MODULATING THE ADHESIVE STRENGTH OF BLOOD CLOTS BY COAGULATION FACTOR XIIIA-BASED TECHNOLOGIES

by

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B.Sc., The University of British Columbia, 2012

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Genome Science and Technology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

September 2019

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Modulating the adhesive strength of blood clots by coagulation factor XIIIa-based technologies

submitted by Karen Ying Tung Chan in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

In Genome Science and Technology

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Abstract

Trauma is the number one killer of people under the age of 45 worldwide. Hemorrhage is the second-leading cause of death after injuries to the central nervous system and constitutes more than 90% of potentially survivable injuries as reported in a military trauma study. Understanding the components and functions of the blood coagulation system has led to advances in the development of hemostatic materials.

Blood clots form plugs over leaking vessels to stop bleeding. They need to be cohesive to resist fracture from the pressures of blood flow, and adhesive to stay localized to the wound site. While the adhesive properties of individual clot components have been well-characterized, the adhesive properties of the bulk clot are still poorly understood. It is unclear how clot components interact with themselves and substrates on the wound surface to mediate attachment of the clot to the wound site.

In this study, we evaluated the adhesive strength of bulk blood clots. We determined which clot components were important in increasing clot adhesive strength to collagen, a common substrate found in wound tissues. We found that fibrin and FXIIIa increased clot adhesive strength in a concentration-dependent manner. Using this knowledge, we designed a formulation containing Q-PEG, a FXIIIa-crosslinkable synthetic macromer. The gelation of Q-PEG was coupled to the coagulation network through FXIIIa, allowing it to copolymerize with blood when clotting was activated. Copolymerizing Q-PEG with blood led to increased clot adhesion, particularly during fibrin-depleted or fibrinolytic conditions. This shows that clot adhesive strength is a property that can be modulated. Similar strategies, of coupling synthetic polymer formation to the coagulation cascade, may be useful for the design of novel hemostatic

materials that improve the mechanical properties of blood clots to help them resist high pressure arterial hemorrhage. A broader application would be in the design of smart, stimuli-responsive materials, using natural biochemical networks as highly sensitive and specific sensors and signal amplifiers to control polymer formation.

Lay Summary

Bleeding is a major health problem worldwide. Understanding the physical properties of blood clots is important for making new drugs to stop bleeding. Clots need to be strong enough to not burst from the pressure of blood flow. They also need to stick to the wound, but how they do this is unclear. Here, we measured the stickiness of various blood clots and found that two components called fibrin and FXIIIa are important for making clots stick. FXIIIa is an enzyme that catalyzes the linkage of fibrin, a fibrous material, to the tissues in the wound. We made a synthetic equivalent of fibrin that can similarly be stuck to wounds by FXIIIa. The synthetic material is stronger and remains sticky under conditions where fibrin would normally not function. The design concept of this material may be used to develop future drugs for treating high-pressure, high-flow bleeds.

Preface

Approval for this study was given by the research ethics boards of the University of British Columbia (mouse blood collection certificate A16-0176, human blood collection certificate H12-01516) and the Canadian Blood Services (certificate REB 2016.006).

Research described in Chapter 2 is yet unpublished. It was a collaborative effort between I (Karen Y.T. Chan), Alyssa S.M. Yong, Dr. James R. Baylis, and Dr. Christian J. Kastrup. I conceived the idea, designed all experiments, carried out all experiments except those described in Figure 2.4b, and analyzed and interpreted all data collected. A.S.M.Y. helped carry out the experiments described in Figures 2.2b-d, 2.3d, and 2.4a. J.R.B. designed and performed the experiment described in Figure 2.4b. C.J.K. helped design experiments and interpret the data. Overall, I contributed roughly 85% of the work described in this chapter.

Chapter 3 has been modified from research published in the *Scientific Reports*: J.H. Yeon*, K.Y.T. Chan*, T.C. Wong, K. Chan, M.R. Sutherland, R.F. Ismagilov, E.L.G. Pryzdial, and C.J. Kastrup (2015). A biochemical network can control formation of a synthetic material by sensing numerous specific stimuli. The article can be accessed at: https://www.nature.com/articles/srep10274 J.H.Y. and I are co-first authors on this paper. I helped conceive the idea, designed experiments, carried out all experiments except those described in Figures 3.1 and 3.2, analyzed and interpreted all data collected, and wrote 80% of the paper. Figure 3.1 did not come from the aforementioned article published in *Sci. Rep.*; it was published along with the data in Chapter 4 in an article in the journal, *Biomacromolecules* (see next paragraph for full citation). Dr. Erika M.J. Siren designed, conducted, analyzed data, and

helped write relevant sections of the experiments described in Figure 3.1. J.H.Y. helped conceive the idea, designed experiments, optimized the procedure for making the Q-PEG material from previous work[1,2,3,4,5], carried out the experiments described in Figure 3.2, helped with the experiments in Figure 3.10b and wrote 5% of the paper. T.C.W. and K.C. performed preliminary experiments that helped formulate Q-PEG into blood plasma. M.R.S. provided the purified herpes simplex virus particles for Figure 3.10b. R.F.I. helped conceive the idea. E.L.G.P. helped design the experiment described in Figure 3.10b and helped write the paper. C.J.K. helped conceive the idea, design experiments, interpret the data, and write the paper. Overall, I contributed roughly 50% of the work described in this chapter.

Chapter 4 has been modified from research published in *Biomacromolecules* and a conference abstract for the 34th Annual Meeting of the Canadian Biomaterials Society. The paper is: K.Y.T. Chan, C. Zhao, E.M.J. Siren, J.C.Y. Chan, J. Boschman, C.J. Kastrup (2016). Adhesion of Blood Clots Can Be Enhanced When Copolymerized with a Macromer That Is Crosslinked by Coagulation Factor XIIIa. It can be accessed at: https://pubs.acs.org/doi/abs/10.1021/acs.biomac.6b00481 The conference abstract is: K.Y.T. Chan, A.S.M. Yong, C.J. Kastrup (2018). FXIIIa-Crosslinkable Synthetic Macromers Improve Stiffness and Adhesion of Hemophilia B Blood Clots under Fibrinolytic Conditions. It can be accessed at: http://biomaterials.ca/#!/abstracts/view/114351 I helped conceive the idea, designed experiments, carried out all experiments, analyzed and interpreted all data collected, and wrote the paper and conference abstract. C.Z. helped design the lap shear adhesive strength assay described. J.C.Y.C. and J.B. helped with data analysis. A.S.M.Y. helped conduct and analyze data for experiments described in Figure 4.5, C.J.K. helped conceive the idea, design

experiments, interpret the data, and write the paper and conference abstract. Overall, I contributed roughly 85% of the work described in this chapter.

I am also a coauthor on two additional papers, listed below:

[Published in *Scientific Reports*] J.H. Yeon*, N. Mazinani*, T.S. Schlappi, K.Y.T. Chan, J.R. Baylis, S.A. Smith, A.J. Donovan, D. Kudela, G.D. Stucky, Y. Liu, J.H. Morrissey, and C.J. Kastrup (2017). Localization of Short-Chain Polyphosphate Enhances Its Ability to Clot Flowing Blood Plasma. This can be accessed at: https://www.nature.com/articles/srep42119

[Published in *Thrombosis Research*] J.R. Baylis, K.Y.T. Chan, and C.J. Kastrup (2016). Halting Hemorrhage with Self-Propelling Particles and Local Drug Delivery. This can be accessed at: https://www.thrombosisresearch.com/article/S0049-3848(16)30362-0/abstract

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List of Abbreviations

α2AP	α2-antiplasmin
APC	Activated protein C
APTT	Activated partial thromboplastin time
CD	Clusters of differentiation
DAPA	Dansylarginine N-(3-ethyl-1,5-pentanediyl)amide
ECM	Extracellular matrix
FACS	Fluorescence-activated cell sorter
FG	Fibrinogen
FG-def	Fibrinogen-deficient
FII	Coagulation factor II (prothrombin)
FIIa	Coagulation factor IIa (thrombin)
FIX	Coagulation factor IX
FIXa	Activated coagulation factor IX
FV	Coagulation factor V
FVa	Activated coagulation factor V
FVII	Coagulation factor VII
FVIIa	Activated coagulation factor VII
FVIII	Coagulation factor VIII
FVIIIa	Activated coagulation factor VIII
FX	Coagulation factor X
FXa	Activated coagulation factor X

FXI	Coagulation factor XI
FXIa	Activated coagulation factor XI
FXII	Coagulation factor XII
FXIIa	Activated coagulation factor XII
FXIII	Coagulation factor XIII
FXIIIa	Activated coagulation factor XIII
GP	Glycoprotein
GPC	Gel permeation chromatography
HSV	Herpes simplex virus
K-PEG	Lysine-donating FXIIIa-crosslinkable polyethylene glycol macromer
LPS	Lipopolysaccharide
NMR	Nuclear magnetic resonance
PAI	Plasminogen activator inhibitor
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PEG	Polyethylene glycol
PEG-mal	Maleimide-modified polyethylene glycol
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
Q-PEG	Glutamine-donating FXIIIa-crosslinkable polyethylene glycol macromer
RBC	Red blood cells
SEM	Scanning electron microscopy

TAFI	Thrombin activatable fibrinolysis inhibitor
TEG	Thromboelastography
TIC	Trauma-induced coagulopathy
tPA	Tissue plasminogen activator
TXA	Tranexamic acid
uPA	Urokinase plasminogen activator
VWF	Von Willebrand factor
WB	Whole blood

Acknowledgements

I would like to thank members of the Kastrup Lab for their help, particularly J. R. Baylis, V. Chan, S.K. Novakowski, J.C.Y. Chan, A.S.M. Yong and my supervisor, Dr. Christian Kastrup. I would also like to thank E.M.J. Siren from the Kizhakkedathu Lab and M.R. Sutherland from the Pryzdial Lab for their help with gathering the data described in this thesis. My thesis supervisory committee has given me much useful input throughout the progress of this dissertation work. Thank you to Dr. Jayachandran Kizhakkedathu, Dr. Boris Stoeber, and Dr. Wilfred Jefferies. I would also like to extend my gratitude to my parents and friends who have been very supportive over these years.

Dedication

To my family and friends and all those who have helped me.

Chapter 1: Introduction

1.1 Background and Literature Review

1.1.1 Hemostasis

Hemostasis is the process that halts blood loss[6]. During this process, a blood clot is formed to seal the leakage in the damaged blood vessel[6]. The blood clot is localized to the site of injury while systemic blood flow is maintained[6]. It is eventually degraded when vascular integrity is restored[6].

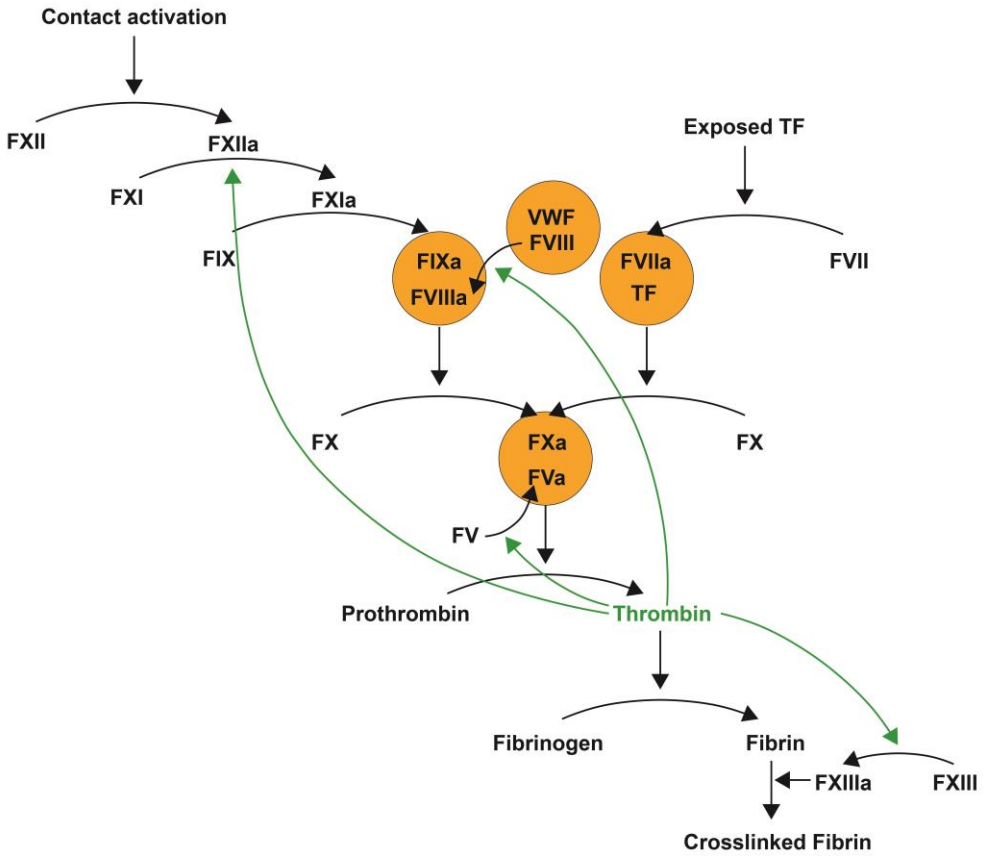


Figure 1.1 Simplified schematic of the classic cascade model of blood coagulation.

The left branch of the cascade is the intrinsic (contact) pathway, while the right branch is the extrinsic (TF) pathway. The two branches converge at the formation of the prothrombinase complex.

A classic model[7] describes hemostasis as a cascade of reactions that activate coagulation factors along two pathways, the extrinsic and the intrinsic pathways. These pathways ultimately converge at the formation of the prothrombinase complex, which consists of the serine protease, Factor (F)Xa, and its cofactor, FVa. This complex catalyzes the proteolytic cleavage and activation of prothrombin to thrombin. The active protease, thrombin, cleaves the blood protein, fibrinogen, converting it to fibrin. Fibrin fibers self-assemble to an insoluble meshwork which serves as a major component of the blood clot. While this model is useful for describing the biochemical interactions between different coagulation factors, it does not explain in detail how these interactions take place *in vivo*[8]. A cell-based model[8] was subsequently developed to better explain *in vivo* observations.

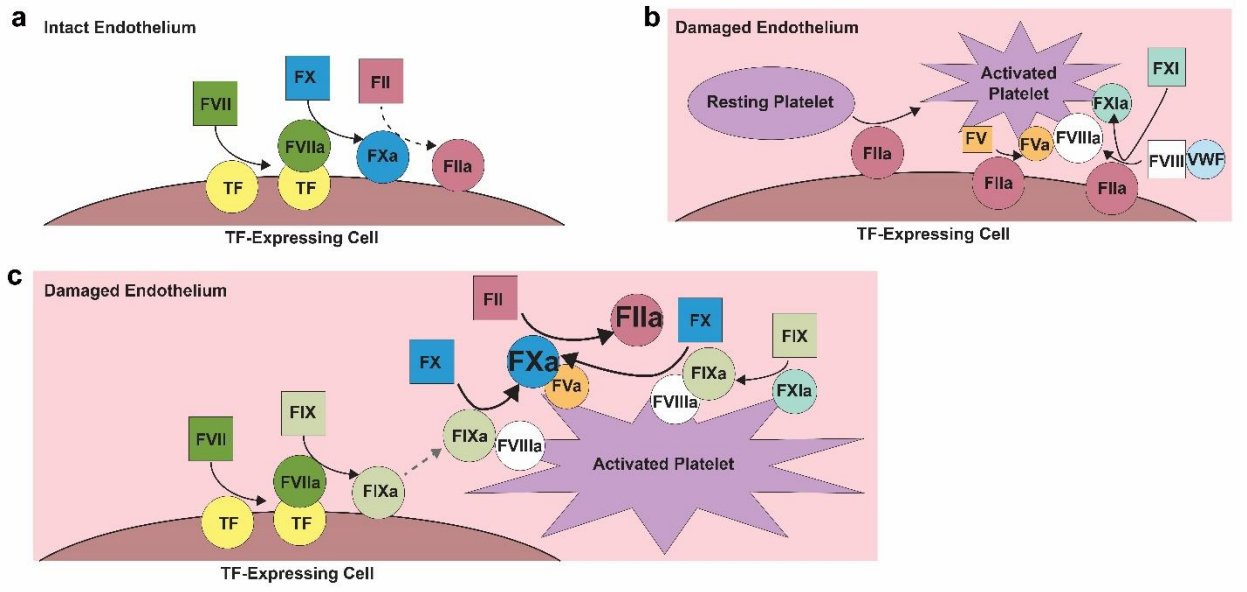


Figure 1.2 Simplified schematic of the cell-based model of blood coagulation.

a) The initiation phase is constantly active, producing very small amounts of thrombin on the surface of TF-expressing cells (reaction shown with a dashed arrow). This does not lead to clotting because of low thrombin concentration and that the endothelium remains intact, separating thrombin from the systemic bloodstream. b) The

amplification phase happens when the endothelium is breached. Platelets adhere and are activated by the thrombin present. Thrombin also helps activate other coagulation factors that assemble on the platelet surface. c) The propagation phase occurs when the prothrombinase complex assembles, generating a burst of thrombin. (Dashed gray arrow indicates the diffusion of FIXa to the platelet surface)

The cell-based model divides blood coagulation into three overlapping phases: 1) initiation, 2) amplification, and 3) propagation.

The initiation phase occurs on cells that express tissue factor (TF). Many cells express TF, including endothelial cells and stromal fibroblasts. These cells are normally separated from the rest of the coagulation system by an intact vessel wall. Some coagulation factors can percolate through the vessel wall and contact these TF-bearing cells, including FVII, FX, and prothrombin. Both the activated forms of these factors as well as their activation peptides can be detected in lymph, suggesting that the TF pathway of coagulation activation may be constantly active. TF binds FVII and promotes its proteolytic activation to FVIIa. The TF:FVIIa complex that is assembled on these cell surfaces then cleaves and activates FX to FXa. FXa alone can cleave and activate prothrombin to thrombin, but at a greatly reduced rate compared to when it is associated with the prothrombinase complex (rate reduced by a factor of 278,000)[9]. This results in a very limited amount of thrombin generation on the surface of TF-bearing cells.

During vascular damage, thrombin generation is amplified. The amplification phase occurs when vascular damage exposes blood components to the subendothelium and the limited amount of thrombin on TF-bearing cells. Platelets adhere to von Willibrand Factor (VWF) in the subendothelium through its GPIb-IX-V receptor. A source of VWF is constitutively associated with subendothelial collagen VI filaments. Plasma VWF can also bind collagen I and III in

deeper layers of the vessel wall. Platelets can also bind directly to subendothelial collagen through its GPVI receptor. The binding and subsequent aggregation of platelets to the subendothelial surface leads to the initial formation of the hemostatic plug. The subendothelial thrombin and collagen help activate the platelets, leading to a release of FV that then gets activated by the thrombin to FVa. Thrombin also releases plasma FVIII from VWF and activates it to FVIIIa. Thrombin also activates FXI to FXIa. FVa, FVIIIa, and FXIa assemble on the surfaces of activated platelets.

During the propagation phase, FIX is activated to FIXa by two ways: cleavage by 1) FXIa on platelet surfaces, or by the 2) TF:FVIIa complex on TF-bearing cell surfaces; this FIXa is then diffused to the surface of platelets. FIXa then complexes with the FVIIIa on platelet surfaces, and the FIXa:FVIIIa complex catalyzes the activation of more FX to FXa. FXa then complexes with its cofactor, FVa, to form the prothrombinase complex, which leads to a large amount of thrombin generation and subsequent conversion of fibrinogen to fibrin, forming the fibrin clot. Thrombin also catalyzes the activation of FXIII to transglutaminase, FXIIIa, which crosslinks fibrin to itself and to antifibrinolytic proteins such as alpha 2-antiplasmin (α2AP), stabilizing the fibrin clot.

1.1.2 Fibrinolysis

Fibrinolysis is the process by which fibrin is degraded, which is a critical step to the clearance of the blood clot during wound healing[10,11]. While the individual reactions in fibrinolysis have been extensively characterized, questions remain in how the reactions localize to the substrate, fibrin, and how fibrinolysis as a process is regulated[10,11].

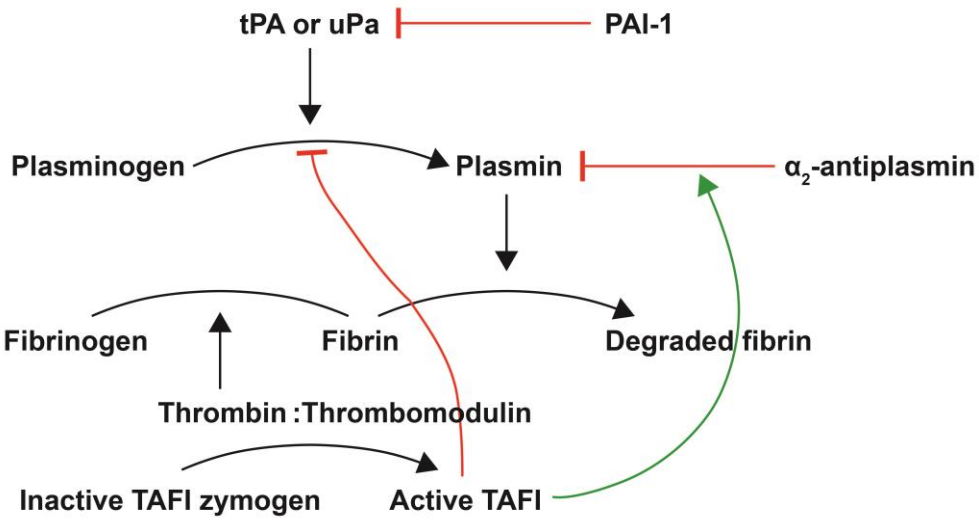


Figure 1.3 Simplified schematic of fibrinolysis.

tPA or uPA can cleave plasminogen to activated plasmin that can then degrade fibrin. tPA or uPa can be directly inhibited by PAI-1. Plasmin can be directly inhibited by α2AP. The thrombin:thrombomodulin complex can activate TAFI which can then remove plasmin from fibrin. This slows down tPA/uPa-catalyzed activation of plasmin and makes plasmin available for inhibition by α2AP.

The enzyme that cleaves fibrin to soluble fragments is plasmin. Plasmin is generated through cleavage of its zymogen form, plasminogen, by one of two physiological plasminogen activators, tissue plasminogen activator (tPA) or urokinase (uPA). Once activated, plasmin can cleave tPA and uPA into a more active form. Furthermore, plasmin cleavage of fibrin exposes carboxy-terminal lysine residues that bind tPA and plasminogen, thus facilitating further plasmin generation. This positive feedback amplifies the fibrinolysis process[10,11].

Several mechanisms exist to inhibit fibrinolysis. Plasminogen activator inhibitor (PAI)-1 can bind and inhibit tPA and uPA, and alpha 2-antiplasmin (α2AP) can inhibit plasmin directly when plasmin is not bound to fibrin. Thrombin activatable fibrinolysis inhibitor (TAFI) can remove carboxy-terminal lysines from fibrin, decreasing the rate of plasmin activation from

plasminogen by tPA, and lowering the amount of fibrin-bound plasmin that is protected from α2AP inhibition[10,11]. FXIIIa, generated from thrombin cleavage of its zymogen form, can crosslink fibrin to itself to make it more resistant to fibrinolysis[12]. Furthermore, it localizes fibrinolysis inhibitors by crosslinking them to fibrin, the most important of which is α2AP[12]. Thus, it can be seen that activation of the coagulation cascade and subsequent fibrin clot formation simultaneously activates and inhibits fibrinolysis. Hemostasis and fibrinolysis exist at a delicate balance where, under physiological conditions, a hemostatic blood clot is formed only when and where it is needed and degraded as vascular integrity is restored with tissue healing[6].

1.1.3 Severe hemorrhage and treatment options

Increased blood loss is correlated with increased mortality risk in hemorrhage[13]. Blood loss beyond 30-40% of the total volume overcomes the body's natural hemostatic responses and requires aggressive treatment[13]. Hemorrhage has many causes, such as trauma[14], surgical procedures[15], cardiovascular diseases[16,17], cancers[18,19,20], and infectious diseases[21,22]. It can be exacerbated by congenital or acquired anomalies in the coagulation and fibrinolytic systems, such as deficiencies in coagulation factors and fibrinolysis inhibitors[23,24,25]. Rapid transport to hospitals is critical for the treatment of severe hemorrhage[26]. Hemostatic devices help stabilize the patients for transport, following which fluid resuscitation and blood product transfusions, coupled with surgical intervention are the golden standards of treatment[13]. There is a demand for novel materials and drugs at both the pre-hospital and hospital settings to stabilize the patient, stop bleeding, and to prevent secondary bleeding.

1.1.3.1 Hemostatic devices for pre-hospital and hospital settings

The first treatment of hemorrhage in a pre-hospital setting is direct compression of the wound[13]. Compression can be provided by packing of the wound pocket with wound dressings[27]. Various different types of dressings exist in additional to plain cotton gauze[27]. Dry fibrin dressings have been shown in multiple animal models of traumatic and surgical hemorrhage to hasten the rate of clot formation and decrease blood loss, but are expensive to produce and mechanically fragile, limiting its application only to the hands of experienced medics[27]. The deacetylated form of chitin, chitosan, has been incorporated into hemostatic dressings (manufactured as HemCon)[27,28,29]. The exact mechanism of action is not completely understood, with possible contributions from the induction of vasoconstriction and binding of red blood cells[27,28,29]. HemCon was used in the US combat operations in Iraq with potential hemostatic benefits[27,28,29]. Dressings containing tissue fluid absorbents that increase the local concentration of coagulation factors have also been developed[27]. These include the powders microporous polysaccharide hemospheres (tradename TraumaDex) and mineral zeolite (tradename QuikClot)[27]. TraumaDex was shown to hasten clot formation in some animal models but failed to show reduced mortality in severe bleeding models such as femoral artery and vein transection[30,31,32]. QuikClot, in contrast, conferred survival benefits to these animal models[27,29,31]. However, QuikClot, when in contact with water, produced an exothermic reaction that caused local tissue injury in animal models[27,29,31]. When used on troops and civilians during US operations in Iraq, it was observed that the material was not adhesive enough to the wound site and was often pushed out by the high pressures of arterial blood flow[27,29,31]. This highlights the importance of clot adhesion and cohesion in the control of severe arterial hemorrhage.

Modifications of QuikClot gauze replaces the active powder with kaolin, which absorbs water into its porous structure without an exothermic reaction like its predecessor[33]. Kaolin also activates FXII of the intrinsic pathway[34]. It has been used by Israeli forces in operations in the Gaza strip in 2009 with a reported 79% efficacy at stopping bleeding on the field[35]. Three ineffective cases were reported due to an inability to apply the material to the location of injury[35].

Recently, expandable dressings and foams have been developed to improve hemostatic efficacy. XStat consists of syringe-applicated compressed mini sponges coated in chitosan[36]. They are applied to deep and narrow wounds in junctional areas of the groin and armpit where manual compression can be difficult[36]. The back pressure exerted by the expandable dressings halts hemorrhage[36]. Efficacy in preventing blood loss and improving survival has been confirmed in a number of animal studies and it is being adapted for use in combat casualty care with promising results[36]. BioHemostat, which contains an expandable hydrogel-forming polyacrylamide core, has also been developed as a topically-applied hemostat, but no *in vivo* studies have been conducted to show efficacy in stopping bleeding[27]. Expandable polyurethane[37], hydrophobically-modified chitosan[38], pressurized fibrin foams[39] have been developed for treating noncompressible truncal hemorrhages, showing reduced blood loss and improved survival in animal models. The advantage of foam products over expandable sponge dressings is that they are not limited in application by wound geometry. However, the pressure of application may cause complications. For instance, the polyurethane foam was reported to distend the bowels upon application to the abdominal cavity, causing lesions[40].

When wound-packing and direct compression are inadequate to stop bleeding, typically in cases of arterial injury, tourniquets may be used[41,42]. Tourniquet use has been debated due to risks of ischemic-perfusion injuries leading to tissue damage and potential amputations, but limiting the amount of time the tourniquet needs to be in place by rapid transport to the hospital has prevented these complications[41,42]. With proper use, tourniquets can be in place for 60-90 minutes without major, irreversible adverse effects[41,42]. Traditional tourniquets can only be used on limb injuries[41,42]. The ideal limb tourniquet design is one with a broad, smooth cuff inflated by air so that the pressure is evenly distributed over the area applied[41,42]. This increases both the hemostatic efficacy and reduces risk of tissue damage from the application of the tourniquet[41,42]. New tourniquet designs have broadened applications to noncompressible junctional hemorrhage[43]. Two junctional tourniquets have been used on human casualties to accomplish hemostasis[43]. The Combat Ready Clamp is a clamp that places direct pressure on the injury through a vertical metal arm[44]. The SAM tourniquet is similar to a blood pressure cuff that is tightened around the waist area and inflated to constrict vessels upstream of the injury[45]. Both junctional tourniquets are designed to stop bleeding from injuries to the groin region[43,44,45].

At the hospital setting, ligation of arteries is a common procedure to stop massive bleeding[46,47,48]. This can be challenging due to difficulties in finding the bleeding arteries in a blood-covered wound, and some wounds may be located deep in difficult to reach regions, such as the pelvis[49]. Various hemostatic devices exist to be used in catheter-directed embolization of arteries. An aortic balloon occlusion technique, REBOA, has been used to treat hemorrhage below the diaphragm[50]. The balloon is placed upstream of the wound site and inflated to cut off aortic blood flow[50]. REBOA is used as a temporary measure until the patient is transported to the

operating room, as aortic blood flow cannot be occluded for extended periods[51]. Other arterial embolization agents include platinum or titanium microcoils[52], and gelatin-based foams (Gelfoam)[53]. Further surgery is often required to repair the injured arteries using traditional suture or vascular clips[54], with stented vascular grafts for severe injuries[55].

1.1.3.2 Fluid resuscitation

Hemorrhage of more than 15% total blood volume require fluid resuscitation to maintain adequate blood pressure for cardiac function and perfusion of major organs[13]. Traditional fluid resuscitation protocols recommend up to 2 L of intravenous crystalloid (e.g. saline) administration, followed by plasma and packed red blood cell transfusion so that normal systolic blood pressure is reached[56,57]. However, the dilution of coagulation factors and increased blood pressure may lead to dilutional coagulopathy and pressure-induced rebleeding due to the bursting or detachment of formed clots at the wound site[58,56,57]. Several studies have suggested that less aggressive fluid resuscitation, maintaining a mean arterial pressure of 50 mm Hg or higher, instead of 65 mm Hg in conventional protocols, may confer survival benefits, but some of these studies lack statistical power or were not blinded in design, thus controversy remains in whether hypotensive fluid resuscitation would reduce mortality risk[56,57].

1.1.3.3 Blood product transfusion

Hemorrhage of more than 30% total blood volume require blood product transfusion[13]. Transfusion protocols differ in different locations, but all include packed red blood cells, plasma, and platelet concentrates[59,60]. Historically, red blood cells were transfused at a higher ratio than the other components to recover oxygen-carrying capacity[59,60]. Recently, retrospective studies

have shown that a 1:1:1 ratio may confer survival benefits[59,60]. Despite the efficacy of blood transfusion, challenges remain. Inadequately warmed blood products may worsen hypothermia[61]. Stored red cells become more acidic over time and, when transfused, may worsen acidosis[62]. Citrate, added as an anticoagulant to keep the blood products from clotting during storage, both dilutes the blood products as well as contributes to coagulopathy once transfused by calcium chelation[61]. All of this leads to lowered rates of fibrin generation and hastened fibrinolysis[61]. To overcome these challenges, coagulation factor concentrates, particularly fibrinogen cryoprecipitates, have been suggested for use[63]. Recombinant FVIIa have been used to treat congenital FVII deficiency, hemophilia A, and hemophilia B[64]. It was investigated as potential treatment for traumatic and surgical hemorrhage with mixed results regarding survival benefit[65,66,67]. In several studies, fibrinogen cryoprecipitates were used to maintain patient fibrinogen levels, and this was correlated with decreased mortality, but larger blinded trials are required to conclude on the effects[68,69,70]. Prothrombinase complex and FXIII administration have also been suggested, but little clinical data regarding their use is available[63].

1.1.3.4 Tranexamic acid

Hyperfibrinolysis is a pathological condition during which fibrin is excessively degraded, thus leading to a bleeding tendency[71]. It can be caused by a variety of congenital or acquired conditions, such as a deficiency in fibrinolysis inhibitors, cancers, liver disease, and severe trauma[71]. In the specific case of severe trauma, up to 25% of patients present with hemostatic abnormalities upon hospital admission[72], of which hyperfibrinolysis is a common finding and indicator of increased mortality risk[73]. The primary treatment being investigated for hyperfibrinolysis in trauma is the use of the lysine analog, tranexamic acid (TXA)[74]. TXA binds

reversibly to lysine receptors on plasminogen to lower the rate of its conversion to plasmin[74]. The efficacy of TXA in preventing hemorrhage deaths has been tested in clinical trials[75,76,77]. The Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage-2 (CRASH-2) trial was the largest to date, enrolling 20,211 adult patients who either received TXA or a placebo within eight hours from trauma[75]. Mortality was reduced in the TXA-treated group only if the TXA was administered within three hours after trauma[75]. It is hypothesized that early TXA treatment is required for survival benefit because fibrinogen stores in the patient must be protected from degradation before they are all consumed and made unavailable for the formation of a stable clot[78].

1.1.3.5 Biomimetic materials

Synthetic materials are novel treatments investigated for hemorrhage. They may have the benefits of decreased demand for donor blood[79], longer storage[80], tunable mechanical and chemical properties[81], and may be an alternative option for those who cannot receive blood product transfusions due to religious reasons[82]. Many synthetic hemostatic materials mimic the mechanisms and functions of the native coagulation system. Synthetic platelets have been created by incorporating platelet and ECM binding peptide sequences to nanoconstructs[83,84,85]. A fibrin-crosslinking polymer, polySTAT, has been created by mimicking the functions of FXIIIa to help stabilize the fibrin clot[86]. These materials increase the mechanical stiffness of blood clots[83,86]. polySTAT has been tested in animal models where it prevented secondary hemorrhage from fluid challenge and confer survival benefits[86]. Similarly, several synthetic platelet formulations have been tested in various animal models and shown to reduce blood loss and improve survival[83,84,85].

1.1.4 Blood clots are adhesive to tissue

Blood clots serve as a physical plug to stop blood from leaking out of a damaged vessel[6]. They need to be mechanically intact to resist rupture by blood flow, and they need to be adhesive to the site of injury to seal the leakage and to avoid detachment and subsequent embolism of the vessel downstream[20]. While the clot's cohesive integrity has been analyzed by a variety of techniques such as TEG, and shown to be useful in guiding transfusion requirements[87,88,89,90,91] which in turn reduces the cost and improves the efficacy of hemostatic treatment, the bulk clot's adhesive properties have been underexplored. Therefore, it may be useful to visualize the blood clot as a tissue adhesive and employ methods previously used for analyzing other glues and sealants on bulk clots to understand their adhesive behavior.

1.1.4.1 Mechanisms of macroscopic adhesion

The adhesion paradox describes a discrepancy in adhesive forces observed at the molecular scale and the macroscopic scale[92]. At the molecular scale, van der Waals interactions lead to strong adhesion between nearby (1-10 nm distance) surfaces[92]. However, this does not translate directly to the adhesion of macroscopic objects, which are mostly non-sticky[92]. This is because most macroscopic objects are not smooth, resulting in few spots between the two surfaces that are in close enough contact for atomic attractive forces to act strongly[93,94]. As a result, macroscopic adhesion depends not only on mechanisms that increase molecular adhesion, but also mechanisms that 1) reduce surface roughness and increase areas of close molecular contact[93,94], and 2) dissipates the force applied to separate two adhesive surfaces, making the adhesive joint less brittle[95]. Thus, strong adhesives are materials that can flow into cracks in the substrate surface to increase area of contact, form strong mechanical and chemical bonds with

the substrate to withstand tearing forces, yet are soft enough to deform elastically to support the stress applied to the adhesive joint.

1.1.4.2 Methods for evaluating the strength of adhesives

Adhesive strength is defined by the stress at which an adhesive fails by detachment from the surface to which it is attached[96]. To measure adhesive strength, an adhesive is applied between and used to attach two substrate surfaces. Force is applied until the adhesive detaches from one of the two surfaces. This force is divided by the surface area over which the adhesive was applied to yield adhesive strength. Assays to measure adhesive strength exists for both microscopic and macroscopic scales.

On a microscopic scale, optical tweezers and atomic force microscopy have been employed to measure the adhesive properties of single fibrin fibers[97] and platelets[98], respectively. On a macroscopic scale, adhesive strength depends heavily on the geometry of the adhesive joint examined, and the type of force applied to the substrates[99]. Two types of adhesive tests are commonly employed for the testing of tissue sealants, a class of materials including fibrin-based sealants: peel tests and lap shear tests. Peel tests are performed on a pair of substrates where at least one is flexible and can be pulled off the other. Different geometries can be used to peel one substrate from the other, such as T-peel[100], 90º peel (L-shaped)[100], and 180º peel (U-shaped)[101]. In a lap shear test, the adhesive is applied between a lap joint and a shearing force is applied to separate the two substrates until failure of the adhesive, either through cohesive breakage, or detachment of the adhesive from one of the substrate surfaces[99]. A modified version of the lap shear test exists called the double lap shear test[102]. It is a symmetrical version of the single lap shear test, in which a movable shaft is sandwiched between two immovable shafts. The two faces

of the movable shaft are coated with the adhesive and stuck to the surfaces of the two outer, immovable shafts. The movable shaft is pulled to apply an equal shearing force to both adhesive joints. This modified lap shear test reduces the amount of eccentric loading that results from a bending of the substrates or rotation of the adhesive joint, thus reducing the amount of peel and tensile stresses on the adhesive[102]. The double lap shear test is designed for measuring an adhesive's response to simple shear stress[102].

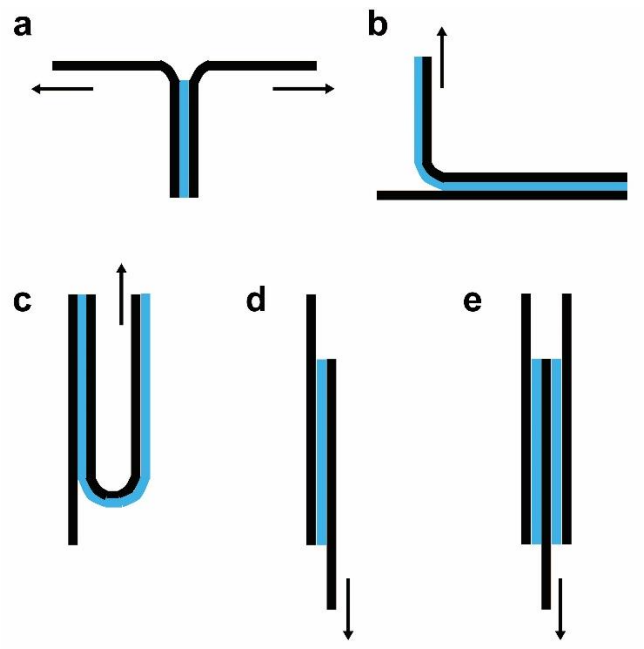


Figure 1.4 Schematic of adhesive tests for bulk materials.

a) T peel test, b) 90º peel test, c) 180º peel test, d) single lap-shear test, e) double lap-shear test. Substrates shown in black. Adhesive shown in blue. Arrows indicate direction of force applied.

No single adhesive test can yield all the information required to predict the behavior of an adhesive subjected to combined loads, such as blood clots that seal wounds of varying geometry, subjected to a variety of different forces such as shear, peel, tension, and compression. Due to the simplicity of using lap shear tests, which do not require any sophisticated equipment for

administration, it has been used in a number of studies on the adhesion of fibrin and fibrin-based adhesives[103,104,105].

1.1.4.3 Adhesive components of blood clots

The mechanical behavior of individual blood components has been well studied[98,97]. Although it remains difficult to model a clot's mechanics based solely on this information due to complexities in the clot's architecture, this knowledge is still a useful first step in understanding the mechanisms behind how clots adhere. Multiple clot components are known to have adhesive properties, including platelets[106], fibrin[103], and several plasma proteins[107].

1.1.4.3.1 Platelets

Platelets are anucleate blood cells that adhere to the injured vasculature to initiate thrombus formation and hemostasis[106]. Under normal physiological conditions, where the blood vessel walls remain intact, platelets do not adhere to the endothelium[106]. When vessel injury occurs, substrates that platelets can adhere to are exposed, including immobilized VWF, collagen, and other proteins such as fibronectin, laminin, fibulin, and thrombospondin[106]. Platelets have a variety of receptors for binding to these materials, and binding depends on the flow conditions within the vessel[106]. At high shear rates (above 500 to 800 sec^{-1}) usually found in arterioles, platelet adhesion depends on the binding between platelet glycoprotein Ibα on the GPIb-IX-V complex with the A1 domain of VWF, either sourced from the subendothelium or initially present in plasma and later immobilized to exposed collagen[108]. Below that threshold shear rate, platelets can also attach to collagen through two main receptors, integrin $\alpha_2\beta_1$ and GPVI[109]. Binding to collagen helps activate platelets, both directly through GPVI and by holding

the platelets to the subendothelium where it is exposed to low concentrations of thrombin, ADP, and thromboxane A_2[6,110]. Activated platelets then sustain their own activation through further secretion of activation agonists[111]. These activated platelets then secrete adhesive molecules like VWF and fibrinogen, as well as express adhesive receptors such as P-selectin and the activation of $\alpha_{IIb}\beta_3$[106,112]. P-selectin is important for adhesion and recruitment of leukocytes that initiate the inflammatory process critical for wound healing[106,112]. $\alpha_{IIb}\beta_3$ mediates platelet adhesion to each other via fibrinogen or VWF[106,112]. This recruits additional platelets to the wound site, a process called platelet aggregation[106,112]. The bound platelets narrow the vessel lumen, which given equal volumetric blood flow through the vessel, would increase the flow velocity of the blood, which increases the shear rate and shear stress[106]. The increased shear rate of fluid flowing close to the vessel wall makes it difficult for more platelets to attach, while the increased shear stress exerted on the vessel wall tears away platelets that are already attached[106]. Thus, as the platelet plug enlarges into the lumen of the vessel, it becomes more difficult for additional platelet accumulation, limiting the size of the platelet plug, localizing it to the injured vessel wall so that blood flow is maintained through the vessel[106].

1.1.4.3.2 Fibrin and coagulation factor XIIIa

Fibrinogen is a 340 kDa blood protein produced primarily by hepatocytes, and also secreted by platelets during hemostasis[113]. It is a dimeric protein with each dimer consisting of the Aα, Bβ, and γ chains bound by disulfide bridges[113]. Proteolytic cleavage of the A and B fibrinopeptides by thrombin leads to a conformational change in the protein, exposing "knobs" on the α chain that can bind to "holes" constitutively present on γ chains through primarily

hydrogen bonds and electrostatic interactions[113]. This leads to the self-assembly of fibrin monomers into a fibrous polymer[113].

Factor XIII is a protein found in plasma (pFXIII) and cellular (cFXIII) forms[12]. cFXIII is found in platelets, megakaryocytes and their precursors, monocytes and their precursors, and monocyte-derived macrophages[12]. cFXIII is composed of dimers of the catalytic A subunit ($FXIIIA_2$), while pFXIII consists of tetramers of two A subunits bound to two inhibitory B subunits ($FXIIIA_2B_2$)[12]. FXIIIA is primarily produced by bone marrow cells, while FXIIIB is primarily produced by hepatocytes[12]. FXIIIB exists in excess in plasma, with roughly 50% circulating in free form without complexing to FXIIIA[12]. Almost all pFXIII circulates bound to fibrinogen[12]. pFXIII is activated during hemostasis by thrombin cleavage of the activation peptide off the A subunits[12]. The A subunits then dissociate from the B subunits in the presence of Ca^{2+} and undergo conformation change to the active transglutaminase, FXIIIa. FXIIIa catalyzes γ-glutamyl-ε-lysyl linkages between many different substrates, one of which is fibrin[12]. It catalyzes fibrin γ chain linkages into γ chain dimers in minutes, and α chain linkages to α chain polymers of varying lengths in minutes to hours[114]. There are also some linkages between α and γ chains, as well as γ trimers and tetramers with prolonged incubation[114]. FXIIIa crosslinking of fibrin increases clot stiffness[114].

Fibrin is an adhesive protein and it has been used as a tissue sealant since the 1940s[103]. It attaches to wound sites through chemical bonds and mechanical interlocking to irregularities in the wound surface[103]. FXIIIa catalyzes covalent crosslinks of fibrin to various substrates in the wound, such as collagen and fibronectin[115]. Fibrin also has RGD motifs on the N- and C- termini

of its α chains, which can bind to receptors on various cells in wounds, such as platelets, megakaryocytes, endothelial cells, and fibroblasts[116].

1.1.4.3.3 Other plasma proteins

Recent findings suggest that immediately after vessel injury and exposure of the subendothelium to the blood stream, plasma fibronectin and possibly other circulating ECM proteins are deposited on the wound surface prior even to the attachment of platelets[107]. As platelets accumulate at the wound site, they further secrete more adhesive proteins, fibronectin, vitronectin, fibrinogen, and VWF[107]. These ECM proteins become incorporated into the subendothelial matrix exposed on the wound site through various mechanisms. For instance, VWF can bind to itself through disulfide linkages and bind to collagen through its A3 domain[117]. Fibronectin has RGD motifs for cellular attachment[118], as well as domains for interacting with collagen[119]. These proteins tether the adhesion of platelets and fibrin to the subendothelium. In addition, they are integrated into the forming blood clot, modulating its mechanical properties[107,115].

1.1.5 Q-PEG, a synthetic FXIIIa substrate

FXIIIa is formulated into fibrin sealants to improve their adhesion to tissues[103]. FXIIIa-crosslinkable synthetic fibrin-mimicking sealants with improved mechanical properties have been developed over the years based upon several landmark studies. In 1999, Schense and Hubbell coupled bifunctional peptides onto fibrin via FXIIIa-mediated crosslinking[120]. Of the peptides used was one containing the FXIIIa-crosslinkable domain derived from the amino acid sequence of α2AP. This was a pioneering work for incorporating novel functions to fibrin using

a synthetic FXIIIa substrate. In 2002, Sanborn *et al.* conjugated polyethylene glycol (PEG) to a peptide substrate of FXIIIa derived from fibrin. The material was shown to form a hydrogel when encapsulated FXIII was released from thermal-responsive liposomes[5]. PEG was chosen as the backbone material due to good water-solubility, low cost, low toxicity, low protein adsorption, and low immunogenicity as a result. In 2013, Mosiewicz *et al.* conjugated a α2AP-derived peptide to PEG[3]; a material similar to the Q-PEG described in this thesis. Combined with a PEG macromer conjugated to a short lysine-donating peptide, K-PEG, the two were crosslinked by FXIIIa to form a hydrogel. By photocaging the K-peptide, hydrogel formation became controlled by light stimulation, which cleaved the cage group and allowed FXIIIa access.

The molecular mechanisms of FXIIIa's interaction with the α2AP-derived Q-peptide were investigated in detail by Pénzes *et al.*[121] In short, FXIIIa's active site Cys314 forms a thioester bond with the acyl group on the second glutamine in the Q-peptide sequence (N**Q**EQVSPLTLLK), releasing an ammonia molecule. In the second step, the acyl group is transferred to the primary amine donor substrate, forming an isopeptide bond between the γ-glutamyl residue on Q-PEG and the primary amine, releasing the covalently-linked product from the enzyme. A multi-armed PEG that is conjugated to multiple Q-peptides can then form multiple linkages to other Q-PEG macromers through the tethering of substrates containing multiple reactive amines, such as multi-armed K-PEG or polyamines like spermidine and cadaverine. Standard ammonia-detection assays can be used to monitor FXIIIa activity[122].

1.2 Rationale

Bleeding remains a major health problem. Approximately 5 million people die of trauma each year worldwide, with a third of these deaths due to exsanguination[14]. Understanding the mechanisms of hemostasis has led to the development of many lifesaving technologies to treat hemorrhage. Since the publication of the cascade model of coagulation in 1964[7], tremendous advances have been made in the understanding of the underlying biochemistry behind blood clotting. Global coagulation assays to analyze the mechanical properties of blood clots have also been developed, such as thromboelastography (TEG), a technique developed in 1948 to trace the shear elastic modulus during clot formation and dissolution[123]. Such global assays have the disadvantage of being nonspecific, making it difficult as a tool to pinpoint which coagulation reaction may be dysfunctional in a patient with clotting pathologies[124]. However, renewed interest in analyzing the bulk mechanical properties of blood clots has developed in recent years after seeing uses in the clinic as a tool to guide the type and number of blood product transfusions needed to treat certain types of hemorrhage including those in cardiac[87,88,89] and hepatic surgery[90,91]. They have also been used as a research tool to evaluate the function of various hemostatic technologies prior to *in vivo* testing[125,126].

Blood clots physically halt bleeding by acting as a plug to stop blood leakage from damaged vessels[6]. It needs to be both cohesive, to resist breakage under the blood pressure it is subjected to, as well as adhesive to the site of injury[58]. While numerous tests have been developed to analyze cohesive properties of blood clots[90,127,128], they do not measure clot adhesive strength to substrates found in vessel walls and surrounding tissues in the wound pocket. The adhesive properties of various components of blood, such as single fibrin

strands[103,129] and platelets[130,98,106], have been extensively characterized, but the complexity of clot architecture makes it difficult to model and predict bulk adhesive properties based upon these observations alone[131]. How various components of a blood clot interact with each other and to the substrate to which it is attached to contribute to the final adhesive strength of the bulk material to the wound site remains to be fully elucidated. The aim of this work is to identify blood components that increase clot adhesive strength and utilize this knowledge to develop technologies for modulating this property.

1.3 Objectives

1.3.1 Identifying blood components that increase clot adhesive strength.

1.3.1.1 Biological question

Blood clots must adhere to the site of injury to achieve hemostasis[58]. Multiple components of clots, such as platelets and fibrin, are known to be adhesive, and their adhesive strength characterized on a molecular level[103,129, 130,98,106]. However, clot architecture is complex and makes it difficult to deduce macroscopic adhesive properties based on these molecular level observations alone[131]. The objective of Chapter 2 was to measure the adhesive strength of bulk clots of various compositions and to identify the components that increase the adhesive strength of clots to collagen, a common substrate on wound surfaces.

1.3.1.2 Significance

The mechanical properties of blood clots are important for their hemostatic function[131]. While the clot's resistance to breakage from various forces have been measured[90,127,128], this is not directly correlated with the clot's adhesive properties, as clot adhesion depends also on the

interaction of various clot components with the substrates on the wound surface. By identifying the clot components that improve macroscopic adhesion, it allows us to develop methods to modulate clot adhesion, with a future goal of creating therapeutics to treat hemorrhage, particularly secondary hemorrhage due to clot detachment.

1.3.2 Formulating a FXIIIa-crosslinkable macromer, Q-PEG, to copolymerize with blood clots during coagulation.

1.3.2.1 Biological question

One method of modulating the mechanical properties of natural biomaterials is to copolymerize them with synthetic materials[132,133]. However, the formation of blood clots is a tightly regulated process, responsive to a plethora of diverse yet specific signals[6]. This tight regulation is required to ensure that clots form only when and where needed to stop bleeding[6]. A synthetic material designed to copolymerize with blood to modulate clot mechanical properties must similarly only form an insoluble polymer when and where specific signals are present to activate the coagulation system. The objective of Chapter 3 was to test the method of coupling a synthetic material, Q-PEG, to the coagulation cascade via FXIIIa. The hypothesis was that Q-PEG, a FXIIIa-crosslinkable macromer, when formulated in blood, would only polymerize into a hydrogel when the coagulation cascade is activated, which would generate the necessary FXIIIa to catalyze the reaction[12].

1.3.2.2 Significance

The coagulation system acts as a complex sensor and amplifier of signals of vascular damage[6]. This system regulates the activation and modification of multiple cells and proteins

leading to the final formation of the hemostatic clot[6]. It is unclear whether the formation of a synthetic hydrogel can be coupled to this system as an additional output of the coagulation cascade. Testing this concept would help in the design of future hemostatic materials that could localize and enhance clotting at the specific site of injury. A broader significance of this work is to examine whether natural biochemical networks could be exploited as sensor systems to control the formation of synthetic materials in response to multiple specific stimuli. This could be useful for the design of novel responsive materials[134].

1.3.3 Examining the adhesive strength of blood clots copolymerized with Q-PEG.

1.3.3.1 Biological question

Blood clots must remain adhered to the site of injury to achieve hemostasis, particularly during fluid resuscitation when increased blood pressure may detach the clot and lead to secondary bleeding[58]. Although adhesion is important for hemostasis, no technologies have been developed to specifically enhance it. In Chapter 4, Q-PEG was copolymerized with blood and the adhesive strength of the resulting copolymer was measured. Various conditions of fibrin deficiency that led to decreased clot adhesion (depletion, tPA-induced fibrinolysis, decreased rate of fibrin formation from congenital FIX deficiency) were modeled and the effect of Q-PEG was explored. It was hypothesized that Q-PEG would recover the adhesive strength of these fibrin-poor clots.

1.3.3.2 Significance

Synthetic copolymers can modulate the mechanical properties of biomaterials. Such polymers have been developed to increase clot stiffness[132,133]. However, to our knowledge, this is the first study where a synthetic copolymer was shown to increase bulk clot adhesive strength to

purified collagen and aortic tissues. This presents a strategy for the development of future hemostatic materials that improve clot adhesion to prevent secondary hemorrhage, particularly in cases where fibrin may be deficient due to congenital conditions, traumatic bleeding, or heightened fibrinolysis[10,135,136]. Fibrin supplementation therapy has been tested in small studies for some of these conditions with promising results[68,69,70] and larger clinical trials are under way[137,138]. However, fibrin concentrates require a high cost for production[139], put a strain on the demand for donor blood supply[140], and may not be available for patients who cannot receive blood product transfusions due to religious reasons[82]. A synthetic fibrin analog similar to Q-PEG may be an alternative treatment option for these patients.

1.4 Hypotheses

The overall hypothesis of this dissertation work is that a synthetic FXIIIa substrate, Q-PEG, can improve the adhesive strength of blood clots to collagen. This hypothesis can be broken into three parts: 1) It is hypothesized that FXIII concentration is positively correlated with the adhesive strength of a fibrin-rich blood clot to collagen. 2) It is hypothesized that a Q-PEG and polyamine formulation can be controllably crosslinked by FXIIIa generated via coagulation cascade activation. 3) It is hypothesized that blending Q-PEG into blood would therefore improve clot adhesion to collagen, which is an amine-donating FXIIIa substrate.

Chapter 2: Fibrin and FXIIIa are Major Contributors to Bulk Blood Clot Adhesion

2.1 Synopsis

Blood clots must be adhesive to the wound site to achieve hemostasis, however, the adhesion of bulk clots have not been explored in detail. Many components of blood clots are known to be adhesive, such as platelets and fibrin, but it is unclear whether their adhesive strength is additive. In this study, we measured the adhesive strength of blood clots of various compositions, in addition to measuring clot stiffness by a clinically-used technique, TEG. We found that red blood cells mildly decreased the clot stiffness and adhesive strength to collagen, but the effects were not statistically significant. While platelets are known to be adhesive to collagen, they had no statistically significant effect on bulk clot adhesion at 1 h except at a very high concentration of 686 x 10^9 cells/L, where a roughly 30% adhesive strength increase was observed. Platelets were, however, required for clot adhesion at an early time point of 2 min. Both clot stiffness and adhesive strength increased as fibrinogen concentration was increased. While FXIII increased both clot stiffness and adhesive strength, adhesive strength increased by 3-times at a supraphysiological concentration of 30 μg/mL compared to the average physiological concentration of 10 μg/mL, while a much more modest (roughly 20%) increase was observed for clot stiffness. This suggests that fibrin and FXIII are major contributors to bulk clot adhesion, and that bulk clot adhesion is not the result of a simple addition of all adhesive components but represents an interaction between these components with themselves and with the wound substrate.

2.2 Background

Blood coagulation is typically effective at sealing damaged blood vessels to achieve hemostasis, but it can be compromised during severe hemorrhage. One of the reasons why coagulation is compromised during severe hemorrhage is that when arteries are cut, strong mechanical forces can be exerted on blood clots that initially form over the wound[58,56]. These forces may increase further with fluid resuscitation due to increased blood pressure[58,56]. As a result, the clots may fail, causing rebleeding[58,56]. Topical hemostatic agents in powder form, such as mineral zeolite, have been reported to be blown off from the wound by the pressure of blood flow[27]. Therefore, there is a demand for strategies to help the clot remain intact and adhered to the site of injury to achieve hemostasis.

Blood clots are sealants[6]. They form a hemostatic plug that closes off the leak in the injured blood vessel to keep blood from further escaping[6]. There are two modes of failure for sealants like blood clots, 1) cohesive failure, and 2) adhesive failure[96]. During cohesive failure, the sealant fractures from the force applied on it. During adhesive failure, the sealant remains mechanically intact, but it is torn off from the substrate to which it is adhered[96]. The critical stress a clot can withstand before fracture, its cohesive strength, is positively correlated with its stiffness as measured by a clinically-utilized technique, TEG[141]. While the factors that contribute to clot stiffness have been extensively characterized[124,90], the adhesive properties of bulk clots have been overlooked.

Several components of blood clots adhere to wound surfaces. For example, platelets can adhere to subendothelial ECM components such as VWF[108] and collagen[109]. Fibrin has been used as a tissue sealant and is known to be able to attach to the wound pocket through mechanical

interlocking, as well as chemical bonds with ECM proteins[103]. The extent to which these contribute to the bulk adhesion of clots has not been determined, and it is not clear if their adhesion is additive or if they compete for adhesion to the substrate. It was hypothesized that platelets, fibrin, and the transglutaminase that mediates fibrin crosslinking to itself and to collagen, FXIIIa[12], would significantly increase clot adhesion to a collagen substrate. Platelets are generally considered to adhere earlier than the wave of fibrin polymerization and attachment during coagulation[130], thus it was expected that it would increase clot adhesion at a similarly early time point (seconds to minutes).

In this study, we developed an assay for measuring the lap shear adhesive strength of microlitre-volume blood clots of varying compositions to evaluate the effect of different blood components on the adhesion of bulk clots to collagen. We also measured clot stiffness using TEG; the way forces are applied in TEG is designed to measure the clot's cohesiveness, such as the amount of crosslinking between fibrin fibers[142] and the aggregation of platelets[143] within the clot. As adhesion is a process that depends both on the cohesiveness of the material to itself as well as its interactions with the substrate it is attached[96], it was expected that bulk clot adhesive strength might be related, but not directly proportional to the stiffness of the blood clot as measured by TEG. We found that red blood cells had no statistically significant effect on clot stiffness or adhesive strength to collagen. Fibrin and FXIIIa increased both the clot stiffness and adhesive strength. FXIIIa increased clot adhesive strength by 3-times at 3-times the physiological concentration[144], while its effect on clot stiffness was less than 20%. Platelets at a mid-range physiological concentration[145] increased clot stiffness by more than 10-times, but had no statistically significant effect on clot adhesion at 1 h after formation. Only at double that

concentration did platelets improve adhesive strength by roughly 30%. Furthermore, clot adhesion to ECM (collagen, fibronectin, laminin) was stronger than to plain glass. Thus, this shows that clot adhesive strength is a property that is dependent not only on the binding of its components to each other but is affected by interactions with the substrate to which it is attached. Fibrin and FXIIIa are especially important for increasing the adhesive strength of bulk clots.

2.3 Methods

2.3.1 Isolation of red blood cells (RBC) from whole blood

This study was approved by the University of British Columbia Research Ethics boards. Informed consent was obtained from all healthy human volunteers prior to whole blood donation. Human whole blood was collected into Vacutainer tubes containing sodium citrate (0.105 M) (BD Biosciences) and centrifuged at 100 g for 20 min to separate the RBC from platelet-rich plasma (PRP)[146]. The RBC fraction is centrifuged at a higher speed of 500 g for 5 min to separate residual plasma from the RBC[147]. The RBC fraction was then washed twice with phosphate buffered saline (Gibco^{TM} PBS Buffer, pH 7.4, Thermo-Fisher)[148].

2.3.2 Preparation of washed platelets

This study was approved by the ethics boards of the University of British Columbia and the Canadian Blood Services (CBS). Informed consent was obtained from all healthy human volunteers prior to whole blood donation. The CBS netCAD facility kindly provided us with PRP isolated from pooled donor blood. The PRP was centrifuged at 250 g for 20 min to pellet the platelets[149]. The platelet-poor plasma was removed from the pellet and the pellet was resuspended in citrate glucose saline buffer (120 mM NaCl, 30 mM D-glucose, 11 mM trisodium

citrate, pH 6.5). The platelets were again centrifuged at 250 g for 10 min, the buffer removed, and the platelets resuspended in Tyrode's HEPES buffer (1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 2.7 mM KCl, 137 mM NaCl, 0.4 mM NaH_2PO_4, 10 mM HEPES, 5.6 mM D-glucose, pH 6.5). The platelets were centrifuged at 250 g for 10 min for a final wash. The buffer was then removed and the platelets resuspended in fresh Tyrode's HEPES to be counted by a XN-550 Automated Hematology Analyzer (Sysmex). Platelet concentration was then adjusted by the addition of more Tyrodes's HEPES as specified in the protocol for each experiment in Table 2.1.

2.3.3 Measurement of the adhesion of blood clots using a lap shear test

Clot reaction mixtures of 50 µL in volume were prepared according to the recipes listed in Table 2.1. Adhesion was measured with a TA Q800 dynamic mechanical analyzer equipped with a shear-sandwich clamp (TA Instruments). Collagen-coated glass coverslips (Corning, all experiments except Figure 2.2e) or other types of glass coverslips (Neuvitro, Figure 2.2e) were attached to the four clamp faces using UV-curable glue (Loon Outdoors). The clot reaction mixture was loaded onto the 10 × 10 mm section that was 0.25 mm thick between each pair of coverslips, and the edges were sealed with fluorocarbon grease (Krytox grease, DuPont) to prevent evaporation (see Figure 2.1 for schematic). Clots were formed for 60 min unless otherwise specified to allow adequate crosslinking by FXIIIa[114]. A force ramp was applied at 0.01 N/min until the clots failed. Clot formation and adhesive strength measurements were performed at 37ºC. Adhesive failure was observed by visual inspection. The normality of data sets was verified with a Shapiro-Wilk test and p values were calculated by Welch's analysis of variance to account for unequal sample sizes and variances[150].

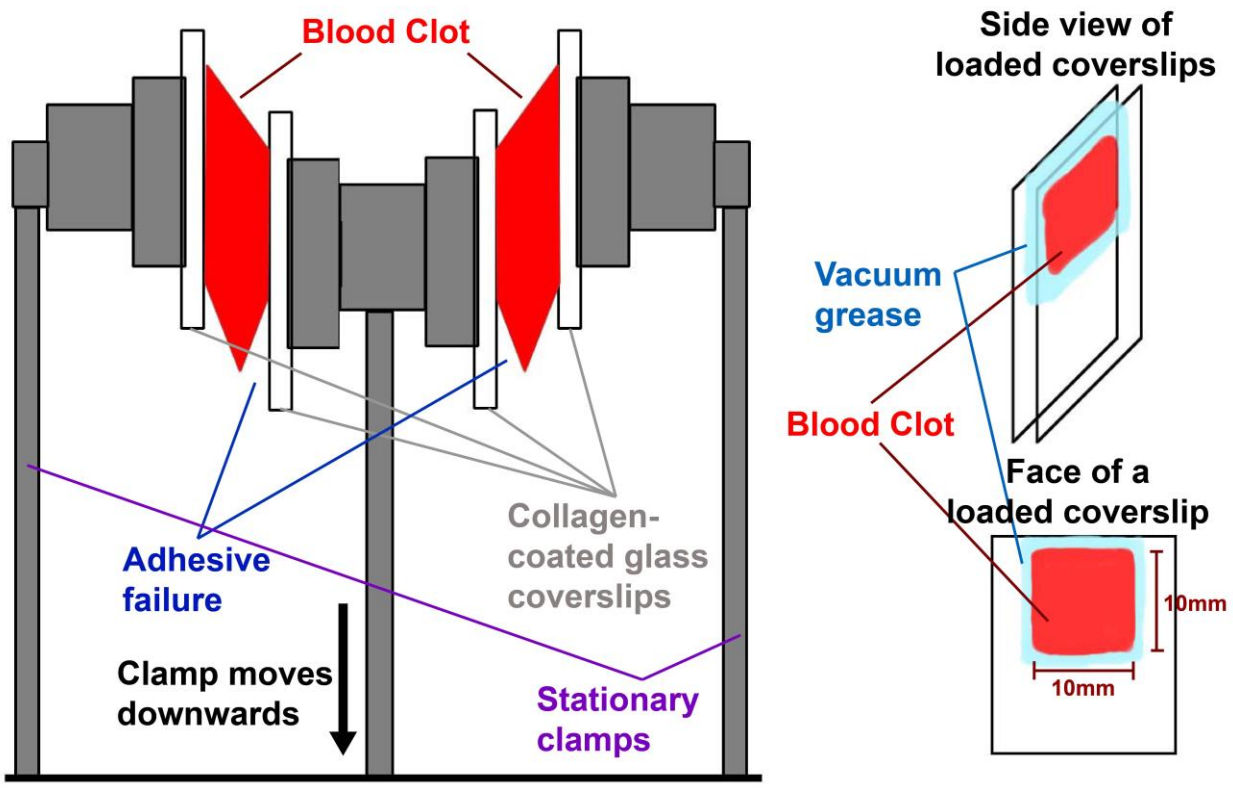


Figure 2.1 Schematic of lap-shear adhesive strength test.

A double lap-shear apparatus was used, where the middle clamp was moved downwards to deliver shearing force on the samples. Blood samples were loaded into vacuum grease-lined wells between collagen-coated glass coverslip pairs. The grease was used to prevent evaporation of samples.

Figure number	Hematocrit (% total volume)	Platelet (10^9 cells/L)	Plasma (% total volume)[†]	$CaCl_2$ (mM)	Innovin[‡] (% total volume)	Other modulators
2.2a	45	variable[§]	51 (donor)	15	2	None
2.2b	0	Platelet concentration varied,	44	15	2	None

[†] Plasmas are from commercial sources unless otherwise specified (Affinity Biologicals)
[‡] Innovin molar concentrations not declared by manufacturer (Dade Behring, Marburg, Germany)
[§] Platelet counts variable between donors and not normalized. PRP removed by centrifugation and reconstituted with or without RBC

		see figure				
2.2c	0	0	56	15	2	Fibrinogen varied, see figure
2.2d	0	0	92	15	2	FXIII varied, see figure
2.2e	Fresh donor whole blood used at 96% v/v			15	2	None
2.3a	0	343	44	15	2	None
2.3b	0	343	44	15	2	Eptifibatide (160 μM), Blebbistatin (300 μM, in 3.2% DMSO)
2.4	0	0	94	15	2	tPA (256 ng/mL)

Table 2.1 Reaction mixture recipes for lap-shear adhesive strength test, thromboelastography, and spectrophotometric clot lysis assay

2.3.4 Measurement of clot stiffness using thromboelastography

A Haemonetics TEG5000 thromboelastograph was used to measure the elastic modulus of the forming clot. The reaction mixture was 360 μL in volume, prepared according to the recipes listed on Table 2.1. The normality of data sets was verified with a Shapiro-Wilk test and p values were calculated by Welch's analysis of variance to account for unequal sample sizes and variances[150].

2.3.5 Quantification of clot lysis using spectrophotometry

Plasma reaction mixes with or without tPA treatment was prepared in a 96-well plate according to the recipes listed on Table 2.1 to a final volume of 100 μL. The plate was read by a GENios microplate reader (Tecan) at 405 nm every minute for 103 min until the absorbance of all reactions have reached the baseline level as indicated by the no-calcium negative control. A_{405}

increases as the clot becomes insoluble through fibrin polymerization and decreases as the clot is lysed[151].

2.3.6 Verification of PRP clot retraction

Standard 0.2 mL microtubes were submerged in 1% Tween 20 for 20 min then removed. Residual Tween 20 was pipetted out and the microtubes were dried. This treatment was to reduce clot adhesion to the walls of the tube[152] to allow for free contraction. PRP clots with or without platelet contraction inhibitors were prepared according to the recipes listed on Table 2.1 and incubated for 1 h before imaging.

2.4 Results

2.4.1 Fibrin and FXIIIa are major contributors to bulk clot adhesion

To test the effect of RBC on the adhesion of bulk clots, whole blood was separated into the PRP and RBC fractions through centrifugation. Clots with or without RBC were formed for 1 h and subjected to a lap shear test till adhesive failure (Figure 2.2a, left panel). While a slight decrease in both clot adhesive strength to collagen and clot stiffness as measured by TEG (Figure 2.2a, right panel) was seen with RBC addition, the differences were not statistically different. Results on clot stiffness agreed with previous literature, where it was found that increased RBC concentration might lead to a small decrease in clot stiffness[153]. This was proposed to be due to interference with the formation of the fibrin network[153]. This slight decrease in clot cohesion might be the reason for similar observations to the clot adhesive strength, as the RBC force the fibrin strands to be farther apart, and in this stretched configuration, they are stiffer and thus less capable of being strained further[154] by the lap shear stress applied. It is important to reiterate that

because the differences were not statistically different, further testing would be required to confirm whether RBC would have an effect on clot adhesive strength.

To test the effect of platelets on the adhesion of bulk clots, PRP clots were prepared by spiking commercially-purchased platelet-poor plasma (PPP) with washed platelets for final concentrations of 173 x 10^9 cells/L to 686 x 10^9 cells/L, which spans the normal physiological range to its very high end[145]. The lap shear adhesive test was performed 1 h after clot formation (Figure 2.2b, left panel). Bulk clot adhesive strengths of PPP control clots and PRP clots at 173 x 10^9 cells/L and 343 x 10^9 cells/L were not significantly different. On the other hand, the clot's stiffness increased by more than 10-fold with the addition of platelets at 343 x 10^9 cells/L (Figure 2.2b, right panel). Only at double the mid-range physiological concentration of 686 x 10^9 cells/L did adhesive strength increase by 30% compared to the no platelet control. This was unexpected, as platelets are known to be adhesive to collagen[109] and is important in mediating the initial adhesion of clot components to the exposed endothelium[130]. The effect of platelets on bulk clot adhesion was further explored in the following section.

As neither RBC nor platelets at a mid-range physiological concentration had an effect on bulk clot adhesion, the plasma component of blood clots was further analyzed. To test the effect of fibrinogen on the adhesion of bulk plasma clots, the 1 h lap shear test was performed on commercially-purchased fibrinogen-deficient plasma, with exogenous fibrinogen spiked in at varying concentrations (Figure 2.2c, left panel). Fibrinogen-deficient clots were not adhesive, and adhesive strength increased with increasing fibrinogen concentration. Similarly, no clot stiffness was detected for fibrinogen-deficient plasma on TEG, and clot stiffness increased with increasing fibrinogen concentration (Figure 2.2c, right panel). Increasing the fibrinogen

concentration from 2 g/L to 4 g/L increased clot stiffness by 4-times, and clot adhesion by less than 50%. This again shows that clot stiffness and clot adhesive strength are not directly proportional quantities. It is likely that to capitalize on the effect of increasing fibrinogen concentration on improving clot adhesion, agents to increase the crosslinking of fibrin to the collagen surface would need to also increase in concentration to mediate attachment of the fibrin clot.

To test the hypothesis of adding fibrin-to-collagen crosslinking agents to improve clot adhesion, FXIII concentration was varied for fibrin-rich plasma clots. The 1 h lap shear test was performed on commercially-purchased FXIII-deficient plasma, with exogenous FXIII spiked in at varying concentrations (Figure 2.2d, left panel). Clot adhesive strength increased by roughly 2-times at a physiological concentration of FXIII (10 μg/mL)[144] compared to FXIII-deficient plasma. Further increase of FXIII concentration to 30 μg/mL led to roughly 3-times increase of adhesive strength compared to physiological FXIII concentration. Clot stiffness showed a modest increase of roughly 20% between 10 μg/mL and 30 μg/mL FXIII (Figure 2.2d, right panel). This indirectly shows that FXIIIa-mediated crosslinking of clot components, namely fibrin, to the substrate surface is likely one of the mechanisms by which the bulk clot attaches to collagen. This is in addition to increases in clot cohesion due to FXIIIa-mediated crosslinking of fibrin to itself that increases clot stiffness.

To confirm that adhesive strength of clots is not entirely dependent on clot cohesion to itself, the substrate to which they are attached was varied. Whole blood clots were formed on plain glass, and glass coated with different ECM proteins, collagen, fibronectin, and laminin (Figure 2.2e). Blood clots were similarly adhesive to all three ECM proteins tested but had

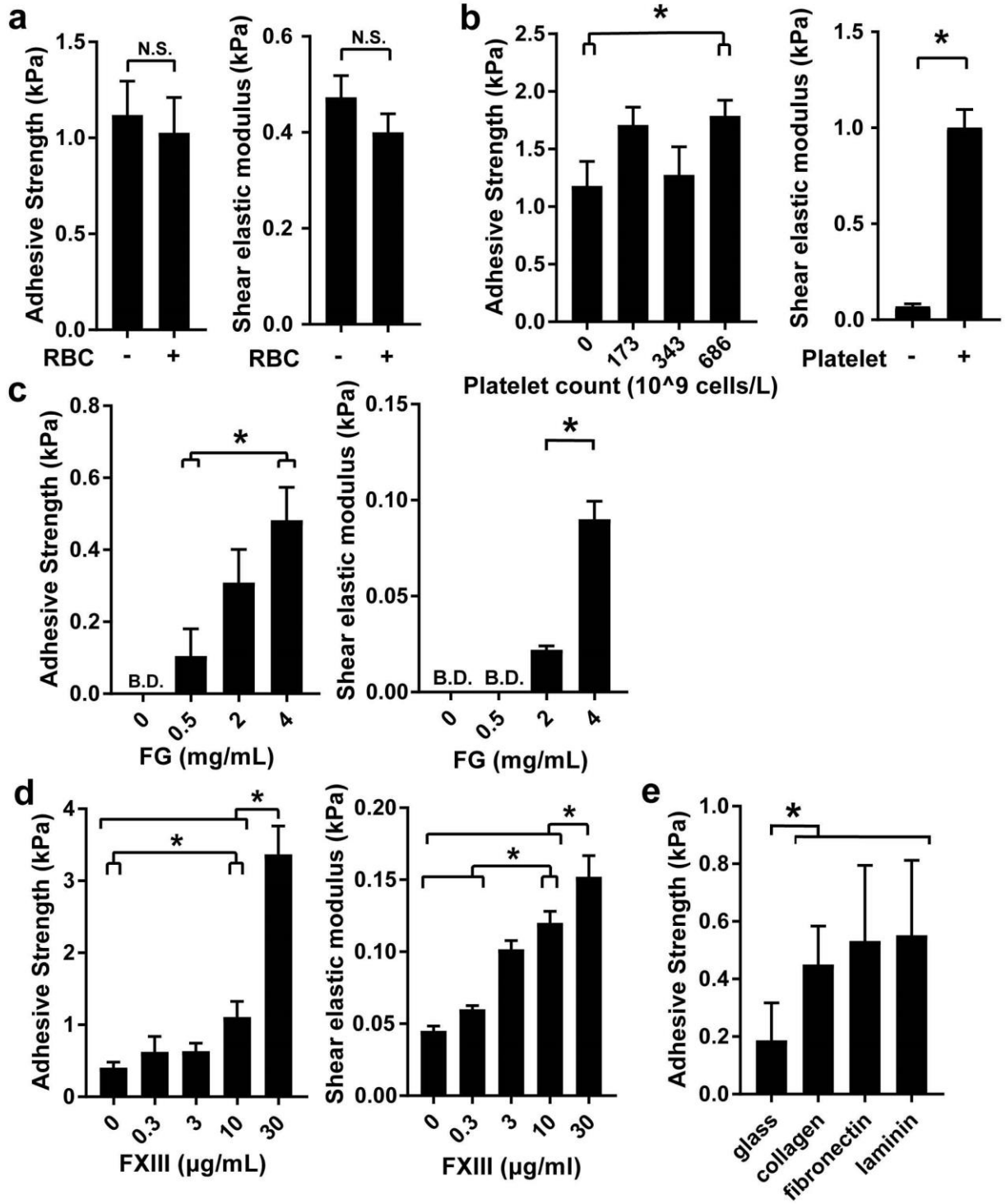


Figure 2.2 The effect of clot components on clot adhesive strength to collagen and maximum clot stiffness on TEG.

a) RBC had no significant effect on clot adhesive strength or clot stiffness. b) Platelets stiffen the clot but increased adhesive strength only at a high concentration. c) Fibrinogen and d) FXIII stiffen plasma clots and made them more adhesive. e) Whole blood clots were more adhesive to ECM proteins. Data bars indicate mean ± standard error of mean, n = 5-7, $*p < 0.05$. N.S. denotes "not significant". B.D. denotes "below detection". Refer Table 2.1 for experimental details.

decreased adhesive strength to plain glass. This could be due to mechanical interlocking of the clot to the ECM proteins, or chemical bonds between clot components with these proteins. For example, FXIIIa could mediate crosslinking between fibrin and these ECM proteins[115].

2.4.2 Platelets enhance early clot adhesion, but are not required for clot adhesion at 1 h

To further investigate the effect of platelets on clot adhesion, PRP (at mid-range physiological platelet concentration) and PPP clots were compared using the lap shear adhesive test. While at 1 h, PRP clots were not more adhesive than PPP clots, they were more adhesive at 2 min. PPP clots were not adhesive at all at that early time point (Figure 2.3a). This could be due to the role of platelets in mediating the attachment of the clot to collagen through direct binding with its GPVI receptor[155], or indirectly tethered by VWF[109]. This could also be due to the platelets' procoagulant functions, which lead to quicker clot formation[130]. To test whether platelet contraction affects clot adhesion, platelets were treated with two inhibitors, eptifibatide, an inhibitor of integrin $\alpha_{IIb}\beta_{III}$, a fibrinogen and VWF receptor[156], or blebbistatin, an inhibitor of myosin II[157]. Both inhibitors stopped platelet contraction (Figure 2.3b), and eptifibatide decreased clot stiffness on TEG (Figure 2.3c), but neither inhibitor affected clot adhesionat 1 h (Figure 2.3d,e). This suggests that platelet contraction does not affect bulk clot adhesive strength. Together, these results suggest that platelets may be involved in the initial attachment of the bulk

clot to the substrate surface but are not required for bulk clot adhesion at a later time point, at least under the static conditions tested in this assay. Based on previous literature, the platelets are important for clot initiation under flow since its GPIbα receptor has a fast on-rate to attach to subendothelial VWF[106]. The platelets subsequently release procoagulant molecules and provide appropriate surfaces for reactions of the coagulation cascade to take place, thus mediating the formation of fibrin that then perfuses and locks onto the ECM matrix[158]. While the platelets can increase the stiffness of the clot by contracting the fibrin fibers, this does not affect the strength to which the fibrin is bound to the ECM.

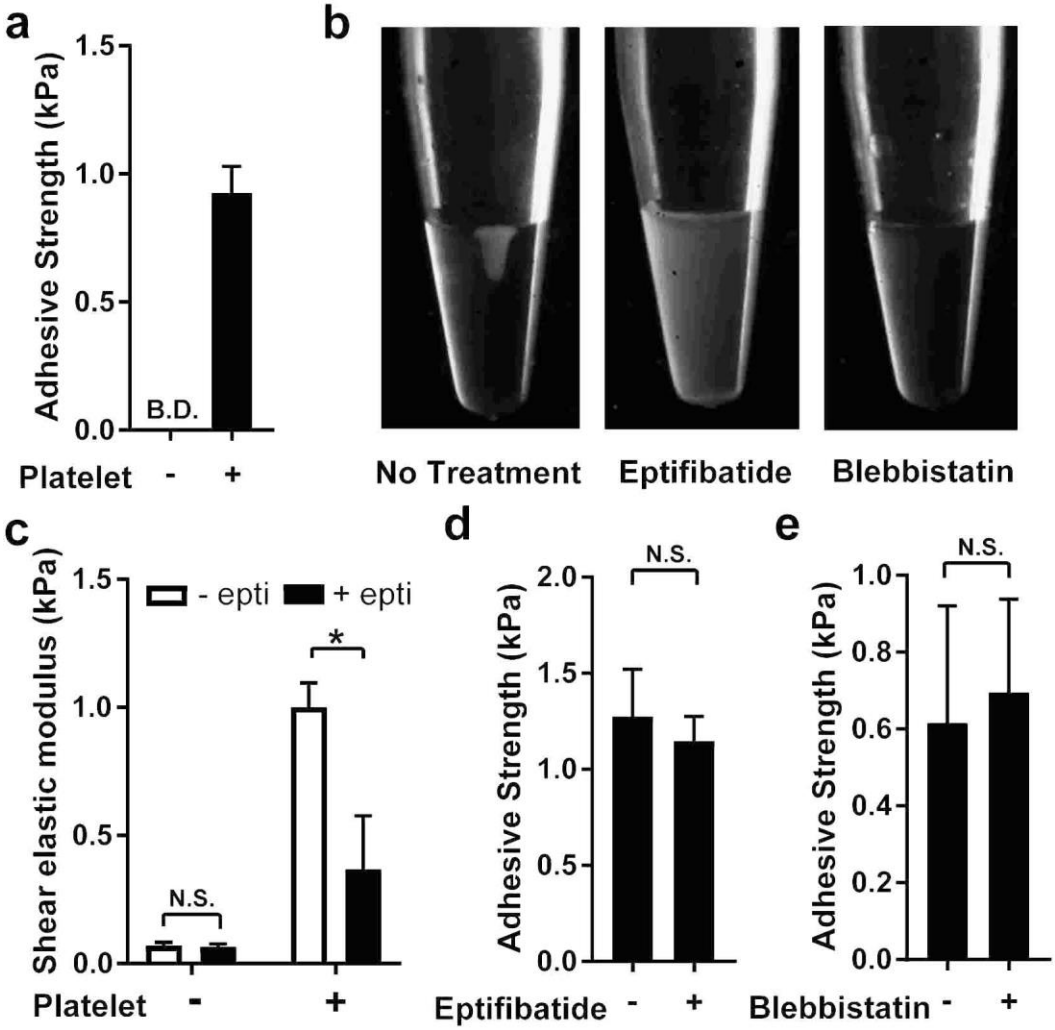


Figure 2.3 The effect of platelets on clot adhesive strength to collagen.

a) PPP clot is not adhesive at 2 min. b) Platelets treated with eptifibatide or blebbistatin did not contract. Representative images of clots (lighter-coloured material) in microtubes shown. Clot formation was confirmed after imaging by poking with a pipette tip. c) Eptifibatide decreased PRP maximum clot stiffness on TEG. d) Eptifibatide and e) blebbistatin have no effect on PRP adhesive strength at 1 h. Data bars indicate mean ± standard error of mean, n = 5-10, $*p < 0.05$. N.S. denotes "not significant". Refer to Table 2.1 for experimental details.

2.4.3 Bulk clots treated with tPA completely lose adhesive strength before full clot lysis

Hyperfibrinolysis is a complication of severe bleeding that affects roughly 25% of patients[72] and is associated with increased mortality risk[159]. Hyperfibrinolysis increases the risk of secondary hemorrhage, and antifibrinolytics as TXA have been shown to decrease this risk[74]. To investigate how fibrinolysis affects clot adhesion, clots were treated with tPA and subjected to the lap shear adhesive strength test at various time points (Figure 2.4a). This was compared to global clot lysis as evaluated by thromboelastography (results below, data not shown) and spectrophotometry (Figure 2.4b). The results show that clots fully lost adhesive strength at an earlier time point (46 min) than complete clot lysis (Lysis time estimate 62.2 ± 2.48 min, n=6 on TEG, and 56 min by spectrophotometry). As tPA is secreted by cells of the endothelium and subendothelium during tissue damage[160], it is expected to be localized to the wound tissues to which clots are attached *in vivo*. This may make the effect of tPA on clot adhesion more prominent, as it activates plasmin locally to degrade the fibrin at the clot-substrate interface. Plasmin also degrades the FXIIIa activated there[161], which would otherwise contribute to the crosslinking of fibrin to collagen and other ECM proteins. Since tPA may cause clots to completely lose adhesive strength even when the clots are still physically present, this may lead clots to fail adhesively and detach from the wound site, causing rebleeding.

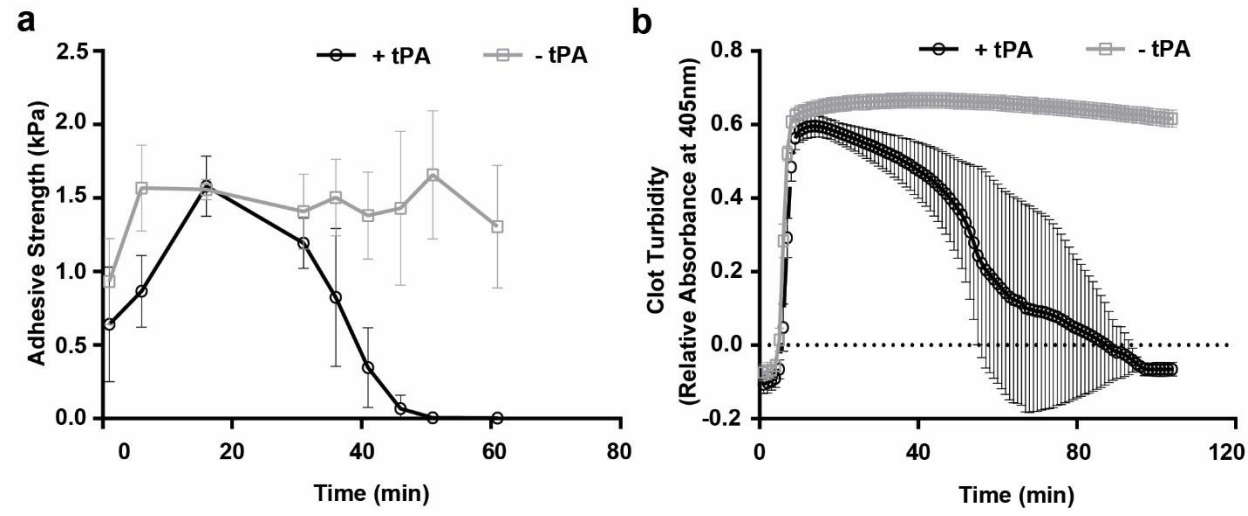


Figure 2.4 Clots lose adhesion before full clot lysis during tPA-induced fibrinolysis.

a) Adhesive strength of tPA-treated clots was lost at 46 min. b) The clots fully lysed at 56 min as observed by spectrophotometry. Absorbance plotted is relative to the no-clotting control without any calcium added to the citrated plasma. Data bars indicate mean ± standard error of mean, n = 3-5. Refer to Table 2.1 for experimental details.

2.5 Discussion

In this study, we developed a lap shear adhesive strength test that could analyze microliter volumes of blood, which allowed us to cost-effectively modify the composition of blood samples measured by adding or subtracting components. We measured bulk clot adhesive strength to collagen, a substrate found in the subendothelium and surrounding tissues in the wound site[162].

Results showed that RBC decreased clot adhesive strength and clot stiffness mildly, although the differences were not significant. It has been reported in previous literature that RBC may decrease clot stiffness by interfering with the binding and contraction of fibrin[153]. While

RBC have adhesive receptors and may bind to endothelial cells and other cell types[163], this adhesion does not seem to significantly change the bulk clot's adhesive strength to collagen.

Platelets increased clot adhesive strength at 2 min after the initiation of clotting, but at a mid-range physiological concentration no statistical differences were detected at 1 h. At double this concentration, platelets increased clot adhesive strength by roughly 30%. Experiments with inhibitors, eptifibatide and blebbistatin, suggested that platelet contraction did not affect clot adhesion. Although effects on adhesive strength were minor, platelets increased maximum clot stiffness drastically, up to 10-times, and this process can be greatly inhibited by inhibiting platelet contraction, decreasing clot stiffness by 3-times. This agrees with previous studies showing that platelets mediate the initial attachment of the clot during primary hemostasis, initiating fibrin formation at the clot-tissue interface, which strengthens clot attachment early in its formation[130].

Fibrin increased both the clot stiffness and adhesive strength in a concentration-dependent manner, with a 4-times increase in clot stiffness, and a less than 2-times increase in clot adhesive strength when the concentration was increased from 2 mg/mL to 4 mg/mL. Interestingly, the addition of FXIII at a supraphysiological concentration (30 µg/mL, compared to 10 µg/mL physiological concentration) increased clot adhesive strength by 3-times, while it increased clot stiffness by roughly 20%. This suggests that while fibrin increases clot stiffness, which reflects increased clot cohesion, its effect on clot adhesion is moderate when there isn't a corresponding increase in FXIII concentration to mediate crosslinking of the fibrin to the substrate surface.

Clot adhesive strength was substrate dependent, with stronger attachment to ECM proteins than plain glass. This was likely due to both mechanical interlocking of the clot to the ECM fibers, as well as chemical bonding to the ECM, which could be mediated by FXIIIa[115]. Furthermore, during tPA-induced clot lysis, adhesive strength was lost before full solubilization of the clot. As the tPA was blended evenly in the clot reaction mix in these experiments, rather than localized to the substrate surface as would be expected at a wound site since tPA is produced by tissue cells[160], the *in vivo* effect on adhesion might be even more pronounced.

Adhesion and cohesion are linked processes[96]. Adhesion depends both on the cohesion of the adhesive as well as its bond to the substrate to which it is attached. Results shown here reflect this relationship. A bulk clot with no stiffness detectable by TEG has no detectable adhesive strength. It is possible that a thin layer of cells and proteins may be able to adhere to the substrate surfaces, but this layer does not bridge the two substrates and thus the bulk clot as a whole cannot withstand the lap shear stress applied in these experiments, and likely cannot withstand the stresses of blood flow during severe hemorrhage either[37]. However, a more internally cohesive clot, here measured as increased stiffness by TEG[164], is not necessarily more adhesive. The results on varying adhesive substrates show that clot adhesion is affected by the interaction between the clot and the substrate to which it is adhered, and not solely a product of the clot binding itself together. Furthermore, the adhesive strength of a bulk clot is not the simple additive contributions of all adhesive blood components. While platelets mediate initial attachment of the clot[130], their effect on the final adhesive strength of the bulk clot material is mild. While fibrin alone is adhesive, FXIIIa-activity improves its adhesion further. Different clot components may be complementary in mediating clot adhesion. Further research would need to

be conducted to reveal more mechanisms behind how different clot components interact with each other and the adhesive substrate to affect the adhesive strength of the bulk clot. A more systematic way of testing the effects of different clot components on adhesive strength would be utilizing factorial experimental design. A challenge to this approach is the high cost and time commitment associated with testing all possible combinations of clot components[165]. One way of mitigating this problem would be to limit the factorial experiments to a fraction of variables based on known biochemical aspects about the coagulation cascade. For instance, knowledge in our current study shows that fibrin crosslinking plays a major role in clot adhesive strength, so further experimentation can focus on other clot and wound tissue components known to be crosslinkable to fibrin.

The results of this research may be useful for the design of therapies for treating secondary hemorrhage due to clot detachment from blood flow. Fibrinogen and FXIII supplementation have been suggested as treatment options for severe hemorrhage[63], with human or animal trials being conducted to evaluate the efficacy of these treatments[69,70,138,166]. For congenital FXIII-deficiency, the only approved indication for FXIII supplementation therapy to date[167], FXIII is usually supplemented to a fraction of its physiological plasma concentration of 10 μg/mL[144]. Our results suggest that a supraphysiological concentration may be beneficial for improving clot adhesive strength. While a systemic concentration of FXIII this high may lead to thrombotic complications[168], topical application to the wound site may reduce this risk and help enhance the effect of FXIII where it can act between the clot and the wound tissues. FXIIIa can be degraded by plasmin[161], which is activated by tPA localized to the wound tissues[160]. Supplementation of FXIII to those areas where it is most lacking may help improve its efficacy.

Whether topical FXIII supplementation at such supraphysiological concentrations is effective at stopping hemorrhage would require *in vivo* testing in animal models.

Our *ex vivo* results are a first step towards understanding how bulk clots adhere to materials in the wound. It is important to note that the lap-shear adhesive strength assay here is a static system that does not fully model the complexity of various forces exerted on clots in wounds via blood flow and external movements, such as those associated with patient transport[169]. Other strength tests could be used to reveal a more comprehensive picture of clot mechanical properties under various stresses, such as peel[101], rotational shear[170], tensile[171], compressive[172], and burst tests[95]. Recent research in microfluidic technologies brings us closer to detailed models of blood vessels and the blood flow within. Collagen and TF coated microfluidic channels have been used to understand the deposition of platelets, fibrin, and other blood proteins. An interesting model was designed by Muthard and Diamond where a vessel injury was modeled as a TF coated collagen matrix on the side of the channel[158]. As citrated blood was run through the channel, some of the blood was perfused into the collagen matrix. Platelets quickly deposited onto the luminal side of the collagen matrix, similar to what was observed for *in vivo* clots. This formed the first hemostatic plug that also acted to limit the growth of thrombus into the lumen of the vessel, as it slowed down the diffusion of procoagulants into the blood stream[173]. Thrombin generation and fibrin polymerization occurred at the interface between the platelets and the collagen matrix, with the fibrin extending into and possibly interlocking with the collagen. These observations might explain why we did not observe major differences in the final clot adhesive strength (at 1h) with platelet addition. In our static system, coagulation factors were not washed out by blood flow, thus were able to accumulate at the collagen surface.

Clotting without platelets was slower, but did occur, and clots that formed could be robustly adhered to the collagen. Under flow, particularly at shear rates over 500 s^{-1}, platelet adhesion receptors are what mediates the activation of the coagulation cascade and subsequent fibrin formation at the wound site[34]. Such a microfluidic model would be interesting to use for analyzing the effect of FXIII on clot adhesion – whether FXIII deficiencies would lead to a tendency for clots to detach. Also, if tPA were perfused into the collagen matrix, would that also lead to a higher rate of clot detachment under flow? Such an assay would also mitigate the limitation of the 2D structure of ECM-coatings used in the current lap-shear adhesive strength test. The lack of 3D adherend structure made it difficult to evaluate potential adhesive mechanisms involving the physical entanglement of fibrin fibers with the fibrous architecture of native ECM in wound tissues[174]. A different way to mitigate this problem and expand the potential applications of this study, which might be less time-consuming and costly to develop than a microfluidics assay, would be to modify the lap-shear adhesive test to use other substrates, such as thin sections of vascular and dermal tissues, or synthetic materials used in medical devices. Ultimately, *in vivo* models of hemorrhage would be required for further understanding of clot adhesive mechanisms during different types of bleeding.

Chapter 3: Formulating a FXIIIa-Crosslinkable Macromer, Q-PEG, to Copolymerize with Blood Clots During Coagulation

3.1 Synopsis

Blood clots are smart biomaterials. The coagulation system can discriminate multiple specific stimuli from a complex environment to induce clotting only when and where it is needed. Native blood clots are generally effective at stopping bleeding but may fail mechanically during severe hemorrhage. Strategies exist to tune the mechanical properties of native biomaterials by copolymerizing with synthetic materials. However, reactions that form synthetic materials have yet to achieve the level of sensitivity towards environmental stimuli as the tailored reaction networks of complex biological systems like that of coagulation. Here we show that the formation of a synthetic material can be coupled to the coagulation cascade so that it is also stimuli-responsive like the native blood clot. Formation of the synthetic material was controlled by the activity of FXIIIa generated from the activation of the coagulation cascade. The functions of the coagulation cascade remained intact when the material was incorporated. Clot-like polymerization was induced in indirect response to distinct small molecules, phospholipids, enzymes, cells, viruses, an inorganic solid, a polyphenol, a polysaccharide, and a membrane protein. This strategy demonstrates for the first time that an existing stimuli-responsive biological network can be used to control the formation of a synthetic material by diverse classes of physiological triggers.

3.2 Background

Biological materials often form in a highly responsive manner, and organisms regulate these processes with precise control by integrating many environmental signals. This responsiveness is often the result of complex biochemical and genetic networks that sense numerous, diverse regulators[175]. These networks can amplify signals from appropriate stimuli with exquisite discretion. The formation of blood clots is one such example. To effectively achieve hemostasis and avoid disseminated thrombosis, the process of coagulation is carefully controlled by a multitude of co-operative on- and off-switches. These switches regulate an intricate network of enzymatic reactions that polymerize and crosslink fibrin. Synthetic materials have not achieved this level of control[175,176], partly because it is difficult to engineer numerous types of molecular recognition into the materials or their crosslinking systems. Current synthetic materials can typically only respond to one signal with high specificity[177,178], or alternatively, many stimuli but with the inability to distinguish between similar signals[179,180,181]. For example, there are synthetic materials that form directly in response to a specific purified crosslinking enzyme[182], but their formation lacks sensitivity to the plethora of stimuli that naturally controls the enzyme in its biological system. For this reason, there is tremendous interest in creating "smart" materials that are responsive to multiple signals, with a goal toward developing materials that can respond to environmental cues[176]. Many successful strategies have been developed[175,183,184], but smart materials do not typically detect distinct stimuli from more than three to four diverse classes of signals[176]. It can be particularly challenging to control the formation of a material through polymerization or self-assembly by multiple diverse stimuli, while the behavior of the material, such as swelling, degradation or localization, can more often

be controlled[179,180,181]. We are unaware of a material whose formation is responsive to specific stimuli from over eight diverse classes of signals. This makes it a challenge to apply copolymers to modify the mechanical properties of blood clots, which is a potential strategy to improving clot function under severe hemorrhage where arterial pressures could break and detach clots from the wound site, causing rebleeding. An ideal copolymer would increase clot cohesion and adhesion locally without increasing thrombosis risk. For this purpose, it would be useful to develop a smart copolymer which could respond like a native blood clot to the diverse yet specific cues of coagulation.

We asked the question: Can the responsiveness of a material be expanded to include coagulation stimuli if its crosslinking enzyme is connected to the coagulation cascade? To test this concept, we used a well-characterized synthetic material, a polyethylene glycol (PEG) hydrogel whose polymerization is catalyzed by coagulation factor XIIIa (FXIIIa)[1,2,3,4,5]. FXIIIa is a transglutaminase that covalently crosslinks glutamine to lysine residues or to other primary amines and is a promiscuous enzyme with many known substrates. PEG macromers conjugated to appropriate glutamine-donating peptides can be crosslinked by purified FXIIIa in buffered systems to form gels (Q-PEG)[1,2,3,4,5]. FXIIIa plays an important role in blood coagulation. During the coagulation process, the zymogen FXIII is cleaved by the protease, thrombin, and activated to form FXIIIa, which then crosslinks and stabilizes fibrin, the natural output of the coagulation system. In blood plasma, the activity of thrombin is controlled by a network of dozens of enzymatic reactions, which constitute a sensor that indirectly controls the activation of FXIII and crosslinking of fibrin[7]. We tested if this ability of the coagulation cascade to control the crosslinking of fibrin could be used to control crosslinking of Q-PEG, by mixing the Q-PEG and

a synthetic polyamine, spermidine, with fibrinogen-depleted plasma. By connecting these well-characterized components, we hypothesized that the coagulation cascade could control Q-PEG to polymerize in a clot-like manner in response to new stimuli.

We found that the Q-PEG hydrogel was formed as a product of the coagulation cascade in place of fibrin. Although the blood coagulation network is composed of dozens of reactions that could have potentially been impeded by replacing fibrinogen with high concentrations of a synthetic macromer, the network remained functional. The network retained the ability to sense the vast and specific repertoire of natural regulators of coagulation to robustly form and degrade this material. The material was then controlled not just by the direct addition of FXIIIa, or FXIII and thrombin, but also indirectly by the multitude of diverse stimuli that can modulate the coagulation network through thrombin and FXIIIa. Q-PEG polymerized in response to all the system's natural input triggers we evaluated, including specific chemicals, materials, cells, and combinations of stimuli. Q-PEG exhibited greater mechanical stiffness than the natural coagulation output, fibrin. In this strategy, the responsiveness of the modified system emerged nearly entirely from the biological network and this responsiveness was passed on to the crosslinking enzyme and then indirectly to the material, leading to its clot-like polymerization. This approach of changing the output of a biological network resembles strategies used in synthetic biology, where networks in microorganisms are altered to produce new biological products or gain new functions[185,186,187]. Although the approach and the results are in some ways rather intuitive and expected, the outcome is remarkable in that it is the first liquid mixture we are aware of that responds to specific modulators from over eight diverse classes of signals to harden into a synthetic material.

3.3 Methods

3.3.1 Synthesizing Q-PEG

The acetyl-NQEQVSPLTLLKKGC (Thermo Fisher Scientific, Waltham, USA) peptide derived from α2-antiplasmin was conjugated to polyethylene glycol maleimide (PEG-mal) (40 kDa, 8-armed) (Creative PEGWorks, Winston Salem, USA). To reduce disulfide coupling of the peptides, the peptide solution (20 mg/mL, 12.5 mM) was run through an immobilized Tris-(2-carboxyethyl)phosphine (TCEP) disulfide reducing gel (Thermo Fisher Scientific). For the conjugation reaction, the peptides were reacted with PEG-mal (50 mg/mL, 1.25 mM) in Tris-EDTA buffer (150 mM NaCl, 50 mM Tris-Base, 10 mM EDTA, pH 8) overnight at 4^0C, stirring continuously. To quench any remaining unreacted maleimide, mercaptoethanol (180 μM) was added to the mixture. This mixture was then dialyzed against Tris-EDTA buffer for 1 day, and then against water for 2 days. To confirm the conjugation of PEG-mal and peptides, Ellman's reagent (5,5'-dithiobis(2-nitrobenzoate) (DTNB), Sigma), a sensitive reagent for measuring free sulfhydryl content, was used to detect free thiols on the peptide. Free thiols on the peptide significantly decreased and were eliminated following conjugation with PEG-mal and subsequent dialysis. The dialyzed solution was purified further using a desalting column (Zeba Spin Desalting Columns, 7k MWCO, Thermo Fisher Scientific) and then the purified conjugates were concentrated by a Centri-prep filter (Amicon®Ultra 10K, Millipore). The final conjugates were lyophilized and stored at -20^0C until use.

3.3.2 Characterization of Q-PEG

The molecular weights of polymers were determined by gel permeation chromatography (GPC) using a DAWN-EOS multi angle laser light scattering monitor from Wyatt Technology Inc., U.S.A. and Optilab RI detectors in an aqueous 0.1 M $NaNO_3$ solution, using a dn/dc of 0.135 (Data not shown). ^{1}H NMR analysis was performed on a 300 MHz Bruker Advance at room temperature. The purity of synthesized polymers and the degree of functionalization was determined using NMR spectral data processed with ACD/Spectrus Processor software (see Figure 4.1). The amount of peptide attached to PEG was quantified by comparing the integrals corresponding to the polymer backbone and the leucine and valine –CH_3 groups of the peptide.

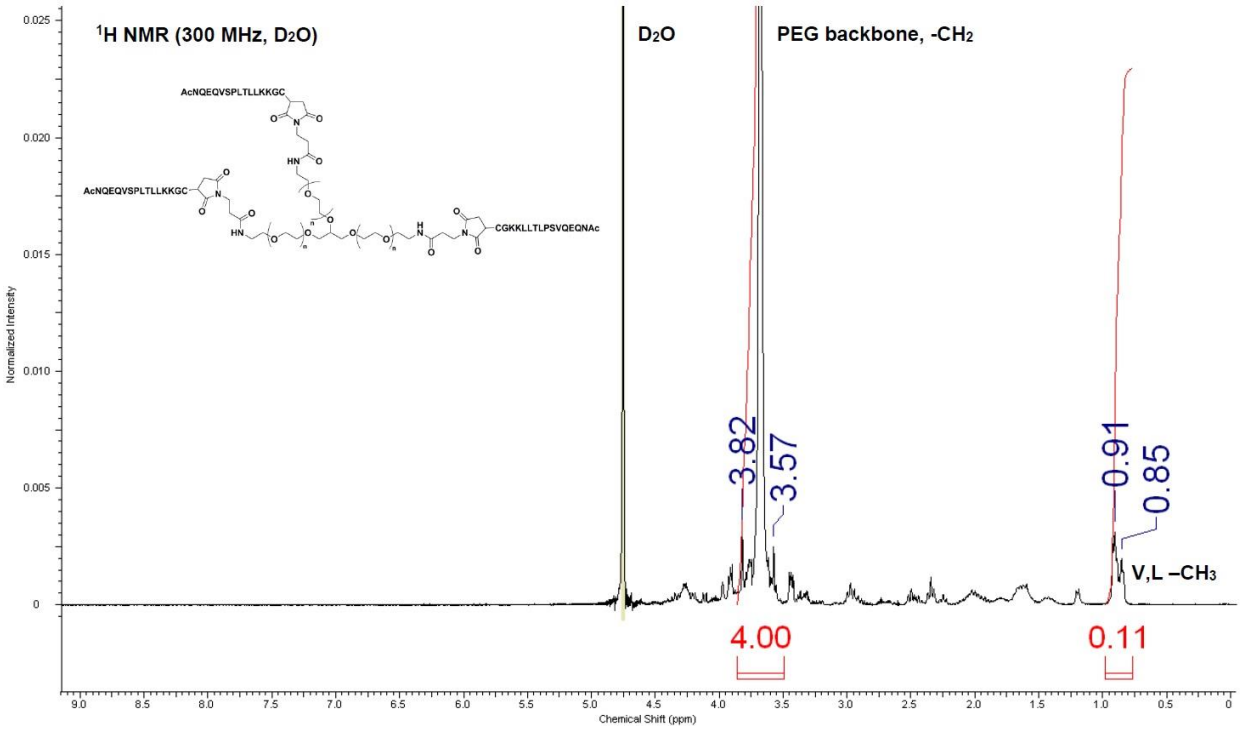


Figure 3.1 Spectral data from ^{1}H NMR (D_2O) of PEG (M_n = 39 kPa) functionalized with peptide (Q-PEG).

The peaks ranging from 3.57 to 3.82 ppm are from the PEG backbone and represent the 4 protons from the ethylene monomer. The multiplet from 0.85 to 0.91 ppm are characteristic of the leucine and valine methyl groups of the peptide (~24 H). The calculated average number of peptides attached to the 8-arm PEG is 4 groups.

3.3.3 Testing the rate of fibrin clot formation in response to known modulators

Frozen normal human plasma was thawed at 37^0C and recalcified. The recalcification solution (90 mM NaCl and 40 mM $CaCl_2$) was added to the plasma in a 1:3 ratio. Each modulator was separately added to this mixture (concentrations stated in Table 3.1) and samples were maintained at 37^0C.

Stimulus	Manufacturer	Final concentration
Human Xa	Haematologic Technologies Inc. (Vermont, USA)	100 µg/mL
Bovine Thrombin	Sigma-Aldrich (St. Louis, USA)	53 units/mL
Thromboplastin	Pacific Hemostasis (Middletown, USA)	6.6 mg/mL
Silica Particulates	"Kontact," Pacific Hemostasis (Middletown, USA)	13.3% v/v
APC	Haematologic Technologies Inc. (Vermont, USA)	50 µg/mL
DAPA	Haematologic Technologies Inc. (Vermont, USA)	100 µg/mL
Hirudin	Sigma-Aldrich (St. Louis, USA)	319 units/mL
Rivaroxaban	Sigma-Aldrich (St. Louis, USA)	13.3 µM
Fondaparinux	"Xarelto," Bayer Healthcare (Berlin, Germany)	13.3 µM
Silica Particulates (for Figure 3.7f)	"SiMAG-Silanol," Chemicell GmbH (Berlin, Germany)	66 µg/mL

Hydroxyl-coated Silica Particulates	"SiMAG-Hydroxyl," Chemicell GmbH (Berlin, Germany)	66 µg/mL
Ellagic Acid	Sigma-Aldrich (St. Louis, USA)	6.6 µM
Rutin Trihydrate	Sigma-Aldrich (St. Louis, USA)	6.6 µM

Table 3.1 Concentrations of coagulation modulators tested in Chapter 3

3.3.4 Testing the formation time of crosslinked Q-PEG hydrogels in response to modulators

A reaction mixture of 5 µL was prepared in buffer (10 mM HEPES, 7 mM sodium citrate, pH 7.4) containing the Q-PEG macromers (49 mg/mL), fibrinogen-deficient plasma (13.3% v/v) (Affinity Biologicals, Ancaster, Canada), purified FXIII (93 µg/mL) (Haematologic Technologies Inc., Vermont, USA), spermidine (1.8 mM) (Sigma-Aldrich, St. Louis, USA), dithiothreitol (DTT; 0.66 mM) (Sigma-Aldrich, St. Louis, USA), and $CaCl_2$ (29 mM) (Acros Organics, New Jersey, USA). The stimuli being evaluated were added to this mixture at the test concentrations stated in Table 3.1 and the reaction was maintained at 37^0C. Fibrinogen-deficient plasma partially depleted for other coagulation factors was used in Figure 3.8d, e, g and Figure 3.10. Specific enzyme activities for this plasma are listed in Table 3.2. A higher concentration of fibrinogen-deficient plasma (26.6% v/v) and lower concentration of supplemented purified FXIII (46.5 µg/mL) was used in Figure 3.8f.

Plasma Type	FII Activity	FV Activity	FVII Activity	Fibrinogen Concentration
Normal Plasma	1.12 U/mL	1.13 U/mL	1.22 U/mL	3.06 g/L
Fibrinogen-deficient plasma (active cascade)	1.02 U/mL	0.30 U/mL	0.64 U/mL	0.06 g/L

Fibrinogen and factors-deficient plasma	0.79 U/mL	0.07 U/mL	0.29 U/mL	0.001 g/L

Table 3.2 Enzyme activities of various plasma types used in Chapter 3.

Plasmas obtained from Affinity Biologicals (Ancaster, Canada). Enzyme activity assays were performed by the manufacturer to determine activities.

3.3.5 Gelling the Q-PEG using purified FXIIIa, without plasma

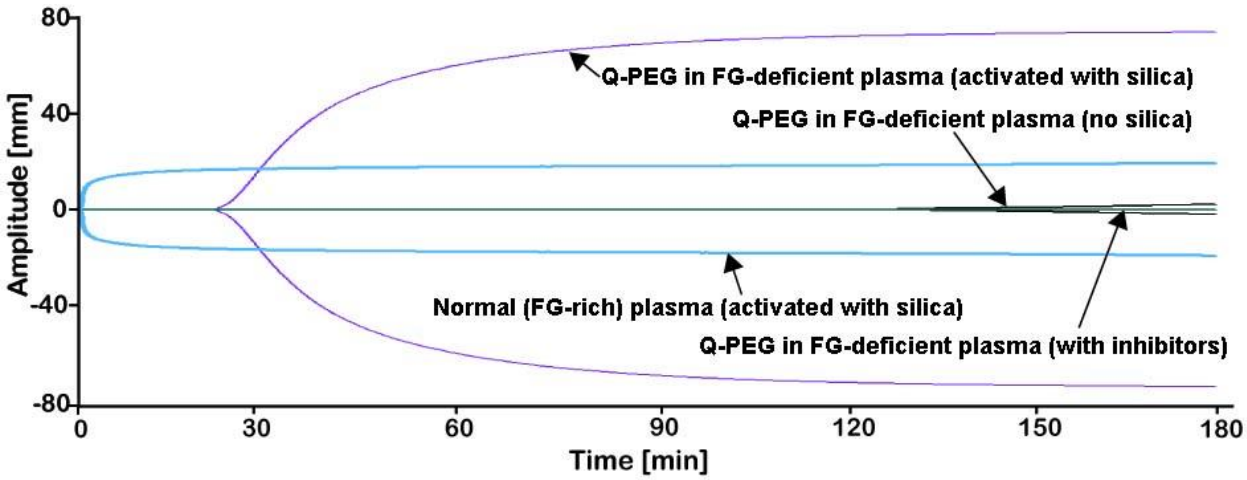
The same protocol for forming the crosslinked Q-PEG hydrogel in plasma was used with minor modifications. The reaction mixture of 5 µL was made in the same citrated buffer containing the Q-PEG macromers (87 mg/mL), human FXIIIa (0.31 mg/mL, unless otherwise stated) (Haematologic Technologies Inc., Vermont, USA), spermidine (2.4 mM), DTT (0.90 mM), and $CaCl_2$ (9.4 mM). No blood plasma was added. In the experiments in which different stimuli were tested, stimuli were added to this mixture at concentrations specified in Table 3.1. The reaction mixture was maintained at 37^0C.

3.3.6 Assessment of the mechanical properties of the synthetic polymer and fibrin clots

The mechanical properties of crosslinked Q-PEG and fibrin clots were measured during the course of their formation by thromboelastography (TEG) (TEG® 5000 Thrombelastograph® Hemostasis System, Haemoscope Corporation, IL) (see Figure 3.2 and Figure 3.6c)[188]. TEG measures properties such as the reaction rate, mechanical strength, and stability of the gel or clot by measuring rotational movement of a pin suspended in a gel mixture or plasma. Reaction time (R) indicates the time that gelation begins. Amplitude (A) is a function of clot strength or elasticity. Maximum amplitude (MA) represents the maximum strength of the gel. The amplitude

can be used to determine the shear elastic modulus (G) of the gel or fibrin clot during its formation. The reaction mixture (300 μL) of Q-PEG in fibrin-poor plasma or normal plasma was prepared as previously described and the reaction was initiated by recalcification and the addition of silica (Kontact reagent) to activate the coagulation network. The effects of an inhibitor of FXIIIa (10 mM 1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride (T101), ZEDIRA GmbH, Germany) or a cocktail of protease inhibitors (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.3 μM aprotinin, 130 μM bestatin, 1 mM EDTA, 14 μM E-64, 1 μM Leupeptin, Sigma-Aldrich) were evaluated. The samples were analyzed at 37°C for 3 hr.

The compressive elastic moduli of the Q-PEG gels were measured by a controlled force compression test (DMA Q800, TA Instruments, DE). The same protocol for forming the Q-PEG hydrogel in fibrin-poor plasma was used as described above, with individual reaction volumes of 5.79 μL. Reaction mixtures were incubated overnight at 37°C to allow them to completely gel. Each gel was removed from its microchamber and placed in a submersion-compression clamp. Its diameter was measured using a caliper to determine the surface area of the sample. Preload force of 0.01 N was applied to the gel to ensure good contact with the compression clamp. The instrument measured initial sample thickness, ramped the compression force at 0.01 N/min and measured the sample's stress and strain. The compressive elastic modulus was calculated by taking the absolute value of the slope of the stress-strain curve at its initial linear portion where elastic deformation was observed (Figure 3.3).



	Reaction time (R, min)	Maximum amplitude (MA, mm)	Elastic modulus (G, X100 N/m^2)
Q-PEG in FG-deficient plasma (activated with silica)	17.8	72.5	13.2
Q-PEG in FG-deficient plasma (no silica)	130.8	5.9	0.3
Normal (FG-rich) plasma (activated with silica)	0.8	17.0	1.0
Q-PEG in FG-deficient plasma with T101 (FXIIIa inhibitor)	179.9	0	0
Q-PEG in FG-deficient plasma with serine protease inhibitors	157	7.2	0.4

Figure 3.2 TEG curves of Q-PEG in plasma.

The shear elastic modulus of Q-PEG in FG-deficient plasma that is activated to clot (purple curve) is greater than the material in unstimulated FG-deficient plasma (black curve), normal plasma with fibrin (blue curve) and the material in FG-deficient plasma treated with a serine protease inhibitor cocktail (green curve) after 3 hr.

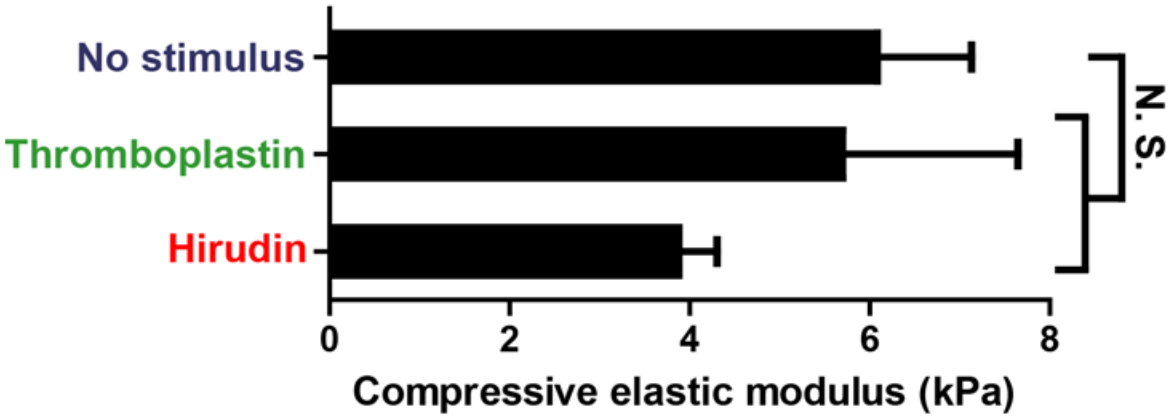


Figure 3.3 Clotting modulators do not influence compressive elastic moduli of Q-PEG in FG-deficient plasma. Activator (green) and inhibitor (red) of clotting influenced the rate of Q-PEG formation, but once Q-PEG did polymerize, the clot stiffness measured under compressive force was the same. Data bars indicate mean ± standard error of mean, n = 3-4. N.S. indicates "not significant", $p > 0.05$.

3.3.7 Assessment of the gelation of Q-PEG and normal plasma clots

The Q-PEG hydrogels or normal plasma clots were formed in polypropylene tubes. The gelation time was determined to be the time it took for the reaction mixture to reach a viscosity at which it could no longer be pipetted (see photos in Figure 3.6b). For measuring the gelation time of many samples simultaneously, a microscope assay was employed. Here, fluorescent beads (Fluoresbrite Plain YG 1.0 Micron Microsphere, Polysciences Inc., Warrington, USA) were suspended in the reaction mixture (6×10^8 particles/mL) and then transferred into individual microchambers. These microchambers were constructed by sandwiching an adhesive silicone isolator (4.5 mm diameter x 0.5 mm depth; Grace Bio-Labs, Bend, USA) between two plastic coverslips (HybriSlip, Grace Bio-Labs, Bend, USA) (Figure 3.4). Brownian motion of the green fluorescent beads within the reaction mixture was monitored via time-course imaging using a

Leica DMI6000B fluorescence microscope. Gelation was considered to be complete when the fluorescent beads stopped moving.

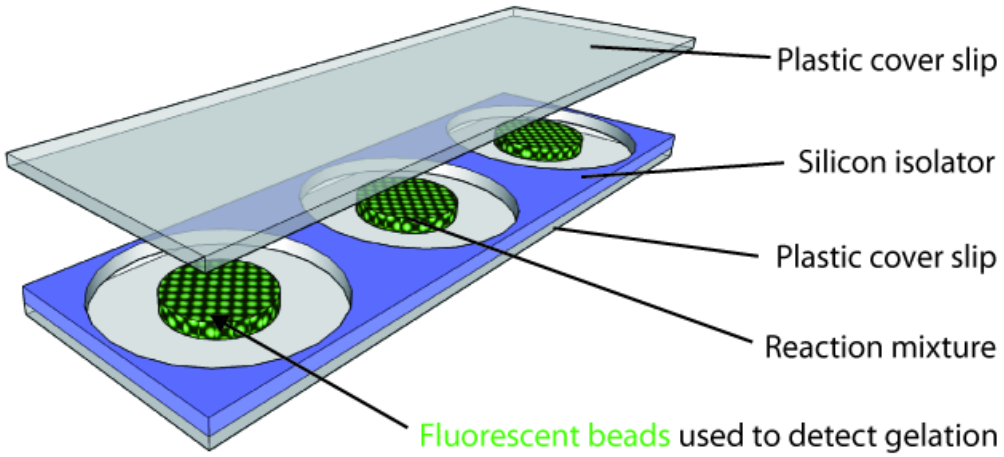


Figure 3.4 Schematic of microchambers used in microscope clotting assay.

Adhesive silicone isolators formed an air-tight seal once sandwiched between two plastic coverslips. Each microchamber held a 5 µL sample. Assay used for measuring the hydrogel formation time of plasma and samples containing Q-PEG.

3.3.8 Imaging the Q-PEG hydrogels and fibrin clots by electron microscopy

Samples for conventional SEM were prepared as described previously[189]. The gels were fixed with formalin and then dehydrated with ethanol. These gels were carbon-coated and then imaged with a Hitachi S-4700 field Emission Scanning Electron Microscope. SEM imaging was replicated three times. Cryo-EM was done following previously published methods with slight modification[190]. The gels were flash-frozen in liquid nitrogen and excess water from the samples was sublimed in a higher temperature vacuum chamber. The samples were then carbon-coated prior to imaging.

3.3.9 Generation of lipid vesicles

Two lipids were used for the vesicles: porcine brain phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, USA) and 1,2-dilauroyl-sn-glycero-3-phosphocholine (PC) (Avanti Polar Lipids, Alabaster, USA). Two types of vesicles were made using these lipids, vesicles containing 100% PC, or negatively-charged PC/PS (molar ratio 4:1) vesicles. To make these, the lipids were dissolved in chloroform within 1.5 mL polypropylene tubes. The chloroform solution was spread evenly over the tube surface, and chloroform was evaporated off the tube surface using a stream of N_2 gas. Residual chloroform was evaporated overnight in a vacuum chamber. The lipids were then resuspended in deionized water (5 mg/mL). To obtain vesicles of a specific diameter (100 nm), samples were passed through membranes of successively smaller pore sizes (Northern Lipids, Burnaby, Canada) three times using a Lipex Thermobarrel Extruder (Northern Lipids, Burnaby, Canada). The vesicles extruded off the final 100 nm membrane were used for the experiments.

3.3.10 Isolation, culture, and FACS analysis of PBMC

This study was approved by the research ethics boards of the University of British Columbia, and informed consent was obtained from all healthy volunteers before whole blood donation. Whole blood was collected into tubes containing sodium citrate (12 mM) before 1:1 dilution in buffer (PBS with 2% FBS). The mix was layered onto lymphocyte isolation density media (Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the PBMC were isolated according to previous literature with minor modifications[191]. The layered blood was centrifuged at 400 x g for 30 min at room temperature. The PBMC layer was collected. Two washes at 200 x g were performed in a buffered solution (PBS with 2% FBS) to remove the

platelets from the supernatant. The PBMC pellet was then resuspended in culture medium (RPMI 1640; 50,000 U/L penicillin; 50 mg/L streptomycin; 25 mM HEPES; 20% FBS) at 10^6 cells/mL. Cultures of 5 mL were either PBS-treated as an unstimulated control or stimulated with 1 μg/mL LPS. The cells were then incubated at 5% CO_2 and 37˚C for 24 h. For each sample, 10^6 cells were spun down and resuspended in 1 μL of HEPES-citrate buffer. The cells were then added to the coagulation-controlled gel reaction mixture. To confirm expression of TF, remaining cells were stained with Anti-Human CD142 PE (eBioscience, San Diego, CA) fluorescent antibody on ice for 30 min. Propidium iodide was also added just before FACS analysis to stain for inviable cells. The cells were analyzed using FACS Calibur (BD Biosciences, San Jose, CA). Based on the light scatter, which indicates the cell size and granularity, PBMC were selected for analysis. Single color controls were used to determine the background fluorescence level of each stain. Based on this information, quadrants were drawn to indicate which cells were fluorescing above the threshold. Target cells, which were both viable and expressed TF, were shown in the bottom right quadrant (Figure 3.5).

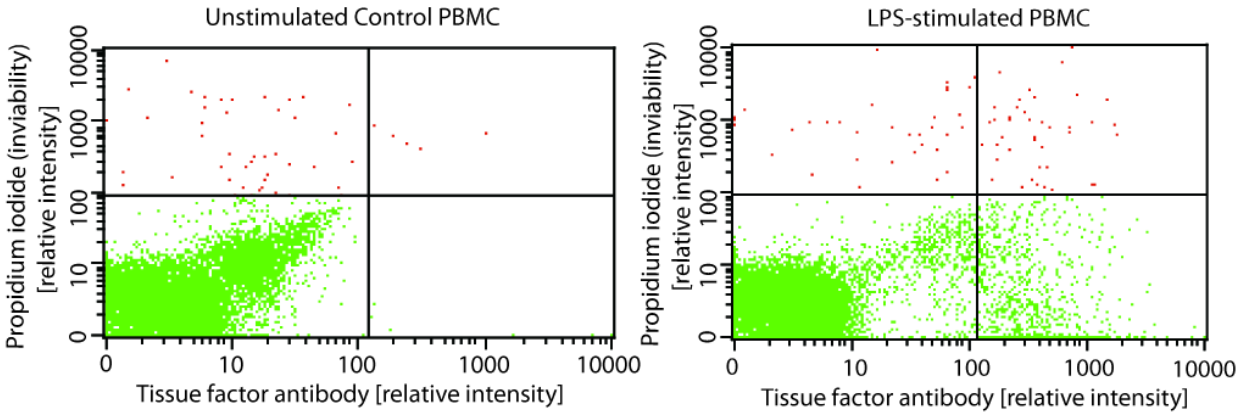


Figure 3.5 PBMC were activated by LPS to express TF.

The bottom right quadrant of both graphs showed target cells that were viable and expressed tissue factor. Viable TF-expressing cells were not consistently observed for the unstimulated control (0.06% of the population, shown on

the plot on the left). A subpopulation of PBMC expressed TF upon stimulation by LPS (6.98% of the population, shown on the plot on the right).

3.3.11 Preparing TF+ HSV-1.

A low-passage clinical isolate, HSV-1 NS, was propagated in human melanoma cell line A7 that either expresses or is deficient in TF. Viral progeny were isolated by sucrose gradient ultracentrifugation[192] and added to the Q-PEG reaction mixture as an initiator.

3.3.12 Measurement of plasmin-induced and bacteria-induced degradation of the Q-PEG hydrogel

Human plasmin (Haematologic Technologies Inc., Vermont, USA) was diluted in HEPES buffer and added to 3 μL BNC-material or fibrin clots (final plasmin concentration 333 μg/mL) pre-formed in microchambers. Microchambers were then sealed with immersion oil (type DF, code 1261) (Cargille Laboratories, Inc., Cedar Grove, NJ) and the gels were monitored for lysis at 37°C. Degradation time was determined by measuring how long it took for the immobilized fluorescent beads to become liberated from the gel mesh and exhibit Brownian motion. To measure degradation by bacteria, a similar procedure was used but plasmin was replaced with bacteria. Glycerol stocks of bacterial strains (*Bacillus thuringiensis* Berliner ATCC #35646, *Bacillus subtilis subsp. spizizenii* Nakamura *et al.* ATCC #6633, *Bacillus cereus* Frankland and Frankland ATCC #10987, *Bacillus circulans* Jordan ATCC #4513, *Staphylococcus epidermidis* (Winslow and Winslow) Evans ATCC #14990, *Staphylococcus aureus subsp. aureus* Rosenbach ATCC #25923) were streaked onto Difco™ LB agar, Lennox (BD Biosciences, San Jose, CA) plates. The bacteria were cultured overnight at 37^0C. Colonies

were picked and weighed to obtain bacterial biomass. The biomass was then resuspended in the gel reaction buffer at 0.1 mg/mL. The suspended bacteria (2.78 μL) were then added to 5 μL pre-formed Q-PEG hydrogels (final bacteria concentration 36 μg/mL) and degradation was measured.

3.4 Results

3.4.1 The biochemical reaction network of blood coagulation is a sensor that can be used to polymerize a synthetic material in a clot-like manner

To test whether the coagulation network could exert control over the formation of a synthetic material that can be polymerized by FXIIIa, the Q-PEG macromer and a polyamine were added to human plasma lacking fibrinogen, the soluble precursor of fibrin (Figure 3.6a). This fibrinogen-deficient plasma enabled us to selectively detect gelling of the synthetic macromer, rather than fibrin. Purified FXIII zymogen was added due to co-depletion of FXIII along with fibrin during commercial preparation of the plasma. The synthetic macromer was constructed as described by others, by conjugating an 8-armed PEG to a peptide sequence found in α2-antiplasmin, a natural, glutamine-donating, plasma protein substrate for FXIIIa[1,2,3,4,5]. Spermidine, a polyamine, was added with the synthetic macromer to fibrinogen-depleted plasma, serving as the amine-donating substrate for FXIIIa. Other polyamines could be substituted for spermidine (Figure 3.7). When blood coagulation was triggered with silica to activate the factor XII branch of the coagulation network, FXIIIa crosslinked the amine- and glutamine-donating substrates (spermidine and the α2-antiplasmin peptides, respectively) polymerizing the Q-PEG in a clot-like manner. The shear elastic modulus increased over 40-times during formation. The Q-PEG hydrogel also displayed mechanical properties that differed from fibrin. The shear elastic

modulus of the Q-PEG hydrogel was over 10-times higher than a fibrin clot (Figure 3.6b,c). Furthermore, the Q-PEG hydrogel was more robust than fibrin when handled. While the fibrin clot collapsed when disturbed by gentle poking of a pipette tip, the Q-PEG hydrogel did not noticeably deform. While more quantitative measurements would need to be performed to obtain the fracture strength of these respective materials, this qualitative observation suggests that Q-PEG has the potential to withstand greater mechanical stresses than the native fibrin clot, which would make it useful for applications in stopping arterial bleeds. Polymer formation in fibrinogen-deficient plasma occurred only when both the Q-PEG macromer and the amine-donating spermidine were present. An inhibitor of FXIIIa or a cocktail of serine protease inhibitors prevented polymer formation, verifying that formation was controlled by the coagulation system. Using both conventional scanning electron microscopy (SEM) and cryo-SEM, the architecture of the Q-PEG hydrogel in fibrinogen-deficient plasma was compared to that of Q-PEG crosslinked with purified FXIIIa in the absence of plasma and other coagulation enzymes (Figure 3.6d–i). The plasma-containing Q-PEG hydrogel better resembled the fibrous and porous structure of fibrin. This fibrous structure may have emerged from plasma proteins providing a template as the synthetic polymer formed, or from the spatial-heterogeneity of enzyme activation that occurs during coagulation[193,194,195,196].

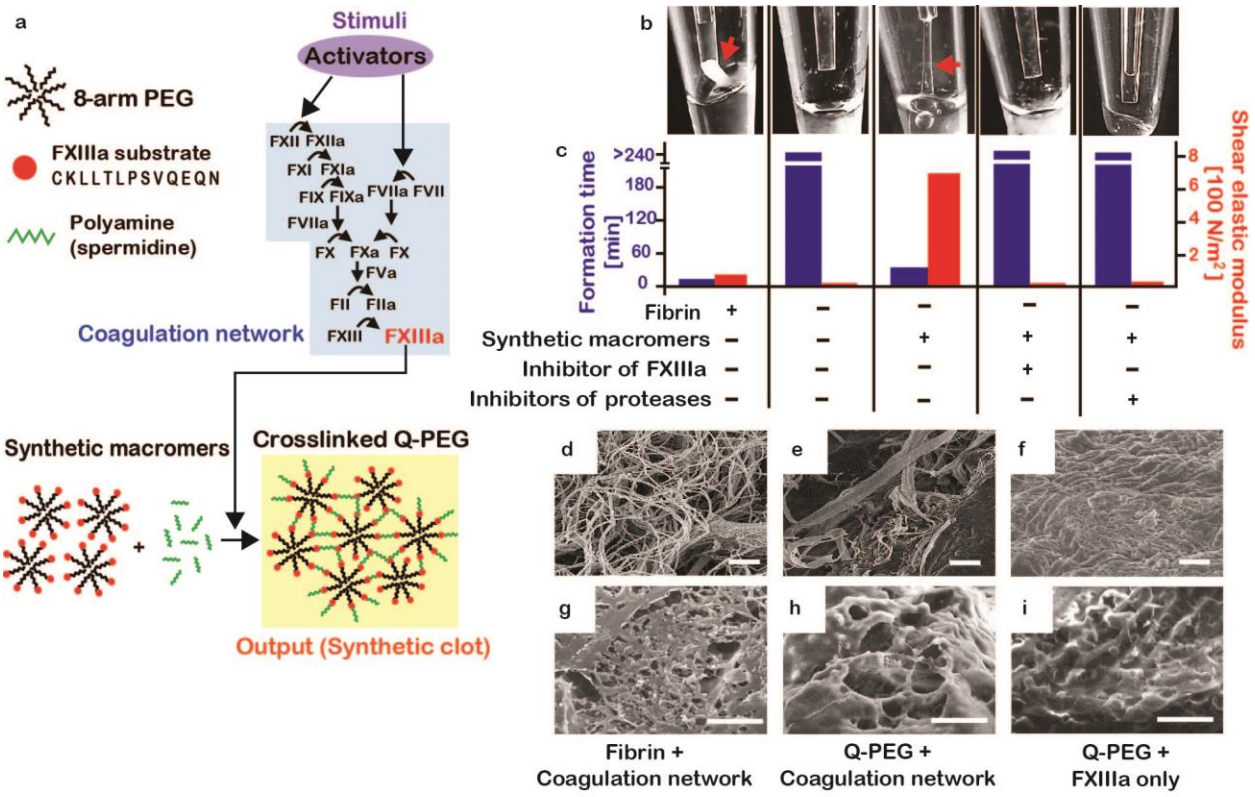


Figure 3.6 Q-PEG can be controllably crosslinked via the activation of the coagulation network.

a) Schematic showing an input of activators (purple) into the coagulation cascade (blue) generating the crosslinking enzyme (FXIIIa) that would crosslink Q-PEG and spermidine into a hydrogel material (yellow). b-c) Clot-like polymerization of liquid mixtures containing the coagulation network (supplied by FG-deficient plasma) and either fibrin or the synthetic macromers. Images of microtubes (b) show clot-like gels adhered to pipette tips and pulled upwards when fibrin or the synthetic macromers were connected to the coagulation network. Gels did not form when a serine protease inhibitor cocktail was used to fully inhibit the coagulation cascade. Neither did gels form when a specific FXIIIa inhibitor was used. The hydrogel formation times (blue bars) and the shear elastic modulus as evaluated by TEG (red bars) are shown in (c). Data bars for formation times indicate mean ± standard error of mean, n = 3-5 d-i) Conventional SEM images (top row) and cryo-SEM images (bottom row) of fibrin in normal plasma (d,g), Q-PEG in FG-deficient plasma (e,h), and Q-PEG polymerized with FXIIIa only (no plasma, f,i).

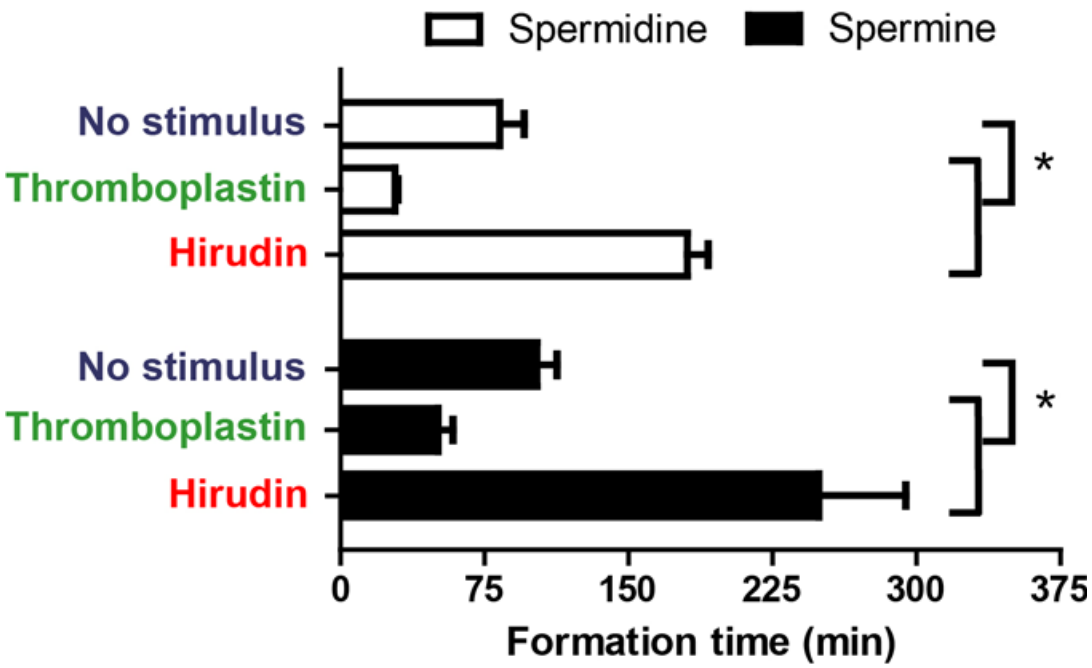


Figure 3.7 Q-PEG can crosslink to two different polyamines.

With either spermidine (used in other figures in this chapter) or spermine, Q-PEG gelled faster or slower than inert controls in response to a known coagulation activator, thromboplastin, or an inhibitor, hirudin, respectively. Data bars indicate mean ± standard error of mean, n = 3-5, $*p < 0.05$.

3.4.2 Clot-like polymerization of the Q-PEG can be controlled by diverse and specific modulators when coupled to a network

Coagulation is responsive to several distinct classes of stimuli, including small organic molecules, divalent metal ions, the extracellular matrix, anionic lipid surfaces, and soluble and transmembrane proteins. To test if the hydrogel formation of Q-PEG indirectly responds to diverse stimuli when coupled to the coagulation network, its gelation time was measured after adding known activators and inhibitors of coagulation. The activators included two enzymes, coagulation factor Xa and thrombin, thromboplastin (a mixture of transmembrane tissue factor (TF) embedded in phosphatidylserine (PS)/phosphatidylcholine (PC)-containing vesicles with

$CaCl_2$), and inorganic silica nanoparticles. The inhibitors included two small molecules, rivaroxaban and dansylarginine N-(3-ethyl-1,5-pentanediyl)amide HCl (DAPA), a small protein, hirudin, an enzyme, activated protein C, and a polysaccharide, fondaparinux. DAPA and hirudin bind and inhibit thrombin, rivaroxaban binds and inhibits FXa, APC is a proteolytic inhibitor of coagulation cofactors, and fondaparinux accelerates coagulation enzyme inhibition. These 9 diverse and specific modulators of coagulation significantly altered the formation time of the Q-PEG hydrogel. Clot-like polymerization occurred in 120 min in the absence of a stimulus, but occurred approximately twice as fast with an activator and significantly slower with an inhibitor (Figure 3.8a,b). These same activators and inhibitors respectively sped up and slowed down fibrin clot formation in normal plasma (Figure 3.9). Although they influenced formation times, these modulators did not significantly affect the final material's physical properties, such as the compressive elastic moduli (Figure 3.3). When the macromer was not coupled to the network, but was polymerized with purified FXIIIa alone, the stimuli did not cause significant differences in hydrogel formation time (Figure 3.8c). Thus, the original FXIIIa-crosslinkable material that was described previously could be made responsive to numerous new stimuli by connecting it to the coagulation network. The sensitivity of the Q-PEG formulation in plasma to activators could be modulated by changing the concentrations of coagulation factors (Table 3.1). By increasing the concentration of coagulation enzymes, another activator, ellagic acid, a plant polyphenol, could be detected by the system to speed up the rate of Q-PEG hydrogel formation. The clot-like polymerization of Q-PEG was highly specific to its modulators and could distinguish between closely related signals (Figure 3.8d–f). Prothrombin and factor X (the respective zymogens of thrombin and FXa), heat-inactivated thromboplastin, silica nanoparticles with hydroxlyated surfaces, and rutin (a plant polyphenol) have similar chemical structures to their counterparts but

led to significantly slower formation of the Q-PEG hydrogel. The enzymes of the coagulation system also specifically require calcium ions as opposed to other divalent cations, as formation of the material did not occur when calcium was replaced with magnesium or strontium.

The coagulation network can sense and respond to combinations of stimuli, which allow clotting to respond appropriately at sites of vascular damage without causing errant clotting and thrombosis. Sensing and responding to specific combinations of stimuli is challenging to engineer in synthetic materials. To evaluate if the coagulation network can be used to extend the repertoire of signals recognized by the original FXIIIa-crosslinkable material to include specific combinations of stimuli, the functional contribution of thromboplastin constituents was dissected. Q-PEG gelation was sensitive to combinations of TF, PS, and PC, and its rate was only faster when all three were combined (Figure 3.8g), which is consistent with the sensitivity of the endogenous coagulation system.

3.4.3 Cells and viruses with specific phenotypes can stimulate Q-PEG to polymerize in a clot-like manner

Materials in nature can respond to subtle differences in the phenotypes of cells and viruses in their environments. We tested if formation of the Q-PEG hydrogel could distinguish between activated and non-activated peripheral blood mononuclear cells (PBMC). A subset of PBMC activate when treated with lipopolysaccharide (LPS), a common bacterial antigen that induces the expression of TF. Formation of the Q-PEG hydrogel occurred faster in the presence of activated PBMC compared to resting PBMC that were not pre-stimulated by LPS (Figure 3.10a). Hydrogel formation in the presence of resting PBMC also occurred faster than without PBMC, but not as fast as with activated PBMC.

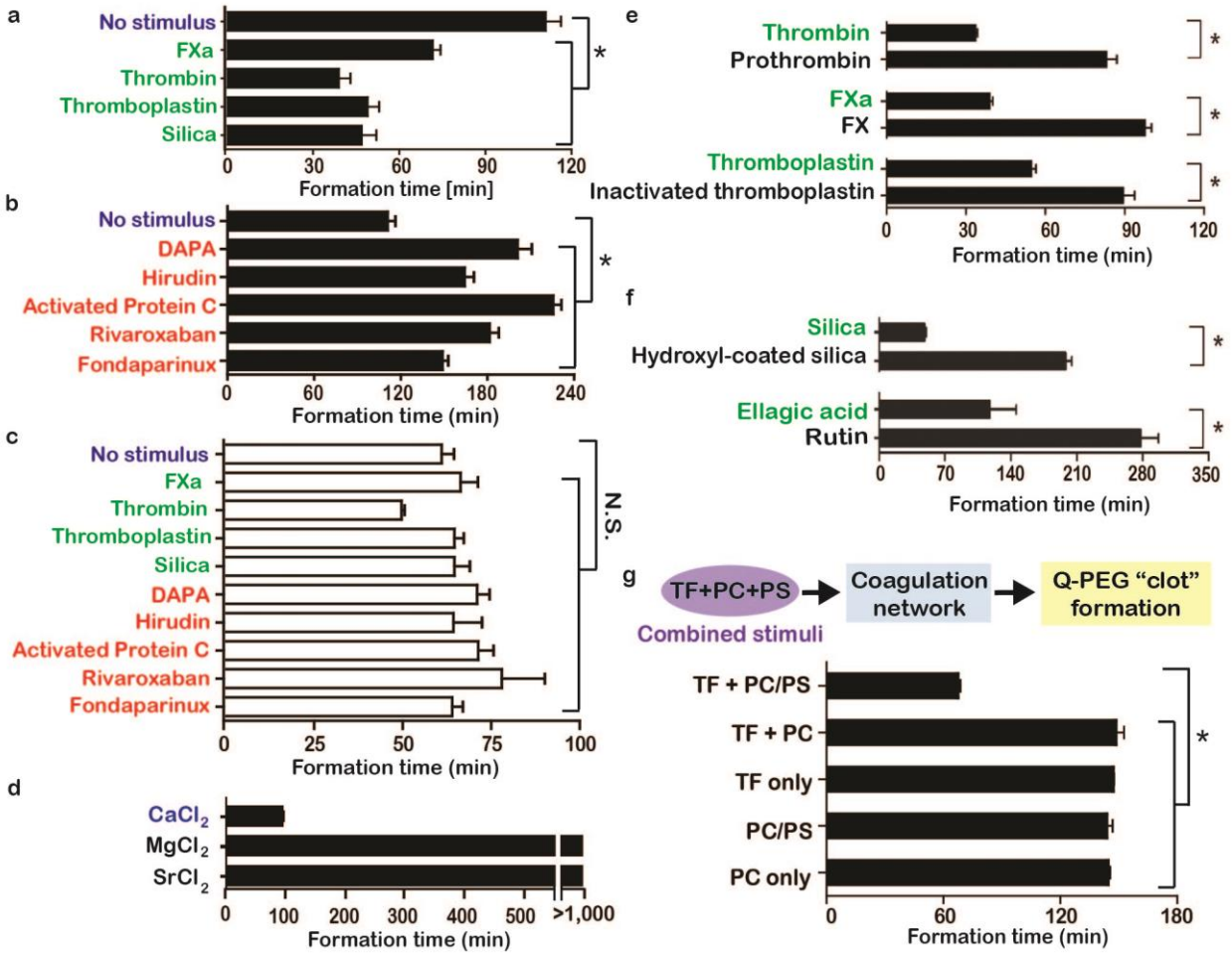


Figure 3.8 The coagulation cascade controls the rate of Q-PEG hydrogel formation in response to diverse and specific stimuli.

a,b) Q-PEG hydrogel formation times in FG-deficient plasma in response to known activators (a) or inhibitors (b) of coagulation, compared to the control without a stimulus. c) Q-PEG hydrogel formation with direct addition of FXIIIa (no plasma) did not respond to the modulators. d-f) Q-PEG hydrogel formation in FG-deficient plasma in response to agents that have similar chemical structures, indicating a specific response to modulators. The rate of hydrogel formation responded specifically to calcium ions (d), the active forms of coagulation enzymes (e), and contact pathway activators (f). g) Schematic and graph showing that Q-PEG hydrogel formation was sensitive to combinations of stimuli, with fastest formation when TF, PS, and PC were combined. Data bars indicate mean ± standard error of mean, n = 3-5, $*p < 0.05$.

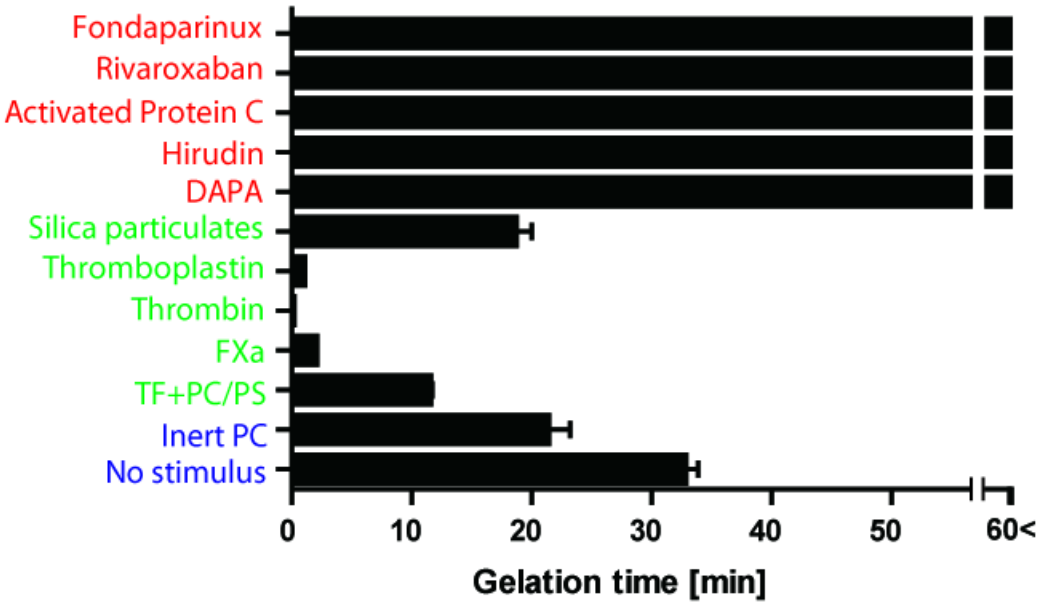


Figure 3.9 The coagulation cascade controls the formation of fibrin clots in response to diverse stimuli. Fibrin clots generated in recalcified normal plasma gelled faster or slower compared to the inert controls (blue) in response to known coagulation activators (green) and inhibitors (red) respectively. Data bars indicate mean ± standard error of mean, n = 3.

To further examine if this Q-PEG containing fibrinogen-deficient plasma formulation retained the ability to detect minute changes in biological stimuli, two variants of herpes simplex virus (HSV) were compared, one with and one without host-cell derived TF incorporated in the envelope surface. HSV is a known stimulus of clotting if the host cell that replicates the virus expresses TF[192]. To produce the two variants of HSV, host cells that were otherwise identical were engineered to either express TF (TF+) or not express TF (TF-) and then were inoculated with HSV. The Q-PEG containing formulation detected this variation between the HSV and formed faster in the presence of the TF+ variant (Figure 3.10b). It is remarkable that formation could detect the small difference between these two viruses, whose genotypes were identical but were grown in host cells with or without a single gene knockout.

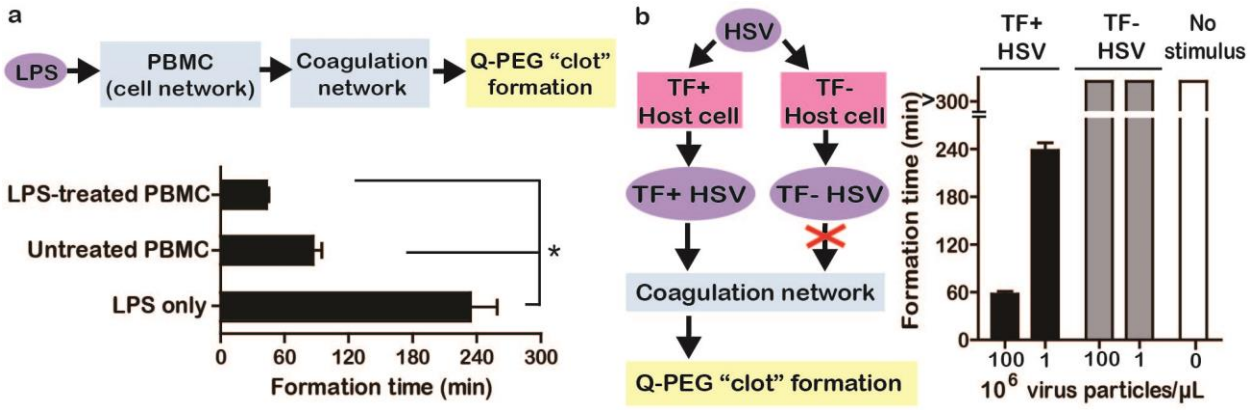


Figure 3.10 Cells and viruses with specific phenotypes can activate the coagulation cascade, leading to quicker gelation of Q-PEG when formulated in FG-deficient plasma.

Q-PEG gelled faster when a) PBMC were stimulated by LPS, and when b) HSV was expressing TF on its surface. Data bars indicate mean ± standard error of mean, n = 3, $*p < 0.05$.

3.4.4 The Q-PEG hydrogel was controllably degraded by specific modulators

In contrast to solidification, there are many examples of synthetic materials that can be degraded in response to particular cells[197,198]. Peptide-containing materials, such as the synthetic macromer used in this report, are sensitive to degradation by proteases. In blood, fibrin is naturally degraded by the serine protease, plasmin, and bacterial proteases[199], all of which degrade a wide range of peptides. We tested if the Q-PEG hydrogels formed in fibrinogen-deficient plasma could also be degraded by plasmin or bacteria (Figure 3.11a). The Q-PEG hydrogel was degraded by exogenous plasmin within 100 min, which was similar to fibrin gels formed under the same conditions (Figure 3.11b). Q-PEG hydrogels formed in the absence of plasma degraded at a similar rate to those that contained plasma, suggesting that cleavage occurred at the synthetic peptides. Degradation of Q-PEG hydrogels was sensitive to bacterial strains. Degradation was not apparent in the presence of two *Staphylococcus* strains, but did

occur by, and could distinguish between, two strains of *Bacilli* (Figure 3.11c). This could be due to differences in the type or concentration of proteases produced by these strains. In the future, altering the peptide sequences in Q-PEG-like materials presents potential for tuning the material's susceptibility to degradation, which could be useful for designing hemostatic materials that could function under conditions of hyperfibrinolysis.

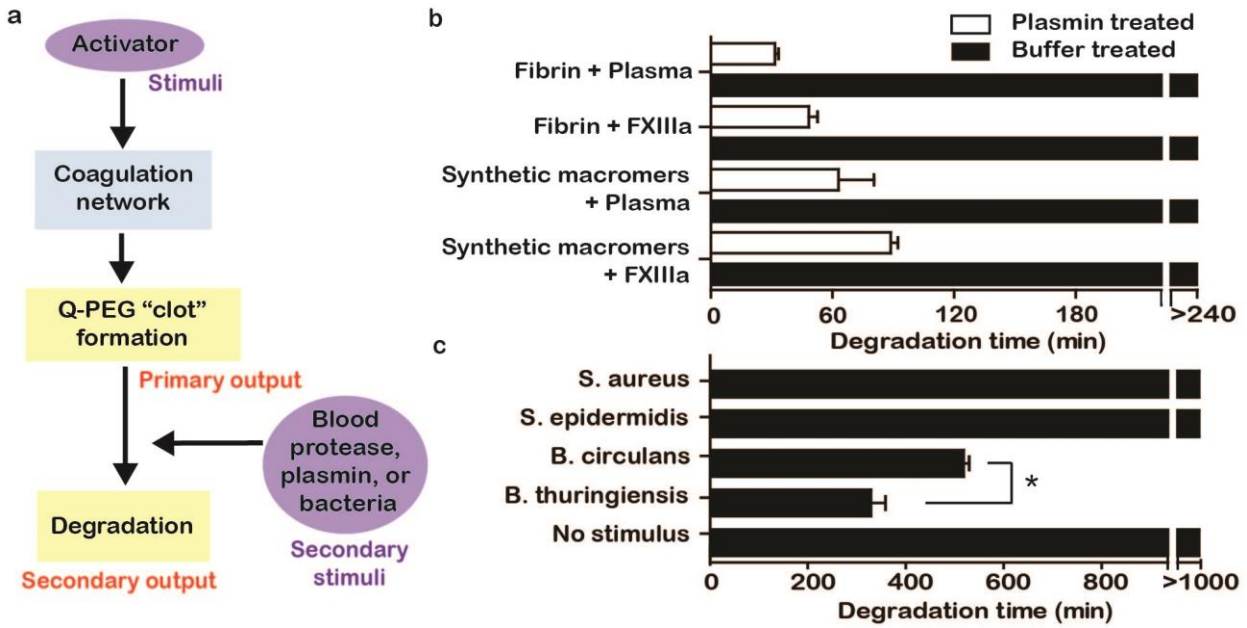


Figure 3.11 Q-PEG can be controllably degraded by specific modulators.

a) Schematic showing the addition of blood protease, plasmin, or bacteria degrades the Q-PEG hydrogel. b) Plasmin degrades Q-PEG hydrogels both when the synthetic macromers were formulated in plasma or when gelled by the addition of FXIIIa alone. C) Q-PEG hydrogels degraded in the presence of *Bacilli* strains, but not in the presence of *Staphylococci* strains. Data bars indicate mean ± standard error of mean, n = 3, $*p < 0.05$.

3.5 Discussion

Here, we demonstrate for the first time that the blood coagulation network can be used to control the formation of a synthetic material, allowing the material to then indirectly respond to specific stimuli from more than 8 diverse chemical and biological classes. Formation indirectly

responded to even subtle changes in the overall phenotype of a virus. These proof-of concept experiments utilized a well-characterized synthetic macromer, a FXIIIa-crosslinkable PEG macromer. Although with or without the biological sensor, the coagulation cascade, the Q-PEG hydrogels were very similar after they were formed, the rate of clot-like polymerization of the material was only modulated by diverse stimuli when plasma was present. It is remarkable that the controllability over a synthetic polymer could reach a level of precision typically only seen in nature simply by mixing these well-characterized components together[175,176]. This approach mimics strategies used in synthetic biology, where microorganisms have been engineered to produce new molecules from their existing biological networks or to produce their metabolites in response to new stimuli[185,186,187]. The simple approach to extending the responsiveness of existing materials described here is different from approaches that chemically modify their backbones[175]. In fact, the responsiveness of the PEG polymerization was increased with the addition of a single peptide sequence. In the approach here, the Q-PEG macromers do not directly respond to multiple stimuli but responds indirectly through the coagulation network which acts as a sensor that modulates the rate of formation of their crosslinking agent, FXIIIa. Modifying the polymer backbone, such as changing the length or branching of PEG, is expected to influence the physical properties of the final material[200], but not the sensitivity of the system to the coagulation modulators. Strategically changing the polymer backbone to add recognition and sensitivity to other modulators is a potential strategy for further increasing the responsiveness of the system[3]. Thus, smart materials based on polymers with responsive backbones could be complementary to the approach herein. The strategy described here is also distinguishable from, and potentially complementary to, approaches used to create functional materials through biomimicry. Biomimicry, imitating the dynamics and functions of biological materials, is a

tested strategy to engineer new and useful materials[201,202]. Together these approaches may be capable of creating multi-functional smart materials that emulate the highly evolved and tailored reaction networks of complex biological processes.

Blood clotting is a tightly-regulated process—normally activating only where and when it is supposed to. Retaining this regulation while replacing the output from a fibrin clot to a synthetic material may be useful for developing smart hemostatic materials with potentially fewer thrombotic complications due to its ability to gel in response to coagulation stimuli. Also, an appropriate synthetic equivalent of fibrin/ogen, developed based on the Q-PEG strategy, would be useful for treating fibrinogen deficiency by replacing fibrin's role in coagulating at vascular damage. Fibrinogen deficiency can occur congenitally and acutely from trauma or sepsis, resulting in severe bleeding episodes[136].

Although there are a number of potential applications, questions remain regarding the feasibility and extensibility of the Q-PEG strategy. This is a proof-of-concept study for the strategy of coupling synthetic materials to the coagulation system. The material, Q-PEG, was chosen for this study because it was a well-characterized material known to be crosslinkable by FXIIIa[1,2,3,4,5], not necessarily for the specific properties conferred by the PEG backbone. While the strategy may see future applications as a hemostatic material, Q-PEG in itself is unsuitable for this purpose due to poor solubility and high molecular weight, which is close to the renal secretion limit[203]. A mitigation strategy would be to replace the PEG backbone with a more soluble and biodegradable material, such as hyaluronic acid[204]. Another limitation of Q-PEG is its slow "clot" formation time compared to native fibrin clots, and the necessity for high concentrations of FXIIIa for its gelation. A potential solution would be to develop a self-

assembling synthetic material that would assemble when an inhibitor group is cleaved off by generated thrombin. Similar self-assembling materials have been developed to gel in response to proteolytic cleavage of peptide branches[205,206]. The physical application of Q-PEG to wound tissues is another question to explore. If applied topically, would a dual-syringe system like that of fibrin glues be adequate for mixing calcium and FXIIIa with Q-PEG and spermidine? The material is very viscous, which might make it difficult to apply[207]. A potential alternative would be to lyophilize the material onto cotton gauze and pack this into wounds. It is unclear whether the Q-PEG would retain activity when formulated into gauze, and whether storage times would be affected by this procedure.

Additional questions remain regarding how to apply the general strategy of controlling synthetic material polymerization via enzymatic signal-sensing and amplification networks. In theory, any biochemical cascade resulting in the output of a crosslinking enzyme, including other transglutaminases, can be exploited to exert biological control over the polymerization of synthetic materials bearing substrates for the enzyme. Furthermore, this strategy can possibly be extended to biochemical cascades that do not produce crosslinking enzymes, such as those that produce proteases capable of activating the self-assembly of materials. A limitation of these strategies is that biological networks typically function under tightly controlled conditions, which may limit their use to specific biological settings. Identifying other biological networks that can modulate the activity of crosslinking agents or self-assembling processes[208], and developing strategies that allow them to function outside their native environments is a potential route to reformulating useful synthetic materials to attain powerful new functions for uses even in environments outside of living organisms.

Chapter 4: Examining the Adhesive Strength of Blood Clots Copolymerized with Q-PEG

4.1 Synopsis

The adhesion of blood clots to blood vessels, such as through the adhesion of fibrin, is essential in hemostasis. While numerous strategies for initiating clot formation and preventing clot lysis are being developed to create improved hemostatic agents, strategies for enhancing clot adhesion have not been widely explored. Here, we show that adhesion of blood clots can be increased by adding a previously characterized synthetic polymer that is crosslinked by coagulation factor XIIIa during clotting. Addition of the polymer to normal plasma increased the adhesive strength of clots by 2-times. It also recovered the adhesive strength of nonadhesive fibrinogen-deficient whole blood clots from <0.06 kPa to 1.9 ± 0.14 kPa, which is similar to the adhesive strength of a fibrinogen-rich clot (1.8 ± 0.64 kPa). The polymer also enabled plasma clots to remain adhered under fibrinolytic conditions. Congenital FIX deficient plasma clots were especially susceptible to fibrinolysis, but copolymerization with Q-PEG helped them maintain clot adhesion during tPA-treatment. By demonstrating that the adhesive strength of clots can be increased with a synthetic material, this provides a potential strategy for creating advanced hemostatic materials, such as treatments for fibrinogen deficiency in trauma-induced or congenital coagulopathies.

4.2 Introduction

Blood clots must adhere to vasculature to stop bleeding effectively. Multiple components of clots, including platelets and fibrin, have adhesive properties that help seal damaged

vessels[106]. Strongly adhered clots are also less likely to detach from the inside of vessels, where they would otherwise flow downstream and, in some cases, embolize smaller vessels[209]. Strategies for enhancing the adhesive strength of clots, particularly when endogenous adhesive components are depleted, may increase the efficacy of existing hemostatic agents, but this has not been widely explored.

Many natural and synthetic tissue adhesives have been developed to close wounds[210]. Fibrin sealants, in particular, are effective for surgical applications when applied topically[103]. The adhesive strength and hemostatic potential of topical fibrin sealants increase with the concentration of fibrinogen[103]. Endogenous clots are also better at achieving hemostasis when fibrinogen is transfused into the bloodstream[211]; fibrinogen replacement therapy has been used both as a prophylactic and acute treatment of bleeding in patients with congenital and acquired fibrinogen deficiency[212,213,214]. This suggests that the natural adhesion of endogenous clots can be increased by adding exogenous fibrinogen; however, there are downsides to this strategy. Fibrin is susceptible to fibrinolysis, which accompanies rebleeding[215]. Furthermore, both recombinant and isolated fibrinogen are expensive to produce, with isolated fibrinogen requiring additional steps to mitigate the risk of blood-borne pathogens[210,103,212]. Transfusions of blood products are also prohibited by some religions[82]. Together, these provide motivation to identify alternative strategies for increasing the adhesion of endogenous clots.

Fibrin is a component of blood clots produced by thrombin-mediated cleavage of its zymogen, fibrinogen, in response to vascular injury. It functions by self-assembling into insoluble fibers, which are crosslinked by coagulation factor XIIIa (FXIIIa) to stabilize and adhere clots to tissue[103]. FXIIIa is a transglutaminase that forms covalent bonds between certain

glutamine- and amine-containing molecules[103,12]. It is highly promiscuous and can covalently link fibrin to many substrates, including collagen, fibronectin, and platelet surface proteins[12,216,217]. Several synthetic FXIIIa-crosslinkable polymers have been developed, including a well-characterized 8-armed polyethylene glycol conjugated to a glutamine-containing peptide derived from α2-antiplasmin (Q-PEG)[1,2,3,4,5,218]. Q-PEG can be crosslinked by FXIIIa to many polyamine substrates, such as spermidine[1,2,3,4,5,218]. This FXIIIa-crosslinkable polymer (Q-PEG and spermidine) can be gelled by the coagulation network when added to fibrinogen-deficient (FG-def) plasma[218]. We hypothesized that copolymerizing blood clots with this polymer would increase the clot's adhesive strength and also restore adhesion of fibrin-poor blood clots, such as clots formed during fibrinogen-depletion or clots exposed to fibrinolytic enzymes.

Adding polymers to fibrin clots is known to increase their mechanical strength by contributing mechanical impedance to the movement of the fibrin network[132,133]. This does not necessarily increase the adhesion of the material, and no quantitative study on increasing clot adhesion by adding a synthetic polymer has been reported. Here, we show that the lap shear strength of blood clots supplemented with a FXIIIa-crosslinkable polymer increased under a wide range of conditions in a FXIIIa-dependent manner. The polymer also restored the adhesive strength of whole blood clots that were fibrin-poor.

4.3 Methods

4.3.1 Synthesis of Q-PEG

Q-PEG was synthesized as previously described in Section 3.4.1. In brief, a peptide derived from α2-antiplasmin, acetyl-NQEQVSPLTLLKKGC (Thermo Fisher Scientific), was

conjugated to 8-armed PEG maleimide (40 kDa, Creative PEGWorks) through the peptide's cysteine thiol. Q-PEG was purified by dialyzing first in Tris-EDTA buffer, then in water. Further purification was achieved using a desalting column (Thermo Fisher Scientific). The purified conjugates were concentrated by a Centri-prep filter (AmiconUltra 10K, Millipore), lyophilized, and stored at −20 °C until use. Q-PEG was characterized as described in Section 3.3.2.

4.3.2 Preparation of FG-def human and mouse whole blood

This study was approved by the University of British Columbia Research Ethics boards. Informed consent was obtained from all healthy human volunteers prior to whole blood donation. Human whole blood was collected into Vacutainer tubes containing K3 EDTA (15% solution) or sodium citrate (0.105 M) (BD Biosciences) and centrifuged at 2000 g for 15 min to pellet the blood cells[108]. The plasma was discarded and blood cells were washed thrice with citrated FG-def human plasma (0.06 mg/mL fibrinogen, Affinity Biologicals) to deplete the fibrinogen. The washed blood cells were resuspended in citrated FG-def human plasma to prepare FG-def human whole blood. To obtain FG-def mouse whole blood, 50% 129SvJ/50% C57Bl/6 animals with a complete FG-γ chain knockout were used[219]. Whole blood was collected via cardiac puncture and citrated (7 mM).

4.3.3 Copolymerization of Q-PEG with blood clots and measuring their adhesion

Whole blood or plasma was diluted to a solution yielding 75% blood or plasma and containing final concentrations of Q-PEG (46 mg/mL), spermidine (1.3 mM, Sigma-Aldrich), $CaCl_2$ (50 mM), purified human FXIII zymogen (0.067 mg/mL, Haematologic Technologies Inc.), and silica particulates/APTT activator (2.5% v/v, Pacific Hemostasis). In experiments

where FXIIIa was inhibited, T101 (FXIIIa inhibitor, 3.8 mM, 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride, ZEDIRA GmbH) was added to the reaction mix. In experiments with excised sheep vessels, the descending aortae were collected from Dorset cross sheep and endothelial layers were removed to expose the subendothelia. Higher concentrations of FXIII (0.13 mg/mL), and bovine thrombin (24 U/mL, Sigma-Aldrich) were used to activate clotting. Blue food coloring (0.5% v/v, Club House) was added to visualize the blood plasma between the translucent blood vessels. Adhesion was measured with a TA Q800 dynamic mechanical analyzer equipped with a shear-sandwich clamp (TA Instruments). Collagen-coated glass coverslips or excised sheep vessels were attached to clamps using UV-curable glue (Loon Outdoors). Q-PEG-containing blood was loaded onto a 7 × 7 mm section that was 0.46 mm thick between the coverslips or excised vessels, and the edges were sealed with fluorocarbon grease (Krytox grease, DuPont) to prevent evaporation. Clots were formed for 90 or 180 min on collagen or vessels respectively to allow the clots to be fully crosslinked by FXIIIa. A force ramp was applied at 0.01 N/min until the clot failed. The normality of data sets was verified with a Shapiro-Wilk test and p values were calculated by Welch's analysis of variance to account for unequal sample sizes and variances[150].

4.3.4 Measurement of the gelation of Q-PEG and collagen in FG-def plasma

In experiments investigating the interaction between Q-PEG and collagen, FG-def plasma (1 μg/mL fibrinogen, Affinity Biologicals) was used. The reaction mixture of 5 μL contained FG-def plasma (14% v/v), Q-PEG (26 mg/mL), rat-tail collagen I (4.1 mg/mL, Sigma-Aldrich), $CaCl_2$ (30 mM), purified human FXIII (0.1 mg/mL), and bovine thrombin (69 U/mL). T101 (6.9 mM) was added in specified samples to inhibit FXIIIa. Green fluorescent beads (6×10^8

particles/mL) were added to the reaction mix to monitor gelation by fluorescence microscopy as previously described in Section 3.3.7.

4.3.5 Measurement of the lysis of Q-PEG-copolymerized clots in normal plasma

Plasma was diluted (58%) in a solution containing Q-PEG (final concentration 34 mg/mL), spermidine (1 mM), $CaCl_2$ (39 mM), and purified human FXIII zymogen (0.07 mg/mL). Ampicillin (1.9 mg/mL, Sigma-Aldrich) was added to the reaction mixture of 5 µL to prevent bacterial-induced clot lysis. Tissue plasminogen activator (tPA, 15 nM, Genentech), a fibrinolytic enzyme, and bovine thrombin (19 U/mL), an activator of clotting, were then added. Clot formation occurred immediately after thrombin was added (<10 min), and shear adhesive strength of the resulting clot on collagen-coated coverslips was measured 90 min later. Clot lysis times were detected by fluorescence microscopy via the Brownian movement of green fluorescent beads (6×10^8 particles/mL) in the reaction mixture.

4.3.6 Measurement of the lysis of FIX-deficient plasma clots by TEG

Plasma was diluted (90%) in a solution containing tPA (257 ng/mL), $CaCl_2$ (15 mM), and Innovin (1/250 dilution, stock concentration not given by manufacturers. Purchased from Dade Behring). For detailed methods on TEG operation, see Section 3.3.6 of this dissertation.

4.3.7 Measurement of the adhesive strength of FIX-deficient plasma clots copolymerized with Q-PEG under fibrinolytic treatment

Plasma was diluted (75%) in a solution containing tPA (257 ng/mL), $CaCl_2$ (15 mM), Innovin (1/250 dilution, stock concentration not given by manufacturers. Purchased from Dade

Behring), purified human FXIII zymogen (0.07 mg/mL), Q-PEG (34 mg/mL), and spermidine (1 mM). The reaction mixtures were loaded onto the dynamic mechanical analyzer and subjected to a lap-shear test according to the method described in Section 2.3.3 of this dissertation.

4.4 Results

4.4.1 Adhesion of normal plasma clots can be increased with a synthetic polymer

To test if adding a FXIIIa-crosslinkable polymer to plasma could increase the adhesion of the copolymerized clot, Q-PEG, spermidine, and additional FXIII zymogen were mixed with human plasma and clotted for 90 min on collagen-coated coverslips or for 180 min on excised blood vessels (Figure 4.1a). Collagen is a major component of the subendothelial layers of blood vessels[155]. The adhesive strength of the clot to the collagen-coated surface was measured, and detachment from the substrate was verified to ensure adhesive failure of the material. The composite clot, containing supplementary FXIII zymogen, Q-PEG, and spermidine, was over 4-times more adhesive than a normal plasma clot and over 2-times more adhesive than a control clot supplemented with the same amount of FXIII (Figure 4.1b). This increase in adhesive strength required both Q-PEG and spermidine, demonstrating that a FXIIIa-crosslinkable polymer can be used to increase the adhesion of a fibrin clot. To test if the increased adhesion to collagen translates to increased adhesion to blood vessels, the adhesion of the composite clots to sheep aortae was tested *ex vivo*. Composite clots containing Q-PEG and spermidine were formed on the exposed subendothelia of excised sheep aortae and their adhesive strengths were measured at 180 min (Figure 4.1c). The adhesive strength of the composite clot was roughly 2-times higher than a clot supplemented with FXIII alone, showing clot adhesion to excised vessels can be increased as well.

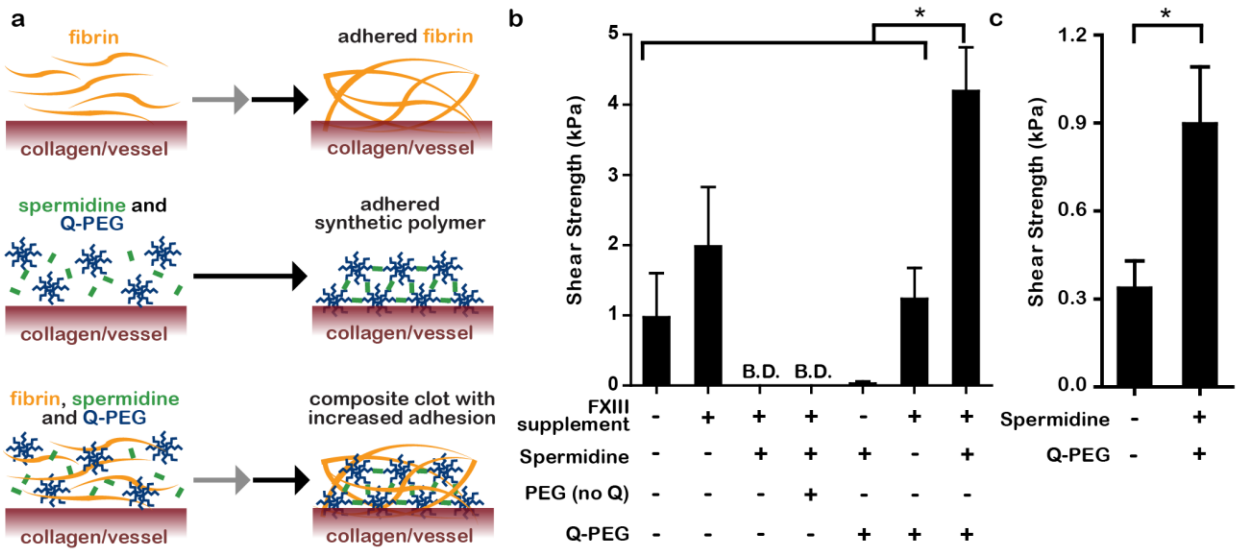


Figure 4.1 The shear adhesive strength of a normal plasma clot to collagen or the surfaces of excised blood vessels can be increased by adding FXIIIa-crosslinkable synthetic macromers.

a) Schematic showing the self-assembly of fibrin (grey arrow) and the copolymerization of FXIIIa-crosslinkable macromers (black arrow) increasing adhesion of the resulting clot. b) Graph shows the maximum shear stress plasma clots withstood before they mechanically failed in a lap-shear adhesion test, with combinations of supplemental FXIII (0.067 mg/mL), spermidine (1.3 mM), a non-crosslinkable PEG (46 mg/mL) and Q-PEG (46 mg/mL). c) Graph shows the shear adhesive strength of FXIII-supplemented (0.13 mg/mL) normal plasma clots formed between two pieces of excised blood vessels. $*p < 0.05$. Data indicate mean ± standard error of mean, n = 5-7. B.D. indicates values below detection (< 0.059 kPa).

4.4.2 Increased adhesion is dependent on FXIIIa

To determine the mechanism of increased adhesion to collagen, we investigated whether collagen and Q-PEG bind to each other in a FXIIIa-dependent manner. Collagen is a known amine-donor for FXIIIa[216]. To test if Q-PEG and collagen bind to each other in plasma, their ability to form a gel together was measured (Figure 4.2a,b). They were added to plasma deficient in fibrinogen and free of spermidine, because FXIIIa is known to crosslink both of these amine

donors to Q-PEG. Without these amine donors or collagen, Q-PEG did not gel. When collagen was added, gelation with Q-PEG did occur, except when T101, an irreversible inhibitor of FXIIIa, was added. This showed that FXIIIa was responsible for binding Q-PEG to collagen, either directly or indirectly through other FXIIIa substrates present in plasma. To test if the Q-PEG and spermidine components of composite clots were also bound to collagen-coated surfaces by FXIIIa activity, their lap-shear strength at 90 min was measured in FG-def plasma (Figure 4.2c). The adhesive shear strength of FG-def plasma alone to collagen was below detection, whereas it was 1.8 ± 0.27 kPa when Q-PEG and spermidine were added, similar to clots of normal plasma (2.0 ± 0.85 kPa, shown in Figure 4.1b). Adhesion was dependent on the activity of FXIIIa, as the shear strength dropped below detection when T101 was added. Together, these results are consistent with a mechanism where increased adhesion of clots containing the FXIIIa-crosslinkable polymer was due to Q-PEG binding to collagen by the transglutaminase activity of FXIIIa.

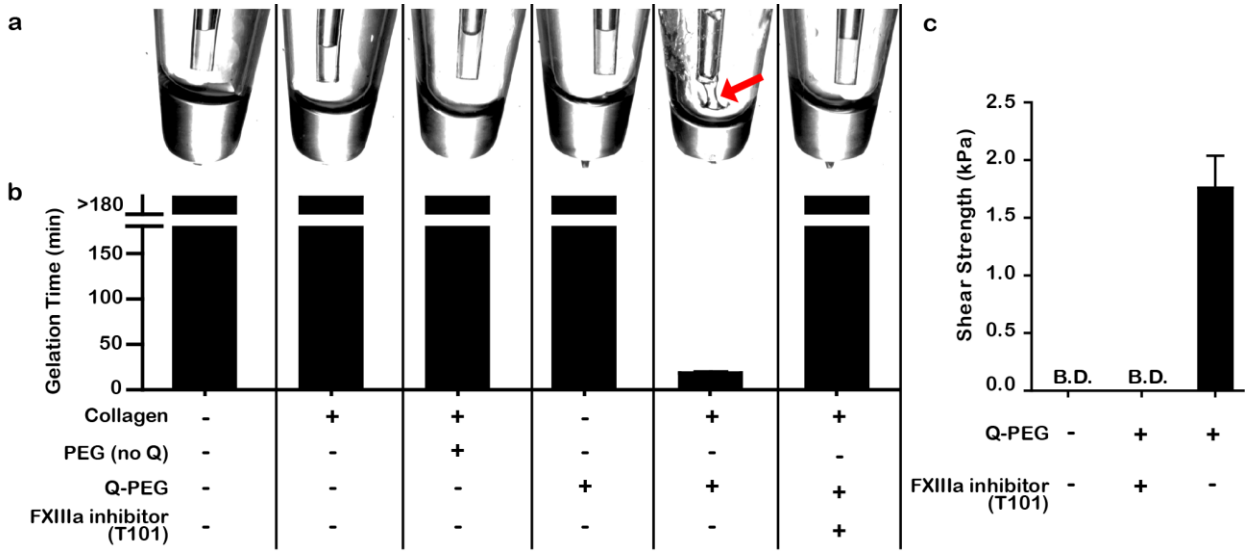


Figure 4.2 FXIIIa activity controls the binding of Q-PEG to collagen.

a) Images show clot-like gelation of Q-PEG and collagen in FG-def plasma. Gelation can be seen in the image with the red arrow, where the gel became stretched by the micropipette tip. b) Gelation times of samples in panel a. n = 3. c) The shear strength of Q-PEG and spermidine gels formed onto a collagen-coated surface with and without T101. n = 5. Data indicate mean ± standard error of mean. B.D. indicates values below detection (< 0.059 kPa).

4.4.3 Adhesive strength of fibrinogen-depleted clots can be restored to that of normal whole-blood clots

We next tested whether the adhesion of whole blood (WB) clots and fibrinogen-deficient whole blood (FG-def WB) clots could be increased by the addition of Q-PEG (Figure 4.3a). Clots were formed for 90 min before subjected to the lap-shear test. We were interested in the adhesion of FG-def WB clots because depletion of fibrinogen is a major cause of uncontrolled bleeding during trauma, which may potentially be addressed by an agent that enhances adhesion[73]. Human WB clots supplemented with Q-PEG, spermidine, and FXIII zymogen did not have adhesive strengths higher than those of human WB clots with supplemental FXIII alone (Figure 4.3b). Human FG-def WB clots were nonadhesive, but adhesion was recovered when Q-PEG and spermidine were added (Figure 4.3c). Remarkably, addition of the synthetic polymer increased the shear strength of FG-def WB clots to those of normal WB clots (1.9 ± 0.14 and 1.8 ± 0.64 kPa, respectively). Similarly, blood clots from mice with congenital fibrinogen-deficiency were nonadhesive, but the addition of Q-PEG and spermidine recovered adhesion to 0.47 ± 0.20 kPa (Figure 4.3d).

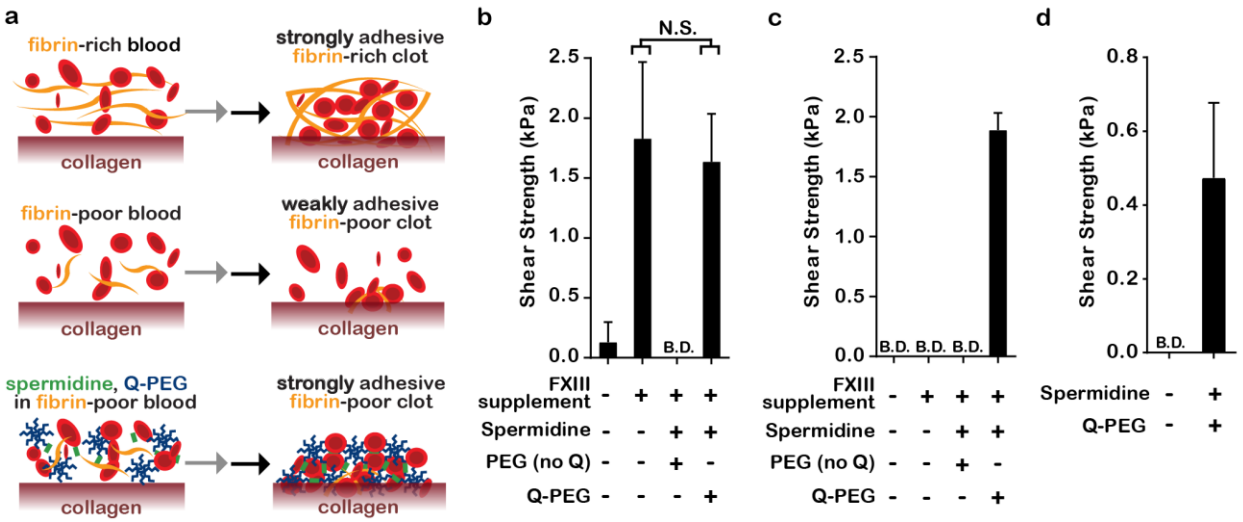


Figure 4.3 The shear adhesive strength of a FG-deficient whole blood clot can be increased by adding FXIIIa-crosslinkable synthetic macromers.

a) Schematic showing FXIIIa-crosslinkable macromers adhering a whole blood clot to a surface of collagen. b) Shear strength of fibrinogen-rich human whole blood clots. No significant difference (N.S., $p > 0.05$) detected between FXIII-supplemented whole blood with and without Q-PEG and spermidine. c) Shear strength of FG-deficient human whole blood clots. d) Shear strength of clots from whole blood collected from congenitally FG-deficient mice and supplemented with FXIII (0.067 mg/mL). Data indicate mean ± standard error of mean, n = 5. B.D. indicates data below detection (< 0.059 kPa).

4.4.4 Clot detachment during fibrinolysis can be inhibited by adding a FXIIIa-crosslinkable polymer

To test if the FXIIIa-crosslinkable polymer can also increase the adhesive strength of clots compromised by fibrinolysis[220], tPA was added to normal plasma with and without Q-PEG and spermidine. The lysis time of normal plasma was substantially extended by adding Q-PEG and spermidine, increasing from 62 ± 8.8 min to >1000 min (Figure 4.4a). Addition of Q-PEG and spermidine also allowed the clot to remain adhesive under tPA-induced lysis (1.6 ± 0.35 kPa,

compared to 1.8 ± 0.26 kPa without tPA treatment, Figure 4.4b). In contrast, without the FXIIIa-crosslinkable polymer, clots easily detached when treated with tPA, and no adhesion was detected at 90 min. This shows that the FXIIIa-crosslinkable polymer is more resistant to tPA-induced lysis and helps prevent the detachment of clots under fibrinolytic conditions.

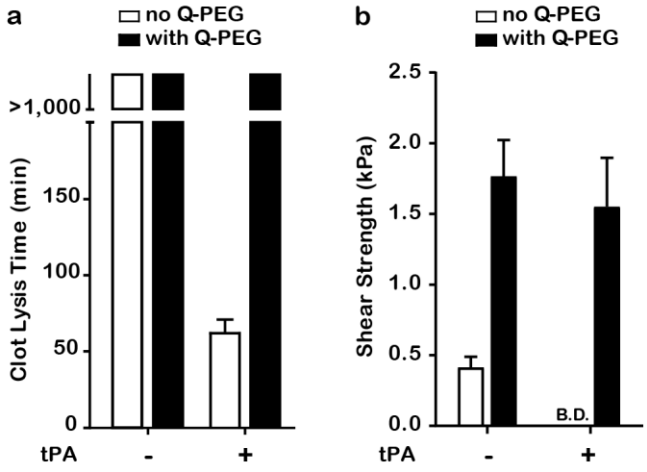


Figure 4.4 The shear adhesive strength of a normal plasma clot can be maintained under fibrinolytic conditions by adding FXIIIa-crosslinkable synthetic macromers.

a) Lysis times of tPA-treated plasma clots with or without Q-PEG. b) Shear strength of tPA-treated plasma clots at 90 min with or without Q-PEG. Data indicate mean ± standard error of mean, n = 5-6. B.D. indicates data below detection (< 0.059 kPa).

4.4.5 Congenital FIX-deficient plasma clots are susceptible to adhesion loss during fibrinolysis but are recovered by copolymerization with Q-PEG

Congenital FIX-deficient plasma clots were more susceptible to tPA-induced clot lysis than normal plasma clots. On TEG, both Ly30 (Figure 4.5b) and Ly60 (Figure 4.5c) percentages of FIX-deficient clots were increased compared to control clots (from 5.3 $\pm$ 1.3 to 77.7 $\pm$ 2.9, and from 43.4 $\pm$ 3.3 to 89.1 $\pm$ 1.4, respectively). FIX-deficient clots were no longer adhesive at 90

min after tPA treatment, but with Q-PEG supplementation, adhesive strength remained detectable at 0.72 ± 0.15 kPa (Figure 4.5d).

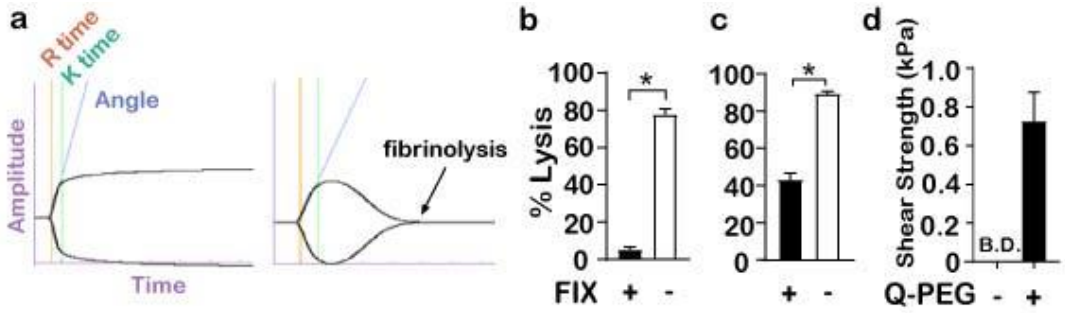


Figure 4.5 FIX-deficient clots are more susceptible to fibrinolysis and Q-PEG can help maintain clot adhesive strength under tPA treatment.

a) Schematic representation of TEG data on FIX-deficient clots. Panel on the left shows a normal plasma clot. Panel on the right shows a FIX-deficient clot with longer clotting time, decreased amplitude indicative of lower clot stiffness, and rapid fibrinolysis when treated with tPA. b-c) Clot lysis percentages obtained from TEG curves at 30 min (b) and 60 min (c). FIX-deficient clots undergo more lysis than FIX-rich clots. d) FIX-deficient clots are not adhesive at 90 min after tPA treatment but copolymerizing with Q-PEG resuscitates adhesion.

4.5 Discussion

In summary, this work shows that copolymerizing blood clots with a FXIIIa-crosslinkable synthetic macromer can increase the adhesion of clots in a FXIIIa-dependent manner. This increase in adhesive strength was found both on collagen surfaces and on the subendothelia of excised blood vessels. Together, the data show the effect Q-PEG has on clots is to link blood components to collagen via FXIIIa. The FXIIIa-crosslinkable polymer recovered the adhesive strength of whole blood clots deficient in fibrinogen and maintained the adhesion of plasma clots treated with tPA, two conditions where clots were otherwise nonadhesive. FIX-

deficient plasma clots were more susceptible to tPA-induced fibrinolysis, but supplementation with Q-PEG made it more adhesive under fibrinolytic conditions.

Within the larger goal of creating advanced hemostatic agents, this work demonstrates that the adhesion of clots to vascular surfaces can be increased with a material besides fibrinogen. This is the first time that the adhesion of plasma-based or whole blood-based clots has been increased with a molecule exogenous to the coagulation system. The specific material used here was chosen for these proof-of-concept experiments because it was already known to crosslink by FXIIIa, not because it met all the criteria for development into a therapeutic. Nevertheless, this discovery-phase work may assist in the conceptualization and design of advanced hemostatic agents that increase the adhesion of clots. Specifically, this concept may enable the design of therapeutics for treating fibrinogen deficiency. Fibrinogen deficiency can occur congenitally due to genetic defects in protein synthesis or its secretion[221], or it can be acquired when antibodies are formed against fibrinogen[222], or when fibrinogen is depleted in trauma-induced coagulopathy (TIC)[136]. Furthermore, the rate of fibrin formation may be impaired in other coagulation deficiencies, such as that of FIX tested in this study.

TIC occurs in one in four trauma cases and is associated with a 4-times increase in patient mortality[73]. Fibrinogen deficiency accompanies TIC and infusions of fibrinogen and FXIII concentrates are treatments being investigated for this condition[212,213]. Our results show that a FXIIIa-crosslinkable material can recover clot adhesion in human whole blood artificially depleted of fibrinogen, and whole blood from congenitally fibrinogen-deficient mice. In contrast, without the polymer, adhesion was not detected. Hyperfibrinolysis also accompanies TIC. The lysis of plasma clots via tPA quickly renders them nonadhesive. This is especially true of FIX-

deficient clots. However, our results show that by supplementing plasma with a FXIIIa-crosslinkable material, clots remained adhesive. With further development, this strategy of increasing adhesion with FXIIIa-crosslinkable materials may lead to a synthetic alternative or a complementary additive to fibrinogen for treating TIC. However, patients with TIC may also suffer impaired platelet function and acidosis[73,223,224,63] and therefore, future experiments using blood samples from patients with TIC or *in vivo* models of TIC would be required for testing the safety and efficacy of this therapeutic strategy. Similarly, while the experiments with the congenital FIX-deficient plasma are a first-step towards analyzing whether Q-PEG and similar technologies could improve the adhesion of hemophilia B blood clots, it is unclear whether it would confer such benefits if the condition was induced by neutralizing antibodies to FIX[225]. Further testing in patient plasma would help yield a clearer picture as to which patient subset might benefit from these technologies.

The strategy of copolymerizing blood clots with a synthetic material was designed to be distinct from technologies that blend a synthetic matrix to interpenetrate with fibrin fiber networks[226]. As fibrin is both a glutamine and amine donor of FXIIIa[227], it is possible that the Q-PEG is covalently crosslinked to fibrin in addition to being crosslinked to the polyamine, spermidine. Further chemical characterization is required to confirm this crosslinking have occurred under the reaction conditions described, as well as to investigate whether this copolymerization would confer different mechanical and lysis properties than two interpenetrating, but chemically non-interacting, polymer matrices.

Chapter 5: Conclusions and Future Studies

5.1 Conclusions

In conclusion, a FXIIIa-crosslinkable substrate, Q-PEG, can be blended into blood to improve the adhesive strength of clots to collagen. This was answered in three parts: 1) In Chapter 2, it was shown that fibrin and FXIIIa are major contributors to bulk clot adhesive strength to collagen. 2) In Chapter 3, Q-PEG, was formulated into blood and found to controllably form a hydrogel in place of fibrin when the coagulation cascade was activated. 3) In Chapter 4, this Q-PEG containing formulation increased clot adhesive strength to collagen even under fibrin-poor conditions.

5.2 Contributions to Current Field of Research and Potential Applications

The adhesive strength of blood clots has not been comprehensively explored in previous works. While the adhesion of individual clot components such as platelets[106] and fibrin[103] have been investigated, it is unclear how these components interact to yield the adhesive properties of the bulk clot. To the best extent of our knowledge, this is the first study that explores how different clot components contribute to the adhesion of the bulk clot. Remarkably, we found that clot adhesion is not directly correlated with clot cohesive strength, which can be estimated through measures of clot stiffness by TEG[141]. Measures of clot stiffness by TEG have been useful as both a tool for tracing the rate of clot formation as well as for predicting how effective the clot can stop bleeding[90]. While the clot must have some degree of stiffness to form an adhesive sealant, adhesion also depends on interactions with the surface of the wound tissues. Thus, materials that increase clot cohesion, such as platelets, may not necessarily improve the

adhesive strength of the formed clot. Similarly, while FXIII does increase clot cohesion, reflected in a TEG-detected stiffness increase of 20% at 3-times the physiological concentration, the increase in clot adhesive strength was much greater. This suggests that the active FXIIIa enzyme may be mediating attachments of the clot to the tissue surface, not just increasing clot adhesive strength by increasing clot cohesion.

Knowledge gained in this study could potentially be applied to the development of hemostatic technologies. It has been proposed that fibrin and FXIII could be supplemented for patients with severe bleeding[63]. Multiple trials are being conducted to test the efficacy of fibrin supplementation[69,70,138]. However, FXIII remains indicated only for patients with congenital FXIII deficiencies[167]. Its efficacy as a hemostatic treatment remains controversial. Based on our results, it is possible that FXIII is more effective as a topical hemostatic material applied at high, supraphysiological concentrations. Such concentrations might have thrombotic complications when applied systemically[168], but when applied locally, this problem might be mitigated, and it might be more effective at overcoming plasmin degradation of FXIIIa at the wound site[161]. This knowledge might also be useful for the design of coatings for the prevention of clot adhesion. Such coatings are often used on medical devices[228,229]. A FXIIIa inhibitor might be useful for preventing thrombus attachment and growth on medical devices. Alternatively, FXIII siRNA therapy[230] might be useful for lowering, but not fully depleting, FXIII concentrations in patients with implanted medical devices.

The specific FXIIIa-mediated strategy for improving clot mechanics we pursued here was the testing of a synthetic FXIIIa-crosslinkable material, Q-PEG, with the ultimate goal of developing a material that could controllably polymerize to a “synthetic clot” to complement the

native clot during hemostasis. This strategy is envisioned as a potential treatment for non-compressible hemorrhage. There is a high demand for therapies to treat this condition, as it remains the main cause of death in traumatic bleeding[26]. The strategy described here may be useful for designing such technologies, as direct pressure is difficult to apply in these cases and the hemostatic treatment must localize to the wound without the aid of compression[27]. Coupling synthetic materials to the coagulation system so that it is likewise responsive to stimuli that signal vessel injury is a potential solution for localizing synthetic hemostatic materials to the wound site. This strategy adds to a growing field of biomimetic hemostatic materials that are also inspired by and capitalizes on the functions of the native coagulation network. One such example is the FXIIIa-mimicking fibrin-crosslinker, PolySTAT[86]. As a fibrin-crosslinker, PolySTAT functions only where fibrin is localized. It increases the stiffness of fibrin clots and makes them more resistant to the pressure of blood flow. PolySTAT is particularly efficient at preventing secondary hemorrhage from fluid resuscitation. Another biomimetic hemostatic technology is a platelet-mimicking nanoconstruct, SynthoPlate[84]. Sequences that allow for platelet adhesion and aggregation are incorporated into synthetic nanoparticles that then attach to each other, to platelets and fibrin, and to the substrates on the wound to mimic and amplify the function of native platelets. Similarly, because of its ability to recognize wound substrates, the nanoconstructs localize to the wound site. These technologies are complementary to traditional blood product transfusion, and the synthetic nature allows fine-tuning of their effects. In our particular study, Q-PEG modified the stiffness of blood clots, giving it new and potentially useful properties. Other properties as its viscosity, elasticity, porosity, and resistance to fracture can potentially be tuned by further modifications of the polymer backbone. In a broader sense, this research also presents a new strategy, that of using native biochemical networks as complex

sensors to control polymer formation, to design stimuli-responsive biomaterials, which are useful for diverse applications beyond hemorrhage treatment, such as drug delivery, tissue engineering, and biosensing[134].

To the extent of our knowledge, this work is the first to demonstrate that synthetic materials can be coupled to the coagulation network to copolymerize and modulate the adhesive properties of bulk blood clots. While it is intuitive that clots must be adhesive to remain at the site of injury, the development of such a strategy to improve clot adhesion is unprecedented. There are a variety of tissue sealants that could be applied to replace the blood clot's role in plugging the vascular leakage[231], but many of these sealants require crosslinkers that may be cytotoxic to cells[232]. Thus, this limits the dosage and location these sealants could be used. Synthetic materials have been blended into blood to modulate its mechanical properties and shown to confer hemostatic benefits[84,86]. This work adds to this body of literature, with a focus on clot adhesion under a variety of fibrin-poor conditions, including not only an artificial depletion of fibrinogen, but also more relevant fibrin-related coagulopathies such as tPA-induced fibrinolysis and congenital FIX-deficiency leading to a decreased rate of fibrin formation and compromised clot architecture[23]. Potential applications include using this strategy to develop hemostatic materials that specifically target the problem of secondary bleeding due to high-pressure hemorrhage. Clot adhesion is especially important in these cases, particularly when fluid resuscitation is administered[58]. This strategy can also be used to tune other properties of the native blood clot, such as its tensile, compressive, and shear strengths, elasticity, and viscosity. These could have applications outside of hemostasis, where the sensitivity of the coagulation system as a sensor is prized but the material properties of the native blood clot are not ideal[27].

For instance, components of the blood clot may provide a good scaffold for tissue culture, with many adhesive substrates and growth factors to help with cell attachment and growth. However, the scaffold may not be strong and bendable enough[233], thus this strategy of modulating clot mechanical properties may be useful.

5.3 Future Directions

One of the future directions would be to further understand the mechanisms by which clot components mediate clot adhesive properties. For instance, is FXIIIa crosslinking fibrin directly to collagen? Or is it crosslinking fibrin to proteins like VWF or fibronectin deposited on the collagen surface? Depleting various adhesive proteins in plasma would be a good way of testing their effect on clot adhesive strength. Would changing the structure of the collagen substrate change the clot's adhesive strength to it? Would a thicker coating of collagen change the clot adhesive strengths measured? The thickness of structural adhesives is known to affect its adhesive strength. At high thicknesses, adhesives are more likely to fail by cohesive breakage due to microdefects in the adhesive material, and greater stresses and poorer redistribution of those stresses along the interface due to fast plastic spreading[234]. However, at low thicknesses, the joint strength depends almost solely on the adhesive interactions at the connecting surfaces between adhesive and adherend, with little contributions from the mechanical interlocking of the two. This could lead to lower joint strength, particularly for elastic materials like blood clots and ECM proteins[235]. Furthermore, would changing the collagen matrix porosity improve penetration of the clot into the pores and mediate stronger mechanical interlocking? Taking sections of the attached clot on a 3D collagen matrix and staining for platelets, fibrin, and other adhesive proteins may help us identify the localization of various clot components and what might be

penetrating into the matrix to mediate clot adhesion. If the substrate were coated with TF to localize fibrin formation to its surface, would that change the clot's adhesive strength? Would changing the rate of thrombin generation (higher rate of thrombin generation leads to the formation of denser fibrin networks with thinner fibers[236]) change the clot's adhesive properties? Those are all questions that could be addressed in future studies to better understand how clots attach to the complex 3D structure of tissues on the wound site.

With regards to Q-PEG, it is unclear what its effects on clot adhesion would be under a more dynamic system resembling *in vivo* conditions as opposed to the static lap-shear test described in this dissertation. Testing of Q-PEG in microfluidic systems would allow us to understand whether it could improve clot adhesion under flow. It would also yield insights as to the behavior of Q-PEG as a moving fluid; whether it could mix evenly with blood to form a homogenous copolymer or would Q-PEG have to be applied by other means, such as formulating it with cotton gauze that would then get packed into the wound and soaked with blood.

Q-PEG's effects on other clot properties have not been comprehensively analyzed. In Chapter 3, it was found that Q-PEG also increased clot stiffness. In Chapter 4, it was found that Q-PEG was more resistant to clot lysis. How Q-PEG affects other functions of blood clots, such as trapping RBC[237], recruiting leukocytes to initiate the inflammatory response, and mediating vascular regeneration[238] is unknown. It is unclear whether Q-PEG would affect these processes by virtue of its modification of the clot's mechanical properties, as well as its own chemical properties. For instance, Q-PEG forms a less porous gel than the native blood clot, as seen in the SEM images on Figure 3.6. Would that impede the migration of fibroblasts during wound

healing[239]? Would its polymer backbone, generally regarded as not biodegradable, make it difficult for cells to grow into the scaffold it provides[240]?

As mentioned in the discussion sections of chapters 3 and 4, Q-PEG is unsuitable for *in vivo* use. Modifications of the macromer backbone is required for improving solubility, lower its viscosity, and improve its biodegradability. The use of a degradable and more hydrophilic backbone, such as hyaluronic acid[204], and lowering the molecular weight of the macromer, could be a potential solution. It could also be formulated in a different way, such as incorporation into cotton gauze, to improve its applicability[27]. Future versions of this material could be tested in animal models of severe arterial hemorrhage, such as the rat femoral artery injury model we are currently investigating as described later in this section. This would help us understand whether materials similar to Q-PEG could reduce adhesive failure of clots *in vivo* when subjected to fluid challenge, and thus reduce blood loss and mortality rates. Other *in vivo* models to consider would be that of hemophilia and dilutional coagulopathy, as our *in vitro* and *ex vivo* models suggest that Q-PEG and similar technologies might confer benefits to clot adhesion in these cases. As Q-PEG is very resistant to tPA-induced fibrinolysis, it might not be ideal for wound healing[241], as the material might not be removed by the fibrinolytic system. While it was shown that Q-PEG could be degradable by high concentrations of plasmin (Figure 3.11), the mechanism has yet to be completely elucidated. It is hypothesized that plasmin cleaves the peptide linkages between Q-PEG macromers. If this hypothesis is supported, then it may be possible to modulate the synthetic material's plasmin-induced lysis time by changing the peptide sequence used, adding more plasmin-recognizable motifs for cleavage[242]. Similar research has been done with the aim of designing multi-responsive biomaterials[243], although challenges remain to ensure all peptide

sequences incorporated do not interfere with one another's functionality. Tuning the lysis time of Q-PEG and future prototypes developed based upon it would allow it to remain intact and adhered during hemostasis, but after the bleeding has stopped, it could be degraded to make way for cells to mediate wound healing.

To understand the effect of FXIII supplementation and future Q-PEG-like materials when applied *in vivo*, testing in animal models would be a first step, followed by clinical testing in humans. An appropriate hemorrhage model would have to be identified. Initial experiments would be performed in murine models. We are currently investigating the effects of topical FXIII in a rat femoral artery hemorrhage model[86]. In this model, we applied a supraphysiological concentration of FXIII to the forming blood clot over the transected artery, allowed the clot to form for 5 minutes, then resuscitated with intravenous fluids to increase the blood pressure and challenge the blood clot. Each instance of secondary hemorrhage from clot failure was recorded, as well as the clot failure mode (adhesive versus cohesive). We are currently analyzing the data collected in this double-blind study to see if FXIII supplementation shifts clot failure mode, whether it prevents secondary hemorrhage, lowers blood loss, and improves survival.

Porcine models more closely resemble the human coagulation system than murine models[244], but due to the larger size of the animal, more of the hemostatic material would need to be produced for such experiments. Cost of supplementary FXIII would be a concern. Partnering with industrial collaborators to conduct experiments using recombinant FXIIIa would be necessary. In addition, the current production of Q-PEG is costly and time-consuming. Moving towards a material with a shorter conjugated peptide but could retain its ability to be coupled to the coagulation system would be ideal for reducing cost.

As previously mentioned, a broader application of biochemical cascade-coupled stimuli-responsive technologies, similar to the coagulation-coupled Q-PEG technology described, is in non-biological environments where such smart sealants could be useful[134]. A mitigation strategy for adapting the Q-PEG technology for use in external environments would be to pick enzymatic cascades that could function at a wider range of temperatures, salinity, and pH[245]. The enzymatic cascades of prokaryotic systems might be worth investigating. For instance, a stimuli-responsive sealant for use under high temperatures might require a thermophilic bacterial enzymatic cascade to serve as its sensor system[246]. Gelation is also not the only output possible for such a design strategy. Enzymatic cascades could be coupled to synthetic materials that fluoresce by cleavage and release of a fluorescent tag from the polymer backbone[247]. Such a stimuli-responsive material that uses fluorescence as its output could have use in developing detection assays. For instance, coupling the complement activation system to such a synthetic material might lead to the development of an assay for detecting a wide range of pathogens[247].

5.4 Closing

In summary, this thesis reveals the importance of fibrin and FXIIIa to the adhesion of bulk blood clots. A FXIIIa-crosslinkable fibrin-mimic, Q-PEG, was demonstrated to be able to copolymerize with blood and improve clot adhesion under a variety of fibrin-poor conditions, such as tPA-induced fibrinolysis, congenital fibrinogen deficiency, and congenital FIX deficiency. This work presents a novel strategy that may be helpful in the future for the design of stimuli-responsive hemostatic materials that could localize to severely bleeding wounds.

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Appendices

Appendix A A Biochemical Network Can Control Formation of a Synthetic Material by Sensing Numerous Specific Stimuli

This paper was the basis for Chapter 3 of the dissertation and can be found here:

https://www.nature.com/articles/srep10274

Appendix B Adhesion of Blood Clots Can Be Enhanced When Copolymerized with a Macromer That Is Crosslinked by Coagulation Factor XIIIa

This paper was the basis for Figure 3.1 and most of the contents of Chapter 4 of the dissertation and can be found here:

https://pubs.acs.org/doi/abs/10.1021/acs.biomac.6b00481

Appendix C FXIIIa-Crosslinkable Synthetic Macromers Improve Stiffness and Adhesion of Hemophilia B Blood Clots under Fibrinolytic Conditions

Some results described in this conference abstract was included in Chapter 4 of the dissertation and can be found here:

http://biomaterials.ca/#!/abstracts/view/114351

Appendix D Localization of Short-Chain Polyphosphate Enhances Its Ability to Clot Flowing Blood Plasma

I was a co-author on this publication in *Scientific Reports*: J.H. Yeon*, N. Mazinani*, T.S. Schlappi, K.Y.T. Chan, J.R. Baylis, S.A. Smith, A.J. Donovan, D. Kudela, G.D. Stucky, Y. Liu, J.H. Morrissey, and C.J. Kastrup (2017). Localized short-chain polyphosphate can speed up the clotting time of flowing plasma. I helped design the microfluidics system used in figures 3 and 4, performed preliminary static experiments showing short-chain polyphosphate localized on particles are more procoagulant than soluble polyphosphate, helped analyze the data and revised the manuscript. This paper can be found here:

https://www.nature.com/articles/srep42119

Appendix E Halting Hemorrhage with Self-Propelling Particles and Local Drug Delivery

I was a co-author on this publication in *Thrombosis Research*: J.R. Baylis, K.Y.T. Chan, and C.J. Kastrup (2016). This is a review paper on novel hemostatic technologies that can localize to the site of injury. I helped with literature research and the writing of the paper, with particular emphasis on sections “Enhanced local drug delivery for managing hemorrhage”, and “Potential applications in treating bleeding with advanced delivery mechanisms”. This paper can be found here:

https://www.thrombosisresearch.com/article/S0049-3848(16)30362-0/abstract