate complex.

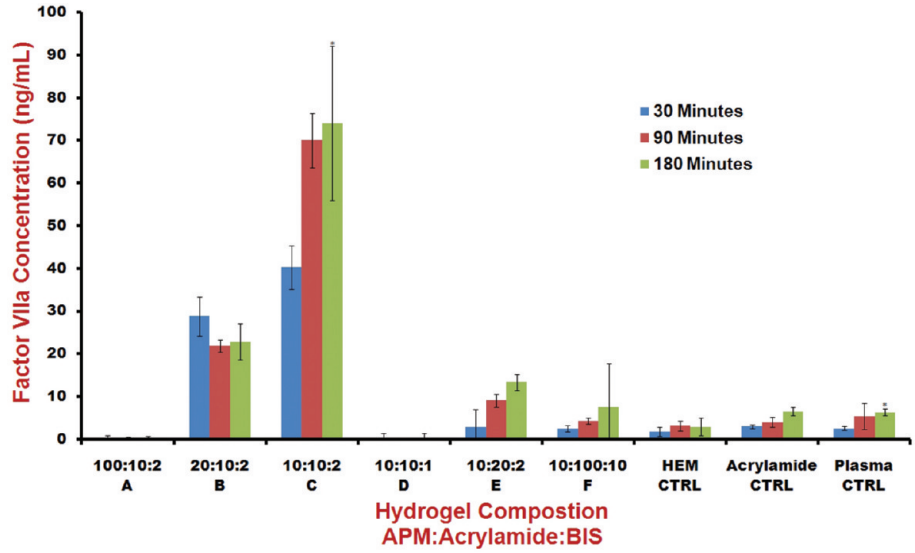

Figure 3. Factor VIIa concentration was measured in citrated (4% (w/v) sodium citrate) human plasma containing various hydrogel compositions at 30, 90, and 180 min. Data is representative of an average and corresponding standard deviation (error bar) of three ($n = 3$) separate sample trials. Asterisk (*) indicates a duplicate sample point.

after 180 min. The data also provides insight into the critical material properties of this activation suggesting that both electrostatic positive charge, for example, primary amine functionality on the APM monomer, and polymer chain rigidity, that is, cross-link density, are both crucial for the activation of FVII. Composition A, which contains a high amount of APM concentration yet low amount of cross-linking monomer, BIS, is unable to induce FVII activation. Similarly, composition F, which is highly cross-linked yet contains a minimal amount of electrostatic functionality, APM monomer, is also unable to induce FVII activation. The data indicates that both electrostatic charge and polymer mechanical properties influence the activation of FVII in citrated plasma.

Factor Deficient and Factor Inhibition Experiment. Although previous experimentation utilizing a FVIIa specific ELISA confirmed the ability of a charged polymeric hydrogel to induce the activation of FVII in citrated platelet poor plasma, the data offered little insight into the biological mechanism by which fibrin formation was occurring. Subsequent experimentation was aimed at investigating the biological mechanism of tissue factor pathway activation and subsequent fibrin formation including the vital biological components and cofactors involved.

Experimentation utilizing various factor deficient and factor inhibited plasmas was completed in order to determine the coagulation factors which were vital to the mechanism by which the hydrogel was inducing fibrin formation (Figure 4). Removal of FVII from plasma severely inhibited the ability of the hydrogel to induce fibrin formation relative to the normal, plasma control, indicating that FVII is indeed vital to the process. The small amount of fibrin formed is believed to be induced by the trace amounts of FVII not sufficiently removed in the purification process. Removal of FX also severely inhibited clotting. When FXa was inhibited using GGACK, complete inhibition of fibrin formation was observed. These results, along with known mechanics of the coagulation cascade, suggest that FX is vital to the mechanism and that the small amount of fibrin formed in the FX-deficient plasma was induced by trace amounts FX not properly removed in the purification (immunodepletion) process. The removal of FII or the chemical inhibition of FIIa using PPACK, led to complete inhibition of fibrin formation. Also, the data seems to agree with suggested theory of the ability of positively charged surfaces to initiate the autoactivation of FVII and further enhance or support the activation of FX and subsequently FII in calcium depleted media.[23,24,26−29]

Removal of FV, a cofactor of FII, had no significant effect on fibrin formation, suggesting that this cofactor is not vital to fibrin formation in the presence of a positively charged surface at depleted calcium levels.[27,29] As predicted, removal of FI, fibrin, resulted in complete inhibition of fibrin formation. Results from the factor deficient and factor inhibited experiments suggest that FVII, FX, FII, and FI are all vital to the process, indicating that the biological mechanism of action of the polymer is via the activation of the tissue factor pathway, which leads to the subsequent activation of the common pathway, eventually resulting in fibrin formation.

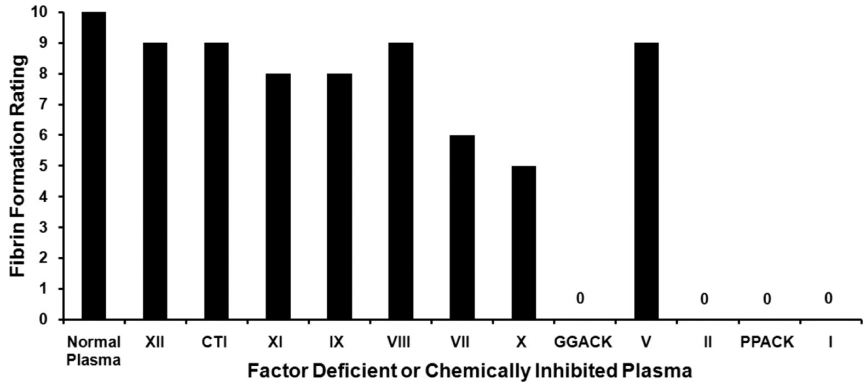

Figure 4. Composition C from Dynamic FVII Activation Experiment (1.5 M APM, 1.5 M acrylamide, 0.3 M BIS) tested in various factor deficient and factor inhibited plasmas. The resulting fibrin formation was visually rated on a scale of 0 (no fibrin formation) to 10 (substantial fibrin formation).

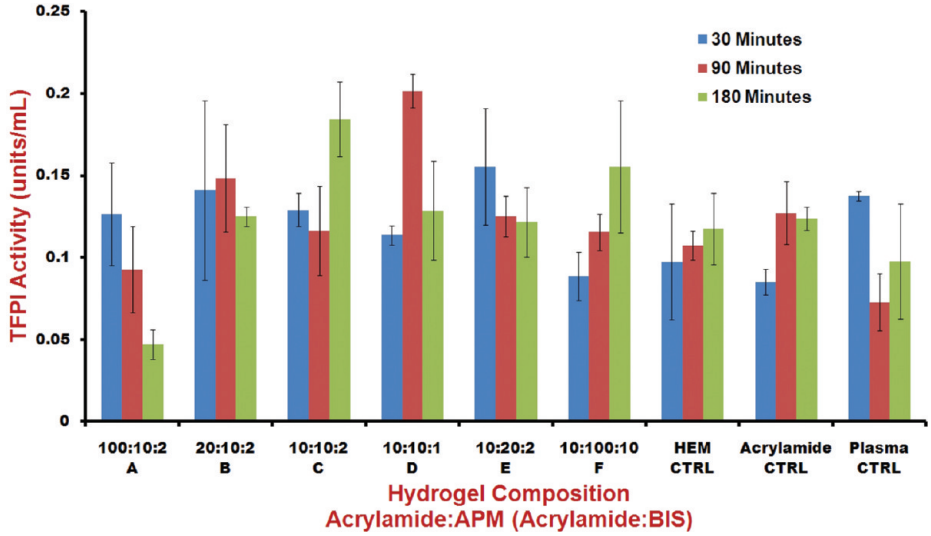

Figure 5. TFPI Activity was measured in citrated (4% (w/v) sodium citrate) human plasma containing various hydrogel compositions at 30, 90, and 180 min. Data is representative of an average and corresponding standard deviation (error bar) of three ($n = 3$) separate sample trials.

Removal of FXII or chemical inhibition of FXIIa using CTI showed little to no effect on fibrin formation when compared to normal human plasma. Similarly, removal of FXI had a negligible effect on the ability of the polymer to induce fibrin formation, indicating that these factors, or rather the contact activation pathway, hold insignificant roles in the process.

Removing FIX from plasma did reduce fibrin formation but did not completely inhibit the process. While removal of FIX had a modest inhibitory effect on fibrin formation, removal of its primary cofactor FVIII had almost no effect on fibrin formation. While the data corroborates previous findings that positively charged surfaces are capable of enhancing the activation of FX by activated FIX in calcium depleted media, it further suggests that this mechanism is secondary to activation by FVII.[39]

TFPI Activity Experiment. After investigating the vital coagulation factors and outlining a general mechanism of fibrin formation in the presence of charged hydrogels, experiments were conducted to understand roles that various cofactors and inhibitors had on this mechanism. Under normal physiologic circumstances, the FVIIa-TF-FX complex is rapidly bound and inhibited by TFPI. A strong electrostatic interaction could prevent TFPI from appropriately binding to its substrates, thereby reducing its inhibitory ability, although in the absence of TF, no TFPI activity is expected. To explore the role of TFPI in this process, a specifically designed TFPI activity assay (American Diagnostica ACTICHROME TFPI Activity Assay) was used. There appeared to be no outlying trend of TFPI activity for any of the experimental samples compared to the control samples (Figure 5), suggesting that the hydrogel is not interfering with the inhibitory activity of TFPI.

Artificial Plasma Experiment. The complete and successful activation of the tissue factor pathway requires the coordination and interaction of a multitude of cofactors including calcium, platelets, and TF. Previous work has shown the ability of positively charged polymers to induce the autoactivation of FVII and to assist in the activation of FX by FVIIa and FIXa in medium with depleted calcium levels.[23−29] Although platelets and TF enhance this process it has been shown that these cofactors are not necessary for these activation steps. To further investigate the role of these cofactors in the biological mechanism, an experiment was conducted using "artificial plasma" consisting of only FVII, FX, FV, FII, FXIII, and FI (fibrin) and explicitly lacking calcium, TF, and platelets.

The hydrogel was able to induce fibrin formation in the artificial plasma within minutes, and by 30 min, a substantial fibrin-based aggregate formed that was confirmed using IHC. Although the artificial plasma was also void of the inhibitory mechanisms naturally present in plasma, the results do indicate that the polymer is capable of inducing fibrin formation through the rapid activation of FVII independent of calcium, platelets, and TF.

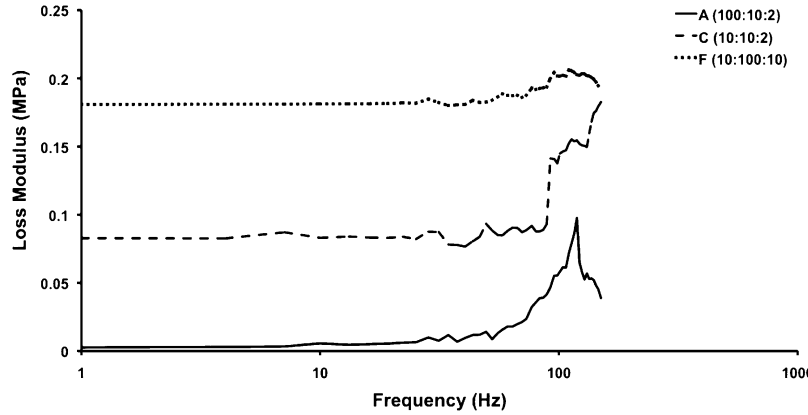

Figure 6. Graph of loss modulus vs frequency of three compositions used in Dynamic FVII Activation Experiment ranging from high APM, low acrylamide, and BIS content (composition A) to low APM, high acrylamide, and BIS content (composition F). Spectra for sample compositions C and F are shifted vertically to avoid overlapping of data. Spectra are representative of data obtained from several samples ($n = 3-5$).

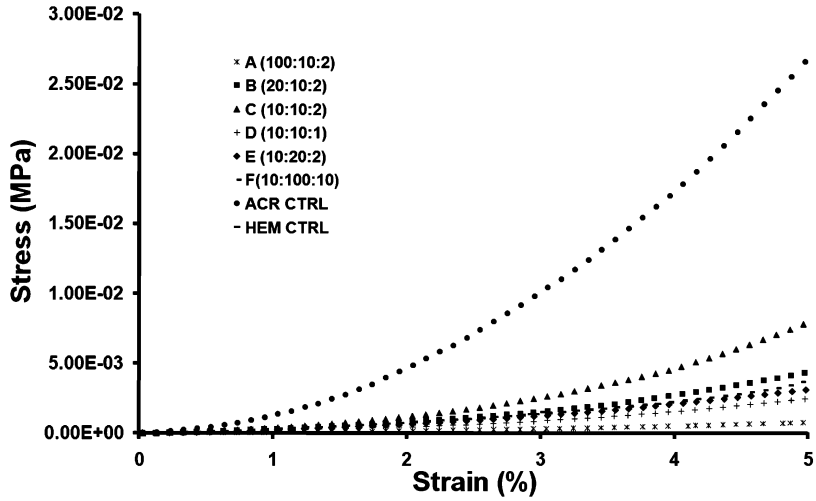

Figure 7. Stress−strain graph of all sample compositions used in the Dynamic FVII Activation Experiment. Curves are representative of data obtained from several samples ($n = 3-5$).

Dynamic Mechanical Analysis. Dynamic mechanical analysis (DMA) was used to investigate the relationship between specific mechanical properties and the ability to activate FVII and induce fibrin formation. DMA measures the mechanical properties of a material as a function of time, temperature, and frequency. There is a direct correlation of measured mechanical properties to microstructure. Multifrequency experiments were conducted to investigate the heterogeneity of the material, while stress−strain experiments were conducted to determine stiffness and bulk mechanical properties.

The storage modulus is related to the stiffness of the material, while the loss modulus to damping and energy dissipation. Low frequency transitions are attributed to liquidlike properties of the material, where flow dominates. High frequency transitions are characteristic of more solidlike behavior, where elastic properties are prevalent. The multifrequency transitions in all hydrogel compositions clearly indicate that each polymer hydrogel is not homogeneous, but consists of various regions of mechanical equivalence. The compositions containing high to medium APM content (compositions A and C) show a transition at a lower frequency around 8 Hz, indicating that these hydrogels contain a softer, more liquidlike region (Figure 6). The transitions of the low APM, high acrylamide composition (composition F) occur only in the higher frequency ranges, consistent with a stiffer material.

Stress−strain experiments were conducted to investigate the relationship between the mechanical stiffness of the hydrogel and its ability to induce FVII activation, and subsequent fibrin formation. As Figure 7 shows, each composition exhibits distinctly different stress−strain behavior. Moduli for both the linear elastic region, between 0 and 1% strain, and the strain hardening region, between 4 and 5%, were determined to establish a quantitative assessment of material stiffness (Table 1).

Table 1. Linear Elastic Modulus (Calculated between 0−1% Strain) and Strain Hardening Modulus (Calculated between 4−5% Strain) Were Calculated for All Sample Compositions Used in the Dynamic FVII Activation Experiment[a]

sample (APM/acrylamide/BIS)	modulus (Pa; 0−1% strain)	modulus (Pa; 4−5% strain)
A (100:10:2)	90 ± 17	433 ± 153
B (20:10:2)	300 ± 71	1540 ± 358
C (10:10:2)	320 ± 45	2760 ± 439
D (10:10:1)	200 ± 100	900 ± 400
E (10:20:2)	160 ± 69	1000 ± 100
F (10:100:10)	200 ± 82	2000 ± 638
ACR CTRL	1225 ± 359	10500 ± 622
HEM CTRL	160 ± 55	N/A (sample yields)

[a] The moduli were calculated by fitting a linear regression to the data for each respective region and obtaining the slope. A total of 3−5 samples were used for each calculation ($n = 3-5$).

The relationship between material stiffness of the hydrogel and its ability to induce FVII activation and fibrin formation is demonstrated in the differences in elastic moduli between

Table 2. Linear Elastic Modulus (Calculated between 0−1% Strain) Was Calculated for Hydrogels Containing a Fixed Amount of Positive Electrostatic Charge (APM Monomer) and Varied Amounts of BIS[a]

BIS (M)	modulus (Pa; 0−1% strain)	fibrin formation
	(A) 2.0 M ACR, 1.0 M APM	
0.300	89 ± 4	Y
0.275	94 ± 7	Y
0.250	61 ± 18	N
0.225	60 ± 14	N
0.200	44 ± 9	N
0.175	61 ± 20	N
	(B) 1.5 M ACR, 1.5 M APM	
0.300	330 ± 167	Y
0.275	180 ± 92	Y
0.250	148 ± 54	Y
0.225	116 ± 51	N
0.200	148 ± 54	N
0.175	127 ± 19	N
	(C) 1.0 M ACR, 2.0 M APM	
0.300	211 ± 82	Y
0.275	200 ± 101	Y
0.250	371 ± 128	Y
0.225	205 ± 8	Y
0.200	269 ± 25	Y
0.175	128 ± 12	N
	(D) 0 M ACR, 2.0 M APM	
0.300	837 ± 387	Y
0.275	1168 ± 690	Y
0.250	403 ± 80	Y
0.225	459 ± 272	Y
0.200	415 ± 236	Y
0.175	358 ± 94	N

[a] The hydrogels were also tested in citrated (4% (w/v) sodium citrate) human plasma to determine if they were capable (Y) or incapable (N) of inducing fibrin formation. Concentration sets: (A) 1.0 M APM, 2.0 M acrylamide; (B) 1.5 M APM, 1.5 M acrylamide; (C) 2.0 M APM, 1.0 M acrylamide; (D) 2.0 M APM, 0 M acrylamide.

samples C and D. Both samples contain the identical amount of positive charge (APM) and differ only in cross-link density (BIS), yet composition C is capable of strongly inducing FVII activation and fibrin formation, while composition D is incapable of inducing FVII activation and fibrin formation. This mechanical dependence is further corroborated by the stress−strain data from compositions A and C. Although, the amount of positive charge in composition A is almost twice that of composition C, composition A is incapable of inducing FVII activation and fibrin formation due to decreased structural integrity. The data clearly demonstrates a structural dependence on the ability of the polymer to induce FVII activation and fibrin formation.

Further dynamic mechanical analysis was completed to investigate the specific mechanical rigidity necessary to induce fibrin formation for a respective amount of positive electrostatic charge. The experimentation aimed to help establish a direct correlation between linear elastic modulus and the ability to induce fibrin formation for a specific amount of positive charge (Table 2). It should be noted that, although FVII activation was not measured directly, previous experimentation showed that fibrin formation was induced through the activation of FVII and, therefore, the presence of fibrin indicated that the polymer was capable of inducing FVII activation. As Table 2 shows, the linear elastic modulus necessary to elicit fibrin formation, or FVII activation, is proportional to the amount of positive charge in the polymer ranging from approximately 100 Pa for 1 M APM (1 M APM, 2 M acrylamide) to approximately 200 Pa for 2 APM (2 M APM, 1 M acrylamide). The data suggests that, as more positive charge is incorporated into the polymer, the stiffness of the polymer must be increased to induce activation. The data further corroborates previous findings of a direct relationship between the structural integrity of the formed hydrogel and ability to induce FVII activation and subsequent fibrin formation. Furthermore, the results indicate that the moduli of a hydrogel can be used as a quantitative measure to predict this ability.

Table 3. Swelling Percent Was Calculated for Hydrogels Containing a Fixed Amount of Positive Electrostatic Charge (APM Monomer) and Varied Amounts of BIS[a]

BIS (M)	swelling percent (%)	fibrin formation
	(A) 2 M ACR, 1 M APM	
0.300	187 ± 16	Y
0.275	182 ± 10	Y
0.250	208 ± 7	N
0.225	213 ± 23	N
0.200	233 ± 7	N
0.175	249 ± 7	N
	(B) 1.5 M ACR, 1.5 M APM	
0.300	157 ± 5	Y
0.275	168 ± 9	Y
0.250	161 ± 10	Y
0.225	219 ± 14	N
0.200	208 ± 5	N
0.175	220 ± 6	N
	(C) 1 M ACR, 2.0 M APM	
0.300	154 ± 10	Y
0.275	172 ± 9	Y
0.250	182 ± 13	Y
0.225	193 ± 24	Y
0.200	186 ± 20	Y
0.175	234 ± 11	N

[a] The hydrogels were also tested in citrated (4% (w/v) sodium citrate) human plasma to determine if they were capable (Y) or incapable (N) of inducing fibrin formation. Concentration sets: (A) 1.0 M APM, 2.0 M acrylamide; (B) 1.5 M APM, 1.5 M acrylamide; (C) 2.0 M APM, 1.0 M acrylamide.

Swelling Experiment. Swelling experimentation was conducted to investigate the effect that swelling, or water uptake, of each hydrogel had on its ability to induce FVII activation, and subsequent fibrin formation. As Table 3 shows, when the swelling of the hydrogel reaches approximately 200% the material becomes incapable of eliciting fibrin formation, regardless of the amount of electrostatic charge, indicating that the water content is a critical parameter to this phenomenon. A swollen hydrogel matrix may be separated into two major components: the polymer hydrogel and the aqueous media. A swelling ratio of 200% means that approximately 2/3 of the matrix (by mass) is composed of water while only 1/3 is taken up by the actual polymer strands. In this state the protein encounters an environment that is dominated by an aqueous species, which contains increasing smaller amounts of physical polymer surface area available for the protein to adhere. These results agree with previous research showing that specific coagulation factors are several magnitudes more effective when bound to a surface.[40,41] Our research suggests that positive charge is important to attract FVII to the polymer surface, yet the physical or structural integrity of the surface must be adequate to induce activation.

Conclusion

Polymer hydrogels containing positively charged functional groups were used to help elucidate a better understanding of the critical material properties necessary for the activation of FVII and subsequent fibrin formation in citrated, platelet-poor plasma. Characterization experiments confirmed the generation of fibrin and also provided insight into the microstructure of clot formation. A FVII ELISA was used to confirm the ability of various charged

polymer hydrogels to induce FVII activation. The results of this experimentation suggested that cross-link density, in addition to positive electrostatic charge, was crucial to FVII activation. Experiments utilizing factor deficient and factor inhibited plasmas showed that FVII, FX, FII, and FI are all vital to the process, outlining the general mechanism of coagulation activation and fibrin formation. DMA was used to help establish a correlation between the mechanics, that is, linear elastic and strain hardening moduli, of the polymer microstructure and its ability to induce fibrin formation via the activation of FVII. Subsequent swelling experiments revealed that the water content of the hydrogel matrix is critical to the process, suggesting the activation of FVII and subsequent proliferation of the coagulation cascade is dependent on the presence of a surface with adequate structural integrity.

Several of the protein factors that play a vital role in the ability of the material to induce fibrin formation, that is, FVII, FX, and FII, are vitamin-K dependent serine proteases. Previous research has found that these proteins may be separated from various other coagulation proteins through the presentation of a highly positively charged surface and furthermore that such positively charged surfaces are capable of enhancing the activation of these coagulation factors.[23−29,42] It is believed that the coordination of these factors occurs through the electrostatic binding between the positively charged surface and the protein's Gla domain, which becomes exposed when not bound to calcium. Although the role of electrostatic charge in this phenomenon is well-known, we have shown that there is also an additional structural parameter that is critical to the process. Dynamic mechanical analysis elucidated that there is a clear correlation between the stiffness of the material, that is, elastic and strain hardening modulus, and the ability of the material to induce FVII activation and further promote subsequent fibrin formation. Swelling experiments further corroborated the relationship between the structural integrity and aqueous content of the hydrogel matrix and ability to induce fibrin formation.

Although this work focuses on the blood coagulation cascade, its broader impact involves a better understanding of the interactions that occur between biological proteins and synthesized polymer hydrogel-based materials which is integral to the development of future biomaterial technology. It is imperative we understand the critical material parameters that influence protein activity to develop more effective and safer blood-contacting biomaterials.

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Supporting Information Available. An experiment was conducted to investigate the relationship between surface area of the polymer hydrogel and FVII activation. A hydrogel capable of inducing FVII activation (Composition C from Dynamic FVII Activation Experiment) along with a control hydrogel (HEM Control from Dynamic FVII Activation Experiment) were synthesized, dried, and ground into four different sizes ranging from 53−425 μm. A total of 3.5 mL of human citrated (4% sodium citrate) plasma were added to a glass vial containing 100 mg dried hydrogel sample. FVIIa concentrations were measured using an IMUBIND Factor VIIa ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Price, G. C.; Thompson, S. A.; Kam, P. C. A. *Anaesthesia* **2004**, *59* (5), 483–492.
(2) Hoffman, M.; Monroe, D. M.; Oliver, J. A.; Roberts, H. R. *Blood* **1995**, *86* (5), 1794–1801.
(3) Mackman, N.; Tilley, R. E.; Key, N. S. *Arterioscler., Thromb., Vasc. Biol.* **2007**, *27* (8), 1687–1693.
(4) Davie, E. W.; Fujikawa, K.; Kisiel, W. *Biochemistry* **1991**, *30* (43), 10363–10370.
(5) Khan, A. R.; James, M. N. G. *Protein Sci.* **1998**, *7* (4), 815–836.
(6) Pike, A. C. W.; Brzozowski, A. M.; Roberts, S. M.; Olsen, O. H.; Persson, E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (16), 8925–8930.
(7) Broze, G. J. *J. Clin. Invest.* **1982**, *70* (3), 526–535.
(8) del Zoppo, G. J.; Yu, J. Q.; Copeland, B. R.; Thomas, W. S.; Schneiderman, J.; Morrissey, J. H. *Thromb. Haemostasis* **1992**, *68* (6), 642–7.
(9) Eddleston, M.; de la Torre, J. C.; Oldstone, M. B.; Loskutoff, D. J.; Edgington, T. S.; Mackman, N. *J. Clin. Invest.* **1993**, *92* (1), 349–58.
(10) Fleck, R. A.; Rao, L. V. M.; Rapaport, S. I.; Varki, N. *Thromb. Res.* **1990**, *57* (5), 765–781.
(11) Flossel, C.; Luther, T.; Muller, M.; Albrecht, S.; Kasper, M. *Histochemistry* **1994**, *101* (6), 449–453.
(12) Lockwood, C. J.; Nemerson, Y.; Guller, S.; Krikun, G.; Alvarez, M.; Hausknecht, V.; Gurpide, E.; Schatz, F. *J. Clin. Endocrinol. Metab.* **1993**, *76* (1), 231–236.
(13) Komiyama, Y.; Pedersen, A. H.; Kisiel, W. *Biochemistry* **1990**, *29* (40), 9418–9425.
(14) Drake, T. A.; Morrissey, J. H.; Edgington, T. S. *Am. J. Pathol.* **1989**, *134* (5), 1087–1097.
(15) Faulk, W. P.; Labarrere, C. A.; Carson, S. D. *Blood* **1990**, *76* (1), 86–96.
(16) Smith, S. A.; Morrissey, J. H. *J. Thromb. Haemostasis* **2004**, *2* (7), 1155–1162.
(17) Baugh, R. J.; Broze, G. J.; Krishnaswamy, S. *J. Biol. Chem.* **1998**, *273* (8), 4378–4386.
(18) Ratnoff, O. D.; Rosenblum, J. M. *J. Lab. Clin. Med.* **1957**, *50* (6), 941–942.
(19) Revak, S. D.; Cochrane, C. G.; Johnston, A. R. *Fed. Proc.* **1973**, *32*, 845.
(20) Smith, S. A.; Mutch, N. J.; Baskar, D.; Rohloff, P.; Docampo, R.; Morrissey, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (4), 903–908.
(21) Smith, S. A.; Morrissey, J. H. *Blood* **2008**, *112* (7), 2810–2816.
(22) Bjorn, S. T. L. *Res. Disclosure* **1986**, *269*, 564–565.
(23) Pedersen, A. H.; Lundhansen, T.; Bisgaardfrantzen, H.; Olsen, F.; Petersen, L. C. *Biochemistry* **1989**, *28* (24), 9331–9336.
(24) Fiore, M. M.; Neuenschwander, P. F.; Morrissey, J. H. *J. Biol. Chem.* **1994**, *269* (1), 143–149.
(25) Griffith, M. J.; Breitkreutz, L.; Trapp, H.; Briet, E.; Noyes, C. M.; Lundblad, R. L.; Roberts, H. R. *J. Clin. Invest.* **1985**, *75* (1), 4–10.
(26) Lundblad, R. L.; Roberts, H. R. *Thrombosis Res.* **1982**, *25* (4), 319–329.
(27) Lundblad, R. L.; Vogel, C. N.; Butkowski, R. J.; Mann, K. G. *Fed. Proc.* **1976**, *35* (3), 757–757.
(28) Miller, K. D.; Copeland, W. H.; Mcgarrahan, J. F. *Proc. Soc. Exp. Biol. Med.* **1961**, *108* (1), 117–&.
(29) Vogel, C. N.; Butkowski, R. J.; Mann, K. G.; Lundblad, R. L. *Biochemistry* **1976**, *15* (15), 3265–3269.
(30) Oliver, J. A.; Monroe, D. M.; Roberts, H. R.; Hoffman, M. *Arterioscler., Thromb., Vasc. Biol.* **1999**, *19* (1), 170–177.
(31) Lyon, M. E.; Drobot, D. W.; Harding, S. R.; Lyon, A. W. *Clin. Chim. Acta* **1999**, *280* (1−2), 91–99.
(32) Motlagh, D.; Yang, J.; Lui, K. Y.; Webb, A. R.; Ameer, G. A. *Biomaterials* **2006**, *27* (24), 4315–4324.
(33) Bajaj, S. P.; Rapaport, S. I.; Brown, S. F. *J. Biol. Chem.* **1981**, *256* (1), 253–259.
(34) Jozefowicz, M.; Jozefonvicz, J. *Pure Appl. Chem.* **1984**, *56* (10), 1335–1344.
(35) K.G., M. *Methods Enzymol.* **1976**, *45*, 123–156.
(36) Tracy, P. B.; Eide, L. L.; Bowie, E. J. W.; Mann, K. G. *Blood* **1982**, *60* (1), 59–63.
(37) Thompson, A. R. *Blood* **1986**, *67* (3), 565–572.
(38) Hedner, U.; Henriksson, P.; Nilsson, I. M. *Scand. J. Haematol.* **1975**, *14* (2), 114–119.
(39) Griffith, M. J.; Breitkreutz, L.; Briet, E.; Trapp, H.; Lundblad, R. L.; Noyes, C.; Roberts, H. R. *Thromb. Haemostasis* **1983**, *50* (1), 204–204.
(40) Gilbert, G. E.; Arena, A. A. *J. Biol. Chem.* **1996**, *271* (19), 11120–11125.
(41) Gilbert, G. E.; Arena, A. A. *Blood* **1995**, *86* (10), 293–293.
(42) Hashimoto, N.; Morita, T.; Iwanaga, S. *J. Biochem.* **1985**, *97* (5), 1347–1355.

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