Fbg αC 389 – 402 modulates Factor XIII crosslinking in the fibrinogen αC region.

Francis Dean Orlina Ablan

University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd

Part of the Biochemistry Commons, and the Other Biochemistry, Biophysics, and Structural Biology Commons

Recommended Citation

This Doctoral Dissertation is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.
Fbg αC 389 – 402 MODULATES FACTOR XIII CROSSLINKING IN THE FIBRINOGEN αC REGION

By

Francis Dean Orlina Ablan
B.S., University of North Carolina at Wilmington, 2012
M.S., University of North Carolina at Wilmington, 2014

A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy in Chemistry

Department of Chemistry
University of Louisville
Louisville, Kentucky

August 2023
Fbg αC 389 – 402 MODULATES FACTOR XIII CROSSLINKING IN THE FIBRINOGEN αC REGION

By

Francis Dean Orlina Ablan
B.S., University of North Carolina at Wilmington, 2012
M.S., University of North Carolina at Wilmington, 2014

A Dissertation approved on

July 31, 2023

by the following Dissertation Committee:

Dissertation Chair
Dr. Muriel C. Maurer

Dr. Eugene G. Mueller

Dr. Andrew J. Wilson

Dr. Michael A. Menze
ACKNOWLEDGEMENTS

I am extremely grateful to my graduate school advisor and mentor, Dr. Muriel Maurer. Thank you for giving me the opportunity to be a graduate student in your lab and to introducing me the fascinating world of blood coagulation. Also, thank you for being a mentor who was present to teach and guide me every step of the way.

I am very thankful for Dr. Eugene Mueller, Dr. Andrew Wilson, and Dr. Michael Menze for taking the time to be a part of my Dissertation committee, as well as for their constructive criticisms to refine my dissertation to where it is today. I would also like to thank Dr. Eugene Mueller for giving me a deeper appreciation for the field of biochemistry, particularly in the engaging, thought-provoking, and entertaining way he taught Biochemistry I.

I would also like to extend a special thank you to David Grimm for training me in the basics of DNA ligation and subcloning, as well as to Will Holmes for introducing me to the In-Fusion seamless cloning technique. As a predominantly protein biochemist, I am grateful for their insight into the wonderful and mysterious world of DNA.

I would like to acknowledge and express my gratitude to my lab mates, both past and present here at the University of Louisville. Thank you, Dr. Ramya Billur and Dr. Boris Anokhin, for welcoming me into your lab when I first arrived and training me in what I needed to know to get started. Thank you, Rameesa Syed Mohammed and Richard Lumata, for being wonderful labmates that supported me intellectually and emotionally. Thank you, Nicholas McCann, Mohammed Hindi, and Renee Stohlmann, for being the best undergraduates that a graduate student could hope to mentor.

To all my friends that I made in graduate school, Dr. Faye Carvajal, Dr. Caleb Calvary, Hari Nambiar, Peter Armstrong, Dr. Kritika Bajaj, Chinmay Potnis, Christine Phipps, Dr. Sagar Mudsinghe, Dr. Emily Kempfer-Robertson, Dr. Dillon Hofsommer, Dr. Dustin Woods, thank you very much for our wonderful times together and helping me stay sane in our graduate school journey.

To my brothers and sisters in Christ in Louisville, Dr. Eric and Tonia Schansberg, Matt Frawley, Keith Gilcreast, Zack Bacon, David and Celeste Bennet, John Cook, Josh and Krista Webber, Gary and Betsy Ricucci, thank you for continually encouraging me and lifting up my spirits.

To my parents, my siblings, and my new in-laws, thank you for your ceaseless support for my endeavors.
To Ginny Quinn Ablan, my beautiful bride and the love of my life, words alone are inadequate to express my gratitude and appreciation for your steadfast love, guidance, support, and inspiration. Also, to my soon-to-be-born son, Franklin Luigino Ablan, I hope that the work in this dissertation would be one of the many things I can do to make you proud.

Lastly, I would like to thank and praise God, in Whose universe I live and study, for the rare opportunity to get a PhD in Chemistry and guiding me to where I am in life today.
Fibrinogen (Fbg) is a coagulation protein critical for clot formation. Coagulation Factor XIII (FXIII) is a calcium-dependent transglutaminase that crosslinks reactive glutamines (Q) and lysines (K) between fibrin and other anti-fibrinolytic proteins. In the presence of Ca$^{2+}$, FXIII could be activated non-proteolytically (FXIII-A°), or proteolytically by thrombin (FXIII-A*). Significant increases in clot stability and red blood cell retention are linked to FXIII activity in the fibrinogen αC region (Fbg Aα 221 – 610). This region contains several FXIII-reactive glutamines and lysines, as well as a binding site for FXIII-A* (Fbg αC 389 – 402) that includes a key binding residue, Fbg αC E396. While FXIII-crosslinked clots maintain hemostasis, they also exacerbate the development of deep vein thrombosis (DVT). The work from this research seeks to aid further drug design against DVT by inhibition of FXIII binding and activity on Fbg αC.

Fbg αC 233 – 425, a “model” αC system that contains three reactive glutamines and the FXIII binding site (Fbg αC 389 – 402), was recombinantly expressed and purified. A series of mutations were subsequently introduced to the αC FXIII binding site to observe
how crosslinking was affected. FXIII activity was monitored through MS-based glycine ethyl ester (GEE) crosslinking and gel-based fluorescence monodansylcadaverine (MDC) crosslinking assays. Fbg αC 389 – 402 was found to selectively enhance Fbg αC crosslinking from FXIII-A* over FXIII-A°. Additionally, Fbg αC 389 – 402 was more instrumental than either αC 403 – 425 or αC 328 – 388 in facilitating FXIII-A* crosslinking. Further work explored αC E395, D390, W391, and F394A as residues within Fbg αC 389 – 402 that could enhance αC FXIII-A* activity by increasing binding affinity. While E395 minimally impacted FXIII-A* activity, αC D390, W391 and F394 were subsequently identified as key residues alongside E396 for promoting FXIII-A* crosslinking. In summary, Fbg αC 389 – 402 was demonstrated to be a major facilitator of FXIII-A* activity on Fbg αC, as well as a potential target for therapeutic inhibition of VTE. Groundwork was laid for future studies through expression and preliminary crosslinking studies on Fbg αC 221 – 425, a new recombinant Fbg αC.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................................................. iii
ABSTRACT ........................................................................................................................................ v
LIST OF TABLES ............................................................................................................................. viii
LIST OF FIGURES ............................................................................................................................ ix

CHAPTER 1: INTRODUCTION ......................................................................................................... 1

CHAPTER 2: REVIEW OF BIOCHEMICAL AND ANALYTICAL METHODS .......... 22

CHAPTER 3: EFFECT OF THE FACTOR XIII BINDING SITE IN FIBRINOGEN αC ON FACTOR XIII CROSSLINKING IN THE FIBRINOGEN αC REGION .............. 39

CHAPTER 4: FIBRINOGEN αC 389 – 402 ENHANCES FACTOR XIII CROSSLINKING IN THE FIBRINOGEN αC REGION VIA ELECTROSTATIC AND HYDROPHOBIC INTERACTIONS......................................................................................... 63

CHAPTER 5: PLASMID CONSTRUCTION, RECOMBINANT EXPRESSION OF FIBRINOGEN, AND FXIII-A* REACTIVITY OF FIBRINOGEN αC 221 – 425 ... 87

CHAPTER 6: RESEARCH SUMMARY AND FUTURE DIRECTIONS ...................... 111

REFERENCES ................................................................................................................................. 119
APPENDICES ................................................................................................................................. 134
CURRICULUM VITAE ...................................................................................................................... 137
LIST OF TABLES

Table 1. List of Fibrinogen αC (233 – 425) E396A and Truncation Variant Primers. ................. 43

Table 2. Comparison of curve fit coefficients from FXIII-A* and FXIII-A° Q237-GEE crosslinking assays involving Fbg αC (233-425, WT), αC (233-425, E396A), and αC (233-338, 389 Stop).......................................................................................................................................................... 51

Table 3. Comparison of one phase exponential decay model coefficients from plots of FXIII-A* Fbg αC Q237-GEE crosslinking assays involving WT, E396A, and truncation variants........... 53

Table 4. Multiple sequence alignment of FXIII-A* binding region in Fbg αC in select species. .66

Table 5. List of Fibrinogen αC (233 – 425) Variant Primers.......................................................... 71

Table 6. Comparison of one phase exponential decay model coefficients from plots of FXIII-A* Fbg αC Q237-GEE crosslinking assays involving WT, 389 Stop, and FXIII-A* binding site variants............................................................................................................................................. 73

Table 7. List of Fbg αC Glutamines and Lysines Reactive to FXIII-A* Crosslinking................. 88

Table 8. List of Fibrinogen αC (221 – 610) Insert Cloning Primers............................................. 95

Table 9. List of Fibrinogen αC (221 – 425) Mutagenesis Primers. ............................................. 97

Table 10. Comparison of curve fit coefficients from 50 nM and 500 nM FXIII-A* GEE crosslinking assays involving the reactive glutamines of Fbg αC WT 221 – 425. ......................... 108
LIST OF FIGURES

Figure 1. Blood coagulation cascade diagram. .................................................................................. 3
Figure 2. Models of fibrin(ogen) structure and polymerization. ...................................................... 6
Figure 3. Crystal structures of a FXIII-A subunit (PDB ID: 1F13) and a FXIII-A° monomer attached to the irreversible inhibitor ZED1301 (4KTY).................................................................................. 8
Figure 4. Diagram of FXIII Activation. .............................................................................................. 8
Figure 5. Mechanism of the transglutaminase reaction of FXIII-A.................................................. 10
Figure 6. Fibrinogen αC Region Amino Acid Sequence (Fbg αα 221 – 610). ................................. 20
Figure 7. Plot of typical bacterial growth curve. ............................................................................... 23
Figure 8. pGEX-6P-1 plasmid sequence map. .................................................................................. 25
Figure 9. Flow chart of Fbg αC variant DNA synthesis and expression........................................... 30
Figure 10. SDS-PAGE Analysis of Fbg αC 233 – 425 WT Purification.............................................. 31
Figure 11. Flow chart of MALDI-TOF sample analyte ionization, differentiation by time-of-flight and detection. ..................................................................................................................... 34
Figure 12. Representative Flow chart of a GEE Crosslinking Assay of Fibrinogen αC WT (233 – 425). ................................................................................................................................. 37
Figure 13. Amino acid sequence of the recombinantly expressed Fbg αC 233 – 425 model system. ........................................................................................................................................ 41
Figure 14. Representative MALDI-ToF MS Spectra of Proteolytic Digests of Fbg αC 389 Stop after FXIII-A°-catalyzed GEE Crosslinking................................................................. 45
Figure 15. Comparison of FXIII A-catalyzed GEE-crosslinking of Q237, Q328, and Q366 between Fbg αC WT (233 – 425), and the FXIII binding site αC variants E396 and 389 Stop. ... 48
Figure 16. Transglutaminase activity of FXIII-A* is more affected by Fbg αC binding site mutations than FXIII-A°. .............................................................................................................. 50
Figure 17. Transglutaminase activities between FXIII-A* and FXIII-A° on Fbg αC Q237 are significantly different........................................................................................................... 52
Figure 18. Effect of Fbg αC 233 – 425 truncation mutations on FXIII-A* transglutaminase activity. .................................................................54

Figure 19. Effect of Fbg αC truncation mutations on FXIII-A*-catalyzed MDC crosslinking. .... 56

Figure 20. PONDR-VLXT Analysis of Fbg αC 233 – 425. .................................................................61

Figure 21. Cartoon model of fibrinogen monomer. ...........................................................................64

Figure 22. Molecular mapping of rFXIII-A2* chemically cross-linked via Lys446 to a synthetic Fbg αC 389 – 402 peptide (PDWGTFFEEVSGNVSC). .................................................................67

Figure 23. Effect of Fbg αC E396D on FXIII-A* transglutaminase activity....................................74

Figure 24. Effects of Fbg αC E395 Variants on FXIII-A* transglutaminase activity. ............76

Figure 25. Effect of Fbg αC D390A on FXIII-A* transglutaminase activity....................................77

Figure 26. Effect of Fbg αC W391A on FXIII-A* transglutaminase activity. ............................79

Figure 27. The effect of Fbg αC F394 on FXIII-A* transglutaminase activity. ............................80

Figure 28. Effect of Fbg αC single mutations on FXIII-A*-catalyzed MDC crosslinking........81

Figure 29. Wheel diagram of unique fibrin Q-K crosslinks identified within in vivo thrombi. .... 89

Figure 30. pET-20B plasmid sequence map. ..................................................................................92

Figure 31. Ligation Subeloning Flow chart .................................................................................94

Figure 32. InFusion seamless cloning flow chart of Fbg Aα 221 – 610 DNA from a pET-20B vector to a pGEX-6P-1 vector ........................................................................................................96

Figure 33. Agarose gel of Fbg αC 221 – 610 insert cDNA. .............................................................100

Figure 34. Agarose gel of pGEX 6P-1 plasmid with Fbg αC 233 – 425 cDNA simultaneously digested by BamHI and SalI. .................................................................................................101

Figure 35. Agarose gel of pGEX 6P-1 plasmid with FXIII-A cDNA digested by NcoI, AatII, or both simultaneously. ............................................................102

Figure 36. Amino acid sequences of recombinant Fbg αC 221 – 610 and the Fbg αC 233 – 425 model system. ..................................................................................................................103

Figure 37. PONDR-VLXT Analysis of Fbg αC 221 – 610. ...............................................................104

Figure 38. Representative MALDI-TOF MS Spectra of Proteolytic Digests of Fbg αC 221 – 425 after FXIII-A*-catalyzed GEE Crosslinking. .................................................................107
Figure 39. Representative MALDI-TOF MS Spectra of Tryptic Digests of Fbg αC 221 – 425 after FXIII-A*-catalyzed GEE Crosslinking........................................................................................................................................ 108

Figure 40. FXIII-A*-catalyzed GEE-crosslinking assays of Fbg αC 221 – 425. ................................. 109
CHAPTER 1: INTRODUCTION

Blood coagulation is an important physiological process in hemostasis in the event of vascular injury.\textsuperscript{1, 2} Through clot formation, organisms are able to mitigate blood loss by preventing blood flow to damaged blood vessels, while simultaneously ensuring that blood continues to flow to the rest of the body. In addition to preventing extensive blood loss, coagulation also serves as an effector of the immune response by preventing the entry and spread of infectious bacteria.\textsuperscript{3} As important as coagulation is to the homeostasis of an individual, this process is suppressed by the body in normal circumstances to prevent thrombosis: the formation of blood clots within arteries (arterial thrombosis) and veins (venous thrombosis).\textsuperscript{1, 4, 5} Thrombosis prevents the proper flow of blood, resulting in hypoxia, tissue damage, and painful inflammation. If thrombosis is left untreated, parts of the clot could potentially break off and travel to the lungs where pulmonary arteries could also be blocked, an event known as pulmonary embolism (PE). A major complication of PE is chronic thromboembolic hypertension, which results in progressive heart failure and death.\textsuperscript{6} Given the danger that thrombosis poses, timely and effective treatment to prevent and safely remove thrombi is warranted.

This introductory chapter aims to provide an overview of the blood coagulation process and a brief review of the different rationales regarding modern thrombosis treatment. Factor XIII (FXIII), a transglutaminase responsible for introducing crosslinks into the fibrin blood clot network, is a key research focus of this dissertation. The inhibition
of FXIII activity has been proposed in recent literature to be a prospective improvement over existing anticoagulants, particularly for the treatment of VTE.\textsuperscript{7-9} The history of anticoagulant development and treatment will also be examined in this chapter to strengthen the case for further FXIII-based anticoagulant design.

Coagulation occurs via a cascade of enzymes activated sequentially by proteolytic action of serine proteases (Figure 1).\textsuperscript{1,10} A majority of these enzymes are synthesized in the liver. This coagulation cascade could be initiated by either two pathways: the tissue Factor (extrinsic) pathway involving coagulation Factor VIIa and tissue Factor (TF), or the contact (intrinsic) pathway by coagulation Factors VIII, IX, XI and XII, plasma prekallikrein (PK), and high molecular weight kininogen (HMWK).\textsuperscript{11,12} The extrinsic pathway is commonly triggered by damage to tissues. On the other hand, the contact pathway is initiated in response to the presence of pathogens or pathogenic material, likely from the infiltration of the body through cuts or wounds. Both pathways culminate in a common pathway where coagulation factor X is activated (FXa).

Coagulation factor X (FX) sits in a pivotal point of the coagulation cascade (Figure 1):\textsuperscript{13,14} Both the extrinsic TF-FVIIa and intrinsic VIIIa-IXa pathways of the coagulation cascade end with the activation of FX to FXa. Additionally, FXa starts the first step of the common pathway: the activation of thrombin. Prior to activation, FX circulates at a concentration of 8 – 10 µg/mL (135 – 170 nM) in plasma as a ~59-kDa glycoprotein consisting of a 139-residue light chain and a 306-residue heavy chain that contains the serine protease domain.\textsuperscript{13,15,16} Upon activation, FXa proteolytically activates coagulation Factor V in plasma (FVa). Both FXa and FVa then bind at mutually exclusive binding sites to the surface of negatively charged phospholipid membranes.\textsuperscript{17,18} While both FXa and
FVa bind to membranes independent of each other, both coagulation Factors bind to each other rapidly in a 1:1 stoichiometric ratio to form the prothrombinase complex in the presence of calcium ions (Ca$^{2+}$). This complex is responsible for binding prothrombin (coagulation Factor II, FII) and activating it to thrombin (IIa). The serine protease domain of FXa acts as the active site for the activation of thrombin, while FVa acts an important cofactor along with Ca$^{2+}$.

**Figure 1.** Blood coagulation cascade diagram. Some coagulation Factors are represented by their roman numeral designation. Zymogen, inactive forms of the coagulation factors, are shown as ellipses, while activated forms are shown as squares and affixed with “a”. PK: plasma kallikrein; HMWK: high molecular weight kininogen.
Thrombin is viewed as the centerpiece of the coagulation cascade.\textsuperscript{19, 20} In its zymogenic prothrombin form, this serine protease is produced in the liver and secreted into blood as a 70-kDa glycoprotein consisting of 622 residues at a concentration of \( \sim 84 \mu \text{g/mL} \) (\( \sim 1.2 \mu \text{M} \)). The prothrombinase complex of FVa-FXa then cleaves prothrombin at two sites: first at R320, then subsequently at R271. These two proteolytic cleavages result in an active enzyme with a molecular weight of 35.5 kDa. From here, thrombin amplifies the coagulation cascade through a positive feedback loop: Thrombin activates FXI of the intrinsic pathway, as well as FX and FV of the common pathway, leading to more generation of thrombin. Thrombin also serves to advance the activation of platelets, as well as the generation of the final product of the coagulation cascade: stable blood clots.\textsuperscript{19, 20} Thrombin promotes platelet activation through its interactions with protease-activated receptors (PARs) and glycoprotein Ib\(\alpha\) (GPIb\(\alpha\)). Upon activation, platelets become adhesive, upon which they bind and aggregate to damaged blood vessels, forming a hemostatic platelet plug to prevent further blood loss. Activated platelets are also involved in the body's inflammatory and immune responses to curb infection.\textsuperscript{21} Activated platelets secrete chemokines that attract leukocytes to sites of injury. Activated platelets have also been observed to form neutrophil extracellular traps (NETs) upon binding with neutrophils.\textsuperscript{21} These NETs are able to capture and sequester pathogens for the immune system to remove later on. However, hemostasis is not solely dependent on activated platelets: Rather, the proteolytic activation of fibrinogen and coagulation Factor XIII by thrombin is also required for the formation of a stable blood clot.
Fibrinogen (Fbg) is a coagulation protein that is vital in the latter stages of clot formation. Each fibrinogen monomer is a 340-kDa heterodimer composed of three chains: alpha (Aα, 66.5 kDa), beta (Bβ, 52.0 kDa), and gamma (γ, 46.5 kDa). These fibrinogen chains are held together by a total of 29 disulfide bonds. A crystal structure of a human fibrinogen illustrates that each set of these three chains extends outward in a coiled coil motif from a central E region toward a distal D region (Figure 2B). The central E region is comprised of a γN-domain, a funnel-shaped domain, and two coiled-coil-E domains. On the other hand, the D regions are each made up of a coiled-coil-D domain, and nodules formed by the beta and gamma chains (known as β- and γ- nodules, respectively). Each of these nodules contain a NH2-terminal A-domain, a central B-domain, and a COOH-terminal P-domain. The C-terminal end of the Aα chain continues past the distal D region as a flexible and relatively disordered chain known as the αC region (Figure 2A). Unfortunately, the αC region is not visible in fibrinogen crystal structures due to the disordered nature of this region.

Fibrinogen is primarily synthesized in the liver and circulates as a soluble protein in human blood at a concentration of approximately 2 – 4 mg/mL (6 – 12 µM). In the absence of any injury or pathogens, fibrinogen has a half-life of 3 – 5 days.

Thrombin initiates the conversion of soluble fibrinogen to insoluble fibrin by cleaving fibrinopeptide A (FpA) from the Aα chain, driving the linear aggregation of fibrin monomers into protofibrils by D-E knob-hole interactions. Subsequent cleavage of fibrinopeptide B (FpB) from the Bβ chain results in release of the αC regions from the E domain, leading to increased intermolecular interactions that coalesce protofibrils into fibrin fibers that make up a “soft clot” (Figure 2C). The conversion of fibrinogen to
fibrin by thrombin cleavage can be summarized by the following net equation: 
\[(\text{A}_\alpha \text{B}_\beta \gamma)_2 \rightarrow (\alpha \beta \gamma)_2 + 2 \text{FpA} + 2 \text{FpB},\]  
where \(\alpha\) and \(\beta\) correspond to the alpha and beta chains without their respective fibrinopeptides.\(^{24, 27}\)

![Figure 2. Models of fibrin(ogen) structure and polymerization. (A) Cartoon model of a fibrinogen monomer: alpha chains, \(\text{A}_\alpha\), blue; beta chains, \(\text{B}_\beta\), green; gamma chains, \(\gamma\), red; central E region, brown circle; distal D region, red circle. (B) Crystal structure of fibrinogen monomer (PDB ID: 3GHG)\(^{26}\): alpha chains, \(\text{A}_\alpha\), blue; beta chains, \(\text{B}_\beta\), green; gamma chains, \(\gamma\), red. Unfortunately, the \(\alpha\)C region is not visible in fibrinogen crystal structures due to the disordered nature of this region.\(^{26}\) (C) Scheme of fibrin polymerization: Thrombin cleavage of fibrinopeptide A (FpA) from the \(\text{A}_\alpha\) chain leads to lateral aggregation of fibrin monomers into protofibrils. Subsequent cleavage of fibrinopeptide B (FpB) from the \(\text{B}_\beta\) chain releases the \(\alpha\)C regions from the E domain and merging of protofibrils into fibrin fibers.]

Fibrin clots are subsequently strengthened against premature fibrinolysis by coagulation Factor XIII (FXIII). FXIII is a calcium-dependent transglutaminase that exists as combinations of a catalytic subunit (FXIII-A) and a protective carrier subunit (FXIII-B).\(^{31, 32}\) FXIII is initially present as a zymogen: It is found as the heterotetramer FXIII-A\(_2\)B\(_2\) in plasma. A significant percentage of FXIII-A\(_2\)B\(_2\) circulates in plasma bound to fibrin(ogen) gamma chain residues 390 – 396 through FXIII-B.\(^{33}\) On the other hand, the transglutaminase exists as the homodimer FXIII-A\(_2\) intracellularly within platelets,
megakaryocytes, macrophages, monocytes, osteoblasts, chondrocytes, and preadipocytes. The FXIII-A subunit is expressed within these cells, while FXIII-B is synthesized and secreted by hepatocytes. FXIII-A is composed of an activation peptide, a β-sandwich domain, a catalytic core domain, and two β-barrel domains (Figure 3), while FXIII-B consists of 10 repetitive sushi domains each held together by two disulfide bonds. The enzyme can be activated proteolytically (FXIII-A*) by thrombin cleavage of an activation peptide in the presence of Ca\(^{2+}\), or non-proteolytically (FXIII-A°) without thrombin in the presence of Ca\(^{2+}\). The zymogenic states of FXIII-A\(_2\)B\(_2\) and FXIII-A\(_2\) were observed, upon activation, to dissociate into FXIII-A monomers by analytical ultracentrifugation and atomic force microscopy, regardless of activation method (Figure 4). The crystal structure of a FXIII-A° monomer bound to the irreversible inhibitor ZED1301 was published by Stieler et al. in 2013. In comparing the crystal structures of a FXIII-A subunit with that of FXIII-A°, notable changes occur to the structure of FXIII-A upon activation: FXIII-A° becomes less compact, the β-barrels swing away from the catalytic core, and the active site is more exposed. Unfortunately, the crystal structure of FXIII-A* is difficult to obtain, due to the propensity of the enzyme to precipitate at high concentrations before crystallization could be performed. Yee et al. in 1995 published a crystal structure purportedly of FXIII-A* existing as a dimer (FXIII-A\(_2\)*) with the activation peptide still present on the enzyme after thrombin cleavage. However, the FXIII species crystallized was likely that of uncleaved FXIII-A\(_2\), the minority species in solution. Analytical ultracentrifugation and kinetic results from Anokhin et al. in 2020 show that the FXIII-A\(_2\) dissociates into FXIII-A* monomers with “a higher conformational flexibility” than FXIII-A°.
Figure 3. Crystal structures of a FXIII-A subunit (PDB ID: 1F13) and a FXIII-A° monomer attached to the irreversible inhibitor ZED1301 (4KTY): activation peptide, dark blue; β-sandwich, cyan; catalytic center, orange; β-barrel 1, red; β-barrel 2, green. Red squares highlight approximate location of active sites.

Figure 4. Diagram of FXIII Activation. Inactive FXIII exists as either a tetramer in plasma (FXIII-A₂B₂) or a dimer within cells (FXIII-A₂). Both forms of FXIII could be activated proteolytically by thrombin under low mM Ca²⁺ into FXIII-A* monomers, or non-proteolytically under high mM Ca²⁺ into FXIII-A° monomers. Adapted from refs. 31, 38, 42, and 43.
Activated FXIII-A “crosslinks” fibrin with the chains of other fibrin fibers and coagulation proteins: FXIII-A crosslinking is the formation of isopeptide bonds between the side chains of reactive glutamines and lysines. The mechanism of FXIII-A crosslinking is detailed in Figure 5. The active site of FXIII-A contains the catalytic triad of cysteine-314 (Cys314), histidine-373 (His373), and aspartate-396 (Asp396). In this triad, the side chain thiol group of Cys314 initiates crosslinking by performing a nucleophilic attack on the amide side chain carbon of a glutamine amino acid residue. This nucleophilic attack results in the release of ammonia and the formation of a tetrahedral intermediate, which collapses to form a thioester intermediate. At this point of the reaction, a catalytic diad composed of histidine-342 (His342) and glutamate-401 (Glu401) deprotonates the amino group of a lysine side chain. The resulting amine group is able to nucleophilically attack the thioester intermediate, briefly forming another tetrahedral intermediate that collapses and releases the newly formed crosslinked product.

In terms of FXIII-A activity on fibrin, γ-γ crosslinking occurs first but the αC region of the fibrinogen alpha chain (Fbg αC 221 – 610) is where substantial FXIII-A crosslinking occurs. This portion of fibrinogen is involved in fibrin polymerization, fibrin clot stability, and binding of different coagulation proteins that regulate fibrinolysis. Crosslinking in this region significantly promotes clot stability and mediates clot growth via red blood cell retention. While sizeable and stable blood clots contribute to vascular recovery, FXIII-A can also stabilize and grow thrombi, resulting in the development of venous thromboembolism (VTE). In the 19th century and early 20th century, VTE was widely believed to be caused by infection that resulted in the inflammation of vein walls. As such, past treatment involved the administration of various antiseptic and anti-
inflammatory agents, along with palliative therapies such as bed rest, elevations of limbs, and application of warm compresses on swelling. In contemporary times where the mechanism of clotting and VTE is better understood, thrombosis is therapeutically treated by the administration of anticoagulants.\textsuperscript{5, 56-58}

\textbf{Figure 5.} Mechanism of the transglutaminase reaction of FXIII-A. Adapted from ref. 8.
Anticoagulants disrupt coagulation by targeting and inhibiting one or more enzymes involved in the coagulation process. The earliest known anticoagulant to be discovered was hirudin, a peptide which was isolated from salivary glands of the medicinal leech *Hirudo medicinalis* in 1884. Hirudin binds directly to the active site of thrombin, preventing it from performing its function as a serine protease. Unfortunately, hirudin had some disadvantages that prevented it from being widely adopted as an anticoagulant: The peptide itself was difficult to extract in adequate quantities. Additionally, it has an unfeasibly short half-life, imposes significant risk of bleeding, and is strongly immunogenic. Hirudin would not be considered as a suitable anticoagulant until its therapeutic properties and production were optimized by genetic engineering and recombinant expression in 1986. Further advances using derivatives of hirudin, otherwise known as direct thrombin inhibitors, will be discussed later in this chapter.

The honor of the first widely used anticoagulant belongs to heparin. Heparin was initially isolated from dog liver in 1916, and purified in crystalline form in the 1930's. Heparin, otherwise known as unfractionated heparin (UFH), is a mixture of polysaccharide chains that bind to and greatly enhance the activity of antithrombin, which inactivates thrombin and coagulation Factor Xa. Heparin has a short half-life of 1.0 – 1.5 hours with peak activity being reached in 4 – 6 hours, and has to be administered intravenously or subcutaneously. Unfortunately, the use of heparin can result in increased bleeding risk and a condition known heparin-induced thrombocytopenia, HIT. HIT is a result of heparin forming a complex with platelet Factor 4 (PF4), which the immune system of certain individuals regards as a dangerous foreign body to be attacked. The resulting immune response results in the destruction of platelets and the development of more
thrombi within blood vessels. Additionally, heparin’s anticoagulant effect was observed to vary widely between individuals, requiring near constant monitoring of patients to ensure the drug has taken effect. Heparin derivatives, such as the low weight molecular weight heparin enoxaparin and the synthetically produced fondaparinux, were developed to have longer half-lives, faster peak activity times, lower bleeding risk, and lower HIT occurrence compared to heparin. However, the possibility of HIT occurrence in patients remains with these heparin derivatives.

While heparin and its derivatives are still used in today's clinical settings as anticoagulants, Vitamin K antagonists (VKAs) arose as not just an alternative anticoagulant to heparin, but also as a complement treatment. The most popular known VKA, warfarin, was initially marketed as a rodenticide in the 1940s, but was eventually transitioned into an anticoagulant in 1954. Vitamin K is an important class of fat-soluble biomolecules for the synthesis of coagulation Factors VII, IX, X, and thrombin. It is recycled from its inactive 2,3-epoxide form by vitamin K epoxide reductase complex subunit 1 (VKORC1) in the liver. VKAs like warfarin competitively inhibit VKORC1, eventually depleting the concentration of vitamin K in circulation, resulting in the reduction of key coagulation Factors. Relative to heparin, VKAs like warfarin have a longer half-life (20 – 60 hours for warfarin) and were the first anticoagulant to be administered orally. However, VKA treatment typically takes 3 – 7 days for the desired anticoagulant effect to manifest. While waiting for VKA treatment to have the desired therapeutic effect, heparin is typically administered intravenously. Other shortcomings to therapeutic treatment with VKAs include major bleeding risk and potential vascular calcification, as well as varying results of efficacy based on patient lifestyle (diet, drug and alcohol use, etc.). In some
cases, the administration of heparin alongside warfarin cannot only result in HIT, but also develop necrosis of the digits.\textsuperscript{64} To account for these side effects and treatment limitations, patients on VKA treatment require careful and restrictive lifestyles with constant monitoring.

Various limitations of heparin and VKA treatments as anticoagulants spurred further efforts to develop and discover anticoagulants with better efficacy, ease of use, and less side effects. As mentioned earlier in this chapter, direct thrombin inhibitors (DTIs) derived from hirudin were developed as anticoagulants in response to the issues and limitations caused by heparin and VKA. DTIs such as desirudin, lepirudin, bivalirudin and argatroban were administered intravenously not just to treat thrombosis, but also HIT.\textsuperscript{57,58,60,64} Lepirudin and desirudin were recombinantly expressed hirudin derivatives which inhibited thrombin by blocking both its active site and exosite I, which it uses to recognize substrates like fibrinogen. However, due to a mixture of adverse hemorrhagic events and manufacturing decisions by pharmaceutical companies, lepirudin and desirudin are no longer used in the United States.\textsuperscript{70} Bivalirudin is a synthetic analog of hirudin that also blocks thrombin at both its active site and exosite I.\textsuperscript{60} However, unlike lepirudin and desirudin, bivalirudin is cleaved by thrombin upon binding. Bivalirudin treatment therefore results in reversible thrombin inhibition that does not result in major bleeding risk. In terms of pharmacokinetics, peak activity of bivalirudin is achieved rapidly (2 – 4 min) with a half-life comparable to that of UFH (1 – 1.5 hours).\textsuperscript{57} Argatroban, in contrast to bivalirudin and other hirudin derivatives, is a small molecule that mimics the Arg-Gly bond in fibrinogen that thrombin cleaves.\textsuperscript{71} These features allow argatroban to selectively inhibit thrombin at high affinity relative to other coagulation Factors and serine proteases, as well
as inhibit both circulating thrombin and clot-bound thrombin. Compared to bivalirudin, argatroban has a much longer time to peak activity (3 – 4 hrs.) and half-life (3 – 5 hrs.).\textsuperscript{57} Anticoagulant treatment with either bivalirudin and argatroban can treat and avoid HIT risk from heparin use. With regard to VKAs, both bivalirudin and argatroban have no lag phase before treatment takes effect and are relatively unaffected by patient lifestyle.\textsuperscript{57} However, both DTIs require intravenous administration, which drastically increases the burden on the patient relative to VKAs that can be taken orally.

The anticoagulants of choice in contemporary times are direct oral anticoagulants (DOACs), which improve upon the therapeutic treatment provided by intravenous DTIs and oral VKAs.\textsuperscript{57, 58, 69, 72} The two currently existing classes of DOACs are direct thrombin inhibitors (e.g. dabigatran etexilate) and direct Factor Xa inhibitors (e.g. rivaroxaban, apixaban, edoxaban). Similar to the intravenous small molecule DTI argatroban, dabigatran etexilate is another small molecule that competitively inhibits both circulating and clot-bound thrombin at its active site.\textsuperscript{60} Dabigatran etexilate is a prodrug that is converted to the active drug molecule dabigatran by carboxylesterase 1 and 2 (CES1 and CES2).\textsuperscript{69, 73} Upon administration, dabigatran takes a maximum of two hours to achieve peak activity and has a half-life of 12 – 17 hours. Anticoagulation and thrombosis treatment expanded to include the therapeutic inhibition of Factor Xa, given its pivotal position in the coagulation cascade and its various roles in inflammation.\textsuperscript{69, 72, 74} The development of Factor Xa DOACs started with lead compounds that mimicked the Glu-Gly-Arg sequence in prothrombin that Factor Xa targets. Current Factor Xa DOACs in the US market are rivaroxaban, apixaban, and edoxaban. Time to peak activity for these inhibitors range from 1 – 4 hours, with half-lives ranging from 5 – 14 hours. Compared to VKAs, these four
DOACs were observed to be noninferior in terms of anticoagulant treatment and thrombosis prevention, but had significantly less incidences of major bleeding. However, risks of major bleeding, particularly intracranial bleeding, remains a concern. Interestingly, Factor Xa DOACs were recorded to be significantly more bioavailable than dabigatran. However, all four DOACs are dependent on the kidneys for excretion, making them unsuitable for patients with renal issues.\(^{57, 69, 72}\)

While current DOACs are easier to administer and have fewer side effects than VKAs and heparin for treating thrombosis, further improvements in the field of anticoagulant treatment could be made. As mentioned previously, the current small molecule DOACs requires functioning kidneys to be excreted by the body, and are thus contraindicated for patients with renal diseases. Increased risk of uncontrollable bleeding remains a concern with DOAC thrombosis treatment. Therefore, future anticoagulant design that successfully improves upon DOACs should either be more efficacious and safer, or at least be as equally effective as a DOAC but much safer to use.\(^{58, 75, 76}\) Recent research in further anticoagulant development has sought to target other key steps and components of the coagulation cascade: the TF-FVIIa complex in the extrinsic pathway,\(^11\) coagulation Factors VIII, IX, XI and XII of the intrinsic pathway,\(^{58, 78}\) and the stabilization of fibrin clots by coagulation Factor XIII in the final phase of the coagulation cascade.\(^{29}\)

As mentioned previously in this chapter, the TF-FVIIa complex drives hemostasis from the extrinsic pathway by activating FX. Additionally, TF-FVIIa was reported to be responsible for initiating thrombosis in arterial, venous and microvascular settings.\(^{79}\) Inhibiting either TF or FVII seemed like potential therapeutic strategies to treat thrombosis.
The human body itself has an endogenous inhibitor for the extrinsic pathway, a protein aptly named tissue Factor pathway inhibitor (TFPI).\textsuperscript{77} By itself, TFPI is a weak inhibitor of the TF-FVIIa complex. However, TFPI forms a complex with FXa, which then significantly inhibits not just the TF-FVIIa complex, but FXa as well. Additionally, some forms of TFPI have been observed to prevent TF-mediated cellular migration, limiting the scope of coagulation in the body. Given the importance of TFPI to regulating hemostasis, a number of synthetic inhibitors of the TF-FVIIa complex were also designed for thrombosis treatment: monoclonal antibodies that compete with FVII/FVIIa binding to TF or bind TF directly, chimeric proteins that act similarly to TFPI, and recombinant human FVIIa with an active site blocked with a covalent inhibitor (FVIIai).\textsuperscript{11, 80} Unfortunately, the clinical trials involving FVIIai resulted in increases of risk of serious bleeding and mortality rates.\textsuperscript{81} As a result, directly inhibiting the TF-FVIIa for clinical use is currently no longer considered.\textsuperscript{11} However, some potential remains for modulating TFPI activity to treat hemophilia.\textsuperscript{77}

While directly inhibiting components of the extrinsic pathway of coagulation did not improve upon DOAC treatment of thrombosis, coagulation Factors in the intrinsic pathway (FVIII, FIX, FXI, and FXII) show promise as therapeutic targets.\textsuperscript{58, 75, 78} Due to the additional roles of kallikreins in regulating blood pressure and inflammation, targeting these proteins carries the risk of off-target effects. A monoclonal antibody inhibitor for FVIII, named TB-402, was developed for intravenous admission to prevent VTE after total knee replacement.\textsuperscript{82} A clinical study compared the efficacy and safety of this FVIII antibody to the Factor Xa DOAC rivaroxaban. Unfortunately, the study found that, while TB-402 was just as effective as rivaroxaban in preventing VTE, incidences of significant
bleeding were higher in patients using TB-402 compared to patients using rivaroxaban. As a result, the study and development of TB-402 was discontinued. Efforts to target FIX involved a small molecule inhibitor, TTP889, to be administered as a DOAC, and the intravenous admission of an RNA aptamer, REG1. Trials with TT889 showed no significant improvement in treating thrombosis compared to patients on placebo. On the other hand, REG1 regrettably failed Phase 2 clinical trials when severe allergic reactions were reported in patients and no improvement in mitigating bleeding risk was seen with respect to the DTI bivalirudin. In comparison to FVIII and FIX inhibitors, more progress in drug development was seen in targeting FXII/FXIIa and FXI/FXIa. FXII was initially believed to be a safer target for thrombosis treatment relative to FXI because congenital FXII deficiency did not increase bleeding risk like congenital FXI deficiency did. However, epidemiological data has shown that FXI is strongly linked with thrombosis, while FXII is only weakly associated. Currently, the parenteral FXI inhibitor abelacimab and the oral FXIa inhibitors asundexian and milvexian are undergoing phase 3 clinical trials after showing promising phase 2 trials where thrombosis was reduced without notable increases in bleeding. While FXIIa might not be a better target for thrombosis treatment than FXIa, a FXIIa monoclonal antibody inhibitor, garadacimab, is currently showing promise in treating angioedema in phase 3 clinical trials.

While FXIa inhibitors were shown to reduce or prevent thrombosis in early clinical trials, the use of these inhibitors may still introduce bleeding risk and unwanted side effects at the same extent as current anticoagulants in clinical use, if not more. These side effects from currently used anticoagulants and FXIa inhibitors are a result of their ultimate impact on the generation or activation of thrombin. As previously mentioned, the roles of
thrombin extend beyond generating stable fibrin clots through fibrin and FXIII activation. Platelet activation, which has roles in inflammation and infection control in addition to forming hemostatic plugs, will be affected by any modulation of thrombin. Therefore, new anticoagulants and VTE treatments would need pharmaceutical targets within the coagulation cascade that have little to no impact on the generation or activity of thrombin.

As mentioned previously in this chapter, the inhibition of fibrin crosslinking by FXIII-A has been explored as a possible path for VTE treatment, namely to circumvent side effects related to thrombin regulation.\textsuperscript{7-9} Epidemiological studies of both FXIII and fibrin(ogen) have shown both proteins are major contributors to VTE.\textsuperscript{29} Monoclonal antibodies that bind to the thrombin cleavage site of the FXIII activation peptide (FXIII-AP) sequence successfully inhibited both proteolytic activation of FXIII and crosslinking activity on fibrin.\textsuperscript{86, 87} Unfortunately, increasing thrombin concentration to the point of excess overcame the inhibitory effect of these FXIII-AP antibodies, limiting their use in therapeutic settings. Lysine mimics such as 5-dansylamidopentamine are typically used \textit{in vitro} to competitively inhibit FXIII-A*, but inhibit other important transglutaminases in the body as well.\textsuperscript{88} Small molecule inhibitors such as thioimidazolium derivatives and cerulenin potently inhibit FXIII-A* by forming a covalent bond with the active site residue Cys314, but are also unable to selectively target FXIII-A* over other transglutaminases like tissue transglutaminase.\textsuperscript{89, 90} Peptidic irreversible FXIII-A* inhibitors, namely ZED1301, ZED3197 and KM93, also target Cys314 and are more selective toward FXIII-A* than thioimidazolium and cerulenin derivatives.\textsuperscript{7, 44, 91} Researchers have also studied tridegin, a peptide found from the saliva of the giant Amazon leech \textit{Haementeria ghilianii},
to see if its ability to bind near and block access to the FXIIIa active site has therapeutic potential.\textsuperscript{92}

However, careless inhibition of FXIII and fibrin(ogen) should be avoided: Cases of autoantibodies against FXIII arising from diseases such as leukemia and liver disease were recorded to result in severe bleeding and death.\textsuperscript{93} FXIII also has overlapping substrates with other transglutaminases like transglutaminase-2, which makes the design of specific inhibitors essential and challenging.\textsuperscript{8} Additionally, the removal of fibrin(ogen) by degradation could result in the formation of toxic fibrin(ogen) degradation products.\textsuperscript{29} Any further drug design aimed at the therapeutic inhibition of FXIII requires a deep and careful understanding of the interactions between FXIII and fibrin.

One strategy for navigating the intricacies of targeting FXIII and fibrin(ogen) for thrombosis treatment is to focus on specific interactions between the two coagulation factors. As mentioned previously, the relatively disordered $\alpha$C region in the fibrin(ogen) alpha chain is a significant area of FXIII crosslinking that stabilizes clots and facilitates clot growth through retention of red blood cells (see Figure 6 for Fbg $\alpha_2$ 221 – 610 sequence). Prior studies by Moaupi et al. in 2016 using a recombinant fibrin(ogen) alpha chain fragment spanning residues 233 – 425 (Fbg $\alpha$C 233 – 425) revealed that FXIII activity towards reactive glutamines is not equal.\textsuperscript{94} Of the three reactive glutamines present in the system, Q237 is the most reactive to crosslinking, followed by Q328 and Q366. This glutamine reactivity order is corroborated by \textit{in vivo} studies by Schmitt et al. in 2019, where Q237 (numbered as Q256 when signaling peptide sequence is included) was crosslinked in high frequency with a variety of fibrin lysine residues within fibrin clots.\textsuperscript{50}
A binding site for FXIII-A* is present in the αC region and is comprised of residues Fbg αC 389 – 402. The identity of these residues is relatively conserved across select species. Fbg αC E396 was reported to be a key binding residue for FXIII-A* that also promoted αC crosslinking. A chemical crosslinking and molecular modeling study by Smith et al. in 2013 predicted that Fbg αC 389 – 402 interacts with select FXIII-A* residues within a cleft exposed by the proteolytic activation of the transglutaminase. Given that this cleft is only accessible upon removal of the FXIII-A activation peptide, Fbg αC 389 – 402 will likely only interact with FXIII-A* and not FXIII-A°. The selective interaction of Fbg αC 389 – 402 with FXIII-A* and not FXIII-A° could explain how FXIII-A* was found to be more active on Fbg αC 233 – 425 than FXIII-A° in a study by Anokhin et al. in 2020. This interaction could potentially be used to design a FXIII-A* based anticoagulant that inhibits transglutaminase activity that stabilizes thrombi, but does not affect the intracellular functions of FXIII-A°.

Figure 6. Fibrinogen αC Region Amino Acid Sequence (Fbg Aα 221 – 610). FXIII-A* reactive glutamines are colored red and numbered with their position in the sequence. The FXIII-A* binding site is underlined. The amino acid residues present in Fbg αC 233 – 425 are highlighted in gray.
The overarching goal of this project was to evaluate the role of the FXIII-A* binding site on FXIII-A crosslinking in the Fbg αC region. The project was carried out in three parts: First, the influence of Fbg αC 389 – 402 on FXIII-A* and FXIII-A° Fbg αC crosslinking was determined. Second, any additional residues to E396 within Fbg αC 389 – 402 that impact αC crosslinking were identified and studied. A model system of recombinantly expressed Fbg αC 233 – 425 was used for the first two parts of the study. The role of Fbg αC 389 – 402 and its different components was studied by site-directed mutagenesis of select residues and regions. FXIII-A* activity was quantified using a previously developed MALDI-TOF mass spectrometry assay and gel-based fluorescence assay. Lastly, a new model system that contains the reactive glutamines Q221 and Q223 (Fbg αC 221 – 425) was expressed and studied to expand upon the findings from Fbg αC 233 – 425.
CHAPTER 2: REVIEW OF BIOCHEMICAL AND ANALYTICAL METHODS

This chapter explains and summarizes the different biochemical and analytical methods used in this dissertation. Fbg αC 233 – 425 was synthesized by recombinant expression in *E. coli* through an auto-induction expression system. Variant DNA of the Fbg αC model system were synthesized through site-directed mutagenesis and also recombinantly expressed. FXIII-A crosslinking activity, defined herein as the amount of crosslinking observed over a given period of time, was monitored using both mass spectrometry-based and fluorescence-based assays.

**Use of *E. coli* for Recombinant Expression of Fibrinogen αC DNA**

The model protein system of Fbg αC 233 – 425 and its variants was synthesized through recombinant expression of corresponding DNA in a pGEX-6P-1 plasmid in *E. coli*. One of the foremost advantages of using recombinant expression is that the availability of the human fibrinogen alpha chain from human whole blood extraction is far limited compared to in-house recombinant *E. coli* production. One would then need to isolate the alpha chain from the other two fibrinogen chains, as well as follow more stringent protocols in handling proteins extracted from patients. In comparison, using a traditional growth media in optimal growth conditions can double the population size of *E. coli* every 20 mins. While the amount of protein yielded from harvesting bacterial cells
is not solely dependent on population size, the ability of *E. coli* to grow rapidly roughly increases the potential yield of the desired protein. However, this exponential rate of growth is not indefinitely sustainable (Figure 7): As nutrients are exhausted, the rate of bacterial death will be equal to the rate of bacterial growth (stationary phase) and eventually overtake it (death phase). Depending on how expression of recombinant proteins is induced, proteins are harvested from bacteria either in the exponential or stationary phases.

![Figure 7](image)

Figure 7. Plot of typical bacterial growth curve. The growth of bacteria is described here as the number of bacteria in log scale as a function of time. Growth occurs in four phases, differentiated by the number of bacteria, and the rates of bacterial growth and death: In the lag phase, the initial population of bacteria acclimates to its surroundings and prepares for reproduction. Population growth in this phase is minimal. The log phase is characterized by exponential growth of bacterial population. Upon depletion of nutrients in the surroundings, the bacterial population enters the stationary phase, where the rates of bacterial growth and death are equal. Eventually, as all available nutrients are consumed, the rate of bacteria death is greater than that of growth, marking the final death phase. Adapted from reference 103.

The pGEX-6P-1 plasmid containing Fbg αC 233 – 425 DNA was received as a gift from Smith et al., which was extensively used in their 2011 study on the fibrinogen αC region and FXIII (Figure 8). This plasmid contains two genetic features that aid in the purification of Fbg αC 233 – 425 after its expression: a gene encoding for β-lactamase, and
another gene encoding for glutathione-S-transferase (GST) with a HRV 3C protease cleavage site located N-terminal to the target protein.

Bacteria that contain genes encoding for β-lactamase are resistant to antibiotics. β-lactams like penicillin and ampicillin are antibiotics that prevent bacterial cell wall synthesis, which is essential for maintaining osmotic pressure during cell division. Without a functioning cell wall, bacteria attempting to divide succumb to lysis and death. Some bacterial strains avoid this fate by expressing β-lactamase, a serine protease that inactivates β-lactam antibiotics through hydrolysis of their central β-lactam ring. Cells containing the pGEX-6P plasmid could grow unabated in nutrient-rich media containing ampicillin, while the antibiotic prevents the growth of unwanted bacteria.

The fusion of GST right before Fbg αC 233 – 425 allows GST to act as a purification affinity tag for the target protein: Given that bacterial cells express a multitude of proteins for its own use, an affinity tag attached to a target protein allows it to be easily isolated and purified from other bacterial proteins once the cells are lysed for target protein recovery. Proteins with a GST-tag strongly bind to affinity columns of glutathione-agarose resin, while other proteins dissolved in the cell lysate simply pass through the resin. The presence of a GST tag also increases the solubility of any attached protein, allowing for greater recovery from insoluble lysate components. GST-tagged HRV 3C protease is added to the column to cleave Fbg αC from its GST tag at the HRV 3C protease cleavage site, allowing Fbg αC to be eluted from the column using Tris-buffered saline (TBS) solution. Any bound GST or GST-tagged proteins left in the glutathione-agarose column could then be eluted by running a TBS solution with 10 – 20 mM reduced glutathione through the column.
Figure 8. pGEX-6P-1 plasmid sequence map. DNA base pairs positions are numbered in the 5' → 3' direction, with different restriction nuclease sites highlighted along with their base pair position in parentheses. Notable genetic regions are highlight by different colored segments and arrows: lac operator, blue segment; GST-sequence, light purple arrow; multiple cloning site (MCS), light blue segment; ampicillin-resistance gene (AmpR) coding for β-lactamase, light green arrow; origin of replication (ori), yellow arrow; lacI gene coding for lac repressor. The figure was taken from: https://www.snapgene.com/plasmids/pgex_vectors (ge_healthcare)/pGEX-6P-1
While *E. coli* has been widely used as a tried-and-true protein expression system, some of the disadvantages of using bacterial cells for the recombinant expression of human or mammalian proteins include the inability to reliably chaperone proper recombinant protein folding or perform post-translational modifications (PTMs).\textsuperscript{101, 106} These deficiencies are to be expected. Recombinant proteins are not expressed in their native environment, where an oxidative cytoplasmic environment, the appropriate enzymes, and molecular chaperones are normally present to facilitate proper disulfide bond formation and protein folding. Additionally, *E. coli* is likely to consign misfolded proteins as inclusion body aggregates rather than expend further energy to correct folding in an increasingly crowded cellular environment.\textsuperscript{101, 106} Fortunately, these shortcomings of *E. coli* recombinant expression have a limited impact on the results of this study. Although the human fibrin(ogen) alpha chain contains PTMs that have effects on fibrin clot formation and structure, studies-to-date have not conclusively shown that these PTMs have significantly impacted the rate or extent of FXIII-crosslinking.\textsuperscript{107} Furthermore, the fibrinogen αC region being studied is intrinsically disordered and would not require folding.\textsuperscript{28}

**Autoinduction for Recombinant Expression of Fibrinogen αC DNA**

Conventionally, expression of recombinant proteins in *E. coli* occurs through the use of *lac* operon control elements in plasmids.\textsuperscript{101, 108} Bacterial cells prefer to use glucose as a source for both energy and carbon, but are also able to break down lactose into glucose and galactose through the enzyme β-galactosidase. To prevent the wanton expression of this enzyme, a *lac* repressor protein is bound to the operon when glucose concentration
within bacteria is sufficiently high to prevent transcription of the *lac* promoter. When glucose is low and lactose is present, any basal β-galactosidase breaks down lactose or converts it to allolactose. Allolactose induces expression of more β-galactosidase by binding to the *lac* repressor protein, causing the *lac* repressor to dissociate from the *lac* promoter and allowing transcription to take place. Eventually, allolactose is hydrolyzed and the *lac* repressor re-binds to its promoter. Biologists and biochemists are able to insert genes coding for proteins they want to express into plasmids containing the *lac* promoter, and permanently induce recombinant protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), a non-hydrolyzable allolactose mimic. However, recombinant proteins can be toxic to the bacterial cells, and overexpression by IPTG strains bacterial metabolism to the point of cell death. Thus, researchers using IPTG-induction strategy attempt induction during the log phase of bacterial growth. The current phase of bacterial growth is estimated by measuring the optical density of the biomass at 600 nm in set time intervals using a spectrophotometer.

In 2005, Studier developed a protein expression induction method called “auto-induction.” This auto-induction method proved suitable for routine overexpression of Fbg αC 233 – 425 in the Maurer lab. Through this method, bacteria with *lac* promoter plasmids are incubated in growth media that notably contains glucose, glycerol, and lactose. Glucose will first be consumed before induction by lactose takes place. The presence of glycerol also serves to continue providing cells with carbon and energy for growth, but does not prevent induction by lactose. The addition of glucose, glycerol and lactose allows cells to progress to the stationary phase of growth at high densities before “automatically” being induced by lactose. Hence, monitoring the biomass for induction is
no longer necessary, and protein yield from autoinduction is significantly higher than traditional IPTG induction.

Synthesis and Expression of Fibrinogen αC 233 – 425 Variant DNA via Site-Directed Mutagenesis and E. Coli Transformation

Fbg αC 233 – 425 variant DNA were generated using site-directed mutagenesis with the QuikChange II Site Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Site-directed mutagenesis is an efficient method to engineer specific mutations in the DNA sequence of a template DNA strand, in this case within a plasmid. Through the use of mutagenic DNA primers, codons of certain amino acids could be modified to code for entirely new amino acids or a stop codon, which truncates the protein at the modified position. Each primer is mostly complementary to the target region of one strand of the template plasmid, but are mismatched to effect the desired mutation. Once the primers anneal to their targets, a high-fidelity DNA polymerase (Pfu polymerase) is used to extend the primer and replicate the rest of the plasmid, resulting in two daughter plasmids with the desired mutation in one strand. Amplification by the polymerase chain reaction (PCR) generates large numbers of plasmid with the mutation in both strands. To eliminate all of the original strands of the initial plasmid, the sample is subsequently treated by DpnI, which hydrolyzes the template methylated DNA, with the freshly synthesized unmethylated variant DNA left unscathed. The purified DNA is used to transformed E. coli for propagation and storage. Successful mutagenesis is confirmed by DNA sequencing, and the new plasmid used to transform an E. coli strain specialized for protein expression. Figure 9 shows a step-by-step flow chart of how site-directed mutagenesis
takes place with the pGEX-6P-1 Fbg αC 233 – 425 plasmid as the template strand, followed by the expression and purification of a Fbg αC 233 – 425 variant.

The pGEX plasmid vector coding for GST-tagged Fbg αC 233 – 425 was used as the template strand for site-directed mutagenesis. Product plasmids were used to transform XL-1Blue supercompetent cells for standard propagation and manipulation. Mutagenesis of sequences was confirmed by DNA sequencing at the CGeMM DNA facility core at the University of Louisville (KY). Plasmids encoding variant proteins were used to transform BL21 Gold DE3 (Agilent Technologies, Inc., Cedar Creek, TX) competent cells for expression.

Fbg αC 233 – 425 and its variants were then expressed via auto-induction and purified by GST-affinity chromatography as described previously.\(^{46, 97, 100}\) SDS-PAGE analysis of the sample at different stages of the purification process was used to assess the overall purity of the eluted protein sample and verify its identity by its molecular weight (~20 kD for GST-cleaved recombinant Fbg αC 233 – 425; see Figure 10 for representative SDS-PAGE purification gel). Protein concentrations of different eluted fractions were quantified by UV-vis spectroscopy at 280 nm.
Figure 9. Flow chart of Fbg αC variant DNA synthesis and expression. This flow chart covers the generation of variant DNA by site-directed mutagenesis and PCR, to the expression and purification of variant Fbg αC. XL-1B cells are a strain of supercompetent cells. BL21 cells are a strain of competent cells engineered for recombinant protein expression.
Figure 10. SDS-PAGE Analysis of Fbg αC 233 – 425 WT Purification. Representative 15% SDS-PAGE gel assessing expression and purification of Fbg αC 233 – 425 WT: MW – molecular weight marker showing standard bands of known masses in kiloDaltons (kD); lysate – clarified lysate; load and wash wastes – column flow-through upon and after lysate load, respectively; GST load – column flow-through upon GST-HRV 3C protease load; 1 and 2 – fractions of purified recombinant Fbg Fbg αC 233 – 425 WT (~20 kD); GST elution and urea waste – removal of leftover proteins using TBS containing 20 mM reduced glutathione and 5M urea, respectively

Monitoring FXIII-A Activity with a MALDI-TOF Mass Spectrometry Kinetic Assay

FXIII-A* activity towards specific reactive glutamines within Fbg αC was tracked using a kinetic assay described in prior studies. This assay utilizes an analytical technique called matrix-assisted laser desorption ionization (MALDI), a mass spectrometry (MS) technique first developed by Karas et al. in 1985. Sample analytes are first combined at a low concentration relative to an easily ionizable compound called a matrix in a predominantly organic solution. Typical solvent systems used to dissolve analytes and the matrix are solutions of varying volumes of acetonitrile and 0.1% trifluoroacetic acid. Acetonitrile is a good solvent for dissolving most organic compounds and easily evaporates. On the other hand, trifluoroacetic acid diluted with water is miscible with acetonitrile and serves to charge any ionizable sites by protonation. The sample mixture is then spotted onto a metal plate where both analytes and matrix co-crystallize
into a solid deposit upon solvent evaporation. This sample deposit is irradiated and partially ablated by a laser firing pulsed light at a wavelength of 337 nm,\textsuperscript{122} which desorbs and ionizes the matrix. In the resulting ablation cloud, which contains both the analyte and matrix, matrix ions are able to ionize analyte molecules through proton transfer. The abundance of the matrix relative to analytes not only increases the ionization and detection of analytes, but also prevents excess energy that would fragment or decompose parent analyte ions.\textsuperscript{117,118} Thus, MALDI MS is a predominantly soft ionization MS technique.

MALDI is often paired with a MS ion detection method known as time-of-flight (TOF) when studying biomolecules such as peptides, proteins and carbohydrates.\textsuperscript{118,120,121} In a MALDI-TOF mass spectrometer, groups of ions are accelerated with a fixed amount of kinetic energy by an electric field towards a detector. Because mass is inversely proportional to velocity, heavier and bigger analytes fly slower towards the detector than lighter and smaller analytes. These differences in times of analyte flight based on size allows for MALDI-TOF to distinguish between analytes of different mass-to-charge ratios (m/z). However, resolution between analytes that differ in m/z could be hampered by a multitude of factors, such as different kinetic energies between ions in the same group, or differing times for ion production and extraction.\textsuperscript{118}

Two instrumental features are present in TOF mass spectrometers that could be used to improve mass resolution: The first of these features is called the reflectron. A reflectron is an ion mirror with an electric field placed in the flight path of sample ions. The electric field of the reflectron corrects for any differences between analyte kinetic energies by increasing the time of flight of ions directly proportional to their kinetic energy before redirecting the ions towards a detector. Figure 11 illustrates the workflow of a
MALDI-TOF MS with a reflectron. The second feature used to improve resolution is delayed extraction. Upon ablation and ionization of the sample by the MALDI laser, ions are formed with differing initial kinetic energies that cause them to travel at varying distances from the MALDI plate. Delayed extraction is the ability of the instrument to put a time delay on the application of the electric grid voltage that accelerates the ions toward the reflectron/detector. Ions with higher initial kinetic energy travel farther from the accelerating grid and are imparted with less acceleration potential than ions with lower initial kinetic energy. With the appropriate delay time, the kinetic energies of ions with the same mass-to-charge ratios are equalized and these ions could reach the detector at the same time, improving peak resolution.

When used to analyze proteins, MALDI-TOF MS can be a very sensitive technique, operating with amounts of the target protein in the picomole range, and detection of as little as 1 femtomol in the best conditions. Furthermore, the application of MALDI-TOF MS for protein or peptide analysis is unrestricted by any level of protein structure (primary, secondary or tertiary). Theoretically, the mass range of analytes capable of being detected by MALDI-TOF MS has no upper limit. However, depending on the matrix used, ion species of higher mass ranges (>10 kDa) can suffer from poor mass resolution or mass accuracy. To circumvent these issues experienced by larger ion species, proteins can be fragmented into smaller ion species through protease digestion prior to MALDI-TOF analysis. Examples of proteases are trypsin (cleaves at the C-terminal side of lysine and arginine), chymotrypsin (cleaves at the C-terminal side of residues with aromatic side chains), and GluC endoproteinase (cleaves at the C-terminal side of negatively-charged residues). Protein fragmentation by digestion also allows proteins to be identified by
comparing the mass spectrum of their fragmentation pattern to a protein database or a theoretical digest program like https://web.expasy.org/peptide_mass. However, the amino acid sequence of the peptides would need to be verified by methods such as fragmentation (MS/MS).

**Figure 11.** Flow chart of MALDI-TOF sample analyte ionization, differentiation by time-of-flight and detection.
In the fields of biochemistry, microbiology or clinical analysis, MALDI-TOF MS is primarily used for detection of key analytes or identification via analyte composition. As mentioned earlier, proteins could be identified by MALDI-TOF MS by being subjected to digestion by proteases and comparing the fragmentation pattern to a known database. The same technique could be used to diagnose diseases based on the presence of metabolites in a sample, detect antibiotic resistance in bacteria, and even image the spatial distribution of plant root components.

MALDI-TOF MS has also been used for applications that require quantitative analysis, such as tracking the progression of an enzymatic reaction. Unfortunately, quantitative analysis with MALDI-TOF MS is impaired by “poor sample-to-sample and shot-to-shot reproducibility” from the heterogeneous nature of the co-crystallization sample prep, as well as differing ionization efficiencies between ion species. One solution to address these drawbacks is the use of internal standards with similar chemical properties as the analytes being studied. For example, in a study by Bungert et al. in 2003, the enzymatic reactions of glucose oxidase and carboxypeptidase A were successfully monitored over time by MALDI-TOF MS with the corresponding internal standards. In the absence of a suitable internal standard, the progress of enzymatic reactions could still be measured by taking into account relative peak intensities of reactant and product analytes with the peak height ratio method. The peak height ratio method is characterized by the following equation:

\[ [\text{Reactant}]_t = [\text{Reactant}]_0 \times \frac{\sum \text{Reactant Peak Heights}}{\sum \text{Reactant Peak Heights} + \sum \text{Product Peak Heights}} \]

[Reactant] \(_t\) is the concentration of reactant at time \(t\), and [Reactant] \(_0\) is the initial concentration of the reactant. Through this method, the progress of the enzymatic reaction
could be described as the concentration of reactant remaining as a function of time. However, an important caveat of using the peak height ratio method is that the reactant and product must have similar ionization efficiencies. Otherwise, the difference in peak intensities between reaction and product ions will not be simply due to reaction progress, but also differences in signal sensitivities.

For this research project, a MALDI-TOF MS-based assay was used to examine the crosslinking activity of FXIII-A on specific reactive glutamines of Fbg αC. In this assay, the lysine mimic, glycine ethyl ester (GEE), is crosslinked by FXIII-A to reactive glutamines over time. The resulting reaction increases the mass of any glutamine containing fragment by 86 m/z, the molecular weight of GEE (~103 g/mol) minus the molecular weight of the byproduct ammonia (~17 g/mol). Additionally, GEE is a neutral, non-aromatic molecule with little to no impact on the ionization efficiency of the glutamine-containing reactant fragments upon crosslinking. At specific time points, aliquots of the reaction mixture are quenched by the addition of ethylenediaminetetraacetic acid (EDTA), which chelates the calcium ions required by FXIII-A to function, and therefore arrests crosslinking activity. Reaction aliquots are then subjected to digestion by different proteases. Digested samples are prepared for and analyzed by MALDI-TOF MS. The signal peak heights of reactive glutamine containing fragments and their corresponding GEE-crosslinked products are measured. The peak height ratio equation is then used to determine the amount of uncrosslinked glutamine present for each time point. A flow chart of a typical GEE-crosslinking assay of Fbg αC 233 – 425 is illustrated in Figure 12.
Figure 12. Representative Flow chart of a GEE Crosslinking Assay of Fibrinogen αC WT (233 – 425). This flow chart describes how the GEE crosslinking assay of Fbg αC WT (233 – 425) was conducted, beginning with sample preparation, to finally describing FXIII-A activity on Q237 by plotting the loss of reactant over time. The mass spectrum in this figure is of a GluC endoproteinase digest of a crosslinking reaction quenched by EDTA 1 minute after the addition of Fbg αC WT to a 50 nM FXIII-A* reaction mixture.
Monitoring FXIII-A Activity with an SDS-PAGE Fluorescence Assay

Key GEE crosslinking assay results were supplemented using a fluorescence-based gel assay modified from prior studies. In these assays, monodansylcadaverine (MDC), a fluorescent lysine mimic, is crosslinked by FXIII-A to reactive glutamines over time. Aliquots of the reaction are quenched at specific time points in a reducing buffer solution, which are then transferred onto a polyacrylamide gel for electrophoresis. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins based on mass, is then used to concentrate Fbg αC in distinct protein bands and remove any uncrosslinked MDC. The GEE crosslinking assays monitor FXIII-A activity at specific reactive glutamines, and the MDC crosslinking fluorescence assays allow for the visual observation of overall FXIII-A activity over time as the increase in fluorescence signal is tracked.

To quantitatively compare MDC crosslinking between wild-type (WT) Fbg αC and its variants, an external standard of WT Fbg αC was crosslinked to MDC concurrently with variant reaction mixes and also subjected to SDS-PAGE. Any sample loss during transfer from the reaction mixture was corrected for by staining the gel with Coomassie Blue dye and normalizing protein band fluorescence intensities to their corresponding dye absorbance. To quantify the effect of mutations on crosslinking, normalized variant fluorescence values were each divided by the normalized fluorescence value of the Fbg αC WT external standard to give a relative fluorescence. Through this method, the reaction progress of each variant could be tracked via the increase in relative fluorescence over time.
CHAPTER 3: EFFECT OF THE FACTOR XIII BINDING SITE IN FIBRIN(OGEN) αC ON FACTOR XIII CROSSLINKING IN THE FIBRIN(OGEN) αC REGION

Introduction

Factor XIII (FXIII) is one of the terminal enzymes of the blood coagulation cascade. As a transglutaminase, FXIII joins or crosslinks proteins together by catalyzing the formation of γ-glutamyl-ε-lysyl peptide bonds between protein substrates.\textsuperscript{31, 41, 128} FXIII has additional roles in wound healing and bone formation, but has also been associated with diseases such as venous thromboembolism and arthritis.\textsuperscript{31, 34, 129, 130} In the human body, FXIII is found in plasma (pFXIII) and within cells (cFXIII) like platelets, monocytes, macrophages, and osteoblasts.\textsuperscript{34} pFXIII circulates as a zymogen composed of two protransglutaminase A subunits and two carrier B subunits (FXIII-A\textsubscript{2}B\textsubscript{2}, Figure 3).\textsuperscript{41, 131} pFXIII is activated by thrombin cleavage and release of an activation peptide (AP), followed by Ca\textsuperscript{2+}-induced conformational rearrangements to dissociate the B subunits and generate FXIII-A\textsuperscript{*}.\textsuperscript{41, 131} cFXIII is a dimer of A subunits, FXIII-A\textsubscript{2}, and is non-proteolytically activated by elevated intracellular Ca\textsuperscript{2+} concentrations to form FXIII-A\textsuperscript{o}.\textsuperscript{40} FXIII-A\textsubscript{2} can also be activated by thrombin in vitro in the presence of Ca\textsuperscript{2+} to produce FXIII-A\textsuperscript{*}.\textsuperscript{132} Both forms of activated FXIII-A have a spectrum of plasmatic and cellular substrates, and are involved in multiple physiological functions.\textsuperscript{31, 34, 128}
In plasma, FXIII-A* crosslinks fibrin γ and α chains, which confers stability against mechanical and proteolytic degradation.\textsuperscript{133, 134} FXIII-A* also crosslinks proteins such as α\textsubscript{2}-antiplasmin (α\textsubscript{2}-AP), and fibronectin (FN) to the fibrin αC region of the α chain (Fbg αC 221 – 610), providing anti-fibrinolytic and wound healing properties to the clot.\textsuperscript{135-137} cFXIII is initially localized to the cytoplasm. Upon agonist stimulation of platelets and monocytes, FXIII-\textsuperscript{A°} is translocated to the membrane.\textsuperscript{138-140} This FXIII-\textsuperscript{A°} has the potential to target the thrombus by crosslinking fibrin and α\textsubscript{2}-AP proteins.\textsuperscript{138, 139} Additionally, FXIII-\textsuperscript{A°} is involved in crosslinking cytoskeletal proteins like actin, filamin, and vinculin upon platelet activation.\textsuperscript{31, 128, 141-143} Analytical ultracentrifugation (AUC) experiments demonstrated that FXIII-A* is more conformationally flexible than FXIII-A°.\textsuperscript{38, 46} Moreover, \textit{in vitro} crosslinking assays suggested that FXIII-A* could exhibit greater transglutaminase activity than FXIII-A°.\textsuperscript{46} Probing the novel characteristics that FXIII utilizes to target its substrates will aid in a better understanding of how to regulate different FXIII crosslinking functions therapeutically.

The αC region of the fibrinogen alpha chain (Fbg αC 221 – 610) is an area where substantial FXIII crosslinking occurs.\textsuperscript{47-51, 99} This region is composed of a disordered αC connector (Aα 221 – 391) and a globular, more structured αC domain (392 – 610).\textsuperscript{144} Crosslinks within the αC region lead to stiffer clots that are resistant to fibrinolysis.\textsuperscript{52} \textit{In vitro}, Fbg variants with truncations at the αC 390 and αC 220 positions led to clots with reduced mechanical and fibrinolytic stability.\textsuperscript{49, 145} \textit{Fbg\textsuperscript{270/270}} mice with truncation at Aα271 exhibited hypofibrinogenemia with protection from thrombosis.\textsuperscript{145} Reactive Fbg αC glutamines Q237, Q328, and Q366 are located within the predominantly unstructured αC connector (221-391). Mass spectrometric crosslinking studies of recombinant Fbg αC
233 - 425 and insoluble fibrin clots identified and ranked αC glutamine reactivities as Q237
>> Q328 ≈ Q366.50, 94 FXIII-A* in the presence of Ca^{2+} has been reported to bind to Fbg
αC 389 – 402, with Fbg αC E396 serving as an important anchoring site.96-98

The aim of the current study was to critically evaluate the abilities of FXIII-A* versus FXIII-A° to crosslink Fbg αC. The impact of the FXIII-A* binding site, Fbg αC 389 – 402, on both activated forms of FXIII-A was assessed by truncations to the model Fbg αC 233 – 425 system (see Figure 13 for amino acid sequence). In-gel fluorescence and mass spectrometric techniques were used to monitor the crosslinking of lysine mimics to Fbg αC 233 – 425 and its truncation variants. Collectively, the results of these studies show that Fbg αC 389 – 402 significantly enhances the transglutaminase activity of FXIII-A* on Fbg αC 233 – 425 over FXIII-A°. The study also highlights the significance of the overall FXIII-A* binding site in boosting FXIII-A* activity on αC.

**Figure 13.** Amino acid sequence of the recombinantly expressed Fbg αC 233 – 425 model system. The italicized GPLGS sequence are leftover GST tag residues after HRV 3C protease cleavage. Reactive glutamines are numbered and highlighted in red. The residues of the FXIII-A* binding site are underlined.
Materials and Methods

Materials

Recombinant human FXIII-A2 was obtained from the late Dr. Paul Bishop (Zymogenetics, Seattle, WA). D-Phe-Pro-Arg-chloromethyl ketone (PPACK) were supplied by Haematologic Technologies (Essex Junction, VT). Bovine thrombin, ferulic acid, α-cyano-4-hydroxycinnamic acid (α-CHCA), monodansylcadaverine (MDC), and glycine ethyl ester (GEE) hydrochloride were obtained from Millipore Sigma (St. Louis, MO). Sequencing grade chymotrypsin and GluC endoproteinase, both from Roche, were supplied by Millipore Sigma (St. Louis, MO). 30% Acrylamide/Bis solution and dual-color molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, CA).

Site directed mutagenesis, expression, and purification of fibrinogen αC (233 – 425) and variants

Site-directed mutagenesis was carried out using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) with GST tagged Fbg αC (233 – 425) DNA serving as the template. The mutagenic primers were ordered from IDT (Coralville, IA). The sequences of the mutagenic primers used in this study are listed in Table 1. All Fbg αC (233 – 425) mutations were verified by DNA sequencing at the CGeMM DNA facility core (University of Louisville, KY). The mutant pGEX-6P based plasmids were transformed into BL21 Gold DE3 (Agilent Technologies, Inc., Cedar Creek, TX) or Acella (EdgeBio, San Jose, CA) competent cells. Fbg αC 233 – 425 and its variants were expressed in *E. coli* and purified as described previously.⁴⁶,⁹⁷
**Table 1.** List of Fibrinogen αC (233 – 425) E396A and Truncation Variant Primers.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers</th>
</tr>
</thead>
</table>
| E396A    | Forward: CAGACTGGGGCACATTTGAAGCGGTGTCAGGAAATGTAAGTCC  
            | Reverse: GGACCTTTACATTTTCCTGCCACACCATTACATTTGAG |
| 389 Stop | Forward: CTGGGAACCGGAGCCCTAACAACCTGAGACTGGGGACACATTTGAAG  
            | Reverse: CTTCAAATGTGCCACCAGTCTCAGTTGTTAGGCTCGCTGTTCCAG |
| 403 Stop | Forward: GTGTCAGGAAATGTAAGTTAAGGGACAAGGAGAGAG  
            | Reverse: CTCTCTCCTTTGCTCCCTGGAACCTACTTTCTGACAC |
| 328 Stop | Forward: GAAGCTGGGCTGCCAGGTACTGGAACACTAAAAACCTGGGAGGCCTAGAC  
            | Reverse: GTCTAGGGCTCCAGGGTTTTAGTTTCCAGTACTTCCAGTTC |

**MALDI-TOF mass spectrometric kinetic assay**

A previously optimized MALDI-TOF MS kinetic assay was used to compare the reactivities of FXIII-A* and FXIII-A° toward crosslinking GEE to glutamines within Fbg αC (233 – 425). FXIII-A2 was activated proteolytically by incubation in MALDI kinetic buffer (50 mM Tris-Acetate, 150 mM NaCl, 0.1 % PEG-8000, pH 7.4) with 8.4 NIH units/mL bovine thrombin and 4 mM CaCl2 at 37 °C for 10 min throughout, followed by the addition of PPACK (final concentration of 1.52 µM). To activate non-proteolytically, 500 nM FXIII-A2 was incubated in MALDI Kinetic buffer with 250 mM CaCl2 for 30 minutes. To initiate crosslinking, GEE (17 mM final concentration) and Fbg αC (13.6 µM final concentration) were added to the assay mixture. The final concentration of active FXIII A was 50 nM while CaCl2 final concentrations were 4 mM (proteolytic) and 25 mM (non-proteolytic), respectively. The reaction was allowed to proceed at 37 °C with 25 µL
aliquots of the reaction mixture removed at different time points and quenched with EDTA (10 mM final concentration). Quenched sample aliquots were digested separately by either chymotrypsin or GluC endoproteinase, purified by C18-tip desalting, and MS analysis. Reaction rates were characterized by applying the peak height ratio method between reactive glutamines and their corresponding GEE-crosslinked products. With these values, the loss of reactant over time could be tracked. Figure 14 shows representative GEE-crosslinking related MS peaks and their identities.

Assays for each experimental condition were performed in triplicate with a maximal reaction time of 30 minutes. Data were reported as mean ± standard deviation, and individual data points were analyzed by the Student's t-test and by P-values (GraphPad Prism 9.5.1). In addition, the data from each GEE-crosslinking assay were fit using a one phase exponential decay model $[Q]_{\text{remaining},t} = ([Q]_0 - [Q]_{\text{plateau}}) e^{(-kt)} + [Q]_{\text{plateau}}$. $[Q]_{\text{remaining},t}$ is the concentration of uncrosslinked reactive glutamine in µM for time point $t$ in minutes, $[Q]_0$ is the theoretical initial concentration of uncrosslinked reactive glutamine in µM, $[Q]_{\text{plateau}}$ is the theoretical concentration of uncrosslinked reactive glutamine in µM when $t = \infty$ minutes, and $k$ is the rate constant in min$^{-1}$. 
Figure 14. Representative MALDI-ToF MS Spectra of Proteolytic Digests of Fbg αC 389 Stop after FXIII-A°-catalyzed GEE Crosslinking. Digests were performed on crosslinking reactions quenched in EDTA 5 minutes after addition of Fbg αC 389 Stop to FXIII-A° reaction mixture. Peaks with m/z = 1349, 1549, 2448 correspond to uncrosslinked Q366-, Q237-, and Q328-containing digest fragments, respectively. The peak height ratio method was used to quantify the amount of uncrosslinked glutamine remaining per assay time point.
Monodansylcadaverine crosslinking fluorescence kinetic assay

Key GEE crosslinking assay results were supplemented using a gel-based fluorescence assay modified from prior studies.\textsuperscript{46, 100} 2.85 µM FXIII-A\textsubscript{2} was proteolytically activated by incubation in Tris-buffered saline (TBS, 50 mM Tris-acetate, 150 mM NaCl, pH 7.4) in the presence of 47.9 NIH units/mL bovine thrombin and 4 mM CaCl\textsubscript{2} at 37 °C for 10 min, followed by thrombin quenching via the addition of PPACK (activation mixture concentration of 9.5 µM). During FXIII-A\textsuperscript{*} activation, reaction mixtures containing 1 mM monodansylcadaverine (MDC), 5 µM Fbg αC, and 4 mM CaCl\textsubscript{2} in TBS were heated to 37 °C at least 5 minutes prior to initiation of MDC crosslinking. Crosslinking reactions were initiated by the addition of FXIII-A\textsuperscript{*} to the reaction mixture. The final FXIII-A\textsuperscript{*} concentration in each reaction mixture was 50 nM. The reaction proceeded at 37 °C with 20 µL aliquots of the reaction mixture removed at select time points and quenched with 5× reducing sample loading buffer, followed by heating at 100 °C for 3 – 5 minutes. With the exception of assays involving Fbg αC WT, a separate MDC crosslinking reaction mixture of Fbg αC WT was also prepared and allowed to react for 10 minutes prior to quenching as a positive control. All samples and the Fbg αC WT control were subjected to SDS-PAGE on a 15% T (37.5:1 Acrylamide:bis-acrylamide) Tris-glycine gel. Gels were transferred into deionized water and immediately subjected to fluorescence imaging on a BioRad Gel Doc XR imager. After fluorescence imaging, gels were stained with 0.1% (w/v) Coomassie blue staining solution. Dye-stained gel images were also recorded on a BioRad Gel Doc XR imager under white light.

Assays were performed for a total time of 10 minutes and in triplicate. The MDC fluorescence and Coomassie stain intensities for each sample time point were quantified.
using ImageJ 1.53e (NIH), as previously described.\textsuperscript{100} Fluorescence intensities were divided by their corresponding Coomassie stain intensities to correct for any variations in sample loading. In assays involving variants, these corrected fluorescence values were divided by the corrected fluorescence value of a Fbg $\alpha$C WT positive control that was concurrently subjected to FXIII-A* MDC crosslinking for 10 minutes. In assays where Fbg $\alpha$C WT was tested, corrected fluorescence values were divided by the corrected fluorescence value of the 10-minute time point. Through this method, the reaction progress of each variant could be tracked via the increase in relative fluorescence over time. Data were reported as mean $\pm$ standard deviation, and subjected to statistical analysis by the Student’s $t$-test with GraphPad Prism 9.5.1.

**Results**

MALDI-TOF mass spectrometry was used to monitor GEE-crosslinking on each reactive glutamine within Fbg $\alpha$C 233 – 425. Prior work had shown that Q237 is the most reactive glutamine for FXIII-A* activity among the three glutamines (Q237, Q328, and Q366) present within Fbg $\alpha$C 233 – 425.\textsuperscript{94} The experimental setup of the assay measured overall FXIII-A activity on $\alpha$C by solely tracking Q237-GEE crosslinking: With 50 nM FXIII-A and 30 minutes of assay reaction time, GEE crosslinking to Q237 prominently increased over time with minimal observable crosslinking to Q328 and Q366 (Figure 15). At a physiological concentration of 50 nM FXIII, Q237-GEE production dominates the mass spectral results.\textsuperscript{94} If desired, Q328 and Q366 reactivities in Fbg $\alpha$C WT (233 – 425) could be followed by raising the activated FXIII concentration to the 500 to 2000 nM range.
Figure 15. Comparison of FXIII A-catalyzed GEE-crosslinking of Q237, Q328, and Q366 between Fbg αC WT (233 – 425), and the FXIII binding site αC variants E396 and 389 Stop. GEE-crosslinking assays were initiated with the activation of 50 nM FXIII-A either (A) proteolytically with thrombin and 4 mM Ca^{2+} (FXIII-A*, left), or (B) non-proteolytically with a final assay concentration of 25 mM Ca^{2+} (FXIII-A°, right). Assays were performed in triplicate with 13.6 µM of the chosen αC fragment and 17 mM GEE, and monitored for 30 minutes each. Results were reported as mean ± standard deviation.

The impact of the FXIII-A* binding region on the crosslinking activities of FXIII-A* and FXIII-A° was assessed using through GEE-crosslinking assays of Fbg αC 233 – 425 WT, the alanine substitution mutant E396A, and the truncation mutant 389 Stop (Fbg αC
The 389 Stop truncation mutation removes residues that encompass the FXIII binding region. On the other hand, Fbg αC E396A replaces E396, an acidic residue that makes an important contribution towards improving FXIII-A* binding affinity for Fbg αC.96,97

The ability of FXIII-A* to catalyze GEE-crosslinking varied between Fbg αC WT, E396A, and 389 Stop (Figure 16, Table 2). The rates of Q237 consumption by FXIII-A* ranked as Fbg αC WT > E396A > 389 stop (Table 2). The disparity in reactivities is highlighted at 15 min, where the concentrations of unreacted Q237 in αC E396A and αC 389 Stop were ~2-fold higher ($P \leq 0.01$) and ~4-fold higher ($P \leq 0.001$) relative to WT, respectively (Figure 16A, 16C). For FXIII-A*, the full FXIII binding region αC (389 - 402) played a more prominent role in promoting Q237-GEE crosslinking than just the key anchoring residue αC E396. In contrast, FXIII-A° reactivity toward αC Q237 was not as affected by the binding site mutations as FXIII-A* (Figure 16B). Rates of substrate consumption were comparable for the FXIII-A° catalyzed reactions with Fbg αC WT > E396A ≈ 389 stop (Table 2). Unreacted Q237 concentrations at t = 15 mins for αC E396A ($P \leq 0.05$) and αC 389 Stop ($P > 0.05$) were slightly higher relative to WT (Figure 16C).
Figure 16. Transglutaminase activity of FXIII-A* is more affected by Fbg αC binding site mutations than FXIII-A°. FXIII-catalyzed Q237-GEE crosslinking assays between WT Fbg αC (233–425, black) and the FXIII binding site variants E396A (green) and 389 Stop (blue) were performed using 50 nM of either (A) FXIII-A* (circles, solid line), or (B) FXIII-A° (triangles, dashed line). Reactions were monitored by measuring the concentration of uncrosslinked Q237 remaining at each time point. Plots represent one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. Data were reported as mean ± standard deviation (N = 3). (C) FXIII GEE-crosslinking activity after 15 minutes of reaction time was compared between Fbg αC WT, E396A, and 389 Stop. Shown here is the mean uncrosslinked Q237 concentration ± standard deviation in µM after 15 minutes of reaction time (N = 3). Statistical significance was determined using the Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001).
Table 2. Comparison of curve fit coefficients from FXIII-A* and FXIII-A° Q237-GEE crosslinking assays involving Fbg αC (233-425, WT), αC (233-425, E396A), and αC (233-338, 389 Stop).

<table>
<thead>
<tr>
<th>Fbg αC</th>
<th>FXIII-A Species</th>
<th>Initial Conc. of Q237, [Q237]₀ (μM)a</th>
<th>Initial Reaction Rate, V₀ (µM/min)</th>
<th>k (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>FXIII-A*</td>
<td>13.20</td>
<td>3.21</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>FXIII-A°</td>
<td>13.10</td>
<td>1.29</td>
<td>0.10</td>
</tr>
<tr>
<td>E396A</td>
<td>FXIII-A*</td>
<td>13.64</td>
<td>2.26</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>FXIII-A°</td>
<td>13.37</td>
<td>0.82</td>
<td>0.06</td>
</tr>
<tr>
<td>389 Stop</td>
<td>FXIII-A*</td>
<td>13.40</td>
<td>0.91</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>FXIII-A°</td>
<td>13.47</td>
<td>1.18</td>
<td>0.09</td>
</tr>
</tbody>
</table>

aCalculated theoretically by fit model. The actual initial conc. of each reactive glutamine in the GEE-crosslinking assays is 13.6 μM.

The abilities of FXIII-A* versus FXIII-A° to catalyze GEE crosslinking reactions with Fbg αC 233 – 425 WT, Fbg αC 233 – 425 E396A, and Fbg αC (233-388) 389 Stop were also critical to examine. A comparison of αC Q237-GEE crosslinking plots revealed that FXIII-A* exhibited an ~2.5 times faster rate of transglutaminase activity toward Fbg αC 233 – 425 WT than FXIII-A° (Figure 17 and Table 2). The largest difference between the two plots is seen at 15 min where the concentration of unreacted αC WT Q237 is ~2-fold higher with FXIII-A° than FXIII-A* (P ≤ 0.05) (Figure 17D). Similarly, experiments with Fbg αC 233 - 425 E396A indicated that the rate of the FXIII-A* catalyzed crosslinking reaction was ~3-fold greater than that with FXIII-A° (Figure 17B, Table 2). At 15 min, the concentration of unreacted αC E396A Q237 was ~2-fold higher with FXIII-A° than with FXIII-A* (P ≤ 0.01) (Figure 17D). Interestingly, the full removal of the αC 389 – 402 binding region modestly increased the crosslinking rate for FXIII-A° with respect to FXIII-A* (Figure 17C, Table 2). At t = 15 mins., the concentration of unreacted Fbg αC (233 – 388) 389 Stop Q237 was lower with FXIII-A° than with FXIII-A* (P ≤ 0.05) (Figure 17D). This second set of crosslinking comparisons revealed FXIII-A* exhibits greater reactivity.
toward Fbg αC 233 – 425 WT and αC E396A than FXIII-A°. In contrast, rates of Q-
substrate consumption are more comparable between FXIII-A* and FXII A° when utilizing
the truncated 389 Stop (Fbg αC 233 – 338). The FXIII binding region within αC 389 –
402 is proposed to be more beneficial for promoting the transglutaminase activities of
FXIII-A* over that of FXIII-A°.

Figure 17. Transglutaminase activities between FXIII-A* and FXIII-A° on Fbg αC Q237 are
significantly different. FXIII-catalyzed Q237-GEE crosslinking assays between (A) WT Fbg αC
(233 – 425, black) and the FXIII binding site variants (B) E396A (green) and (C) 389 Stop (blue)
were performed using 50 nM of either FXIII-A* (circles, solid lines), or FXIII-A° (triangles, dashed
lines). Fbg αC WT, E396A and 389 Stop data were taken from Figure 16 and rearranged to compare
the FXIII-A* and FXIII-A° crosslinking data for each variant side by side. Reactions were
monitored by measuring the concentration of uncrosslinked Q237 remaining at each time point.
Plots represent one phase exponential decay fits of uncrosslinked Q237 concentrations as a function
of time. Data were reported as mean ± standard deviation (N = 3). (D) FXIII GEE-crosslinking
activity after 15 minutes of reaction time was compared between Fbg αC WT, E396A, and 389
Stop. Shown here is the mean uncrosslinked Q237 concentration ± standard deviation in µM after
15 minutes of reaction time (N = 3). Statistical significance was determined using the Student’s t-
test (*P < 0.05, **P < 0.01).
The impact of the entire FXIII binding region (Fbg αC 389 – 402) on αC FXIII-A* activity was further assessed by comparing Q237-GEE crosslinking of truncation mutants 403 Stop (Fbg αC 233 – 402) and 328 Stop (Fbg αC 233 – 327) with wild-type (WT), 389 Stop (Fbg αC 233 – 388), and E396A. All truncation mutants experienced markedly slower rates of crosslinking relative to wild-type (Figure 18, Table 3). The mean unreacted Q237 concentration of 403 Stop was ~2-fold higher ($P \leq 0.01$) compared to WT, while 389 Stop and 328 Stop both had ~5-fold higher ($P \leq 0.001$) reactant concentrations remaining (Figure 18B). Crosslinking activity was similar between E396A and 403 Stop while the truncation mutants that did not contain the FXIII binding region (αC 389 – 402), namely 389 Stop and 328 Stop, experienced considerably slower FXIII-A* activity than E396A and 403 Stop ($P \leq 0.01$). At $t = 15$ mins., unreacted Q237 concentrations between E396A and 403 Stop were similar, while remaining reactant concentrations of 389 Stop and 328 Stop were ~2-fold higher relative to both E396A and 403 Stop ($P \leq 0.01$, Figure 18C). Figure 18C also shows similar Q237-GEE crosslinking activities between 389 Stop and 328 Stop.

**Table 3.** Comparison of one phase exponential decay model coefficients from plots of FXIII-A* Fbg αC Q237-GEE crosslinking assays involving WT, E396A, and truncation variants.\(^a\)

<table>
<thead>
<tr>
<th>Fbg αC</th>
<th>Initial Conc. of Q237, [Q237]₀ (µM)(^a)</th>
<th>Initial Reaction Rate, $V₀$ (µM/min)</th>
<th>$k$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>13.06</td>
<td>3.13</td>
<td>0.24</td>
</tr>
<tr>
<td>389 Stop</td>
<td>13.40</td>
<td>0.91</td>
<td>0.07</td>
</tr>
<tr>
<td>328 Stop</td>
<td>13.27</td>
<td>0.92</td>
<td>0.07</td>
</tr>
<tr>
<td>403 Stop</td>
<td>13.02</td>
<td>2.52</td>
<td>0.19</td>
</tr>
<tr>
<td>E396A</td>
<td>13.64</td>
<td>2.26</td>
<td>0.17</td>
</tr>
</tbody>
</table>

\(^a\)Calculated theoretically by fit model. The actual initial conc. of each reactive glutamine in the GEE-crosslinking assays is 13.6 µM.
Figure 18. Effect of Fbg αC 233 – 425 truncation mutations on FXIII-A* transglutaminase activity. (A) Plot of FXIII-A*-catalyzed Q237-GEE crosslinking assays of Fbg αC WT (233 – 425, black) and variants E396A (233 – 425, green), 403 Stop (233 – 402, cyan) 389 Stop (233 – 388, blue), and 328 Stop (233 – 327, dashed line and dark blue). Data were reported as mean ± standard deviation (N = 3), and plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. (B – C) Mean uncrosslinked Q237 concentrations ± standard deviation (in µM) of Fbg αC WT, E396A, 403 Stop, 389 Stop, and 328 Stop after 15 minutes of reaction time. (N = 3, **P < 0.01, ***P < 0.001). Fbg αC E396A and 389 Stop data was taken from Figure 17. Panel (B) compares WT results to those of E396A and the Stop variants. Panel (C) compares E396A results to the Stop variants.
A fluorescence-based gel assay utilizing MDC was performed on Fbg αC 233 – 425 WT and the truncation variants to confirm their effects on FXIII-A* activity. The fluorescence assay also provided an opportunity to work with another lysine mimic and visually observe FXIII-A* activity over time. This method allowed for the simultaneous and direct comparison of Fbg αC variant reaction progress to that of a Fbg αC WT positive control.

The MDC crosslinking assays of Fbg αC WT and the truncation mutants in this study (Figure 19) corroborate the results seen in the corresponding Q237-GEE mass spectrometry crosslinking assays (Figure 18): All truncation mutants experienced distinctly slower increases in fluorescence over time relative to WT, which corresponds to less MDC crosslinking catalyzed by FXIII-A*. Relative fluorescence in the 403 Stop MDC crosslinking assays exhibited on average only a modest decrease over time compared to that of WT (Figures 19B and 19E). MDC crosslinking into αC 389 Stop and 328 Stop were relatively similar, but both were significantly lower relative to WT across all time points (***P ≤ 0.001, Figures 19C, 19D and 19F). Unfortunately, attempts to subject the MDC crosslinking data to a one-phase association model resulted in poor fits to the truncation variant data (R² < 0.90). Standard deviation tended to be higher after 5 minutes of reaction time, which systematically reduced the quality of fitting. The deviation of fluorescence signals at latter time points could be attributed to varying amounts of signal loss at higher intensities due to photobleaching and quenching. Additional replicates (N > 3) could reduce the standard deviation at each time point and improve fits. Poor fitting of MDC crosslinking data to the one-phase association model could also be caused by the participation of more than one reactive glutamine, each with varying K_m and k_cat values.
Figure 19. Effect of Fbg αC truncation mutations on FXIII-A*-catalyzed MDC crosslinking. Representative images of MDC crosslinking SDS-PAGE gels of Fbg αC (A) WT, (B) 403 Stop, (C) 389 Stop, and (D) 328 Stop showing fluorescence under UV light (top) and protein bands under white light after Coomassie blue staining (bottom). (E – F) Plots of MDC crosslinking assays of Fbg αC WT (233 – 425, black), 403 Stop (233 – 402, cyan), 389 Stop (233 – 388, blue), and 328 Stop (233 – 327, dark blue). Data were reported as mean ± standard deviation (N = 3), and plotted as relative fluorescence as a function of time. (*P < 0.05, **P < 0.01, ***P < 0.001).
Discussion

The transglutaminase FXIII can be activated both proteolytically (FXIII-A*) and nonproteolytically (FXIII-A°). Following activation, the catalytic FXIII-A interacts with an array of substrates in different environments. FXIII-A* has been recorded to have higher transglutaminase activity than FXIII-A°. The current project aimed to characterize in greater depth the reactivities of FXIII-A* versus FXIII-A° toward Fbg αC 233 – 425, as well as the relevance of the FXIII-A* binding site, Fbg αC 389 – 402.

GEE crosslinking assays exposed differences between FXIII-A* and FXIII-A° transglutaminase activities toward Fbg αC 233 – 425. For Fbg αC 233 – 425 WT, FXIII-A* exhibited higher activity than FXIII-A°, with Q237 as the dominant reactive glutamine for both activated forms of FXIII-A (Figures 15 – 16). In terms of αC Q237 reactivities, WT > E396A > 389 Stop for FXIII-A*. On the other hand, αC Q237 reactivities between WT, E396A and 389 Stop were nearly similar for FXIII-A°. Current research suggests that the FXIII-A* binding site (Fbg αC 389 – 402), with its key anchoring residue E396, plays a novel role in controlling FXIII-A substrate specificity. Through the Fbg αC 389 Stop mutation, the Q237-GEE crosslinking between FXIII-A* and FXIII-A° are now comparable. FXIII-A* is hypothesized to adopt a conformation that can take advantage of a FXIII binding site region within Fbg αC 389 – 402. The electrostatic anchoring of Fbg αC E396 is also proposed to play a contributing role in FXIII-A* reactivity. Using peptides derived from Fbg αC 389 – 402, Smith et al. reported binding to the FXIII-A* AP cleft that includes R158, a key residue for the electrostatic interaction. This cleft region is located within the β-sandwich domain and becomes exposed following thrombin-catalyzed release of FXIII AP. Biswas et al. performed docking studies with the entire Fbg
α chain, and active site inhibited FXIII-A°. Docking contacts were reported within the FXIII-A β-sandwich and catalytic core, including amino acids in the 160s, 170s, and 200s regions.¹⁴⁶

The current study also explored the impact αC truncations would have on crosslinking. Patients with Fbg αC truncation mutations have been documented to suffer from decreased levels of fibrinogen, abnormal formation of fibrin fibers, and impaired hemostasis.¹⁴⁷-¹⁵⁰ A study by McPherson et al. in 2021 on truncations of the Fbg αC region in whole fibrinogen systems reported that the loss of Fbg αC 390 – 610 resulted in clots with thinner fibrin fibers and less mechanical stability.⁴⁹ The same study also demonstrated that the absence of the entire Fbg αC region resulted in underdeveloped fibrin fibers that form clots with atypical network structures. Interestingly, a study by Hur et al. in 2021 showed that mice with Fbg αC truncated at residue 271 were protected from venous thrombosis formation with the hemostatic potential of fibrinogen preserved, albeit fibrinogen levels were drastically reduced.¹⁵¹

Figures 18 and 19 highlight the substantial influence of Fbg αC 389 – 402 on αC Q237-GEE and MDC crosslinking relative to αC 403 – 425 and αC 328 – 388. While the loss of 23 residues in 403 Stop (Fbg αC 233 – 402) reduced crosslinking relative to WT, its impact on Q237 crosslinking is similar to the alteration of a singular key binding residue, αC E396, via the E396A substitution (Figure 18C). This modest loss of FXIII-A* activity from the 403 Stop truncation is also observed in MDC crosslinking assays between WT and 403 Stop (Figure 19E). The 389 Stop (Fbg αC 233 – 388) and 328 Stop (Fbg αC 233 – 327) truncations result in distinctly less Q237-GEE and MDC crosslinking relative to WT and 403 Stop (Figures 18A – 18C, 19). However, FXIII-A* activity is similar between
389 Stop and 328 Stop. The loss of two reactive glutamines (Q328 and Q366) and 62 residues in 328 Stop do not appear to further impact Q237-GEE and MDC crosslinking relative to 389 Stop. These sets of results imply that the differences in Q237-GEE and MDC crosslinking between 389 Stop and 328 Stop relative to WT and 403 Stop are mainly caused by the absence of the FXIII-A* binding site (Fbg αC 389 – 402). Additionally, the similarities of FXIII-A* activity on αC Q237 between 389 Stop and 328 Stop suggest that Q328 and Q366 have minimal effects on Q237 crosslinking. A prior study by Mouapi et al. in 2019 hypothesized that Q237 crosslinking might promote Q328 and Q366 crosslinking. While Q237 crosslinking impacts FXIII-A* activity toward Q328 and Q366, results suggest that Q328 and Q366 do not have the same influence on Q237 crosslinking.

Conformational differences between FXIII-A* and FXIII-A° are important to consider for elucidating possible sources of substrate specificities. AUC results demonstrated that FXIII-A* exhibits a more flexible conformation, whereas FXIII-A° has a tighter, more homogeneous conformation stabilized by higher mM Ca^{2+} binding. With FXIII-A*, the cleaved FXIII AP segment may be partially or fully displaced from the FXIII surface. With FXIII-A°, the unhydrolyzed FXIII AP segment is still present and may even play an obstructive role.

X-ray crystallography on active site inhibited FXIII-A° revealed that the β-barrel 1 and 2 domains rotate away from the catalytic core domain and are directed upwards towards the β-sandwich domain (Figure 3). Both X-ray crystallography and HDX MS studies have documented that the unhydrolyzed AP can remain associated with FXIII-A° in the presence of Ca^{2+}. A well-defined crystal structure for the less soluble, active
site inhibited FXIII-A* is not yet available. However, a more exposed AP cleft of FXIII-A* is predicted to contribute to Q-substrate binding and have the potential to promote higher transglutaminase activity. In addition, the more flexible FXIII-A* may contribute further conformational states for effective substrate binding.

PONDR-VLXT analysis has revealed Fbg αC 233 – 425 is predominantly disordered, although some potential for structural order is present in an area encompassing Fbg αC 389 – 402 (Figure 20).\textsuperscript{144} The interaction between Fbg αC 389 – 402 and FXIII-A could likely be a function of the position of Fbg αC 389 – 402 within the overall Fbg αC region: Fbg αC 389 – 402 starts at the end of the disordered αC connector, αC 221 – 391, and extends into the structured C-terminal portion, αC 392 – 610.\textsuperscript{144} Removal of the αC 389 - 402 may thus hinder or eliminate a valuable interaction between FXIII-A* and Fbg αC. Hence, both FXIII forms interact similarly with Fbg αC and exhibit comparable activity. FXIII-A* may take more advantage of the Fbg αC 389 – 402 segment to position itself for access to reactive glutamines. Alternatively, the interaction with Fbg αC 389 – 402 promotes a FXIII-A* conformational change leading to enhanced reactivity. In contrast, zymogen FXIII A\textsubscript{2} does not bind to Fbg αC 233 – 425. The binding sites are only exposed upon activation, such as for FXIII-A* in the presence of Ca\textsuperscript{2+}\textsuperscript{98}. In contrast, FXIII-A° is proposed to have less need for the FXIII-A* binding site region, or the FXIII-A° conformation cannot take advantage of this binding region. In further support, Hornyak et al. reported that among the different forms of FXIII-A\textsubscript{2}, FXIII-A* had the strongest binding affinity for fibrin, followed by FXIII-A°, and FXIII-A\textsubscript{2} with the weakest binding affinity.\textsuperscript{156}
Figure 20. PONDR-VLXT Analysis of Fbg αC 233 – 425. The intrinsic disorder propensity of the different residues of Fbg αC 233 – 425 was calculated by PONDR-VLXT [www.pondr.com] as a function of position in the primary structure. A higher PONDR score is associated with a higher propensity towards being in a disordered region. The positions of FXIII-A reactive glutamines are labeled, while the position of the Fbg αC 389 – 402 sequence is marked gray.

Conclusion

FXIII is an intriguing transglutaminase that can be activated both proteolytically (FXIII-A*) and nonproteolytically (FXIII A°). FXIII-A* adopts a flexible conformation upon cleavage of the FXIII activation peptide. In contrast, FXIII-A° remains an intact protein with a tighter, more homogeneous conformation stabilized by additional Ca$^{2+}$ binding. Fbg αC 233 – 425 experiences more crosslinking activity with FXIII-A* over FXIII-A°. However, differences in crosslinking between the two activated forms of FXIII-A are diminished with Fbg αC E396A and further hindered upon removal of the putative FXIII-A* binding region (Fbg αC 389 – 402) in Fbg αC 389 Stop (233 – 388). Such results suggest FXIII-A* relies on this FXIII binding region to generate greater transglutaminase
activity than FXIII-A°. Overall, the unique conformational properties of FXIII-A* and A° are proposed to enhance transglutaminase activity toward substrates with different structural properties and environmentally driven features. The novel characteristics of FXIII-A* versus A° are valuable to consider for future drug designs.¹

¹ This chapter is reproduced in part from the following manuscripts:
CHAPTER 4: FIBRIN(OGEN) αC 389 – 402 ENHANCES FACTOR XIII
CROSSLINKING IN THE FIBRIN(OGEN) αC REGION VIA ELECTROSTATIC AND HYDROPHOBIC INTERACTIONS

Introduction

The formation of blood clots after vascular injury is an essential process to prevent further blood loss and assist blood vessel recovery. Fibrinogen (Fbg) is a coagulation protein that is vital in the latter stages of clot formation. Each fibrinogen molecule is a heterodimer composed of three chains: Aα, Bβ, and γ. All three fibrinogen chains extend outward in a coiled coil motif from a central E region toward distal D regions. The Aα chains continue past the distal D region as a flexible and relatively disordered chain known as the αC region (Figure 21A). Thrombin initiates the conversion of fibrinogen to fibrin by cleaving fibrinopeptide A (FpA) from the Aα chain, driving the linear aggregation of fibrin monomers into protofibrils by D-E knob-hole interactions. Subsequent cleavage of fibrinopeptide B (FpB) from the Bβ chain results in release of the αC regions from the E domain, leading to increased intermolecular interactions that coalesce protofibrils into fibrin fibers that make up a “soft clot”.

63
Fibrin clots are subsequently strengthened against premature fibrinolysis by coagulation Factor XIII (FXIII). FXIII is a transglutaminase that can be activated
proteolytically (FXIII-A*) by thrombin cleavage of an activation peptide in the presence of Ca$^{2+}$, or non-proteolytically (FXIII-A°) without thrombin in the presence of Ca$^{2+}$. Upon activation, FXIII-A crosslinks reactive glutamines and lysines between fibrin and other coagulation proteins. The αC region of the fibrinogen alpha chain (Fbg αC 221–610) is an area where substantial FXIII crosslinking occurs. This portion of fibrinogen is involved in fibrin polymerization, fibrin clot stability, and binding of different coagulation proteins that regulate fibrinolysis. Crosslinking in this region significantly promotes clot stability and mediates clot growth via red blood cell retention. While sizeable and stable blood clots contribute to vascular recovery, FXIII can also stabilize and grow thrombi, resulting in the development of venous thromboembolism (VTE). The inhibition of FXIII has therefore been explored as a potential avenue of VTE treatment. Further drug design aimed at the therapeutic inhibition of FXIII would be supported by a deeper understanding of the interactions between FXIII and fibrin.

A binding site for FXIII-A* is present in the Fbg αC region and is comprised of residues Fbg αC 389–402 (Figures 21A and 21B). The identity of these residues is relatively conserved across select species (Table 4). Prior studies by Smith et al. reported Fbg αC E396 as a key binding residue for FXIII-A* that promoted αC crosslinking. Additionally, Fbg αC 389–402 was predicted to have interactions with select FXIII-A* residues within a cleft exposed by the proteolytic activation of the transglutaminase (Figure 21C). The current study sought to identify additional residues within Fbg αC 389–402 that impact αC crosslinking using a recombinantly expressed Fbg αC 233–425 model system. Fbg αC D390, W391, F394, and E395 were hypothesized to have an effect on FXIII-A* activity toward αC based on a chemical crosslinking and molecular modeling
The effect of these residues on crosslinking was now assessed by site-directed mutagenesis, followed by quantification of FXIII-A* activity using a previously designed MALDI-TOF mass spectrometry assay. Key findings were further supported using a fluorescence-based SDS-PAGE assay. The results of these studies identified key residues in αC 389 – 402 that increase αC crosslinking. The study also highlights the significance of the overall FXIII-A* binding site in boosting FXIII-A* activity on αC.

Table 4. Multiple sequence alignment of FXIII-A* binding region in Fbg αC in select species. The key binding glutamate residue (E396 in the human sequence) is highlighted in red.

<table>
<thead>
<tr>
<th>Species</th>
<th>FXIII-A* Binding Region Fbg αC Amino Acid Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>389PDWGTFEEVSGNVS402</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>389PDWGTFEEVSGNVS402</td>
</tr>
<tr>
<td>Horse</td>
<td>496PDWGTFEEVSGSVS510</td>
</tr>
<tr>
<td>Rat</td>
<td>332PDWGEFSEFGGSSS346</td>
</tr>
<tr>
<td>Pig</td>
<td>475PDWGTFKEVSGSVS489</td>
</tr>
<tr>
<td>Hamster</td>
<td>323PDWGEFSEFTGSSS402</td>
</tr>
<tr>
<td>Mouse</td>
<td>336PNWGVFSEFGDSSS350</td>
</tr>
</tbody>
</table>

aSequences and alignments obtained from UNIPROT
Figure 22. Molecular mapping of rFXIII-A2* chemically cross-linked via Lys446 to a synthetic Fbg αC 389 – 402 peptide (PDWGTFEEVSGNVSC). The lysine at rFXIII-A2* residue 446 is shown (black rectangle) chemically crosslinked to the peptide cysteine (C). Potential attachment sites involving key FXIII-A2 residues Tyr167, Val169, Arg171, Glu24, Arg158, and Lys156 are also shown. The electrostatic interaction between FXIII-A* R158 and Fbg αC E396 is highlighted with a red circle. The electrostatic interaction between FXIII-A* K156 and Fbg αC D390 is highlighted with an orange circle. The positions of Fbg αC W391 and F394 are highlighted with green and pink circles, respectively. Reprinted and minimally altered (circles to highlight key interactions/residues) from Blood, 121, Kerrie A. Smith, Richard J. Pease, Craig A. Avery, Jane M. Brown, Penelope J. Adamson, Esther J. Cooke, Søs Neergaard-Petersen, Paul A. Cordell, Robert A.S. Ariëns, Colin W.G. Fishwick, Helen Philippou, and Peter J. Grant, “The activation peptide cleft exposed by thrombin cleavage of FXIII-A2 contains a recognition site for the fibrinogen α chain”, Pages 2117 – 2126, Copyright © 2013, with permission from Elsevier.

Materials and Methods

Materials

Bovine thrombin, ferulic acid, monodansylcadaverine (MDC), glycine ethyl ester (GEE) hydrochloride, and sequencing grade GluC endoproteinase from Roche were acquired from Millipore Sigma (St. Louis, MO). d-Phe-Pro-Arg-chloromethyl ketone
(PPACK) was attained from Haematologic Technologies (Essex Junction, VT). 30% Acrylamide/Bis solution, and dual-color molecular weight standards were procured from Bio-Rad Laboratories (Richmond, CA).

**MALDI-TOF mass spectrometry kinetic assay**

FXIII-A* activity was tracked by measuring GEE crosslinking to glutamines within Fbg αC 233 – 425 via a MALDI-TOF MS kinetic assay.\(^94, 97, 100, 116\) 50 nM FXIII-A\(_2\) was proteolytically activated by incubation in MALDI kinetic buffer (50 mM Tris-Acetate, 150 mM NaCl, 0.1 % PEG-8000, pH 7.4) in the presence of 8.4 NIH units/mL bovine thrombin and 4 mM CaCl\(_2\) at 37 °C for 10 min, followed by reaction quenching via the addition of PPACK (final concentration of 1.5 µM). The crosslinking reaction was commenced by the addition of GEE (final concentration of 17 mM) and Fbg αC (final concentration of 13.6 µM) to the previous solution. The reaction proceeded at 37 °C with 25 µL aliquots of the reaction mixture removed and quenched with 10 mM EDTA at select time points. Sample aliquots were digested by GluC endoproteinase, subsequently desalted with C18 tips, and analyzed by MALDI-TOF MS. Unreacted glutamine concentrations were calculated by applying the peak height ratio method between reactive glutamines and their corresponding GEE-crosslinked products.\(^116\) Through this method, reaction progress was monitored via the loss of reactant over time.

Assays for each αC species were performed for 30 minutes and in triplicate. Data were reported as mean ± standard deviation, subjected to statistical analysis by the Student’s *t*-test, and were fit using a one phase exponential decay model, \([Q]_{\text{remaining},t} = ([Q]_0 - [Q]_{\text{plateau}})(e^{-kt}) + [Q]_{\text{plateau}},\) with GraphPad Prism 9.5.1 as previously described.\(^100\)
[Q]_{\text{remaining},t} is the concentration of uncrosslinked reactive glutamine in µM for time point \( t \) in minutes, [Q]_0 is the theoretical initial concentration of uncrosslinked reactive glutamine in µM, [Q]_{\text{plateau}} is the theoretical concentration of uncrosslinked reactive glutamine in µM when \( t = \infty \) minutes, and \( k \) is the rate constant in min\(^{-1}\).

**Monodansylcadaverine crosslinking fluorescence kinetic assay**

Key GEE crosslinking assay results were supplemented using a gel-based fluorescence assay modified from prior studies.\(^{46, 100}\) 2.85 µM FXIII-A\(_2\) was proteolytically activated by incubation in Tris-buffered saline (TBS, 50 mM Tris-acetate, 150 mM NaCl, pH 7.4) in the presence of 47.9 NIH units/mL bovine thrombin and 4 mM CaCl\(_2\) at 37 °C for 10 minutes, followed by thrombin quenching via the addition of PPACK (activation mixture concentration of 9.5 µM). During FXIII-A* activation, reaction mixtures containing 1 mM monodansylcadaverine (MDC), 5 µM Fbg αC, and 4 mM CaCl\(_2\) in TBS were heated to 37 °C at least 5 minutes prior to initiation of MDC crosslinking. Crosslinking reactions were initiated by the addition of FXIII-A* to the reaction mixture. The final FXIII-A* concentration in each reaction mixture was 50 nM. The reaction proceeded at 37 °C with 20 µL aliquots of the reaction mixture removed at select time points and quenched with 5× reducing sample loading buffer, followed by heating at 100 °C for 3 – 5 minutes. With the exception of assays involving Fbg αC WT, a separate MDC crosslinking reaction mixture of Fbg αC WT was also prepared and allowed to react for 10 minutes prior to quenching as a positive control. All samples and the Fbg αC WT control were subjected to SDS-PAGE on a 15% T (37.5:1 Acrylamide:bis-acrylamide) Tris-glycine gel. Gels were transferred into deionized water and immediately subjected to
fluorescence imaging on a BioRad Gel Doc XR imager. After fluorescence imaging, gels were stained with 0.1% (w/v) Coomassie blue staining solution. Dye-stained gel images were also recorded on a BioRad Gel Doc XR imager under white light.

Assays were performed for a total time of 10 minutes and in triplicate. The MDC fluorescence and Coomassie stain intensities for each sample time point were quantified using ImageJ 1.53e (NIH), as previously described. Fluorescence intensities were divided by their corresponding Coomassie stain intensities to correct for any variations in sample loading. In assays involving variants, these corrected fluorescence values were divided by the corrected fluorescence value of a Fbg αC WT positive control that was concurrently subjected to FXIII-A* MDC crosslinking for 10 minutes. In assays where Fbg αC WT was tested, corrected fluorescence values were divided by the corrected fluorescence value of the 10-minute time point. Through this method, the reaction progress of each variant could be tracked via the increase in relative fluorescence over time. Data were reported as mean ± standard deviation, and subjected to statistical analysis by the Student's t-test with GraphPad Prism 9.5.1.

**Generation of fibrinogen αC 233 – 425 and its variants via site directed mutagenesis, expression and purification**

Fbg αC 233 – 425 variant DNA were generated using site-directed mutagenesis with the QuikChange II Site Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA), as described previously. A pGEX plasmid vector coding for GST-tagged Fbg αC 233 – 425 was used as the template strand. Primer design sequences are listed in Table 5. Mutagenesis was confirmed by DNA sequencing at the CGeMM DNA facility core at
the University of Louisville (KY). Mutant plasmids were subsequently transformed into BL21 Gold DE3 (Agilent Technologies, Inc., Cedar Creek, TX) competent cells for expression. Fbg αC 233 – 425 and its variants were expressed via auto-induction and purified as described previously.\textsuperscript{46,100}

**Table 5.** List of Fibrinogen αC (233 – 425) Variant Primers.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>E396A</td>
<td>Forward: CAGACTGGGGCACACATTAGCTGAGGGATGTAAGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>E396D</td>
<td>Forward: CAGACTGGGGCACACATTAGCTGAGGGATGTAAGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>389 Stop</td>
<td>Forward: CTGGAAAGGGAGGCTCAACTAGACTGGGGCACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGAACACACCATGGGGTGGATGTAAGTCC</td>
</tr>
<tr>
<td>E395A</td>
<td>Forward: CAGACTGGGGCACACATTAGCTGAGGGATGTAAGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>E395S</td>
<td>Forward: CAGACTGGGGCACACATTAGCTGAGGGATGTAAGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>E395K</td>
<td>Forward: CAGACTGGGGCACACATTAGCTGAGGGATGTAAGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>D390A</td>
<td>Forward: GGAACGGCGAGGCCTCAACAACCCACCTGGGGGACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>D390A, E396A</td>
<td>Forward: GGAACGGCGAGGCCTCAACAACCCACCTGGGGGACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>W391A</td>
<td>Forward: GGAACGGCGAGGCCTCAACAACCCACCTGGGGGACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>W391A, E396A</td>
<td>Forward: GGAACGGCGAGGCCTCAACAACCCACCTGGGGGACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>F394A</td>
<td>Forward: GGAACGGCGAGGCCTCAACAACCCACCTGGGGGACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
<tr>
<td>F394A, E396A</td>
<td>Forward: GGAACGGCGAGGCCTCAACAACCCACCTGGGGGACACATTAGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTCTTTCTTCTGACACGGGCTCAGGTCAGGAAATGTAAGTCC</td>
</tr>
</tbody>
</table>
Results

Similar to the prior study, MALDI-TOF mass spectrometry was used to monitor GEE-crosslinking on each reactive glutamine within Fbg αC 233 – 425. With Q237 as the most reactive glutamine for FXIII-A* activity among the three glutamines (Q237, Q328, and Q366) present within Fbg αC 233 – 425,94 the experimental setup of the assay was able to measure overall FXIII-A* activity on αC by solely tracking Q237-GEE crosslinking: With 50 nM FXIII and 30 minutes of assay reaction time, GEE crosslinking to Q237 prominently increased over time with minimal observable crosslinking to Q328 and Q366 (Figure 15).

The impact of select residues within the FXIII-A* binding region (Fbg αC 389 – 402) on αC FXIII-A* activity was assessed by comparing Q237-GEE crosslinking of their variants with wild-type (WT), 389 Stop (Fbg αC 233 – 388), and the alanine substitution mutant E396A.100 As mentioned previously in Chapter 3, the 389 Stop truncation mutation removes residues that encompass the FXIII binding region. On the other hand, Fbg αC E396A replaces E396, an acidic residue that makes an important contribution towards improving FXIII-A* binding affinity for Fbg αC. Both E396A and 389 Stop were previously shown in Chapter 3 to significantly lower FXIII-A* crosslinking activity toward Fbg αC relative to WT using 50 nM FXIII-A* (Figures 16 – 17).100 The GEE-crosslinking activity of each variant was fitted to a one phase exponential decay model (see Table 6 for model coefficients of each variant).
Table 6. Comparison of one phase exponential decay model coefficients from plots of FXIII-A* Fbg αC Q237-GEE crosslinking assays involving WT, 389 Stop, and FXIII-A* binding site variants.\(^a\)

<table>
<thead>
<tr>
<th>Fbg αC</th>
<th>Initial Conc. of Q237, ([Q237]_0) (μM)(^a)</th>
<th>Initial Reaction Rate, (V_0) (µM/min)</th>
<th>(k) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>13.06</td>
<td>3.13</td>
<td>0.24</td>
</tr>
<tr>
<td>389 Stop</td>
<td>13.40</td>
<td>0.91</td>
<td>0.07</td>
</tr>
<tr>
<td>E396A</td>
<td>13.64</td>
<td>2.26</td>
<td>0.17</td>
</tr>
<tr>
<td>E396D</td>
<td>12.68</td>
<td>2.41</td>
<td>0.19</td>
</tr>
<tr>
<td>E395A</td>
<td>12.65</td>
<td>2.28</td>
<td>0.18</td>
</tr>
<tr>
<td>E395K</td>
<td>12.74</td>
<td>3.06</td>
<td>0.24</td>
</tr>
<tr>
<td>E395S</td>
<td>12.55</td>
<td>3.26</td>
<td>0.26</td>
</tr>
<tr>
<td>D390A</td>
<td>13.00</td>
<td>1.56</td>
<td>0.12</td>
</tr>
<tr>
<td>D390A, E396A</td>
<td>12.89</td>
<td>1.42</td>
<td>0.11</td>
</tr>
<tr>
<td>W391A</td>
<td>12.79</td>
<td>1.28</td>
<td>0.10</td>
</tr>
<tr>
<td>W391A, E396A</td>
<td>13.12</td>
<td>1.44</td>
<td>0.11</td>
</tr>
<tr>
<td>F394A</td>
<td>12.55</td>
<td>1.63</td>
<td>0.13</td>
</tr>
<tr>
<td>F394A, E396A</td>
<td>12.76</td>
<td>1.28</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\(^a\)Calculated theoretically by fit model. The actual initial conc. of each reactive glutamine in the GEE-crosslinking assays is 13.6 μM.

When E396 was replaced via the nonconservative mutation E396A, FXIII-A* binding significantly decreased, and αC crosslinking was reduced, but not abolished.\(^96, 97\)

In the current work, the role of E396 in αC crosslinking was further probed by expressing and analyzing αC E396D. The αC E396D mutation preserves the electrostatic nature of the residue, but should interfere with any salt bridge formation by shortening the side chain by one methylene group. Figures 23A and 23B show that Q237-GEE crosslinking in αC E396D did not significantly differ from αC WT. Other traditional semiconservative mutations, like E396Q and E396N, were not performed because of unwanted variables they would introduce into the αC model system: A E396Q mutation would create an artificial
reactive glutamine for FXIII-A*. Furthermore, native and variant asparagine residues located within α2-antiplasmin derived glutamine-containing substrate peptides were previously shown to affect FXIII-A* crosslinking.157, 158

![Diagram](image)

**Figure 23.** Effect of Fbg αC E396D on FXIII-A* transglutaminase activity. (A) Plot of FXIII-A*-catalyzed Q237-GEE crosslinking assays of Fbg αC WT (233 – 425, black) and variants E396A (233 – 425, green), 389 Stop (233 – 388, blue), and E396D (233 – 425, red). Data were reported as mean ± standard deviation (N = 3), and plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. (B) Mean uncrosslinked Q237 concentrations ± standard deviation (in µM) of Fbg αC WT, E396A, 389 Stop and E396D after 15 minutes of reaction time. (N = 3, **P < 0.01, ***P < 0.001). Fbg αC WT, E396A and 389 Stop data was taken from Figure 18.

αC E395 was another glutamate residue in the FXIII-A* binding region with the potential to form favorable electrostatic interactions with FXIII-A* and contribute to crosslinking activity. To determine if αC E395 has any role in enhancing FXIII-A* activity on αC, the mutant E395A was expressed and analyzed. Additionally, substitution mutants of E395 that mimicked the FXIII-A* binding region sequences of rodents (E395S) and pigs (E395K) were also expressed and analyzed (see Table 4 for a comparison of FXIII-A* binding region sequences). As seen in Figures 24A – 24C, Q237-GEE crosslinking in all
the E395 mutations generated in this study had little variation relative to WT. At 15 min, mean uncrosslinked Q237 concentrations of E395A, E395S and E395K were not significantly different from WT (Figures 24E – 24G). Comparisons of Q237-GEE crosslinking between the E395 variants show little variation of FXIII-A* activity (Figure 24D). Mean uncrosslinked Q237 concentrations of E395S and E395K were statistically different from E395A ($P \leq 0.001$); however, mean uncrosslinked Q237 concentrations differed by less than 1 µM (Figure 24H).

$\alpha$C D390 is another negatively charged residue within the FXIII-A* binding region that potentially advances crosslinking of $\alpha$C. To determine its contribution to the enhancement of FXIII-A* activity on $\alpha$C, D390A was expressed and studied. Comparisons of Q237-GEE crosslinking between WT, E396A and D390A indicate that the absence of either D390 or E396 distinctly slow FXIII-A* activity to similar extents relative to WT (Figure 25A). An examination of the mean unreacted Q237 concentrations at $t = 15$ mins. shows ~3-fold higher reactant remaining for both D390A and E396A relative to WT ($P \leq 0.001$, Figure 25B). $\alpha$C D390 exhibited a similar impact on Q237-GEE crosslinking as $\alpha$C E396. An $\alpha$C mutant containing both the D390A and E396A mutations ($\alpha$C D390A, E396A) was subsequently expressed and examined to explore the effect of losing these two key residues on crosslinking. Unexpectedly, $\alpha$C (D390A, E396A) experienced similar Q237-GEE crosslinking as the single substitution mutants $\alpha$C D390A and $\alpha$C E396A (Figures 25C – 25D).
Figure 24. Effects of Fbg αC E395 Variants on FXIII-A* transglutaminase activity. (A – D) Plots of FXIII-A*-catalyzed Q237-GEE crosslinking assays of Fbg αC WT (233 – 425, black) and variants E396A (233 – 425, green), 389 Stop (233 – 388, blue), E395A (233 – 425, purple), E395S (233 – 425, light green), and E395K (233 – 425, orange). Data were reported as mean ± standard deviation (N = 3), and plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. (E – H) Mean uncrosslinked Q237 concentrations ± standard deviation (in µM) of Fbg αC WT, E396A, 389 Stop, E395A, E395S, and E395K after 15 minutes of reaction time. (N = 3, **P < 0.01, ***P < 0.001). Fbg αC WT, E396A and 389 Stop data was taken from Figure 18. Panels (E), (F), and (G) compare WT results to those of E396A, the Stop variants, and individual E395X mutants. Panel (H) compares E395A results to those of E395S and E395K.
Figure 25. Effect of Fbg αC D390A on FXIII-A* transglutaminase activity. (A, C) Plots of FXIII-A*-catalyzed Q237-GEE crosslinking assays of Fbg αC WT (233 – 425, black), E396A (233 – 425, green), 389 Stop (233 – 388, blue), D390A (233 – 425, light purple), and (D390A, E396A) (233 – 425, burgundy). Data were reported as mean ± standard deviation (N = 3), and plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. (B, D) Mean uncrosslinked Q237 concentrations ± standard deviation (in µM) of Fbg αC WT, E396A, 389 Stop, D390A, and (D390A, E396A) after 15 minutes of reaction time (N = 3, **P < 0.01, ***P < 0.001). Fbg αC WT, E396A and 389 Stop data was taken from Figure 18. Panel (B) compares WT results to those of D390A, (D390A, E396A), E396A, and 389 Stop. Panel (D) compares the acidic residue series D390A, E396A, and (D390A, E396A).

αC residues W391 and F394 are two aromatic amino acid residues within the FXIII-A* binding region that could boost FXIII-A* activity through potential favorable nonionic
interactions with FXIII-A*. The roles of αC residues W391 and F394 on FXIII-A* activity were examined by expressing and analyzing the mutants αC W391A and αC F394A. Q237-GEE crosslinking of αC W391A and αC F394A were distinctly slowed relative to WT to an extent similar to that of αC E396A (Figures 26A, 27A). At t = 15 mins., unreacted Q237 concentrations for W391A, F394A and E396A were both ~3-fold higher compared to WT ($P \leq 0.01$, Figures 26B, 27B). Further examinations of the newly discovered roles of W391 and F394 in boosting FXIII-A* activity in αC were carried out by expressing and studying double mutants αC (W391A, E396A) and (αC F394A, E396A). Surprisingly, Q237-GEE crosslinking in αC (W391A, E396A) was not statistically different compared to crosslinking of either αC W391A or E396A (Fig 26D). On the other hand, Figure 27B showed that Q237-GEE crosslinking diminished in αC (F394A, E396A) relative to both αC F394A ($P \leq 0.01$) and E396A ($P \leq 0.01$).
Figure 26. Effect of Fbg αC W391A on FXIII-A* transglutaminase activity. (A, C) Plots of FXIII-A*-catalyzed Q237-GEE crosslinking assays of Fbg αC WT (233 – 425, black), E396A (233 – 425, green), 389 Stop (233 – 388, blue), W391A (233 – 425, dark gray), and (W391A, E396A) (233 – 425, brown). Data were reported as mean ± standard deviation (N = 3), and plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. (B, D) Mean uncrosslinked Q237 concentrations ± standard deviation (in µM) of Fbg αC WT, E396A, 389 Stop, W391A, and (W391A, E396A) after 15 minutes of reaction time (N = 3, **P < 0.01, ***P < 0.001). Fbg αC WT, E396A and 389 Stop data was taken from Figure 18. Panel (B) compares WT results to those of W391A, (W391A, E396A), E396A, and 389 Stop. Panel (D) compares the variant series W391A, E396A, and (W391A, E396A).
Figure 27. The effect of Fbg αC F394 on FXIII-A* transglutaminase activity. (A, C) Plots of FXIII-A*-catalyzed Q237-GEE crosslinking assays of Fbg αC WT (233 – 425, black), E396A (233 – 425, green), 389 Stop (233 – 388, blue), F394A (233 – 425, pink), and (F394A, E396A) (233 – 425, orange). Data were reported as mean ± standard deviation (N = 3), and plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time. (B, D) Mean uncrosslinked Q237 concentrations ± standard deviation (in µM) of Fbg αC WT, E396A, 389 Stop, F394A, and (F394A, E396A) after 15 minutes of reaction time (N = 3, **P < 0.01, ***P < 0.001). Fbg αC WT, E396A and 389 Stop data was taken from Figure 18. Panel (B) compares WT results to those of F394A, (F394A, E396A), E396A, and 389 Stop. Panel (D) compares results among the series F394A, E396A, and (F394A, E396A).

Fluorescence-based gel assays utilizing MDC were performed on variants that significantly impacted Q237-GEE crosslinking to confirm their importance to FXIII-A*
activity. Reduced MDC crosslinking was recorded across all time points for the αC substitution variants D390A, W391A, and F394A compared to WT (Figure 28). These results are in alignment with the loss of FXIII-A* activity observed in GEE crosslinking assays of D390A (Figure 25), W391A (Figure 26), and F394A (Figure 27).

**Figure 28.** Effect of Fbg αC single mutations on FXIII-A*-catalyzed MDC crosslinking. Representative images of FXIII-A*-catalyzed MDC crosslinking SDS-PAGE gels of Fbg αC (A) WT (from Figure 19A), (B) D390A, (C) W391A, and (D) F394A showing fluorescence under UV light (top) and protein bands under white light after Coomassie blue staining (bottom). (E – G) Plots of MDC crosslinking assays of Fbg αC WT (233 – 425, black, from Figure 19), D390A (purple), W391A (gray), and F394A (pink). Data were reported as mean ± SD (N = 3), and plotted as relative fluorescence as a function of time. (*P < 0.05, **P < 0.01).
Discussion

In this study, MALDI-TOF MS-based GEE-crosslinking assays established the significant influence of Fbg αC 389 – 402 on αC crosslinking. Additionally, the importance of select electrostatic (D390, E395, and E396) and hydrophobic (W391 and F394) αC residues towards accelerating FXIII-A* activity was scrutinized. The results from the GEE-crosslinking assays were further supported by fluorescence-based MDC-crosslinking assays.

*FXIII-A* crosslinking activity on αC Q237 is maintained with Fbg αC E396D in relation to WT*

In 2013, Smith et al. used chemical crosslinking studies and computational docking methods between FXIII and Fbg αC 233 – 425 to study where Fbg αC 389 – 402 binds FXIII-A (Figures 21B – C, 22). The formation of a critical salt bridge between αC E396 and FXIII-A* R158 was predicted (Figures 21C, 22). In the current work, the contributions of this putative salt bridge were probed by expressing and analyzing αC E396D. The αC E396D mutation preserves the electrostatic nature of the residue, but should interfere with salt bridge formation by shortening the side chain by one methylene group. As seen in Figures 23A and 23B, Q237-GEE crosslinking was relatively unaffected by the conservative mutation. Any favorable interaction that αC E396 has with FXIII-A* to promote crosslinking is likely a net result of the negatively charged carboxylate ion in its side chain.
Fbg αC E395 does not significantly impact FXIII-A* crosslinking activity on αC Q237 compared to WT

The role of Fbg αC E395 in boosting FXIII-A* activity was examined in this study, given its proximity to E396 and potential for forming additional favorable negative electrostatic interactions with FXIII-A*. E395 is not consistently conserved between mammalian species (Table 4): In rodents, the corresponding αC residue to E395 is serine (S), an amino acid with a hydroxyl group in its side chain. In pigs, the equivalent residue is lysine (K), which, in contrast to glutamate, has an amino group in its side chain that is positively charged upon protonation. Prior studies have shown that rodents and pigs form clots faster and stronger than humans. However, based on similar crosslinking results between all the E395 variants and αC WT (Figure 24), these physiological effects appear to be independent of αC crosslinking, at least with human FXIII-A*. Thus, E395 does not appear to play a major role in boosting FXIII-A* activity in Fbg αC.

Fbg αC D390 significantly impacts FXIII-A* crosslinking activity on αC Q237 compared to WT

αC D390 is a negatively charged residue within the FXIII-A* binding region that putatively forms a salt bridge with FXIII-A* K156 (Figures 21C and 22). The formation of this salt bridge is hypothesized to promote FXIII-A* binding and crosslinking of αC. Furthermore, with the exception of mouse Fbg αC, D390 is conserved across the FXIII-A* binding regions in all species listed in Table 4. Figures 25A and 25B illustrate the considerable reduction in Q237-GEE crosslinking in the D390A mutation relative to WT. This reduction in FXIII-A* activity stands in contrast to the minimal effects that
substitution mutations on E395 had on crosslinking. This effect on Q237-GEE crosslinking was corroborated in Figures 28B and 28E, where MDC crosslinking in D390A is significantly reduced relative to WT. Between E396A and D390A, the extent of Q237-GEE crosslinking was similar (Figures 25C and 25D), and D390A still experienced more FXIII-A* activity than the 389 Stop truncation (Figures 25A and 25B). Additionally, when D390 and E396 were doubly substituted with alanines, (D390A, E396A), no further reduction in Q237-GEE crosslinking was apparent. Thus, the contributions of αC D390 and αC E396 toward enhancing FXIII-A* activity on αC do not appear to be cumulative. Both acidic residues are part of the same broad FXIII-A*-Fbg αC binding interface that boosts the crosslinking of Fbg αC.

*Fbg αC W391 and F394 significantly impact FXIII-A* crosslinking activity on αC Q237 compared to WT*

Amino acids with large hydrophobic side chains like tryptophan and phenylalanine have the potential to enhance enzymatic activity and modify substrate specificity by providing critical hydrophobic contacts that increase binding affinity between the enzyme and substrate. Figures 26A and 27A show that Q237-GEE crosslinking was adversely affected by the W391A and F394A αC mutations, relative to WT. Interestingly, the reduction in Q237-GEE crosslinking on αC E396A was similar to that of W391A and F394A. MDC crosslinking assays of W391A (Figures 28C and 28F) and F394A (Figures 28G and 28H) also show that FXIII-A* was distinctly diminished by these mutations. The hydrophobic interactions provided by W391 and F394 appear to be just as significant for crosslinking as the electrostatic interaction that αC E396 has with FXIII-A*. As was the
case with D390A, Q237-GEE crosslinking was still higher in W391A and F394A than in the 389 Stop truncation. The double alanine substitution of W391 and E396, (W391A, E396A), did not further reduce FXIII-A* activity significantly with respect to either W391A or E396 (Figures 26C and 26D). On the other hand, the double alanine substitution of F394 and E396, (F394A, E396A), markedly decreased crosslinking relative to F394A and E396A (Figures 27C and 27D). The contributions of F394 and E396 toward enhancing FXIII-A* crosslinking in αC appear to be cumulative, whereas the enhancement of crosslinking between W391 and E396 are not. A possible explanation for the different effects on crosslinking between (W391A, E396A) and (F394A, E396A) is that both W391 and E396 interact with the same broad binding pocket on FXIII-A*, whereas F394 is part of another FXIII-A* binding pocket. In this scenario, (F394A, E396) impedes FXIII-A* binding and activity on Fbg αC by affecting both putative binding pockets.

**Conclusions**

This study demonstrates that Fbg αC (233 – 425) crosslinking is enhanced by both electrostatic and hydrophobic interactions between αC 389 – 402 and FXIII-A*. In addition to the previously reported E396, Fbg αC residues D390, W391, and F394 were now identified as key residues that enhance FXIII A* activity in Fbg αC 233 – 425. By contrast, the impact of E395 on Fbg αC crosslinking was minimal. The enhancement of FXIII-A* activity by Fbg αC 389 – 402 cannot be systematically reduced by “knocking out” E396 (E396A) followed by subsequent alanine substitutions of D390 and W391. An interesting exception was the double mutant (F394A, E396A) that may target distinct, non-
overlapping regions on FXIII. The results from this study are expected to aid further designs of FXIII-A inhibitors to treat coagulation-related diseases such as VTE. Recent trends in FXIII-A* inhibition are focused on directly targeting and reducing FXIII-A* activity. However, potential inhibitors could be designed to specifically reduce FXIII-A* activity on Fbg αC by targeting the enzyme’s interaction with αC 389–402. Fibrinogen-specific and complement C3-specific affimers decreased and promoted fibrinolysis, respectively. Synthetic peptides that mimic the amino acid sequence of Fbg αC 389–402 also inhibited FXIII-A* binding and crosslinking of fibrin between its Aα and γ chains. An inhibition strategy of this nature could treat and prevent VTE while mitigating potential side effects on other functions of FXIII.

---

2 This chapter is reproduced in part from the following published manuscript: Ablan, F. D.O., Maurer, M.C. 2023. "Fbg αC 389–402 Enhances Factor XIII Crosslinking in the Fibrinogen αC Region via Electrostatic and Hydrophobic Interactions." Biochemistry. 62, 2170–2181.
CHAPTER 5: PLASMID CONSTRUCTION, RECOMBINANT EXPRESSION OF FIBRIN(OGEN), AND FXIII-A* REACTIVITY OF FIBRIN(OGEN) αC 221 – 425

Introduction

Fbg αC 233 – 425 has been used extensively as a model system to characterize FXIII-A binding and reactivity toward the physiological αC region (Fbg Aα 221 – 610). Fbg αC 233 – 425 encompasses a majority of the disordered αC connector (Aα 221 – 391) and a part of the more structured αC domain (Aα 392 – 610). This model αC system also contains three reactive glutamines (Q237, Q328 and Q366) and the FXIII-A* binding site (Fbg αC 389 – 402).47, 50, 95, 99 Using Fbg αC 233 – 425, Smith et al. were able to identify Fbg αC E396 as a key binding residue for FXIII-A* and the region in FXIII-A* where Fbg αC 389 – 402 is bound.96, 98 Various researchers from the Maurer lab used Fbg αC 233 – 425 to determine FXIII-A* reactivity differences between αC glutamines, the impact of Fbg αC 389 – 402 on transglutaminase activities on the different activated forms of FXIII-A, and select residues within Fbg αC 389 – 402 that enhance FXIII-A* activity.46, 94, 97, 100, 111 While much has been learned about FXIII-A and Fbg αC through the Fbg αC 233 – 425 model system, a deeper and more comprehensive understanding of crosslinking-related interactions between Fbg αC and FXIII-A necessitates expanding the current Fbg αC model system.
Crosslinking identification studies on the αC region show that two (potentially three) reactive glutamines are not covered by the Fbg αC 233 – 425 model system, namely Q221/Q223 and Q563. Fbg αC Q221 and Q223 are found toward the N-terminal portion of the αC region and the αC connector, while Q563 is in the middle of the αC domain within the vicinity of reactive lysines such as K539 and K556. A 2019 crosslinking-mass spectrometry study of plasma clots and in vivo thrombi by Schmitt et al. identified multiple unique crosslinks formed with Q221 and Q563 (Figure 29), while unique crosslinks involving Q223 and Q563 were reported in a similar in vitro crosslinking-mass spectrometry study by Wang in 2011. An up-to-date list of known Fbg αC reactive glutamines and lysines with their αC crosslinking partners in vivo is listed in Table 7. As mentioned previously in Chapter 3, in vivo studies using whole fibrinogen with the Fbg αC region truncated at residues 271 and 390 altered fibrin clot formation and stability. An expanded Fbg αC model system that includes Q221 and Q223 could be used to study the impact of these truncation mutations on FXIII-A* crosslinking.

**Table 7.** List of Fbg αC Glutamines and Lysines Reactive to FXIII-A* Crosslinking

<table>
<thead>
<tr>
<th>Fbg αC Reactive Lysines</th>
<th>Fbg αC Reactive Glutamines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q221/Q223</td>
</tr>
<tr>
<td>K413</td>
<td>X</td>
</tr>
<tr>
<td>K418</td>
<td>X</td>
</tr>
<tr>
<td>K427</td>
<td>X</td>
</tr>
<tr>
<td>K429</td>
<td>X</td>
</tr>
<tr>
<td>K444</td>
<td>X</td>
</tr>
<tr>
<td>K448</td>
<td>X</td>
</tr>
<tr>
<td>K508</td>
<td>X</td>
</tr>
<tr>
<td>K539</td>
<td>X</td>
</tr>
<tr>
<td>K556</td>
<td>X</td>
</tr>
<tr>
<td>K562</td>
<td>X</td>
</tr>
<tr>
<td>K580</td>
<td>X</td>
</tr>
<tr>
<td>K601</td>
<td>X</td>
</tr>
<tr>
<td>K606</td>
<td>X</td>
</tr>
</tbody>
</table>

47, 50, 99
While an ideal model system would include as many natural reactive glutamines and lysines as possible, the presence of numerous reactive lysines in the αC domain (Fbg Aα 392 – 610) could interfere with the lysine mimics of the GEE and MDC crosslinking assays. Two reactive lysines, K413 and K418, are already present within the current Fbg αC 233 – 425 model system. Both the MDC and GEE crosslinking assays in the previous two chapters of this study used an excess of their corresponding lysine mimics to minimize the interference of either K413 or K418 in crosslinking assays. However, the 11 other reactive lysines present in Fbg Aα 221 – 610 could collectively increase the possibility that unwanted αC Q-K crosslinking could occur and interfere with the current lysine mimic crosslinking assays. Additionally, the recombinant expression and purification of the entire

Figure 29. Wheel diagram of unique fibrin Q-K crosslinks identified within in vivo thrombi. Adapted from ref. 50.
Fbg αC region (Fbg Aα 221 – 610) construct using *E. coli* is more difficult than the current Fbg αC 233 – 425 construct. Fbg Aα 221 – 610 is prone to proteolysis and, possibly because of misfolding issues involving the ordered αC domain, tends to sequester as insoluble aggregates within inclusion bodies after expression. Current approaches in the literature towards recombinantly expressing either the whole fibrinogen alpha chain or the Fbg αC region typically involve mammalian cell cultures like Chinese hamster ovary (CHO) cells. However, in the interest of avoiding expensive mammalian cell upkeep and subsequent post-translational modifications, the decision was made to generate and express Fbg αC 221 – 425 using the current *E. coli* system. Future projects could systematically expand to the use of a Fbg Aα 221 – 610 recombinant system, particularly in the study of FXIII-reactive lysines and Q563.

This chapter will detail the processes of the synthesis and expression of a pGEX-6P-1 plasmid construct that contains Fbg αC 221 – 425 DNA. Fbg αC 221 – 425 contains the FXIII-A* binding site, Fbg αC 389 – 402, and four (potentially five) reactive glutamines (Q221, Q223, Q237, Q328, Q366). Similar to Fbg αC 233 – 425, this new model Fbg αC system is predominantly disordered and does not include the ordered αC domain. The formation of the Fbg αC 221 – 425 pGEX-6P-1 plasmid was initially attempted by traditional subcloning methodology. The Fbg αC 221 – 425 pGEX-6P-1 plasmid was eventually synthesized through the In-Fusion seamless cloning method by Takara Bio. The mass spectra profiles of trypsin, chymotrypsin, and GluC digests of the recombinant Fbg αC 221 – 425 were elucidated. The glutamine reactivity rankings of Fbg αC Q221, Q223, Q237, Q328 and Q366 in the new model system were also determined using the mass spectrometry-based GEE crosslinking assay.
Materials and Methods

Materials

Bovine thrombin, ferulic acid, α-cyano-4-hydroxycinnamic acid (α-CHCA), glycine ethyl ester (GEE) hydrochloride, sequencing grade GluC endoproteinase, chymotrypsin and trypsin were acquired from Millipore Sigma (St. Louis, MO). d-Phe-Pro-Arg-chloromethyl ketone (PPACK) was attained from Haematologic Technologies (Essex Junction, VT). 30% Acrylamide/Bis solution, and dual-color molecular weight standards were procured from Bio-Rad Laboratories (Richmond, CA). High-fidelity restriction endonucleases BamHI, SalI, NcoI, and AatII, nuclease-free water, 10 mM deoxynucleotide triphosphates (dNTPs), 10× rCutSmart buffer, 5× PCR reaction buffer, Q5 DNA polymerase, T4 DNA ligase, and 10× ligase buffer were purchased from New England Biolabs, Inc. (Ipswich, MA). 2× PrimeSTAR HS Premix, 5× In-Fusion Snap Assembly Master mix and Stellar competent cells used for the InFusion seamless cloning reaction were acquired from Takara Bio USA (San Jose, CA). A pET-20b vector containing Fbg Aα 221 – 610 cDNA (see Figure 29 for sequence map) was a generous gift provided by Dr. Leonid Medved and Dr. Kenneth Ingham (University of Maryland at Baltimore, USA), while a pGEX-6P-1 vector containing Fbg αC 233 – 425 cDNA was charitably supplied by Dr. Robert Ariens and Dr. Helen Philippou (University of Leeds, UK).
Figure 30. pET-20B plasmid sequence map. DNA base pairs positions are numbered in the 3' → 5' direction, with different restriction nuclease sites highlighted along with their base pair position in parentheses. Notable genetic regions are highlight by different colored segments and arrows: T7 promoter, white arrow; pelB signal sequence, purple arrow; multiple cloning site (MCS), light blue segment; C-terminal His tag sequence (6xHis), light purple arrow; T7 terminator sequence, white segment; f1 bacteriophage origin of replication (f1 ori); ampicillin-resistance gene (AmpR) coding for β-lactamase, light green arrow; origin of replication (ori), yellow arrow; rop gene coding for repressor of primer protein, purple arrow. The figure was taken from: https://www.snapgene.com/plasmids/pet_and_duet_vectors_(novagen)/pET-20b(%2B)
Subcloning of Fbg Aα 221 – 610 DNA from a pET-20b Vector into a pGEX-6P-1 Vector

The general steps of the typical subcloning method are illustrated in Figure 31. The Fbg Aα 221 – 610 pET-20B plasmid was used as a template to generate Fbg αC 221 – 610 insert DNA, while the Fbg αC 233 – 425 pGEX-6P-1 was used as the destination vector plasmid. Primers containing BamHI and SalI restriction sites were designed to generate insert cDNA encoding for Fbg Aα 221 – 610 (Table 8) by PCR. A PCR mixture (200 µL) of 200 µM dNTP, 500 nM forward and reverse primers, 40 ng pET-20b Fbg Aα 221 – 610 plasmid DNA, and Q5 DNA polymerase in 1× PCR reaction buffer was divided into 50-µL aliquots for PCR. The following parameters were used for PCR: initial denaturation at 98 °C for 30 sec; 35 cycles of 98 °C for 10 sec and 72 °C for 2 min, a final extension at 72 °C for 2 min, and a final hold at 4 °C. The PCR product was separated on a 1% agarose gel with 0.5 µg/mL ethidium bromide. The gel was visualized under UV light, and the desired DNA band of ~1200 bp was cut and extracted from the gel using the Monarch® DNA gel extraction kit from New England Biolabs, Inc.

1 – 3 µg of Fbg Aα 221 – 610 DNA insert and pGEX-6P-1 Fbg αC 233 – 425 vector DNA were double digested with BamHI and SalI for 2 hours at 37 °C, followed by heat inactivation of restriction endonucleases at 65 °C for 20 mins. The insert and vector digests were separated on a 1% agarose gel containing 0.5 µg/mL ethidium bromide. The gel was visualized under UV light, and the desired DNA bands (~1200 bp for insert, ~ 5000 bp for vector) were cut and extracted from the gel. Attempts were made to ligate digested Fbg Aα 221 – 610 DNA insert into the digested pGEX-6P-1 vector at insert:vector molar ratios of 3:1, 5:1, and 7:1 with T4 DNA ligase both at 4 °C overnight or 4 hours at room temperate. The ligation reactions were used to transform XL-1Blue supercompetent cells.
Table 8. List of Fibrinogen αC (221 – 610) Insert Cloning Primers.

<table>
<thead>
<tr>
<th>Type of Cloning</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcloning</td>
<td>Forward: TGACGGATCCATGCAGCTTCAGAAGGTACCCCCAGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCCGTCGACTCAGACCCGGGCGAGATTTAGCATGGCCTCT</td>
</tr>
<tr>
<td>In-Fusion Cloning</td>
<td>Forward: GGATCTGCGGCCATGGATGCAGCTTCAGAAGGTACCCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAAAGTGCCACCTGACGTCTCAGACAGGGCGAGATTTAGC</td>
</tr>
</tbody>
</table>

In-Fusion seamless cloning of Fbg αC 221 – 610 DNA from a pET-20b Vector into a pGEX-6P-1 Vector

The general steps of the In-Fusion seamless cloning method are illustrated in Figure 32. The Fbg Aα 221 – 610 pET-20B plasmid was used as a template to generate Fbg αC 221 – 610 insert DNA, while the Fbg αC 233 – 425 pGEX-6P-1 was used as the destination vector plasmid. Primers containing NcoI and AatII restriction sites were designed with the Takara Bio Primer Design Tool (San Jose, CA) to generate Fbg Aα 221 – 610 insert cDNA (Table 8). The 5′ and 3′ ends of the In-Fusion seamless cloning insert cDNA are each homologous to the destination pGEX-6P-1 vector by 15 base pairs each. A PCR mixture (50 µL) of 15 pmol forward and reverse primers, and 1 ng pET-20b Fbg Aα 221 – 610 plasmid DNA was prepared in 1× PrimeSTAR HS Premix (a Takara Bio USA proprietary mixture of PrimeSTAR HS DNA Polymerase, PCR reaction buffer and dNTPs). The following parameters were used for PCR: initial denaturation at 98 °C for 30 sec; 35 cycles of 98 °C for 10 sec, 60 °C for 5 sec and 72 °C for 1.5 min, a final extension at 72 °C for 2 min, and a final hold at 4 °C. The PCR product was separated on a 1% agarose gel with 0.5
µg/mL ethidium bromide. The gel was visualized under UV light, and the desired DNA band of ~1200 bp was cut and extracted from the gel using the Monarch® DNA gel extraction kit from New England Biolabs, Inc.

~1 µg of pGEX-6P-1 FXIII-A vector DNA was double digested with NcoI and AatII for 1 hour at 37 °C, followed by heat inactivation of restriction endonucleases at 80 °C for 20 mins. The vector digests were separated on a 1% agarose gel with 0.5 µg/mL ethidium bromide. The gel was visualized under UV light, and the desired DNA bands (~5000 bp for vector) were cut and extracted from the gel using the Monarch® DNA gel extraction kit from New England Biolabs, Inc. The In-Fusion Fbg Aα 221 – 610 DNA insert was combined with the digested pGEX-6P-1 vector at an insert:vector molar ratio of 2:1 in 1× In-Fusion Snap Assembly Master mix and incubated at 50 °C for 15 min. This proprietary master mix contains poxvirus DNA polymerase with 3’ → 5’ exonuclease activity that selectively targets linear duplex DNA.\textsuperscript{173-175} The resulting In-Fusion reaction product was used to transform Stellar competent cells.

\textbf{Figure 32.} InFusion seamless cloning flow chart of Fbg Aα 221 – 610 DNA from a pET-20B vector to a pGEX-6P-1 vector.
Generation of fibrinogen αC 221 – 425 and its variants via site directed mutagenesis, expression and purification

Fbg αC 221 – 425 DNA was generated using site-directed mutagenesis with the QuikChange II Site Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA), as described previously. The pGEX plasmid vector coding for GST-tagged Fbg αC 221 – 610 was used as the template. Primers were used to put the cDNA encoding for Fbg αC 221 – 610 in the same reading frame as the GST affinity tag and to replace the G426 codon with a stop codon (Table 9). Mutagenesis was confirmed by sequencing at the CGeMM DNA facility core at the University of Louisville (KY). Variant plasmids were subsequently used to transform BL21 Gold DE3 (Agilent Technologies, Inc., Cedar Creek, TX) competent cells for expression. Fbg αC 221 – 425 was expressed via auto-induction and purified as described previously in Chapter 2.

Table 9. List of Fibrinogen αC (221 – 425) Mutagenesis Primers.

<table>
<thead>
<tr>
<th>Frameshift Correction</th>
<th>Forward: GGATCTCGGCCCATGGGCATGCAGCTTCAGAAGG</th>
<th>Reverse: CCTTCTGAAGCTGCATGCCCATGGCCGCAGATCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>426 Stop Truncation</td>
<td>Forward: GATAAAAGACGGCTCAGGGACCTTGGAAGAAAGGTACCTGTTG</td>
<td>Reverse: CCACAGGTGACCTTTCTTTCAAGTCTGAGCTTTATC</td>
</tr>
</tbody>
</table>

MALDI-TOF mass spectrometry kinetic assay

FXIII-A* activity was tracked by measuring GEE crosslinking to glutamines within Fbg αC 221 – 425 via a MALDI-TOF MS kinetic assay. 50 or 500 nM FXIII-A2 was proteolytically activated by incubation in MALDI kinetic buffer (50 mM Tris-Acetate, 150 mM NaCl, 0.1 % PEG-8000, pH 7.4) in the presence of 8.4 NIH units/mL.
bovine thrombin and 4 mM CaCl\(_2\) at 37 °C for 10 minutes, followed by reaction quenching via the addition of PPACK (1.52 µM final concentration). The crosslinking reaction was commenced by the addition of GEE (17 mM final concentration) and Fbg αC (13.6 µM final concentration) to the previous solution. The reaction proceeded at 37 °C with 25 µL aliquots of the reaction mixture removed and quenched with 10 mM EDTA at select time points. Sample aliquots were digested by either chymotrypsin, GluC endoproteinase, or trypsin. Sample digests were subsequently desalted with C18 tips, and analyzed by MALDI-TOF MS. Unreacted glutamine concentrations were calculated by applying the peak height ratio method between reactive glutamines and their corresponding GEE-crosslinked products.\(^{116}\) Through this method, reaction progress was monitored via the loss of reactant over time. Glutamine-containing fragments were determined using the Peptide Mass theoretical digest tool at expasy.org ([https://web.expasy.org/peptide_mass](https://web.expasy.org/peptide_mass)). However, the contents of these fragments will need to be verified by MS/MS methods at a future date.

Assays for each αC species were performed for 30 minutes and in triplicate. Data were reported as mean ± standard deviation, subjected to statistical analysis by the Student’s \(t\)-test, and were fit using a one phase exponential decay model, 

\[
[Q]_{\text{remaining},t} = ([Q]_0 - [Q]_{\text{plateau}})e^{kt} + [Q]_{\text{plateau}},
\]

with GraphPad Prism 9.5.1 as previously described.\(^{100}\) 

\([Q]_{\text{remaining},t}\) is the concentration of uncrosslinked reactive glutamine in µM for time point \(t\) in minutes, \([Q]_0\) is the theoretical initial concentration of uncrosslinked reactive glutamine in µM, \([Q]_{\text{plateau}}\) is the theoretical concentration of uncrosslinked reactive glutamine in µM when \(t = \infty\) minutes, and \(k\) is the rate constant in \(\text{min}^{-1}\).
Results and Discussion

Construction and Expression of Fbg αC 221 – 425 pGEX-6P-1 Plasmid

The pET-20b vector containing Fbg Aα 221 – 610 cDNA (see Figure 30 for sequence map) supplied by Dr. Leonid Medved and Dr. Kenneth Ingham was previously used in a study by their research group on the biochemical and biophysical properties of FXIII-A* crosslinking on the Fbg αC region. In order to express Fbg αC 221 – 425 with the Maurer group methods, Fbg αC 221 – 610 cDNA from this pET-20b vector was to be subcloned into pGEX-6P-1 vector currently used to express Fbg αC 233 – 425, followed by site-directed mutagenesis of the G426 into a stop codon (G426X). The pET-20b vector used by Matsuka et al. codes for Fbg αC 221 – 610 cDNA to be expressed C-terminally attached to a His tag, which does not allow for the recovery of Fbg αC 221 – 425 after expression. The pGEX-6p-1 vector, on the other hand, contains a N-terminal GST tag which allows for the retrieval of Fbg αC 221 – 425 by GST-affinity chromatography after expression, as previously described in Chapter 2.

The target pGEX-6P-1 vector from the University of Leeds has Fbg αC 233 – 425 cDNA inserted between BamHI and SalI restriction sites (see Figure 8 for sequence map), while the pET-20b vector from the Medved research group has Fbg αC 233 – 425 cDNA inserted between NdeI and HindIII restriction sites (see Figure 30 for sequence map). The subcloning primers in Table 8 were designed to generate insert Fbg αC 221 – 610 cDNA from the pET-20b vector via PCR amplification. The forward primer was designed to introduce a BamHI restriction site N-terminally adjacent to the initiation codon for downstream ligation into pGEX-6P-1. The reverse primer similarly introduces a SalI restriction site C-terminally adjacent to the stop codon. Gel analysis of the insert Fbg αC
221 – 610 PCR products showed bands ~1.2 kb in size, corresponding to the expected size of the insert DNA (1182 bp, Figure 3).

**Figure 33.** Agarose gel of Fbg αC 221 – 610 insert cDNA. Leftmost lane labeled MW corresponds to DNA ladder standard. The five other lanes are PCR samples of Fbg αC 221 – 610 insert cDNA.

Both the target pGEX-6P-1 vector and the Fbg αC 221 – 610 cDNA insert were initially digested with both *Bam*HI and *Sal*I at 37 °C for 2 hours. Gel analysis of the Fbg αC 233 – 425 pGEX-6P-1 plasmid digests showed two distinct bands (Figure 34): the ~5kb band corresponds to the expected size of the opened pGEX-6P-1 vector DNA (4983 bp); the ~0.6 kb band is the expected Fbg αC 233 – 425 cDNA fragment (579 bp). Ligations of the digested pGEX-6P-1 vector and Fbg αC 221 – 610 cDNA were attempted using insert:vector molar ratios of 3:1, 5:1 and 7:1, but these efforts were unsuccessful. Ligation subcloning is known to be challenging to troubleshoot and optimize.176
Subcloning of Fbg α 221 – 610 into a pGEX-6P-1 vector was again attempted using the ligase-independent In-Fusion seamless cloning method.\cite{175,177} pGEX-6P-1 with FXIII-A cDNA inserted between NcoI and AatII restriction endonuclease sites was used as the target vector. Similar to the ligation subcloning method, In-Fusion seamless cloning requires both an insert cDNA and a destination vector plasmid. However, unlike traditional insert cDNA, the 5′ and 3′ ends of the In-Fusion seamless cloning insert cDNA are each homologous to the destination pGEX-6P-1 vector by 15 base pairs each. The In-Fusion cloning insert cDNA primers (Table 7) were also designed to be homologous to the restriction endonuclease sites (NcoI for forward primer and AatII for reverse primer). Gel electrophoresis analysis of the insert Fbg αC 221 – 610 PCR products showed bands of ~1.2 kb in size, similar to Figure 32 and corresponding to the expected size of the insert DNA (1200 bp). The pGEX-6P-1 vector was acquired in this instance through double

---

**Figure 34.** Agarose gel of pGEX 6P-1 plasmid with Fbg αC 233 – 425 cDNA simultaneously digested by *Bam*HI and *Sal*I. Leftmost lane labeled MW corresponds to DNA ladder standard. Lane 1 shows an undigested plasmid, while lanes 2 – 7 are digested samples.
digestion by *Nco*I and *Aat*II. Gel electrophoresis of the FXIII-A pGEX-6P-1 digest resulted in two distinct bands (Figure 35): a ~5kb band that corresponds to the expected size of opened pGEX-6P-1 vector DNA (4983 bp) and a ~2 kb band as the expected size of FXIII-A DNA (2199 bp).

**Figure 35.** Agarose gel of pGEX 6P-1 plasmid with FXIII-A cDNA digested by *Nco*I, *Aat*II, or both simultaneously. Leftmost lane labeled MW corresponds to DNA ladder standard. Lane 1 shows an undigested plasmid. Lane 2 shows plasmid digested by *Nco*I, while lane 3 shows plasmid digested by *Aat*II. Lanes 4 and 5 shows plasmid digested by *Nco*I and *Aat*II simultaneously.

While information regarding the In-Fusion cloning method is mostly proprietary, the method is known to involve poxvirus DNA polymerase capable of 3′ → 5′ exonuclease activity that selectively targets linear duplex DNA. This exonuclease activity creates “sticky ends” at the 3′ positions of both the linearized pGEX-6P-1 vector and the Fbg Aα 221 – 610 insert cDNA, allowing complementary base pairing and annealing to occur between the vector and insert (Figure 32). The resulting In-Fusion reaction product is used to transform into Stellar competent cells, where the annealed products are ligated to intact plasmids by cellular machinery.
Although the overall procedure was successful, sequencing of the transformed pGEX-6P-1 plasmid showed that the inserted Fbg Aα 221 – 610 cDNA was out of frame with the GST affinity tag. One notable problem in using restriction endonuclease-based subcloning on a previously subcloned plasmid is the potential to form “scar sequences,” leftover nucleotides from restriction endonuclease digestion that alter the reading frame of the plasmid. Site-directed mutagenesis was performed to insert two nucleotides just upstream of the start codon of Fbg Aα 221 – 610 cDNA using frameshift correction primers (Table 9), and success was confirmed by DNA sequencing. The resulting amino acid sequence of the frameshift correction is shown in Figure 36A. Subsequent site-directed mutagenesis successfully introduced a stop codon at G426, creating a pGEX-6P-1 vector that expresses GST-tagged Fbg αC 221 – 425 (Figure 36B). The new Fbg αC 221 – 425 was successfully expressed by auto-induction and purified by GST affinity chromatography using the standard Maurer group methods (Chapter 2).

**Figure 36.** Amino acid sequences of recombinant Fbg αC 221 – 610 and the Fbg αC 233 – 425 model system. (A) Fbg αC 221 – 610; (B) Fbg αC 221 – 425. The italicized GPLGS sequence contains leftover GST tag residues after HRV 3C protease cleavage. The italicized AAMGM sequence is from a combination of scar sequence residues from prior subcloning of FXIII-A into the pGEX vector (AAM), and residues introduced from frameshift correction (GM). Reactive glutamines are numbered and highlighted in red. The residues of the FXIII-A* binding site are underlined.
PONDR-VLXT analysis of Fbg α 221 – 610 suggests that the region comprising residues 221 – 440 is predominantly disordered, while overlapping segments of order and disorder are present among residues 440 – 610 (Figure 36). As mentioned previously, the mainly disordered nature of Fbg αC 221 – 425 potentially avoids solubility issues related to protein misfolding and is believed to be a main contributor to its successful recombinant expression and purification from a bacterial system.179-181

![Image](173x298.png)

**Figure 37.** PONDR-VLXT Analysis of Fbg αC 221 – 610. The intrinsic disorder propensity of the different residues of Fbg αC 233 – 425 was calculated by PONDR-VLXT ([www.pondr.com](http://www.pondr.com)) as a function of position in the primary structure. A higher PONDR score is associated with a higher propensity towards being in a disordered region. The positions of FXIII-A reactive glutamines are labeled, while the position of the Fbg αC 389 – 402 sequence is marked gray.

**MALDI-TOF Mass Spectrometry Analysis and GEE-Crosslinking Assays of Fbg αC 221 – 425**

As performed previously with Fbg αC 233 – 425, MALDI-TOF mass spectrometry was used to monitor GEE-crosslinking on each reactive glutamine within Fbg αC 221 – 425. Figure 14 has previously illustrated that Q328 and Q366 were negligibly crosslinked.
when 50 nM FXIII-A* was used in this assay with Fbg αC 233 – 425. To ascertain overall glutamine reactivity order between Q221/Q223, Q237, Q328 and Q366, GEE-crosslinking assays using 50 nM and 500 nM FXIII-A* were performed with Fbg αC 221 – 425. Chymotrypsin, GluC endoproteinase and trypsin digests were performed on all GEE-crosslinking sample aliquots to determine which digest best reported on each reactive glutamine.

With Fbg αC 233 – 425, GluC digests were used to monitor GEE crosslinking at Q237 and Q366, and chymotrypsin digests to monitor Q328 (Figure 15). In the Fbg αC 221 – 425 model system, the only reactive glutamine-containing MS fragment suitable for GEE-crosslinking measurement from chymotrypsin digestion was that of Q328 (315NSGSSGTGSTGNQNPSPRPGSTGTW341, m/z = 2448, m/z = 2534 with GEE, Figure 38C). Mass spectra of Fbg αC 221 – 425 GluC digests show three reactive glutamine-containing fragments (Figure 38D – 38E): 1. Q366 (358SSVSGSTGQWHSE370, m/z = 1348, m/z = 1434 crosslinked to GEE); 2. Q237 (227WKALTDMPQMRME241, m/z = 1637, m/z = 1723 crosslinked to GEE); 3. Q221/Q223/Q237 (GPLGSAAMGM21QLQKVPPEWKALTDMPQMRME241, m/z = 3428 uncrosslinked, m/z = 3514 with one GEE, m/z = 3600 with two GEEs). Because more than one GluC digest fragment contains Q237, Q237-GEE crosslinking would be challenging to monitor using GluC digestion. Furthermore, the only GluC digest to contain Q221/Q223 also includes Q237, meaning GEE-crosslinking of Q221/Q223 cannot be quantified using GluC digestion either. The m/z = 3514 fragment most likely contains mixture of Q221/Q223-GEE and Q237-GEE that could be determined by MS/MS. Therefore, only Q366-GEE crosslinking could be measured through GluC digestion. Fortunately, tryptic digests of the
recombinant Fbg αC 221–425 system (Figures 38A–38B have suitable GEE-crosslinking fragments that exclusively contain either Q221/Q223 (GPLGSAAMGMGQ221QLQKQ224, m/z = 1389, m/z = 1475 with GEE) and Q237 (G231ALTDMPQMRQ239, m/z = 1063, m/z = 1149 with GEE).

GEE-crosslinking for each reactive glutamine was monitored over time using the appropriate digests. Representative GEE crosslinking assay plots of Q237 and Q221/Q223 show the decrease of their respective reactant peak heights and increase of their respective product peak heights over time (Figure 39). 500 nM FXIII-A* GEE-crosslinking assays were performed to determine the overall glutamine reactivity rankings in the new Fbg αC 221-425 model system (Figure 40A, Table 10). These assays have shown that the most reactive glutamine to FXIII-A* is Q237, followed by Q221/Q223, then Q328 about equal to Q366. Rapid Q237-GEE crosslinking occurred approximately 20 seconds after the reaction was initiated, as previously observed in the 500 nM FXIII-A* GEE-crosslinking assays of Moaupi et al. The glutamine reactivity order, then, is Q237 > Q221/Q223 >> Q328 ~ Q366. In terms of known Fbg αC lysine crosslinking partners (Table 7), Q237 and Q328 have the most partners, followed by Q221/Q223, and Q366. However, the number of known Fbg αC lysine crosslinking partners for each reactive glutamine does not take into account the frequency of each unique Q-K crosslink. Q221/Q223 could still have more overall crosslinking with less Fbg αC lysines than Q328. Additionally, unlike Q328 and Q366, Q221/Q223 has distinct and measurable GEE-crosslinking at 50 nM FXIII-A* (Figure 40B, Table 10). Q221/Q223-GEE crosslinking is still less than that of Q237-GEE crosslinking at all time points. Nevertheless, the 50 nM FXIII-A* GEE-crosslinking assays corroborates the glutamine reactivity order of Q237 > Q221/Q223 >> Q328 ~ Q366.
Figure 38. Representative MALDI-TOF MS Spectra of Proteolytic Digests of Fbg αC 221 – 425 after FXIII-A*-catalyzed GEE Crosslinking. Digests were performed on crosslinking reactions quenched in EDTA 5 minutes after addition of Fbg αC 221 – 425 to 500 nM FXIII-A* reaction mixture. (A – B) Trypsin digest spectra highlighting Q221/Q223 and Q237 fragment peaks. (C) Chymotrypsin digest spectrum highlighting Q328 fragment peaks. (D – E) GluC digest spectra highlighting Q237, Q366 and Q221/Q223/Q237 fragment peaks.
Figure 39. Representative MALDI-TOF MS Spectra of Tryptic Digests of Fbg αC 221–425 after FXIII-A*-catalyzed GEE Crosslinking. Tryptic digests were performed on crosslinking reactions quenched in EDTA at 0, 5 and 15 minutes after addition of Fbg αC 221–425 to (A) 50 nM FXIII-A* or (B) 500 nM FXIII-A* reaction mixture. Panels (A) and (B) highlight the GEE-crosslinking of Q237 and Q221/Q223 over time, respectively.

Table 10. Comparison of curve fit coefficients from 50 nM and 500 nM FXIII-A* GEE crosslinking assays involving the reactive glutamines of Fbg αC WT 221–425.

<table>
<thead>
<tr>
<th>Reactive Glutamine</th>
<th>FXIII-A* Conc. (nM)</th>
<th>Initial Glutamine Conc., [Q]₀ (µM)ᵃ</th>
<th>Initial Reaction Rate, V₀ (µM/min)</th>
<th>k (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q221/Q223</td>
<td>50</td>
<td>13.87</td>
<td>2.63</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>11.92</td>
<td>2.37</td>
<td>0.20</td>
</tr>
<tr>
<td>Q237</td>
<td>50</td>
<td>11.99</td>
<td>3.40</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7.70</td>
<td>15.31</td>
<td>1.99</td>
</tr>
<tr>
<td>Q328</td>
<td>500</td>
<td>10.20</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>Q366</td>
<td>500</td>
<td>13.37</td>
<td>4.74</td>
<td>0.35</td>
</tr>
</tbody>
</table>

ᵃCalculated theoretically by fit model. The actual initial conc. of each reactive glutamine in the GEE-crosslinking assays is 13.6 µM.
Figure 40. FXIII-A*-catalyzed GEE-crosslinking assays of Fbg αC 221 – 425. (A) 500 nM FXIII-A* GEE-crosslinking plots of Q221 (solid black circle), Q237 (solid black square), Q328 (red diamond), and Q366 (blue open circle). (B) 50 nM FXIII-A* GEE-crosslinking plots of Q221 (circle, solid line) and Q237 (square, dashed line). Data were reported as mean ± standard deviation (N = 3). Both the 50 nM and 500 nM FXIII-A* GEE-crosslinking assays were plotted as one phase exponential decay fits of uncrosslinked Q237 concentrations as a function of time.

Conclusion

A Fbg αC 221 – 425 pGEX-6P-1 plasmid was successfully constructed by In-Fusion seamless cloning, Fbg αC 221 – 425 was expressed, and a new fibrinogen crosslinking model system was established. The glutamine FXIII-A* reactivity ranking order of Q237 > Q221/Q223 >> Q328 ~ Q366 was determined by both 50 nM and 500 nM FXIII-A*. Further work on Fbg αC 221 – 425 should first establish if Q221 and Q223 are
both reactive to FXIII-A*, or if only one of these two glutamines experience crosslinking. Prior work by Mouapi et al. in 2016 was able to ascertain glutamine reactivity in Fbg αC 233 – 425 by monitoring changes in crosslinking after substituting each glutamine with asparagine.⁹⁴ Upon accounting for the reactivity of each glutamine to FXIII-A*, this new model Fbg αC could be used to study the impact of the FXIII-A* binding site on Q221/Q223.
CHAPTER 6: RESEARCH SUMMARY AND FUTURE DIRECTIONS

Venous thromboembolism (VTE) is consistently reported to be among the top five most common vascular diseases worldwide, with approximately 1.2 million cases annually in the United States since 2021.\textsuperscript{182-184} In a 2019 study by Troy et al., millions of Americans on Medicare were reported to be using anticoagulants, and spending on these drugs totaled billions of dollars each year.\textsuperscript{185} The same study reported that warfarin use is being steadily replaced by direct oral anticoagulants (DOACs) since 2011, with DOAC use surpassing warfarin use in the United States in 2017. However, side effects from DOAC treatment, like increased bleeding risk, persist as a result of their mechanism of action: inhibiting the generation or activation of thrombin. Coagulation FXIII is believed to be a better drug target for DVT treatment and anticoagulation, as only the stability and size, but not the formation, of clots are affected.\textsuperscript{8, 29} However, as mentioned in the Chapter 1 of this dissertation, diseases that prevent the generation or overall activity of FXIII were reported to exacerbate bleeding and toxic fibin(ogen) degradation product generation.\textsuperscript{8, 29} Additionally, impairment of FXIII crosslinking in the fibrin(ogen) gamma chain increased the incidence of pulmonary emboli in murine models.\textsuperscript{186} Research towards an effective FXIII-based anticoagulant that mitigates bleeding risk would benefit from focusing on the main contribution of FXIII to thrombi formation and stability: crosslinking in the fibin(ogen) $\alpha$C region.\textsuperscript{54}
The research in this dissertation studies the role and significance of a structural feature in the Fbg αC region that could selectively inhibit FXIII crosslinking in Fbg αC: the FXIII-A* binding region (Fbg αC 389 – 402). Chapter 3 of this dissertation shows that Fbg αC 389 – 402 significantly and selectively promotes FXIII-A* crosslinking over FXIII-A° in Fbg αC. Additionally, Chapter 4 describes how select hydrophobic and aromatic residues within Fbg αC 389 – 402 were systematically identified by site-directed mutagenesis to be important in advancing FXIII-A* crosslinking on Fbg αC. The key findings from Chapters 3 and 4 were discovered using a recombinant Fbg αC model spanning residues 233 – 425. The outcomes of these chapters could be further studied and elaborated upon using a new model system, Fbg αC 221 – 425. Chapter 5 details the synthesis and preliminary FXIII-A* crosslinking studies of Fbg αC 221 – 425. The following sections will summarize the findings from each chapter and provide perspectives for future studies on the role of Fbg αC 389 – 402 on FXIII-A* αC crosslinking.

Selective Enhancement of FXIII-A* αC Crosslinking by Fbg αC 389 – 402

Coagulation FXIII is a unique enzyme that exists in the human body in two different forms: FXIII-A2B2 in plasma and FXIII-A2 in select cells like platelets and osteoblasts. Although both forms can be activated proteolytically or non-proteolytically, FXIII-A2B2 is likely to be activated proteolytically as FXIII-A* in physiological conditions, and FXIII-A° is expected to be activated non-proteolytically as FXIII-A°. Both activated forms of FXIII-A have distinct physiological substrates in the body. For example, FXIII-A* crosslinks fibrin chains and anti-fibrinolytic proteins to each other while FXIII-A° crosslinks cytoskeletal proteins.50, 52, 100, 141-143 A FXIII-based anticoagulant for VTE
treatment could avoid increased bleeding risk by specifically inhibiting FXIII-A* crosslinking on fibrin(ogen) while having little or no impact on FXIII-A° activity. The results from Chapter 4 posits that such an anticoagulant could be made by targeting the interaction between FXIII-A* and Fbg αC 389 – 402.

MDC crosslinking assays by Anokhin et al. in 2020 showed that FXIII-A* had more crosslinking activity with Fbg αC 233 – 425 than FXIII-A°. The GEE crosslinking assays of Fbg αC 233 – 425 WT, the E396A substitution mutant, and the 389 Stop truncation mutation elaborated upon this observation by revealing that Fbg αC 389 – 402 was promoting FXIII-A* crosslinking over FXIII-A°. When the FXIII-A* binding site was altered by “the removal of key binding residue E396,” the difference between FXIII-A* and FXIII-A° crosslinking decreased. Once the FXIII-A* binding site was removed with 389 Stop, FXIII-A* and FXIII-A° crosslinking activities were roughly equivalent.

The truncation mutants 403 Stop and 328 Stop were synthesized and studied to clarify the impact of Fbg 389 – 402 in enhancing FXIII-A* crosslinking. The 403 Stop mutation notably decreased FXIII-A* crosslinking but was on par with “the loss” of one FXIII-A* binding residue and experienced more crosslinking than 389 Stop. Another FXIII-A* enhancing interaction is likely present in Fbg αC 403 – 425 but does not impact FXIII-A* as significantly as Fbg αC 389 – 402. The 328 Stop mutation, which removes roughly 50% of the model Fbg αC 233 – 425 system and two reactive glutamines (Q328 and Q366), had similar Q237-GEE and MDC crosslinking with 389 Stop. These results have shown that Fbg αC 389 – 402 has a greater role in promoting FXIII-A* αC crosslinking of αC Q237 than Fbg αC 238 – 425. Interestingly, FXIII-A* crosslinking
occurred in αC Q237, even when nearly 50% of Fbg αC 233 – 425 was removed via 328 Stop.111

In terms of future FXIII-A* drug design, the results of Chapter 3 provide two insights: First, Fbg αC 389 – 402 significantly enhances FXIII-A* crosslinking over FXIII-A° in Fbg αC but is not required for any crosslinking to occur. Second, Fbg αC 389 – 402 is an important promoter of FXIII-A* Fbg αC crosslinking. While other favorable interactions between FXIII-A* and Fbg αC may eventually be found, Fbg αC 389 – 402 is clearly a suitable target for an FXIII-A* based anticoagulant design. A drug that interferes with the interaction between FXIII-A* and Fbg αC 389 – 402 could inhibit FXIII-A* crosslinking in Fbg αC while leaving Fbg γ crosslinking and FXIII-A° activity intact. Although some Fbg αC crosslinking will still occur, the goal of this hypothetical FXIII-based anticoagulant is to hamper the stabilization of thrombi but still allow clot formation.

**Fbg αC 389 – 402 Promotes FXIII-A* αC Crosslinking by Hydrophobic and Electrostatic Interactions**

For a potential anticoagulant to interfere with the favorable interactions between FXIII-A* and Fbg αC 389 – 402, a deeper understanding of these interactions at the residue level is required. Previous work has shown that αC E396 is a key binding residue for FXIII-A* but is not solely responsible for controlling or enhancing crosslinking in Fbg αC.96, 97 A comparison of FXIII-A* GEE crosslinking assays between E396A and 389 Stop in Chapter 4 have shown that other residues within Fbg αC 389 – 402 likely contribute towards the overall promotion of Fbg αC crosslinking. An experimental and computational study on the binding interaction between Fbg αC 389 – 402 and FXIII-A* by Smith et al. in 2013 laid the foundation for which residues within Fbg αC 389 – 402 to examine.98
The FXIII-A* binding site residues D390, W391, F394, and E395 were studied by subjecting each residue to site-directed mutagenesis and studying the resulting variants by GEE and MDC crosslinking assays. In spite of being adjacent to key binding residue E396, E395 had a negligible impact on FXIII-A* crosslinking. On the other hand, D390, W391 and F394 were found to have similar impacts on FXIII-A* crosslinking as E396. Interestingly, the promotion of FXIII-A* activity was not systematically reduced by “knocking out” E396 (E396A) followed by subsequent alanine substitutions of D390 and W391. On the other hand, the double mutant (F394A, E396A) further reduced FXIII-A* activity relative to either F394A or E396A. αC D390, W391 and E396 could potentially belong in one broad binding pocket with FXIII-A*, whereas F394 belongs to a distinct, non-overlapping region. Nevertheless, αC D390, W391 and F394 were identified, along with E396, as residues that significantly enhance FXIII-A* activity on Fbg αC. An effective anticoagulant would interfere with the binding of FXIII-A* and Fbg αC 389 – 402 by disrupting the favorable interactions provided by D390, W391, F394, and E396.

**Synthesis and Preliminary Study of Fbg αC 221 – 425**

Fbg αC 233 – 425 was extensively used by the Maurer lab and other research groups to study FXIII-A* activity on Fbg αC. The Fbg αC 221 – 425 model system was envisioned as a more comprehensive αC model than Fbg αC 233 – 425 by including two other potentially reactive glutamines, Q221 and Q223. A pGEX-6P-1 plasmid containing Fbg αC 221 – 610 cDNA was successfully generated by In-Fusion seamless cloning of Fbg αC 221 – 610 cDNA into a pGEX-6P-1 vector previously used to express FXIII-A. Site-directed mutagenesis of the G426 codon into a stop codon resulted in a pGEX-6P-1 plasmid that could express Fbg αC 221 – 425 with an N-terminal glutathione-
S-transferase tag via auto-induction, allowing for downstream purification by GST-affinity chromatography.

Preliminary GEE crosslinking assays of Fbg αC 221 – 425 revealed that the reactivity order among the reactive glutamines present in the new model system was Q237 > Q221/Q223 >> Q328 ~ Q366. Additionally, Q221/Q223 crosslinking was observed to progress over time with 50 nM FXIII-A*, but no crosslinking of Q328 or Q366 was detected at this FXIII-A* concentration. None of the enzymatic digests used (trypsin, GluC, chymotrypsin) were able to show fragments that exclusively contained either Q221 or Q223. In the future, GEE crosslinking assays utilizing MS/MS analysis of the digests or site-directed mutagenesis of either Q221 or Q223 could determine whether one or both crosslinked. Additionally, while m/z values of glutamine-containing fragments of Fbg αC 221 – 425 very closely match the theoretical values predicted by PeptideMass (https://web.expasy.org/peptide_mass/), the identity of these fragments could be profitably verified by MS/MS analysis.

**Future Research on the Interactions Between Fbg αC 389 – 402 and FXIII-A**

The results in this dissertation show that Fbg αC 389 – 402 is a significant promoter of FXIII-A* crosslinking of the reactive glutamines of Fbg αC 233 – 425. This increase in FXIII-A* crosslinking is aided by favorable interactions from hydrophobic (W391 and F394) and anionic (D390 and E396) residues within Fbg αC 389 – 402. The research presented in this dissertation has also shown that Fbg αC 389 – 402 selectively enhances FXIII-A* crosslinking over FXIII-A°. A compound or antibody designed to disrupt the favorable interactions between Fbg αC 389 – 402 and FXIII-A* has the potential to be an anticoagulant with lower bleeding risk and fewer side effects than current anticoagulants.
However, further research on the interactions of Fbg αC 389 – 402 and FXIII-A* needs to be done for an anticoagulant with this mechanism of action to be developed.

First and foremost, Fbg αC model systems different from Fbg αC 233 – 425 would elucidate the impact of the FXIII-A* binding site beyond Q237, Q328 and Q366. Fbg αC 221 – 425 (Chapter 5) adds one or two reactive glutamines to study (Q221 and/or Q223). Reactive lysines in the αC domain (Fbg αC 392 – 610) also warrant study as the second component of FXIII-A* crosslinking. A recombinant Fbg αC fragment such as Fbg αC 389 – 610 would be suitable for studying the impact of Fbg αC 389 – 402 on the FXIII-A* crosslinking of these reactive lysines. Work in this manner could eventually expand to the use of full length Fbg αC region (Fbg Aα 221 – 610) or whole fibrinogen chains.

Second, other residues within Fbg αC 389 – 402 could also significantly impact crosslinking in a similar fashion to D390, W391, F394 and E396. Double alanine substitutions of (D390A, E396A), (W391A, E396A) and (F394A, E396A) did not inhibit FXIII-A* crosslinking as significantly as 389 Stop. However, not every residue in Fbg αC 389 – 402 will have a pronounced effect on FXIII-A* crosslinking, as evidenced by negligible impact of E395 mutations. Additionally, successive alanine substitutions or different combinations of alanine substitutions of D390, W391, F394 and E396 other than those covered in this study (e.g. (W391A, F394A)) may be worth examining.

Overall, in this dissertation I sought to advance the design of an FXIII-A* based anticoagulant for treatment or prevention of VTE without increased bleeding risk or other unwanted side effects. Coagulation FXIII is a multifunctional enzyme with a variety of substrates. For a FXIII-A based anticoagulant to be feasible, drug design should avoid side effects incurred by current anticoagulants such as warfarin. An ideal FXIII-A*
anticoagulant would prevent specific interactions between FXIII and fibrin(ogen) that promote thrombus stability, such as fibrin α-α crosslinking. At the same time, this drug would avoid hampering FXIII-A° activity, which is critical for the enzyme’s intracellular functions. Understanding the fundamental biochemical and biophysical interactions between Fbg αC 389 – 402 and FXIII-A* might lead to a new generation of safe and effective VTE treatment.
REFERENCES


Organisms. Part I: Recombinant Analogues and Their Antithrombotic Activity In Vitro, Biomedicines 10.


fibrinogen AalphaIVS4 + 1G>T mutation and an AalphaGln328 truncation (fibrinogen Keokuk), Blood 103, 2571-2576.


132
APPENDICES

APPENDIX 1

Amino Acid Chart

## APPENDIX 2

### Amino Acid Codon Chart

<table>
<thead>
<tr>
<th>First base in codon</th>
<th>U</th>
<th>C</th>
<th>A</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>UUU Phe</td>
<td>UCU Ser</td>
<td>UAU Tyr</td>
<td>UGU Cys</td>
</tr>
<tr>
<td></td>
<td>UUC</td>
<td>UCC</td>
<td>UAC</td>
<td>UGC</td>
</tr>
<tr>
<td></td>
<td>UUA Leu</td>
<td>UCA</td>
<td>UAA STOP</td>
<td>UGA STOP</td>
</tr>
<tr>
<td></td>
<td>UUG</td>
<td>UCG</td>
<td>UAG</td>
<td>UGG Trp</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>CCC</td>
<td>CAA Gln</td>
<td>CGU Arg</td>
</tr>
<tr>
<td></td>
<td>CUU Leu</td>
<td>CCA</td>
<td>CAG</td>
<td>CGC</td>
</tr>
<tr>
<td></td>
<td>CUC</td>
<td>CCG</td>
<td>CAG</td>
<td>CGA</td>
</tr>
<tr>
<td></td>
<td>CLA</td>
<td>CUA Arg</td>
<td>CGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CUG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>AUU Ile</td>
<td>ACC Thr</td>
<td>AUA Asn</td>
<td>AGU Ser</td>
</tr>
<tr>
<td></td>
<td>AUC</td>
<td>ACA</td>
<td>AAG Lys</td>
<td>AGC</td>
</tr>
<tr>
<td></td>
<td>AUA</td>
<td>ACG</td>
<td>AAG</td>
<td>AGG</td>
</tr>
<tr>
<td></td>
<td>AUG Met (start)</td>
<td></td>
<td>Arg</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>GUU Val</td>
<td>GCU Ala</td>
<td>GAU Asp</td>
<td>GGU Gly</td>
</tr>
<tr>
<td></td>
<td>GUC</td>
<td>GCC</td>
<td>GAC GGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GUA</td>
<td>GCA</td>
<td>GAA GGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GUG</td>
<td>GCG</td>
<td>GAG GGG</td>
<td></td>
</tr>
</tbody>
</table>

## APPENDIX 3

### Commonly Used Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>C/Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>D/Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E/Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>F/Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Fbg</td>
<td>Fibrin(ogen)</td>
</tr>
<tr>
<td>FXIII</td>
<td>Coagulation FXIII</td>
</tr>
<tr>
<td>FXIII-A</td>
<td>Coagulation FXIII A-subunit</td>
</tr>
<tr>
<td>FXIII-A*</td>
<td>FXIII-A activated proteolytically by thrombin in the presence of Ca²⁺</td>
</tr>
<tr>
<td>FXIII-A°</td>
<td>FXIII-A activated non-proteolytically by high Ca²⁺ concentrations (&gt;25 mM)</td>
</tr>
<tr>
<td>G/Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GEE</td>
<td>Glycine ethyl ester</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>K/Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization – time of flight</td>
</tr>
<tr>
<td>MDC</td>
<td>Monodansylcadaverine</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NIH units</td>
<td>National Institute of Health units</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>PPACK</td>
<td>D-phenylalanyl-prolyl-arginyl chloromethyl ketone</td>
</tr>
<tr>
<td>Q/Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R/Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>S/Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>W/Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
CURRICULUM VITAE

Francis Dean Orlina Ablan
Department of Chemistry, University of Louisville, Louisville KY 40208
dinoablan@gmail.com
(919) 432-8177

EDUCATION

PhD. in Chemistry
University of Louisville, Louisville, KY
August 2023

MS in Chemistry
University of North Carolina at Wilmington, Wilmington, NC
December 2014

B.S. in Chemistry (A.C.S. Certified)
University of North Carolina at Wilmington, Wilmington, NC
May 2012

RESEARCH EXPERIENCE

Graduate Research Assistant – Department of Chemistry, University of Louisville

Research Mentor: Dr. Muriel C. Maurer
Louisville, Kentucky, 2018 – Present

• Studied the dependence of the different activated forms of blood coagulation Factor XIII-A on a binding site in the fibrinogen alpha chain for transglutaminase activity

• Identified key residues on the FXIII-A binding site of the fibrinogen alpha chain that could be utilized for future anticoagulant design

• Designed and optimized 2 time-dependent activity assays using biochemical and analytical methods from literature, such as MALDI-TOF mass spectrometry, gel electrophoresis, and fluorescence

• Proficient in recombinant protein expression in E. Coli and purification via affinity chromatography

• Proficient in entire process of PCR site-directed mutagenesis, from DNA primer design to bacterial cell transformation

• Presented findings to both experts and non-experts in lab meetings, conferences, and publications

• Successfully trained and mentored 3 undergraduates in different lab projects and protocols
Research Assistant – Department of Chemistry and Biochemistry, University of North Carolina at Wilmington

Research Mentor: Dr. Paulo F. Almeida
Wilmington, North Carolina, 2011 – 2014

• Demonstrated that the charge distribution on membrane active peptides affects their ability to penetrate and move across phospholipid vesicles
• Studied the oligomerization state of membrane active peptide δ-lysin using fluorescence anisotropy
• Employed both lipid extrusion and electroformation to synthesize lipid vesicles
• Analyzed membrane active peptide penetration of vesicles using laser scanning confocal microscopy and ImageJ imaging software
• Verified peptide and lipid concentrations using UV-Vis
• Presented findings to both experts and non-experts in meetings, conferences, and publications
• Successfully trained and mentored an undergraduate in different lab projects and protocols

INDUSTRY EXPERIENCE

R & D Chemist I – Generic Products Division, Mayne Pharma LLC
Greenville, North Carolina, 2015 – 2017

• Independently developed and evaluated methods of analysis for chemical entities and drug formulations
• Designed and authored method validation protocols, reports, analytical methods, and specifications
• Extensively performed HPLC, GC, and UV-Vis methods for known chemical entities and dosage forms for quality control
• Followed all regulatory, cGLP, cGMP and Environmental Health and Safety guidelines and regulations as required by the job function
• Served as a technical liaison between Generic Products Division and metrology for routine performance qualifications and troubleshooting
• Reviewed and witnessed laboratory notebooks; provided feedback as needed
• Suggested improvements of laboratory testing procedures, techniques and/or instrumentation
• Trained and assisted laboratory personnel with the preparation and execution of laboratory investigations
TECHNICAL SKILLS:

- **Spectroscopy**: Infrared, UV-Vis, Fluorescence
- **Chromatography**: HPLC, UPLC, Gas Chromatography, Affinity, Size-Exclusion
- **Mass Spectrometry**: MALDI-TOF
- **Protein Biochemistry**: Gel Electrophoresis, Dialysis, Site-Directed Mutagenesis, Recombinant Protein Expression by Bacteria, DNA Ligation Subcloning, In-Fusion Seamless Cloning
- **Microscopy**: Laser-scanning confocal
- **Industry-Related**: Dissolution, Karl Fischer, Loss on Drying, Particle Size Analysis
- **Software**: ImageJ, ChemDraw, EMPOWER 2, MasterControl, Pymol, Swiss-Pdb Viewer, Microsoft Office, Microsoft Teams, Loom, Sequence Scanner, GraphPad Prism

PUBLICATIONS

1. **Ablan, F. D.O., Maurer, M. C.** "Fbg αC 389 – 402 Enhances Factor XIII Crosslinking in the Fibrinogen αC Region via Electrostatic and Hydrophobic Interactions.” *Biochemistry*. **2023.** 62, 2170-2181


PRESENTATIONS

1. **Ablan, F. D. O., Maurer, M. C.** “Fibrinogen αC 389 – 402 Enhances Factor XIII Crosslinking in the Fibrinogen αC Region via Electrostatic and Hydrophobic Interactions”. Biophysical Society 67th Annual Meeting Poster Session (2023), San Diego, CA


**TEACHING EXPERIENCE**

**Teaching Assistant**: Department of Chemistry, University of Louisville, 2018 - 2019
- Taught laboratory classes for Organic Chemistry I and Biochemistry
- Prepared reagents and performed quality control of experiments
- Administered and graded tests in Organic Chemistry I

**Teaching Assistant**: Department of Chemistry and Biochemistry, UNCW, 2012 - 2014
- Taught laboratory classes for General Chemistry I & II, and Organic Chemistry I & II
- Assisted in administering and grading lecture exams of General Chemistry I & II, and Organic Chemistry I & II
- Developed PowerPoint presentations and planned weekly curriculum