Catch FXIa

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Catch FXIa

Stan van der Beelen

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Catch FXIa

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Promotor

Prof. dr. T.M. Hackeng

Copromotor

Dr. S.M. Agten

Dr. B.M.E. Mees

Beoordelingscommissie

Prof. dr. ir. Y.M.C. Henskens (voorzitter)

Dr. W.L. van Heerde (Radboud UMC Nijmegen)

Prof. dr. M. Honing

Prof. dr. P. Timmerman (Universiteit van Amsterdam)

Paranimfen

Hannah van der Beelen

Dennis Suylen

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Chapter 1

General introduction

Chapter 1

In the 17th century Wiseman postulated that a blood clot, also known as thrombus, is formed due to abnormalities in blood.(1) The formation of a blood clot that results in a reduced blood flow in a vessel is known as thrombosis. The hypothesis of Wiseman has led to many scientists trying to find these abnormalities and explain their association with thrombosis. Over centuries a multitude of techniques have been developed to correlate differences in lifestyle or protein function and concentration with thrombosis. Recent findings suggest that an increased protein concentration of clotting factor XIa (FXIa) in blood could be a risk factor for thrombosis, however, the abilities to quantify FXIa in circulation are limited.(2,3)

Clotting

Blood clotting or coagulation is the transformation of blood from liquid to gel. Coagulation is governed by an intricate equilibrium between pro- and anticoagulant processes, of which a misbalance results in either thrombosis or hemorrhage. However, intentional coagulation is part of hemostasis, a process to stop blood loss upon injury and which eventually results in vessel and wound repair. Hemostasis is initiated upon vessel damage by the exposure of collagen and tissue factor to blood.(4) As a response, platelets are activated by exposed collagen and form a platelet plug to stop the vessel from bleeding, a process also known as primary hemostasis.(4) Subsequently, secondary hemostasis, a complex mechanism which utilizes the coagulation cascade to generate fibrin polymers stabilizes the platelet plug.(4)

The coagulation system is a cascade of proteins that are by convention numbered with roman numerals, and in which activated proteins are indicated by the suffix “a”.(5) In short, the coagulation cascade can be activated by two pathways, the extrinsic pathway and the intrinsic pathway (Figure 1).(6) The extrinsic pathway is activated upon vascular injury that exposes tissue factor from the sub-endothelial tissue, which together with factor VIIa (FVIIa) activates factor X (FX) of the common pathway.(7) Alternatively, exposure of negatively charged surfaces to factor XII (FXII) will activate FXII and thereby initiate the intrinsic pathway.(8) FXIIa is responsible for the activation of factor XI (FXI)(9) which subsequently activates factor IX (FIX) that in complex with cofactor VIIIa (FVIIIa) forms the intrinsic tenase complex that activates factor X (FX).(10,11) In the common pathway factor Xa along with cofactor Va converts prothrombin into thrombin.(12) Finally, thrombin cleaves fibrinogen into insoluble fibrin, which cross-links platelets to stabilize the clot.(13) In addition, thrombin is also able to

activate factor XI on the surface of platelets which thereby functions as a positive feedback loop within the coagulation cascade.(14)

Intrinsic pathway

negatively charged surface

FXII → FXIIa

FXI → FXIa

FIX → FIXa

platelets

FX → FXa

FVa, Ca²⁺, PL

Thrombin

Fibrinogen → Fibrin

Extrinsic pathway

Trauma

FVIIa ← FVII

TF ← Trauma

FX → FXa

FVa, Ca²⁺, PL

Prothrombin

Common pathway

Figure 1, Coagulation cascade. The intrinsic pathway (blue) and the extrinsic pathway (green) both end up in the common pathway (red) which results in fibrin formation and thereby clot stabilization.

Factor XI structure and function

Factor XI (FXI) is a zymogen within the intrinsic pathway. The homodimeric multidomain protein (160 kDa) contains one inactive catalytic domain per monomer along with four apple domains, and circulates in blood at a concentration of 30 nM, of which the majority is in complex with high molecular weight kininogen (HMWK).(15,16) FXI was first identified by Robert Rosenthal in 1953 and was designated plasma thromboplastin antecedent. (17,18)

Interactions of FXI with other proteins are governed by the apple domains, which form binding surfaces for various protein-protein interactions: apple domain 1 binds to thrombin, apple domain 2 to HMWK, and apple domain 3 to FIX as well as part of the GP1b-IX-V complex (GP1b) on platelets.(10,16,19,20) Apple domain 4 enables dimerization of FXI via a

hydrophobic surface, salt bridges and a disulfide bond *via* cysteine-321.(21,22) Furthermore, FXI has two anion binding sites that are located on apple domain 3 and the catalytic domain, for binding to polyphosphates, heparin and nucleic acids.(23,24)

Each apple domain consists of a single α -helix that is supported by seven β -strands, arranged in an antiparallel curved sheet. Together the apple domains form a flat surface upon which the catalytic domain is positioned. The tertiary structure of the multidomain protein thereby resembles a cup-and-saucer shape (Figure 2). Upon dimerization, the quaternary structure is aligned in an inverted V-shape, where the catalytic clefts point away from each other. The structure of the catalytic domain has a chymotrypsin like topology, which consists of 2 β -barrels connected through a central loop. (18)

Since FXI is a homodimer, activation by FXIIa or thrombin proceeds through an intermediate with only one active subunit (1/2-FXIa). Upon cleavage of the peptide bond between arginine-369 and isoleucine-370, a light chain (30 kDa) containing the catalytic domain and a heavy chain are formed, which are covalently bound to each other *via* a disulfide bond (C362 to C381).(15,18) Additionally, upon activation isoleucine-370 moves 20 Å from its position into the active site, and thereby forms the oxyanion hole, which stabilizes the transition state of the substrate, and is characteristic for the serine protease family. (18)

Figure 2, Structure of the dimeric form of FXI, the apple domains 1-4 are depicted in green, and the catalytic domain is depicted in blue. The transparent surface shows the secondary structures of FXI. Two FXI monomers are bound to each other in an inverted V-shape with a 70-degree angle.

FXIa deficiencies and clinical antithrombotic strategies limiting FXI

Deficiencies in coagulation factors can lead to serious bleeding phenotypes, and most well-known are deficiencies in factors VIII or IX which lead to the bleeding disorders hemophilia A and B, respectively.(25,26) In contrast, deficiencies in FXI only lead to a mild bleeding phenotype (hemophilia C), resulting in posttraumatic or postoperative bleeding in tissue with strong fibrinolytic activity such as oral cavity and nose.(27) Interestingly, it was found that patients deficient in FXI have a low incidence of venous thromboembolism (VTE) and ischemic stroke (IS).(28–30) The mild bleeding phenotype in FXI-deficient patients suggests the hemostatic role of FXI is modest.

The combination of a mild bleeding phenotype, and the low incidence of pathologic thrombus formation in FXI-deficient patients, makes FXI an interesting target for novel and safe anticoagulant drug development. These are needed because current antithrombotic therapies rely either on the activation of antithrombin, or on the inhibition of FXa, thrombin, or vitamin K epoxide reductase, and require close monitoring of patients to avoid hemorrhage. Currently, different strategies are being pursued to inhibit or reduce FXI in blood plasma ranging from inhibitory small molecules to monoclonal antibodies and antisense oligonucleotides. Currently, 80% of the patented FXIa inhibitors are small molecules, tripeptides or derived peptidomimetics, and are mainly focused on direct inhibition of the active site. Analysis of these molecules showed that those containing an arginine- or phenylalanine derivative to target the S1-pocket of FXIa have the best affinity for FXIa.(31,32) To date, FXIa active site inhibitors have been developed with an affinity lower than 0.1 nM. (32)

In addition, allosteric small molecule inhibitors of FXIa have been found. The most potent allosteric inhibitor is a sulfated pentagalloylglucoside, which targets the heparin binding site of FXIa ($K_i=400$ nM) and is 200-fold more selective for FXIa than other relevant serine proteases (thrombin, FXa, FIXa)(33) Recent advances in allosteric inhibition have shown that a dual binding strategy can increase the selectivity even further. In this strategy, binding to the heparin binding site via anionic sulfate groups is complemented with binding to an adjacent hydrophobic patch.(34) Enzymes lacking the heparin binding site or the hydrophobic patch are therefore not inhibited by this molecule.(34) Although allosteric inhibitors can be

more selective towards their target since they bind to less conserved regions of the protein, the affinity of the current allosteric inhibitors is lower compared to competitive inhibitors.

Another important class of FXIa inhibitors are polypeptides found in nature. Anticoagulant proteins can be found in a range of organisms, the venom of some snakes for example contains a cocktail of anticoagulants that will lead to internal hemorrhages in its prey, ultimately killing it.(35) Alternatively, blood feeding insects such as ticks have developed anticoagulant proteins to prevent the blood from clotting in order to feed.(36) Some examples of FXIa inhibitors produced by nature are Desmolaris, a naturally Kunitz1-deleted form of Tissue Factor Pathway Inhibitor (TFPI) originating from a vampire bat *Desmodus Rotundus*; AcaNAP10, a 41 amino acid peptide from the nematode *ancylostoma caninum*; and Clavatadine A, a small molecule produced by the sponge *Suberea clavata*.(37–39) However, one of the most potent examples is Fasxiator, a small Kunitz-type protease inhibitor isolated and sequenced from the venom of the snake *Bungarus Fasciatus*.(40) Native Fasxiator binds the catalytic domain of FXIa with an affinity of 20 nM. The potency and selectivity of Fasxiator was greatly increased through mutagenesis; more specifically a double mutation (N17R, L19E) of residues near its binding surface. The potency of Faxiator_{N17R,L19R} was demonstrated in a ferric chloride-induced thrombosis model in mice. No occlusion of the common carotid artery was observed in the Fasxiator_{N17R,L19R} (0.3 mg/mouse) treated mice 30 minutes after ferric chloride induction, while complete occlusion of the common carotid artery was observed after 10 minutes in the control group.

In addition to these naturally found inhibitors, a number of companies have described the development of antibodies targeting FXI/FXIa for the development of antithrombotic therapies. These antibodies either inhibit protein-protein interactions with FXI by binding to the apple domains, or they bind directly to the active site and thereby compete for its substrate. One of these antibodies is developed by Bayer pharmaceuticals (BAY1213790), which recently successfully completed a phase 2 study.(41) In contrast to other antibodies on the market, BAY1213790 is currently the only selective antibody for the activated form of FXI.(42)

Finally, an alternative strategy can be found in the use of antisense oligonucleotides (ASO) which leads to a decrease of circulating FXI-levels in patients. These small strands of single stranded DNA bind the mRNA of FXI and thereby induce degradation of the hybridization product. An ASO-strategy was evaluated in a phase 2 study where it is studied in patients

undergoing total knee replacement. It was shown that FXI ASO compared to FXa inhibitor enoxaparin decreased peri-operative thrombotic events from 30% to 4% while bleeding only occurred in 3% compared to 8% with enoxaparin.(43)

Elevated FXIa as a potential biomarker for thrombosis

Despite its modest role in hemostasis, increased levels of FXI are reported to cause a pro-thrombotic state.(44) FXI antigen levels were determined in the Leiden thrombophilia study and showed a linear increase in VTE risk up to two-fold with increased FXI levels.(44) Elevated FXIa levels in blood were first observed in patients supplemented with intravenous immunoglobulin preparations. These preparations were contaminated with trace amounts of FXIa (0.001 U/ml) and led to thromboembolic events.(45) Later, increased FXIa-levels were found in patients suffering from acute coronary syndrome or myocardial infarction.(2,3,46) The effect of the increased FXIa-levels in combination with the fact that the contact activation pathway plays an important role in thrombus formation, could make FXIa an important risk factor for thromboembolic events.(47) Therefore, elevated levels of FXIa could function as biomarker for prediction of thrombosis.

FXIa diagnostics

To date there are three methods to quantify FXIa in solution. The first method mimics part of the coagulation cascade by the addition of purified coagulation cascade proteins to FXIa in a buffered solution. To a FXIa containing sample, factor IX, VIII, X, thrombin, phospholipids (PL), calcium (Ca^{2+}), and chromogenic substrate are added in three steps. First FXIa activates FIX into FIXa, then thrombin-activated factor VIIIa together with FIXa, PL and Ca^{2+} activates FX, which converts FXa-specific chromogenic substrate. The activation cascade from FXIa through FIXa to FXa leads to an exponential amplification, resulting in a low detection limit of 0.1 pM.(48) This method has been commercialized by Biophen and Rossix.^{40,41} However, the main disadvantage of this method is that it cannot be used to quantify FXIa in complex mixtures such as plasma.

Alternatively, FXIa-inhibitor complexes can be quantified using an ELISA assay.(46,50) For this ELISA, wells are coated with an antibody against FXI/FXIa, which bind FXIa:inhibitor complexes from plasma. Subsequently, a detection antibody is added specific for a natural inhibitor of

FXIa, allowing quantification of the inhibitor complex. The inhibitor complex ELISA is optimized for all four natural inhibitors of FXIa (α_2 -Antiplasmin, C1-inhibitor, α_1 -Antitrypsin, Antithrombin). The detection limit for the complexes varies from 41 pM for the FXIa: α_2 AP complex down to 9 pM for the FXIa:C1INH complex.(50) These inhibitor complex ELISAs showed that C1-inhibitor is the predominant FXIa inhibitor responsible for 47% of the FXIa inhibition, followed by α_2 AP(25%), α_1 AT(23%), and AT(5%). However, the complex ELISA's do not quantify the amount of free FXIa.(46,50)

The last method to quantify FXIa in plasma is *via* a thrombin generation based assay. Calibrated automated thrombin generation is a global coagulation assay that measures the activity of thrombin in plasma over time by a fluorogenic substrate. The use of different triggers to start coagulation allows to focus on either the intrinsic or the extrinsic pathway. In contrast to the prothrombin time (PT) test and the activated partial thromboplastin time (aPTT) assays, thrombin generation assays can be used to quantify single protein concentrations in plasma.(51,52) To quantify FXIa in plasma *via* this model, a plasma sample is used which contains a contact activation inhibitor in the blood drawing tube that prevents activation of FXI by contact activation during blood drawing. The free FXIa in a plasma sample then functions as a trigger for thrombin generation, and allows detection down to 1 pM in plasma.(2,3) However, the main disadvantage is that free FXIa in plasma is susceptible to inhibition by natural serpins. Due to these inhibitors, the half-life of free FXIa in plasma is 15 minutes.(50) Once FXIa is inhibited it will no longer function as an initiator for thrombin generation. Thus, FXIa quantification by thrombin generation is affected by the time schedule of the experiment and the age of the plasma sample. Therefore, currently no adequate assays for XIa are available.

Aim and outline of this thesis

The aim of this thesis is to design a robust and reliable assay to quantify FXIa in circulation. This would enable a more elaborate exploration of a possibly significant role of FXIa in thrombosis. Moreover, this assay could be used to evaluate FXIa inhibition strategies currently being developed. As a starting point, **chapter 2** reviews current bio-orthogonal imine-based bio-conjugation strategies, of which some insights will be used later in **chapter 6**. The chapter will cover multiple aspects of the topic such as functionalization of the

protein/peptide, different types of imine chemistry, applications described in literature, and catalysis of the reaction.

Chapter 3 will study a proof of concept for a catch-and-release assay that quantifies FXIa. The assay isolates FXIa from plasma/buffer via a biotin/desthiobiotin-based multivalent dynamic construct. The multivalent inhibitor binds both catalytic domains of the FXIa dimer via a synthetic multimer of a FXIa inhibitor (Fasxiator) derived from the snake *Bungarus Fasciatus*. Subsequent dissociation of the construct results in the release of FXIa in a buffered system.

Chapter 4 will evaluate three different assay set-ups for their sensitivity and limit of detection to quantify FXIa in a buffered system. The limit of detection and sensitivity will be evaluated for an in-house set-up ELISA, a chromogenic substrate assay, and a commercially available amplification assay.

Chapter 5 will examine whether peptide nucleic acids can be used as basis for a dynamic multivalent construct to optimize the catch-and-release assay described in **chapter 3**. The proof of concept study showed great potential, however, the dynamic construct is hindered by biotin present in plasma. Two complementary PNA strands (9-mer) will be studied for their binding affinity, and their ability to dissociate from each other upon addition of an excess complementary strand. In addition, the length of the PNA strands will be optimized for its affinity via isothermal titration calorimetry.

Finally, **chapter 6** will address an important drawback of the catch-and-release assay, namely that quantification of FXIa must be done in the presence of a FXIa inhibitor. By coupling steric hindrance near the FXIa-binding site of Fasxiator, an attempt will be made to develop a reversal strategy for the FXIa-Fasxiator interaction. Three amino acids that are positioned near the FXIa binding-site (E12, G14 and K48) will be studied for their ability to be varied without losing affinity for FXIa. Subsequent oxime ligation with a bulky molecule to these sites is hypothesized to disrupt the FXIa-Fasxiator interaction.

References

1. Hurt et al. Deep Vein Thrombosis: History and Evolution of Treatment. Clin Advis [Internet]. 2018 Apr 13 [cited 2020 Mar 31]; Available from: <https://www.clinicaladvisor.com/home/features/deep-vein-thrombosis-history-and-evolution-of-treatment/>
2. Loeffen R, Oerle R van, Groot PG de, Waltenberger J, Crijns HJ, Spronk HMH, et al. Increased factor XIa levels in patients with a first acute myocardial infarction: The introduction of a new thrombin generation based factor XIa assay. *Thromb Res*. 2014 Dec;134(6):1328–34.
3. Loeffen R, Oerle R van, Leers MPG, Kragten JA, Crijns H, Spronk HMH, et al. Factor XIa and Thrombin Generation Are Elevated in Patients with Acute Coronary Syndrome and Predict Recurrent Cardiovascular Events. *PLOS ONE*. 2016 Jul 15;11(7):e0158355.
4. Gale AJ. Current Understanding of Hemostasis. *Toxicol Pathol*. 2011;39(1):273–80.
5. Wright IS. The Nomenclature of Blood Clotting Factors. *Can Med Assoc J*. 1962 Feb 24;86(8):373–4.
6. Smith SA, Travers RJ, Morrissey JH. How it all starts: initiation of the clotting cascade. *Crit Rev Biochem Mol Biol*. 2015;50(4):326–36.
7. Kirchhofer D, Nemerson Y. Initiation of blood coagulation: the tissue factor/factor VIIa complex. *Curr Opin Biotechnol*. 1996 Aug;7(4):386–91.
8. Naudin C, Burillo E, Blankenberg S, Butler L, Renné T. Factor XII Contact Activation. *Semin Thromb Hemost*. 2017 Nov;43(8):814–26.
9. Geng Y, Verhamme IM, Smith SB, Sun M, Matafonov A, Cheng Q, et al. The dimeric structure of factor XI and zymogen activation. *Blood*. 2013 May 9;121(19):3962–9.
10. Gailani D, Geng Y, Verhamme I, Sun M, Bajaj SP, Messer A, et al. The mechanism underlying activation of factor IX by factor XIa. *Thromb Res*. 2014 May 1;133:S48–51.
11. Grover Steven P., Mackman Nigel. Intrinsic Pathway of Coagulation and Thrombosis. *Arterioscler Thromb Vasc Biol*. 2019 Mar 1;39(3):331–8.
12. Whelihan MF, Zachary V, Orfeo T, Mann KG. Prothrombin activation in blood coagulation: the erythrocyte contribution to thrombin generation. *Blood*. 2012 Nov 1;120(18):3837–45.
13. Stubbs MT, Oschkinat H, Mayr I, Huber R, Anglikar H, Stone SR, et al. The interaction of thrombin with fibrinogen. A structural basis for its specificity. *Eur J Biochem*. 1992 May 15;206(1):187–95.
14. Walsh PN. Roles of platelets and factor XI in the initiation of blood coagulation by thrombin. *Thromb Haemost*. 2001 Jul;86(1):75–82.
15. Bouma BN, Griffin JH. Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. *J Biol Chem*. 1977 Sep 25;252(18):6432–7.
16. Renné T, Gailani D, Meijers JCM, Müller-Esterl W. Characterization of the H-kininogen-binding site on factor XI: a comparison of factor XI and plasma prekallikrein. *J Biol Chem*. 2002 Feb 15;277(7):4892–9.
17. Rosenthal RL, Dreskin OH, Rosenthal N. New hemophilia-like disease caused by deficiency of a third plasma thromboplastin factor. *Proc Soc Exp Biol Med Soc Exp Biol Med N Y N*. 1953 Jan;82(1):171–4.

18. Papagrigoriou E, McEwan PA, Walsh PN, Emsley J. Crystal structure of the factor XI zymogen reveals a pathway for transactivation. *Nat Struct Mol Biol.* 2006 Jun;13(6):557–8.
19. Ho DH, Baglia FA, Walsh PN. Factor XI binding to activated platelets is mediated by residues R(250), K(255), F(260), and Q(263) within the apple 3 domain. *Biochemistry (Mosc).* 2000 Jan 18;39(2):316–23.
20. Baglia FA, Walsh PN. A binding site for thrombin in the apple 1 domain of factor XI. *J Biol Chem.* 1996 Feb 16;271(7):3652–8.
21. McMullen BA, Fujikawa K, Davie EW. Location of the disulfide bonds in human coagulation factor XI: the presence of tandem apple domains. *Biochemistry (Mosc).* 1991 Feb 26;30(8):2056–60.
22. Wu W, Sinha D, Shikov S, Yip CK, Walz T, Billings PC, et al. Factor XI homodimer structure is essential for normal proteolytic activation by factor XIIa, thrombin, and factor XIa. *J Biol Chem.* 2008 Jul 4;283(27):18655–64.
23. Yang L, Sun M, Gailani D, Rezaie AR. Characterization of a heparin-binding site on the catalytic domain of factor XIa: mechanism of heparin acceleration of factor XIa inhibition by the serpins antithrombin and C1-inhibitor. *Biochemistry (Mosc).* 2009 Feb 24;48(7):1517–24.
24. Ho DH, Badellino K, Baglia FA, Walsh PN. A binding site for heparin in the apple 3 domain of factor XI. *J Biol Chem.* 1998 Jun 26;273(26):16382–90.
25. Cormier M, Batty P, Tarrant J, Lillicrap D. Advances in knowledge of inhibitor formation in severe haemophilia A. *Br J Haematol.* 2020 Apr;189(1):39–53.
26. Horava SD, Peppas NA. Recent advances in hemophilia B therapy. *Drug Deliv Transl Res.* 2017;7(3):359–71.
27. Seligsohn U. Factor XI deficiency in humans. *J Thromb Haemost.* 2009;7(s1):84–7.
28. Salomon O, Steinberg DM, Koren-Morag N, Tanne D, Seligsohn U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. *Blood.* 2008 Apr 15;111(8):4113–7.
29. Salomon O, Steinberg DM, Zucker M, Varon D, Zivelin A, Seligsohn U. Patients with severe factor XI deficiency have a reduced incidence of deep-vein thrombosis. *Thromb Haemost.* 2011 Feb;105(2):269–73.
30. Preis M, Hirsch J, Kotler A, Zoabi A, Stein N, Rennert G, et al. Factor XI deficiency is associated with lower risk for cardiovascular and venous thromboembolism events. *Blood.* 2017 Mar 2;129(9):1210–5.
31. Schumacher WA, Seiler SE, Steinbacher TE, Stewart AB, Bostwick JS, Hartl KS, et al. Antithrombotic and hemostatic effects of a small molecule factor XIa inhibitor in rats. *Eur J Pharmacol.* 2007 Sep 10;570(1–3):167–74.
32. Hu Z, Wong PC, Gilligan PJ, Han W, Pabbisetty KB, Bozarth JM, et al. Discovery of a Potent Parenterally Administered Factor XIa Inhibitor with Hydroxyquinolin-2(1H)-one as the P2' Moiety. *ACS Med Chem Lett.* 2015 May 14;6(5):590–5.
33. Al-Horani RA, Desai UR. Designing Allosteric Inhibitors of Factor XIa. Lessons from the Interactions of Sulfated Pentagalloylglucopyranosides. *J Med Chem.* 2014 Jun 12;57(11):4805–18.
34. Karuturi R, Al-Horani RA, Mehta SC, Gailani D, Desai UR. Discovery of Allosteric Modulators of Factor XIa by Targeting Hydrophobic Domains Adjacent to its Heparin-Binding Site. *J Med Chem.* 2013 Mar 28;56(6):2415–28.

35. Kini RM. Anticoagulant proteins from snake venoms: structure, function and mechanism. *Biochem J*. 2006 Aug 1;397(3):377–87.
36. Mans BJ, Neitz AWH. Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective. *Insect Biochem Mol Biol*. 2004 Jan;34(1):1–17.
37. Ma D, Mizurini DM, Assumpção TCF, Li Y, Qi Y, Kotsyfakis M, et al. Desmolaris, a novel factor XIa anticoagulant from the salivary gland of the vampire bat (*Desmodus rotundus*) inhibits inflammation and thrombosis in vivo. *Blood*. 2013 Dec 12;122(25):4094–106.
38. Li D, He Q, Kang T, Yin H, Jin X, Li H, et al. Identification of an anticoagulant peptide that inhibits both fXIa and fVIIa/tissue factor from the blood-feeding nematode *Ancylostoma caninum*. *Biochem Biophys Res Commun*. 2010 Feb 5;392(2):155–9.
39. Buchanan MS, Carroll AR, Wessling D, Jobling M, Avery VM, Davis RA, et al. Clavatadine A, a natural product with selective recognition and irreversible inhibition of factor XIa. *J Med Chem*. 2008 Jun 26;51(12):3583–7.
40. Chen W, Carvalho LPD, Chan MY, Kini RM, Kang TS. Fasxiator, a novel factor XIa inhibitor from snake venom, and its site-specific mutagenesis to improve potency and selectivity. *J Thromb Haemost*. 2015;13(2):248–61.
41. Weitz JI, Bauersachs R, Becker B, Berkowitz SD, Freitas MCS, Lassen MR, et al. Effect of Osocimab in Preventing Venous Thromboembolism Among Patients Undergoing Knee Arthroplasty: The FOXTROT Randomized Clinical Trial. *JAMA*. 2020 14;323(2):130–9.
42. Schaefer M, Buchmueller A, Dittmer F, Straßburger J, Wilmen A. Allosteric Inhibition as a New Mode of Action for BAY 1213790, a Neutralizing Antibody Targeting the Activated Form of Coagulation Factor XI. *J Mol Biol*. 2019 Dec 6;431(24):4817–33.
43. Büller HR, Bethune C, Bhanot S, Gailani D, Monia BP, Raskob GE, et al. Factor XI Antisense Oligonucleotide for Prevention of Venous Thrombosis. *N Engl J Med*. 2015 Jan 15;372(3):232–40.
44. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. *N Engl J Med*. 2000 Mar 9;342(10):696–701.
45. Roemisch J, Kaar W, Zoechling A, Kannicht C, Putz M, Kohla G. Identification of activated FXI as the major biochemical root cause in IVIG batches associated with thromboembolic events. *WebmedCentral Immunother*. 2011 Jan 1;2:WMC002002.
46. Murakami T, Komiyama Y, Masuda M, Karakawa M, Iwasaka T, Takahashi H. Evaluation of factor XIa-alpha 1-antitrypsin in plasma, a contact phase-activated coagulation factor-inhibitor complex, in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol*. 1995 Aug;15(8):1107–13.
47. Gruber A. The role of the contact pathway in thrombus propagation. *Thromb Res*. 2014 May;133 Suppl 1:S45-47.
48. Rossix | Rox Factor XIa [Internet]. [cited 2020 Mar 24]. Available from: https://www.rossix.com/Rox_Factor_XIa.html
49. BIOPHEN™ Factor XIa | HYPHEN BIOMED [Internet]. [cited 2020 Mar 24]. Available from: <https://www.hyphen-biomed.com/products/factor-assays/fxia-clotting-chromogenic-and-immunological-assays/biophen-factor-xia>
50. Wuillemin WA, Minnema M, Meijers JC, Roem D, Eerenberg AJ, Nuijens JH, et al. Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role for C1-inhibitor. *Blood*. 1995 Mar 15;85(6):1517–26.

51. Hemker HC, Giesen P, Dieri RA, Regnault V, Smedt E de, Wagenvoord R, et al. Calibrated Automated Thrombin Generation Measurement in Clotting Plasma. *Pathophysiol Haemost Thromb*. 2003;33(1):4–15.
52. Hemker HC, Al Dieri R, De Smedt E, Béguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. *Thromb Haemost*. 2006 Nov;96(5):553–61.

Chapter 2

Bio-orthogonal imine chemistry in chemical protein synthesis

Stijn M. Agten, Ingrid Dijkgraaf,
Stan H. E. van der Beelen, Tilman M. Hackeng

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Introduction

Previous chapters have discussed multiple methods for native chemical protein synthesis in which end-products will solely contain natural building blocks. In contrast, this chapter will discuss how orthogonal imine chemistry can be used for chemical protein synthesis in conjunction with previously described methods to result in proteins bearing one or more non-natural elements.

Scheme 1, General imine reaction scheme. A condensation reaction of a carbonyl (aldehyde or ketone) with an amine. Resulting products are imines, oximes (X = O) or hydrazones (X = NH)

Reported syntheses of functional protein analogues containing unnatural elements have demonstrated that there is no need to be restricted to the formation of native peptide bonds in order to obtain biologically active proteins. Syntheses utilizing a combination of peptide bond formation and imine ligations (Scheme 1) have resulted in highly complex protein structures with site-specific modifications; three of such products will be highlighted to introduce the potential of this approach. First, Kent and coworkers have focused on naturally occurring noncovalent homo- and hetero-dimers of proteins cMyc and Max that bind DNA and regulate gene expression. (1) These dimers could not be accessed by recombinant techniques and therefore a fully synthetic approach was used to gain access to covalent C-terminal-linked versions of a cMyc-Max heterodimer and a Max homodimer. Monomers were assembled using chemical ligation resulting in thioester linkages and subsequently the desired covalent dimer was synthesized by oxime ligation of two monomer subunits (Figure 1A). Second, a synthetic protein-polymer version of erythropoietin (EPO) was synthesized which displayed potent biological activity and increased duration of action as compared to isolated EPO. (2) In this construct (50 kDa), sequential C to N native chemical ligation for synthesis of the protein part and oxime ligation to site-specifically introduce the polymer part were combined (Figure 1B). Finally, chemical protein synthesis was used to synthesize a covalent branched heterodimer of two chemokines RANTES and PF4 implicated to play a role in progression of atherosclerosis. (3) Oxime linking was achieved by reacting site-specifically modified amino acid side chains, thus keeping the biologically important termini free. Similar

to synthetic EPO, a combination of native chemical ligation and oxime ligation was used; in addition, the resulting heterodimer showed increased biological activity compared to the mixed separate chemokines (Figure 1C).

Figure 1, Examples of chemical protein synthesis that have used imine type reactions to introduce unnatural protein connections.

These examples show that imine chemistry can be particularly useful in chemical protein synthesis. In this chapter, all necessary techniques to combine chemical peptide bond formation and imine ligations will be discussed. This will include incorporation of necessary reactive moieties into proteins and peptides, different ligations that can be performed (oxime, hydrazone, Pictet-Spengler), and methods to catalyze these reactions.

Carbonyl functionalization

To undergo an imine-type ligation, respective proteins must contain a carbonyl functionality in the form of an aldehyde/ketone or an α -nucleophile. The following section will discuss the introduction of carbonyl moieties into proteins and peptides, whereas the next paragraph will cover incorporation of α -nucleophiles such as alkoxyamines and hydrazides.

The increased attention for - and use of - oxime/hydrazone ligations have led to multiple methods of carbonyl introduction. However, introduction of ketones or aldehydes into peptides or proteins remains a limiting step in the use of imine ligations. Methods for carbonyl introduction range from total chemical synthesis approaches to genetic incorporation of the desired ketone or aldehyde. In this section, strategies will be discussed to introduce

ketone/aldehyde moieties and various methods for N-terminal, C-terminal or site-specific modification of a polypeptide will be described.

The bio-orthogonal nature of imine-type ligations is one of the biggest advantages of these reactions; reactive moieties are generally not found in proteins (glycosylated proteins are an exception and will be discussed below) and will therefore only react with each other even in absence of protective groups. It has to be noted, however, that ketones and aldehydes are common in nature which may lead to unspecific reactions in some systems. Absence of ketone or aldehyde carbonyls in proteins limits the use of this functional group as a starting point for modifications. N-terminal modification of proteins was one of the early methods to generate an aldehyde or ketone in a protein. Oxidation using sodium periodate is the most used and most well-known of these reactions and is now common in many biological labs (Scheme 2AB). In proteins, this reaction takes advantage of the extreme selectivity of 1, 2-aminoalcohols or vicinal diols towards hypervalent iodine species such as metaperiodate. This functional group is exclusively found at the site of *N*-terminal serine or threonine and hydroxylysine. (4-6) Although other amino acids may be prone to oxidation the selectivity of this reagent ensures a highly specific reaction. The resulting glyoxylyl moiety can subsequently be used to react with various functionalized labels. (7-10) The necessity of a serine or threonine at the *N*-terminal position can be considered a drawback, but these residues can easily be introduced through mutagenesis or chemical synthesis. The limitation of *N*-terminal serine or threonine residues has nevertheless led to research into transamination approaches that can be used to introduce ketones and aldehydes into a wider spectrum of naturally occurring proteins. Early studies found that proteins treated with glyoxylate and a bivalent metal ion such as Cu(II) at near neutral pH, underwent transamination to the desired carbonyl species (Scheme 2C). (6) Multiple cofactors were evaluated in this study and interestingly, the biological cofactor frequently involved in transamination reactions, pyridoxal 5'-phosphate (PLP), was found to be inferior to glyoxylate. In more recent research, PLP was found to be useful for performing mild *N*-terminal transaminations of intact proteins, does not require the presence of metal ions and can be performed at neutral pH and 37°C in good yields (Scheme 2D). (11, 12) Any *N*-terminal amino acid other than Pro can be transaminated and will result in the corresponding glyoxylyl (Gly) or ketoamide. However, not all terminal amino acids will give rise to similar

transamination yields; His, Trp, Pro and Lys proceed in lower yields due to an adduct with PLP itself, in the case of His and Trp most likely through a Pictet-Spengler type reaction (see below). (13) In addition, some amino acids are found to lead to high transamination yields, but subsequent reactions cannot be performed. In the case of glutamine, high yields of transaminated product are observed but this product does not react to an oxime, with a stable intermediate of the ketone with the glutamine side chain as the most likely explanation. (13) Interestingly, the transamination reaction and subsequent ligations can be optimized by varying amino acids in positions 2 and 3. For example, a lysine at position 3 generally leads to high reaction yields with exception of earlier mentioned terminal amino acids. This may be explained by a synergistic effect of the internal sequence that leads to the observed difference in reaction yield. (13)

In addition to using oxidation or transamination methods, *N*-terminal ketone incorporation can be achieved by taking advantage of the natural machinery present for the synthesis of members of the lanthipeptide family. These ribosomal synthesized small peptides are post-translationally modified to contain thioether bridges (lanthionines). Biosynthesis of these bridges is achieved by dehydration of serine and threonine residues to dehydroalanine and dehydrobutyrine, respectively (Scheme 2E). Subsequently, a reaction with free thiols present on cysteine residues in the sequence will form the lanthionines. It was found that selected lanthipeptides contain an *N*-terminal modification in the form of a ketone as a result of a dehydrated residue at position 1 in the final sequence which is not used to form a thioether bridge. The dehydration of threonine and removal of the leader peptide in this case leads to spontaneous hydrolysis of the *N*-terminal enamine to a α -ketoamide. Interestingly, this modification was accepted in a range of lanthipeptides and the resulting *N*-terminal ketone could be modified using imine chemistry. (14)

Scheme 2, Methods for N-terminal introduction of carbonyl moieties. N-terminal aldehydes or ketones can be introduced via oxidation with meta periodates, reaction with glyoxylate in presence of a metal ion, reaction with biological cofactor pyridoxal 5'-phosphate (PLP) or via the machinery present in bacteria to produce lanthipeptides

In addition to selective *N*-terminal modifications, methods have also been developed to selectively introduce ketone/aldehyde functionalities at the *C*-terminus of a peptide. Interest in peptide-based cysteine protease inhibitors, which require an electrophilic functionality, has led to development of *C*-terminal carbonyl introduction. Peptidyl resin can be modified with hydrazide or semicarbazide functionalities which subsequently can be used to introduce a protected carbonyl moiety. Solid-phase peptide synthesis to this hydrazone or semicarbazone linked residue followed by final cleavage under acidic conditions will lead to *C*-terminal carbonyl modified peptides (Scheme 3AB). (15-17) Alternatively, peptides can be synthesized on Weinreb amide resin where final treatment with DIBAL-H leads to a *C*-terminal aldehyde or addition of a Grignard reagent results in the corresponding ketone product (Scheme 3C). (18) Furthermore, aldehydes have been introduced in peptides using a glyoxylal moiety. This functional group can be introduced at the *C*-terminus using special peptidyl resins or at the *N*-terminus using specialized amino acid building blocks. (19, 20)

Scheme 3, C-terminal carbonyl introduction through solid phase resin approaches. C-terminal ketone synthesis can be performed via semi-carbazone or hydrazone linked building blocks. Alternatively, Weinreb amide resin cleaved with DIBAL-H or LiAlH₄ will result in peptide aldehydes and cleavage with Grignard reagents in peptide ketones.

Apart from chemical synthesis approaches, C-terminal modification can be achieved by taking advantage of the high specificity of naturally occurring enzymes. Enzyme tubulin tyrosine ligase (TTL) attaches a tyrosine residue to the carboxy terminal end of α -tubulin. Minor changes in its tyrosine substrate are accepted by TTL, where 3-formyltyrosine represents an interesting aldehyde containing substrate. (21) This enzyme was used to label α -tubulin with a C-terminal aldehyde in living cells followed by addition of a hydrazine-containing label. (22) Interestingly, the enzyme can be used to introduce 3-formyltyrosine in any protein as long as a recognition site (Tub-tag, 14 aa) is present at the C-terminus of the protein of interest (Scheme 4A). (21) Alternatively, protein farnesyltransferase (PFTase) can be utilized to achieve C-terminal carbonyl introduction. (23) In nature, this enzyme catalyzes the addition of a farnesyl isoprenoid group to a cysteine present in a recognition site at the C-terminus of a protein. The recognition site is the CaaX box, where C is the cysteine to be prenylated, a is any aliphatic amino acid and X is M, S, Q, A or C. The farnesyl substrate was shown to accept modifications with either a simple aldehyde or an aryl aldehyde, both of which are still recognized by PFTase (Scheme 4B). (24) A final enzymatic approach was found in using lipolic

acid ligase (LplA), naturally present in *E. coli* bacteria. The enzyme catalyzes the addition of lipoic acid to a 13 amino acid LAP recognition site, which can be fused to either the *N*- or *C*-terminus of a protein site. Modified substrates incorporating unnatural functional groups have been shown to be accepted in the mutated substrate pocket of the enzyme and have extensively been used to label proteins in various environments. (25-28) The lipoic acid substrate was modified to include an aryl aldehyde after mutation of Trp 37 in LplA. Subsequently, hydrazine probes were used to site-specifically label the desired protein (Scheme 4C). (29) A drawback in the use of enzymes is the necessity of acceptor sites which must be introduced in the protein of interest. Moreover, limited accessibility of the enzymes restricts most of these acceptor sites to be introduced at *N*- or *C*-terminal ends of a protein target. An exception to this is formylglycine generating enzyme (FGE) which catalyzes the site-specific conversion of a cysteine residue into formylglycine. (30, 31) The required consensus sequence was found to be CXPXR, where X stands for any amino acid and can be introduced at any accessible position. FGE will subsequently convert the cysteine residue into a formylglycine that can be used for subsequent oxime, hydrazone or Pictet-Spengler ligations. (32) Although a formylglycine generating enzyme is naturally present in *E. coli*, the yield is best when co-expressed with an additional prokaryotic FGE (Scheme 4D). (31)

Scheme 4, Enzymatic approaches for carbonyl introduction in proteins. Several enzymes have been used for carbonyl introduction and all require some sort of recognition sequence. Enzyme mutations are required to incorporate carbonyl-modified substrates.

While these enzymatic approaches have been proven to be very useful, they require specific peptide sequences to be introduced thereby changing the native sequence and/or folding of the target protein. Modifications can be restricted to a single amino acid residue by codon reassignment. This expansion of the genetic code, where an unused codon is recognized by a tRNA/aminoacyl-tRNA synthetase pair equipped with a non-standard amino acid, has gained increased interest over the last decades (33, 34). Genetic code expansion has been used to introduce dozens of non-standard amino acids that could subsequently be used to tailor the target protein properties or functions. (35, 36) Ketone and aldehyde functionalities have been introduced mainly through amber codon suppression (Figure 2). Early work used *p*-acetyl-L-phenylalanine to introduce an aryl ketone group into proteins. (37, 38) This functional group could subsequently be used to site-specifically label proteins both *in vitro* and *in vivo*. An analogue of *p*-acetyl-L-phenylalanine, namely *m*-acetyl-L-phenylalanine was later used for a similar purpose. (39) Ketone incorporation in expressed proteins was used for multiple bioconjugations such as PEGylation, spin-labeling and modification of Fab fragments. (40-42) A limitation of these aryl ketones is that they are relatively unreactive as compared to alkyl ketones. (43) This will benefit stability of the formed oxime bond in a cellular environment, but also leads to requirement of large excesses of label and prolonged reaction times. Stability can be further enhanced by introduction of a β -diketone which will even react with primary amines to form stable enamines. (44)

Figure 2, Examples of modified amino acid building blocks to site-specifically introduce carbonyl moieties via amber codon suppression

While ribosomal synthesis has clear advantages to produce large proteins, chemical synthesis and/or semi-synthesis allow protein modifications that are not compatible with biological assembly machinery. Ketones or aldehydes can be incorporated at any desired position in a protein when using solid-phase peptide synthesis (SPPS). Newly synthesized ketone-containing amino acid building blocks were used for this purpose (Figure 3). The aforementioned *p*-acetyl-L-phenylalanine, when suitably protected, was also incorporated

using SPPS. Alternatively, ketoproline can be used which can be synthesized from the commercially available hydroxyproline by Jones oxidation and was shown to be accepted in the collagen triple helix. (45) The collagen helix could subsequently be labeled with a biotin tag via oxime ligation. (46) In addition, advantage was taken from orthogonally protected lysine residues which can site-specifically be introduced in a peptide sequence. After deprotection, aldehydes can be introduced by coupling of a serine or threonine residue followed by periodate oxidation as discussed above. (47) Alternatively, these orthogonally protected residues can be used for site-specific ketone incorporation by amide bond formation with selected keto acids. Frequently used building blocks for this purpose are pyruvic acid and levulinic acid. (1, 2, 48-50) However, the choice of keto acid is not arbitrary; recent research found that the levulinoyl moiety is prone to an intramolecular cyclization reaction. (43) This leads to a 5-membered heterocycle that is unable to react with an hydroxylamine to form an oxime bond. Alternatives for levulinic acid were therefore investigated and it was found that elongation of the carbon chain with a single carbon atom was sufficient to prevent the cyclization reaction. (43)

Figure 3, Examples of modified amino acids building blocks and structural elements to site-specifically introduce carbonyl moieties via total chemical synthesis

The disordered nature of small peptide fragments has led to the development of multiple stabilizing elements typically to constrain peptides in an α -helical structure. (51) These elements are mostly inert, but incorporation of a ketone can be achieved by macrocyclization using dichloroacetone (DCA). (52) DCA was shown to react with thiol nucleophiles present in the peptide sequence. The stapled peptide, incorporating a ketone, could then be modified with alkoxyamines. (52)

Finally, glycosylated proteins can be applied to imine bioconjugation strategies. Although none of the natural 20 amino acids contain a reactive or free carbonyl moiety, glycosylated

proteins contain carbohydrate groups that can be used as starting points for carbonyl incorporation. First, the cyclic form (hemiacetal) of every free saccharide is in equilibrium with its linear form (carbonyl-containing) which can be used for modification using oxime ligation (Scheme 5A). (53, 54) Interestingly, the cyclic structure of the carbohydrate can be retained after oxime ligation. (54, 55) Next, synthetic approaches can be used to generate glycopeptides with site-specific sugar incorporation. An *O*-linked glycopeptide was synthesized in this manner using a GalNAc-modified Ser/Thr building block. Enzymatic conversion of this galactose derivative by galactose oxidase into a C-6 aldehyde was used to introduce an oligosaccharide using oxime ligation (Scheme 5B). (56) Advantage can further be taken of sialic acids frequently found at the end of sugar chains on glycoproteins. Sialic acids contain a diol that can be oxidatively cleaved to an aldehyde using sodium periodate. This method was first used as a means of quantifying sialic acid contents in biological mixtures. The resulting aldehyde could be used to generate stable chromogens when reacted with resorcinol. (57) In a similar approach, sialic acid oxidation and subsequent modification was used to introduce radioactive labels in glycoconjugates or novel functional groups on glycoproteins (Scheme 5C). (58-60) Interestingly, the approach could be used for live cell labelling when sialic acids are present on cell surface glycans. (61) Finally, live cell modification can be achieved by presenting cells with unnatural derivatives of natural carbohydrates. A ketone-containing derivative of *N*-acetyl-mannosamine was presented to cells and is subsequently converted into an unnatural sialic acid and incorporated into glycans present on the cell surface (Scheme 5D). These ketone groups present on the cell surface could be labeled with hydrazines. (62)

Scheme 5, Glycoprotein modification. Glycans can be utilized in several ways to incorporate carbonyl functionalities in proteins. Examples include the natural equilibrium between cyclic and linear forms of glycans, enzymatic conversion of GalNAc building blocks, oxidation of sialic acids and metabolic conversion of carbonyl-modified glycans.

Aminoxy, hydrazine and hydrazide functionalization

In contrast to carbonyl functionalization, introduction of α -nucleophiles such as alkoxyamines or hydrazides is mostly limited to synthetic proteins and peptides. Similar to carbonyl introduction, this section will describe developed methods for *N*-terminal, *C*-terminal and site-specific introduction of these moieties in proteins and peptides. Synthetic peptides can be *N*-terminally modified with hydrazide or alkoxyamines using tri-Boc-hydrazino acetic acid or Boc-aminooxy acetic acid respectively (Figure 4). These building blocks have frequently been used for last stage introduction in peptide synthesis, although evidence has been reported that increased reactivity of the *N*-protected aminoxy function may lead to *N*-overacylation and subsequent heterogeneous peptides. (63) To overcome this problem, studies into use of base have been performed and more exotic protecting groups have been used. (64, 65)

Figure 4. Examples of protected building blocks for the N-terminal introduction of α -nucleophiles

C-terminal modification and especially C-terminal hydrazides have found great interest since they can be converted into thioesters and be used for native chemical ligation next to their use in hydrazone forming reactions (66). C-terminal hydrazide modification can be achieved with peptide synthesis on hydrazide-functionalized resins with final cleavage either using standard acidic conditions or photolytically (Scheme 6ABC). (67, 68) Alternatively, C-terminal hydrazides can be generated by peptide cleavage from Wang resin using hydrazine (Scheme 6D). (69)

Scheme 6, C-terminal α -nucleophile introduction through solid phase resin approaches. Methods have been developed using CTC, hydrazide functionalized and Wang resins.

In addition to these synthetic methods suitable for small peptides, larger proteins can be decorated with a C-terminal hydrazide when a terminal X-Cys motif is treated with an hydrazine source (Scheme 7). (70)

Scheme 7, C-terminal hydrazinolysis of protein containing a C-terminal X-Cys motif.

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The earlier described enzyme, lipoic acid ligase, (see above) was also mutated to accept hydrazide substrates. (29) Finally, site-specific incorporation of alkoxyamines or hydrazides can be achieved in synthetic peptides using special amino acid building blocks (Figure 5). (47, 71-74)

Figure 5, Examples of α -nucleophile unnatural amino acids used for the site-specific introduction of hydrazide or alkoxyamine functionalities

Oxime ligation

The first of the imine-type bonds to be discussed in this chapter is the oxime bond. Formation of an oxime bond, or oximation, is the result of a reversible reaction of a ketone or aldehyde with an alkoxyamine (Scheme 8).

Scheme 8, General oxime forming reaction scheme.

While oxime bonds can be hydrolyzed to obtain their starting products, they are generally considered to be stable at neutral pH. (75, 76) For this reason, oxime bonds have been used for multiple applications in protein chemistry which will be highlighted below. (77, 78) Rose and coworkers pioneered in using oximation in protein chemistry in 1994 when an artificial protein consisting of a poly-aldehyde scaffold decorated with multiple aminooxy modified peptides was constructed. (7) A similar approach was used to synthesize peptide dendrimers on an aldehyde-modified lysine core. (79) The chemoselective nature of the oxime reaction allowed use of unprotected peptides and combination with other chemoselective reactions. This property was later exploited in the synthesis of a variant of human erythropoietin, where native chemical ligation was used to construct the protein and oxime ligation was used to introduce two negatively charged polymers to the protein. (2) In a similar approach chemokine CCL5 was PEGylated site-specifically after purification and folding using an

orthogonally protected aminoxy moiety that could be deprotected under mild conditions (Scheme 9). (80)

Scheme 9, RANTES modification with a PEG-moiety. RANTES was PEGylated after selective deprotection of a isopropylidene moiety followed by oximation with an aldehyde PEG.

These unnatural modifications were mainly introduced to generate proteins with prolonged circulating lifetime and to mimic or improve natural glycosylation. However, since the natural open-chain form of saccharides contains a free aldehyde, glycosylation approaches using oximes were also investigated and utilized for glycoprotein assembly. (81, 82) In addition to using this open-chain form, aminoxy sugars were reacted with ketone containing peptides (Scheme 10A). The antimicrobial peptide drosocin was modified with an *O*-linked GalNac mimetic via oxime chemistry and was shown to exhibit the same glycosylation-dependent properties as the native protein (Scheme 10B). (83) In addition, it was found that coupling of unprotected sugars with secondary alkoxyamines would lead to unnatural, *N*-glycosylated peptides that have been termed neo-glycopeptides (Scheme 10C). (55) This reaction was exploited for the generation of a library of cardiac glycosides to explore the anticancer properties of digitoxin. (84)

Scheme 10, Glycoprotein oximation approaches. Glycans have been modified with both alkoxyamines and carbonyl moieties to enable glycoprotein assembly.

In addition to protein and glycoprotein assembly, oxime ligation has frequently been used for the introduction of radiolabels. These radiolabeled peptides can subsequently be used for diagnosis of disease and monitoring of pathological processes. The properties of ^{18}F , such as its half-life of 109.8 min and low β^+ -energy (0.64 MeV), are almost ideal for positron emission tomography (PET) and have led to its widespread use. Although newly developed ^{18}F -labeling techniques, such as Al^{18}F and isotope exchange have been proven to be very suitable, ^{18}F -labeling of peptide and proteins via prosthetic groups has been the most common methodology for years. (85, 86) In general, these prosthetic group-based ^{18}F -fluorination reactions comprise multistep radiosyntheses of the ^{18}F -labeled synthon which are time-consuming and result in moderate to even low yields. In addition, for conjugation of the radiofluorinated prosthetic group the use of protected peptide or protein precursors is required which requires subsequent deprotection. Radiolabeling via oxime ligation has been proven to be a fruitful approach, because it is highly chemoselective, allows the use of unprotected aminoxy precursors (e.g. peptides and proteins), and is feasible in aqueous media under mild conditions (pH 4-7). Since the oxime bond is hydrolytically stable, oxime-conjugated radiopharmaceuticals are suitable for in vitro and in vivo studies. This has indeed been proven by successful application of ^{14}C -labeled polyamines and radioiodinated monoclonal antibodies in radiometric assays and biodistribution studies in tumor-bearing

mice, respectively. Several synthons for ^{18}F introduction by oxime ligation have been developed (Scheme 11).

Scheme 11, ^{18}F radiolabeling of proteins and peptides via oxime forming reactions with fluorobenzaldehyde (A), fluorodeoxyglucose (B) or fluorodeoxyribose (C).

Radiolabeling by oxime conjugation was demonstrated by reacting aminoxy-modified peptides with 4- ^{18}F fluorobenzaldehyde (^{18}F FBA), 2- ^{18}F fluoro-2-deoxyglucose (^{18}F FDG), or 5- ^{18}F fluoro-5-deoxyribose (^{18}F FDR) (Scheme 11). (87) The peptide labeling efficiency of ^{18}F FBA has been evaluated for a variety of aminoxy-functionalized peptides such as minigastrin, RGD, and octreotide analogues and it appeared to be strongly dependent on pH, peptide concentration, reaction time, and temperature. (88) However, it has been demonstrated that oxime ligation with ^{18}F FBA is suitable strategy for the production of ^{18}F -labeled peptides with excellent pharmacokinetic properties in preclinical studies in the case of RGD peptides. (89-91) These include RGD-multimers (92), as well as a glycosylated Tyr3-octreotate analog, Cel-S-Dpr(^{18}F FBOA)TOCA. (93) One of these PET tracers, ^{18}F Fluciclatide, has been successfully translated into the clinic for the visualization of $\alpha\text{v}\beta 5$ - and $\alpha\text{v}\beta 3$ -positive tumors. (91, 94-96) In addition, this PET tracer demonstrated that it may be suitable for molecular imaging of asymptomatic abdominal aortic aneurysm (97) and is potentially useful for investigating healing responses after myocardial infarction. (98)

The ^{18}F FBA-labeling approach is not limited to small peptides as demonstrated by site-specific ^{18}F -labelling of leptin (16 kDa) using a combination of expressed protein ligation and oxime ligation. (99)

Due to the conjugation of [^{18}F]FBA as a prosthetic group to peptides, the lipophilicity of the peptide and thus the radiotracer is increased which may result in clearance via the hepatobiliary route. The preferred route of clearance of peptide-based radiopharmaceuticals is via the kidneys. There are various methods for modifying the pharmacokinetics of peptides, such as PEGylation and introduction of hydrophilic amino acids residues. A suitable method to improve the pharmacokinetics of a peptide is glycosylation since it generally enhances urinary excretion and reduces non-specific binding. Therefore, oxime ligation of [^{18}F]FDG or [^{18}F]FDR to a peptide or protein is an effective approach to radiofluorinate and modulate the pharmacokinetics of a peptide simultaneously.

In 2008, it was already mentioned that the open chain aldehyde form of [^{18}F]FDG should allow chemoselective aldehyde group-based reactions. (100) In that study, glutathione (GSH) was radiofluorinated with [^{18}F]FDG via maleimidehexyloxime and the resulting product [^{18}F]FDG-Maleimidehexyloxime ([^{18}F]FDG-MHO) was subsequently coupled to Annexin A5 (anxA5). (100) Direct labeling of peptides using the FDG aldehyde functionality at position 1 was then investigated for RGD analogs. (101) Although this labeling strategy is effective, it has some limitations such as the high temperature (100 °C) and acidic pH conditions which are required to obtain high yields. These conditions are tolerated for the small unprotected (cyclic) peptides but may be pernicious for the functionality of large peptides and proteins.

For labeling of peptides with [^{18}F]FDG, it is necessary to remove the excess of D-glucose which is normally present in routinely produced clinical grade [^{18}F]FDG. (102) Using no-carrier-added (n.c.a.) [^{18}F]FDG obtained via HPLC removal of excess glucose, afforded [^{18}F]FDG-RGD in reasonable yields (56–93%; decay corrected), allowing to compare [^{18}F]FDG-RGD with “gold standard” [^{18}F]galacto-RGD in preclinical studies. [^{18}F]FDG-RGD had higher tumor accumulation compared to [^{18}F]galacto-RGD. However, both compounds had comparable tumor-to-organ ratios due to a slightly increased uptake of [^{18}F]FDG-RGD in non-target organs.

An interesting alternative to [^{18}F]FDG for radiofluorination of peptides is [^{18}F]FDR. Bioconjugation of this ribose is performed in one step under mild conditions (pH 4.6–7.0). (103) Compared to [^{18}F]FDG, ligation of [^{18}F]FDR is fast, apparently due to the location of the fluorine at C-5 and an increased reactivity of 5-membered over 6-membered aldol sugars with alkoxyamines.

Ease of alkoxyamine or aldehyde introduction in oligonucleotides has resulted in oxime ligation being a preferred method for DNA-peptide conjugates. (105) Moreover, the therapeutic potential of short DNA or RNA fragments but difficulty in crossing cell membranes have led multiple oligonucleotide-peptide conjugate techniques to be developed (Scheme 12). (106) Oligonucleotides were modified to contain a 5' or 3' diol which subsequently were oxidized under mild conditions without affecting the unprotected oligonucleotide chain. (107-109) One or more peptides with an aminoxy moiety were then used to obtain desired conjugates. (110) Generated aldehydes could be used in conjunction with alkynes for a bis conjugation strategy with two different peptides. (111)

Scheme 12, Oximation approaches for the synthesis of oligonucleotide (ODN) – protein complexes. All methods make use of a vicinal diol on either '5 or '3 end of the desired ODN. Oxidation and subsequent oximation result in ODN-protein complexes.

The versatility of the oxime bond has furthermore been used for the generation of cyclic peptide libraries. (49) In a head to side-chain approach, an *N*-terminal alkoxyamine was coupled to a levulinoyl moiety introduced on the side chain of an orthogonally protected lysine. A final highlighted example is found in the total synthesis of Lispro insulin, in which a temporary oxime linker between A and B chains was used to facilitate protein folding (Scheme 13). (50)

Scheme 13, Synthesis of Lispro insulin. Modification of the N-terminus of insulin A-chain and C-terminus of the B-chain allowed for a temporary oxime link between two chains. Subsequent folding and enzymatic removal of the temporary linker resulted in chemical synthesis of desired insulin variant.

Hydrazone ligation

A hydrazone bond is the result of a condensation reaction of a ketone or aldehyde with a hydrazide (Scheme 14). Like oximes, hydrazones are employed in versatile conjugation methods for multiple purposes.

Scheme 14, General hydrazone forming reaction scheme.

A key difference between oximes and hydrazones lies in the hydrolytic stability of the linkage. Whereas an oxime bond is considered stable at neutral pH, a hydrazone bond will hydrolyze to a large extent at pH 7. (76, 112) The hydrazone linkage is therefore less useful for covalent labeling purposes, but can, in contrast to oximes, be useful in dynamic covalent chemistry. (113) The hydrazone equilibrium can be pushed in either direction by variation of neighboring groups. First, hydrazone bonds formed by reaction of a hydrazide with an aldehyde are generally less stable than those formed by reaction with a ketone due to steric effects. Next,

acyl hydrazones or those with other electron withdrawing groups are less stable and therefore useful in dynamic chemistries, whereas without these the formed bonds are more stable. (76, 112) The hydrolytic stability of the hydrazone bond can be greatly improved by reduction with cyanoborohydride to yield a hydrazide. (114-116) In addition, acid catalyzed hydrolysis plays a bigger role in the stability of a hydrazone as compared to an oxime. (117, 118) This property is, however, valuable for the controlled release of bioactive molecules. For example, an antibody drug conjugate was equipped with a hydrazone which would hydrolyze as it is taken up by the target cell due to low pH in endosomes (pH 5.5-6.2) or lysosomes (pH 4.5-5.0) compared to physiological environment (pH 7.4). (119) The conjugate hydrazone linker was designed in such a way that the half-life dropped from 183h at pH 7.2 to 4.4h at pH 5. (120)

Hydrazone linkages were used in protein chemistry as early as 1986, before native chemical ligation was invented. King and coworkers designed a method to construct protein dimers, trimers and even higher order oligomers from hydrazide and aldehyde decorated proteins. (121) Hydrazides were randomly introduced in proteins of interest by reaction with NHS-activated bromoacetyl moieties followed by introduction of N-acetylhomocysteiny hydrazide to yield multiple hydrazides per protein. Aldehyde incorporation was achieved by reaction with NHS-activated *p*-carboxybenzaldehyde groups. The functionalized proteins were subsequently mixed at acidic pH to yield the desired oligomers. Interestingly, presence of multiple hydrazone bonds per conjugate led to a higher than expected stability of the conjugation. Hydrazones were later used in protein chemistry to construct chimeras with defined, site-specific linkages. Proteins of interest were enzymatically cleaved at a Lys-Ser site to produce 2 fragments; one fragment could be oxidized to generate an N-terminal aldehyde whereas a C-terminal hydrazide was introduced in the other fragment using reversed proteolysis. (4) Fragments of different proteins were next combined to yield novel proteins which can be tuned to have desired properties. In both of the above described examples reduction with NaBH_3CN was used to stabilize the formed hydrazones.

As mentioned above, hydrazones are less useful for labeling purposes because of their decreased stability. However, they are continued to be used to attach various payloads to peptides and proteins. Researchers at Pfizer early on recognized the viability of hydrazone bonds to introduce biotin or fluorescent labels to unprotected peptides, but also report on

the lability of the bond especially at lower pH. (5) Hydrazone labeling was later used to introduce various fluorescent labels to proteins and even oligonucleotides. (22, 24, 108) Interestingly, hydrazone formation with fluorescent labels can also be used for live-cell imaging. (29) In addition, hydrazone formation and subsequent reduction to the corresponding hydrazide was used to synthesize a library of peptides incorporating an N-substituted glycine residues. (116)

An advantage of the hydrazone over an oxime is found in dynamic covalent chemistry. The lability of the bond has been exploited for various applications. Cytotoxic agent doxorubicin was ligated to a dendrimer system via a pH-sensitive hydrazone bond. Subsequent internalization of the dendrimer leads to the release of the drug. (122) In a comparable approach doxorubicin was ligated to the block copolymers that build up a micelle via hydrazone bonds. Again, a decrease in pH upon internalization would lead to release of the cytotoxic agent. (123) The reversibility and transimination properties of the hydrazone bond have furthermore been utilized to construct dynamic covalent libraries. (124) Pseudo-peptide building blocks, incorporating both a hydrazide and an aldehyde, were mixed in the presence of an acid catalyst (Scheme 15). A large library of macrocyclic species of different sizes could be generated. These libraries were subsequently used to find new cyclic pseudopeptide receptors for small substrates such Li^+ ions or ammonium salts by adding these compounds as templates. (125, 126) Interestingly, upon addition of acetylcholine this led to formation of a [2]-catenane receptor for acetylcholine. (127)

Scheme 15, Construction of dynamic covalent libraries. Pseudo-peptide building blocks were functionalized with a hydrazide and protected aldehyde functionality. Macrocyclic species of up to 6 of these building blocks ($n=2$ shown here) were detected upon deprotection. Addition of a ligand (i.e. acetylcholine) can lead to selective populations to be formed.

Pictet Spengler

The last imine-type reaction to be discussed in this chapter is the Pictet-Spengler reaction. This reaction was identified in 1911 by Ame Pictet and Theodor Spengler upon heating a mixture of dimethoxymethane and β -phenylethylamine in the presence of hydrochloric acid (Scheme 16A). The resulting tetrahydroisoquinoline opened a new route to the synthesis of a large variety of heterocyclic compounds. Almost 20 years later the amine component was substituted for the electron-rich tryptamine to yield tetrahydro- β -carbolines (Scheme 16B).

Scheme 16, General Pictet-Spengler reaction scheme. A) Reaction of β -phenylethylamine with protected formaldehyde to yield a tetrahydroisoquinoline via an imine intermediate product. B) Reaction of tryptamine with acetaldehyde to yield a tetrahydro- β -carboline via an imine intermediate product

The Pictet-Spengler reaction has become an extremely successful synthetic strategy for the synthesis of isoquinolines and indole alkaloids. (128) In contrast to the earlier discussed oximes and hydrazones, the final product of a Pictet-Spengler reaction does not contain a hydrolysable C=N double bond but instead an intramolecular reaction, after initial imine formation, results in a stable carbon-carbon linkage. Protein conjugates that require long-term stability, especially under dilute conditions greatly benefit from this increased stability. In protein chemistry the Pictet-Spengler reaction was first used to *N*-terminally label myoglobin using modified tryptamine analogues. (129) However, the required high concentrations of both protein and label combined with long reaction times (18h) may not be suitable for every protein target. Bertozzi and coworkers developed a Pictet-Spengler ligation analogous to the Pictet-Spengler reaction but replaced the amine functionality by a *N*-methylated aminooxy moiety and placed this at the 2-position of the aromatic system. (130) In this manner the more nucleophilic 3 position can be utilized for ring closing. This resulted in a rate enhancement of 4-5 orders of magnitude as compared to the canonical Pictet-

Spengler ligation. Subsequently, this Pictet-Spengler ligation was used to modify various proteins and an antibody with biotin or fluorescent moieties (Scheme 17A). In addition, a hydrazine-based reaction was developed analogous to this reaction that proved to be more suitable for conjugation at near neutral pH (Scheme 17B). (131)

Scheme 17, Developed Pictet-Spengler ligation strategies. A) Modified tryptamine substrate incorporating a N-methyl aminoxy functionality at the 2-position reacts with an aldehyde functionalized protein via a oxime intermediate. B) Analogous to previous reaction a methylated hydrazide substrate reacts with an aldehyde protein via a hydrazone intermediate

Catalysis of oxime and hydrazone ligations

As discussed in the sections above, imine-type reactions can be used to modify a large variety of biomolecules. However, conjugation to peptides or proteins is often limited by their low availability and/or solubility leading to slow reactions. In addition, conjugations usually require (aqueous) physiological conditions in order to protect protein integrity. To overcome these challenges, methods have been developed to catalyze oxime and hydrazone reactions. The following section will discuss these methods.

In early studies, Jencks et al. studied semicarbazone formation by general acid catalysis and showed that multiple acids could catalyze the reaction and that this could be predicted based on their pKa value. (132) Remarkably, it was found that aniline, a weak base, and derivatives thereof accelerated the reaction 10-1000 times more effectively than their pKa predicted. Underlying mechanistic studies showed that the rate enhancement was due to a condensation reaction of aniline with the carbonyl, forming an aniline Schiff base. (133)

Subsequent transimination of this aniline Schiff base with the desired semicarbazide occurred rapidly under acidic aqueous conditions (Scheme 18).

Scheme 18, General scheme for the nucleophilic catalysis of oxime or hydrazone reactions

Observations on rate enhancement by nucleophilic catalysis with semicarbazone formation suggested that this approach could also be useful for oxime and hydrazone ligations. Dawson et al. studied the effect of aniline on the reaction rate of oxime and hydrazone ligations, using model peptides at a concentration of 0.1 mM, since the concentration of biological macromolecules is often limited to this concentration. (114, 134) It was shown that oxime formation was increased 30-fold by the addition of 10 mM aniline and 400-fold by the addition of 100 mM aniline at pH 4.5.

While oxime ligations can be greatly enhanced at decreased pH, some proteins are only stable under more physiological pH. Oxime ligations at pH 7 are known to be very slow with reaction half-times of more than 7 days. Addition of aniline derivative *p*-methoxyaniline (**1**, selected because of the elevated pKa of 5.3), drastically decreased the reaction half-time to 280 minutes, enabling oxime ligation to proteins at physiological pH. (134)

In line with the positive results obtained with *p*-methoxyaniline, multiple groups have subsequently optimized oxime and hydrazone nucleophilic catalysis with aniline derived catalysts (Figure 6). Efforts were made to increase reaction rate, increase the water solubility, and decrease cell toxicity of the catalysts. First, use of phenylenediamines increased the reaction rate, however care had to be taken with regards to the position of the additional amine. Meta- and para substituted phenylenediamines (**2**, **3**) led to an increase in reaction rate as compared to aniline whereas ortho-phenylenediamine showed a decrease in catalytic activity. (42, 135, 136) A drawback of the use of phenylenediamines is its oxidative instability, which results in conversion to benzoquinones or poly-phenylenediamines. Next, owing to the poor water solubility of aniline, efforts were made to find derivatives with increased water solubility. In addition to its increased reaction rate it was found that *m*-phenylenediamine had

an increased water solubility of up to 900 mM. Novel water-soluble catalysts were found in amino benzoic acids. More specifically, 5-methoxy anthranilic acid (**4**) and 3,5 diamino benzoic acid (**5**) were found to show increased catalytic activity as compared to aniline especially at neutral pH. (137) Interestingly, the amphoteric nature of anthranilic acids is also postulated to increase reaction rate by a general acid catalysis mechanism. A proton donor in the ortho position could assist in transferring a proton to the imine transition state with subsequent elimination of water. This aspect was further investigated by varying the pKa of the proton donor and found 4-methyl-2-phosphoaniline (**6**) to be a superior catalyst. (138) A drawback of these ortho substituted anilines is that these are less efficient in reactions with bulky ketones, most likely due to steric hindrance. Alternatives, that are less susceptible to this disadvantage, were found in 2-amino-4-nitrophenol (**7**) and 2-(aminomethyl) benzimidazole (**8**). (139) Finally, the biocompatibility of the catalysts was addressed since some proteins and/or cells do not tolerate high concentrations of aniline. 4-aminophenylalanine (**9**) was used to catalyze reactions to an unstable protein where it was found to show decreased toxicity to human cells. (140, 141)

Figure 6, Aniline and derivatives used for oxime and hydrazone nucleophilic catalysis

In addition to aniline-based nucleophilic catalysis to enhance reaction rates of oxime and hydrazone formation, an alternative, catalyst-free method for reaction rate acceleration was found. Counterintuitively it was discovered that slow freezing of a reaction mixture containing ketone and aminoxy modified peptides would lead to an increased oxime formation. (142) Using a model system, a minor increase in reaction rate compared to aniline catalyzed reactions was observed at pH 4.5. However, at pH 7.0 a 25-fold increase in reaction rate was detected. Using freeze/thaw cycles it proved to be possible to push oxime reactions to completion. It was shown that this does not only work for the peptide model system but could also be used to label a chemokine at a low concentration of 10 μM . The unusual phenomenon of rate increase upon freezing was explained by reactant expulsion and concentration increase in the liquid phase upon ice crystal formation.

References

1. Canne, L.E., et al., *Total Chemical Synthesis of a Unique Transcription Factor-Related Protein - Cmyc-Max*. Journal of the American Chemical Society, 1995. **117**(11): p. 2998-3007.
2. Kochendoerfer, G.G., et al., *Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein*. Science, 2003. **299**(5608): p. 884-7.
3. Agten, S.M., et al., *Probing Functional Heteromeric Chemokine Protein-Protein Interactions through Conformation-Assisted Oxime Ligation*. Angew Chem Int Ed Engl, 2016. **55**(48): p. 14963-14966.
4. Gaertner, H.F., et al., *Construction of protein analogues by site-specific condensation of unprotected fragments*. Bioconjugate chemistry, 1992. **3**(3): p. 262-8.
5. Geoghegan, K.F. and J.G. Stroh, *Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine*. Bioconjugate chemistry, 1992. **3**(2): p. 138-46.
6. Dixon, H.B.F., *N-terminal modification of proteins—a review*. Journal of Protein Chemistry, 1984. **3**(1): p. 99-108.
7. Rose, K., *Facile Synthesis of Homogeneous Artificial Proteins*. Journal of the American Chemical Society, 1994. **116**(1): p. 30-33.
8. Ng, S., et al., *Quantitative synthesis of genetically encoded glycopeptide libraries displayed on M13 phage*. ACS chemical biology, 2012. **7**(9): p. 1482-7.
9. Kitov, P.I., et al., *Rapid, hydrolytically stable modification of aldehyde-terminated proteins and phage libraries*. Journal of the American Chemical Society, 2014. **136**(23): p. 8149-52.
10. Tjhung, K.F., et al., *Silent Encoding of Chemical Post-Translational Modifications in Phage-Displayed Libraries*. Journal of the American Chemical Society, 2016. **138**(1): p. 32-5.
11. Gilmore, J.M., et al., *N-terminal protein modification through a biomimetic transamination reaction*. Angewandte Chemie, 2006. **45**(32): p. 5307-11.
12. Scheck, R.A. and M.B. Francis, *Regioselective labeling of antibodies through N-terminal transamination*. ACS chemical biology, 2007. **2**(4): p. 247-51.
13. Scheck, R.A., et al., *Optimization of a biomimetic transamination reaction*. Journal of the American Chemical Society, 2008. **130**(35): p. 11762-70.
14. Bindman, N.A. and W.A. van der Donk, *A general method for fluorescent labeling of the N-termini of lanthipeptides and its application to visualize their cellular localization*. Journal of the American Chemical Society, 2013. **135**(28): p. 10362-71.
15. Lee, A., L. Huang, and J.A. Ellman, *General solid-phase method for the preparation of mechanism-based cysteine protease inhibitors*. Journal of the American Chemical Society, 1999. **121**(43): p. 9907-9914.
16. Vazquez, J. and F. Albericio, *A convenient semicarbazide resin for the solid-phase synthesis of peptide ketones and aldehydes*. Tetrahedron Letters, 2006. **47**(10): p. 1657-1661.
17. Wood, W.J.L., L. Huang, and J.A. Ellman, *Synthesis of a diverse library of mechanism-based cysteine protease inhibitors*. Journal of Combinatorial Chemistry, 2003. **5**(6): p. 869-880.
18. O'Donnell, M.J., et al., *UPS on Weinreb resin: A facile solid-phase route to aldehyde and ketone derivatives of "unnatural" amino acids and peptides*. Journal of Combinatorial Chemistry, 2000. **2**(2): p. 172-181.
19. El-Mahdi, O. and O. Melnyk, *alpha-Oxo aldehyde or glyoxylyl group chemistry in peptide bioconjugation*. Bioconjugate chemistry, 2013. **24**(5): p. 735-65.
20. Far, S. and O. Melnyk, *Synthesis of glyoxylyl peptides using an Fmoc-protected alpha, alpha'-diaminoacetic acid derivative*. Journal of peptide science : an official publication of the European Peptide Society, 2005. **11**(7): p. 424-30.
21. Schumacher, D., et al., *Versatile and Efficient Site-Specific Protein Functionalization by Tubulin Tyrosine Ligase*. Angewandte Chemie, 2015. **54**(46): p. 13787-91.

22. Banerjee, A., et al., *Site-specific orthogonal labeling of the carboxy terminus of alpha-tubulin*. ACS chemical biology, 2010. **5**(8): p. 777-85.
23. Turek, T.C., et al., *Synthesis of farnesyl diphosphate analogues containing ether-linked photoactive benzophenones and their application in studies of protein prenyltransferases*. The Journal of organic chemistry, 2001. **66**(10): p. 3253-64.
24. Rashidian, M., et al., *Chemoenzymatic reversible immobilization and labeling of proteins without prior purification*. Journal of the American Chemical Society, 2012. **134**(20): p. 8455-67.
25. Baruah, H., et al., *An engineered aryl azide ligase for site-specific mapping of protein-protein interactions through photo-cross-linking*. Angewandte Chemie, 2008. **47**(37): p. 7018-21.
26. Liu, D.S., et al., *Diels-Alder cycloaddition for fluorophore targeting to specific proteins inside living cells*. Journal of the American Chemical Society, 2012. **134**(2): p. 792-5.
27. Uttamapinant, C., et al., *A fluorophore ligase for site-specific protein labeling inside living cells*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(24): p. 10914-9.
28. Fernandez-Suarez, M., et al., *Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes*. Nat Biotechnol, 2007. **25**(12): p. 1483-7.
29. Cohen, J.D., P. Zou, and A.Y. Ting, *Site-specific protein modification using lipoic acid ligase and bis-aryl hydrazone formation*. Chembiochem : a European journal of chemical biology, 2012. **13**(6): p. 888-94.
30. Dierks, T., B. Schmidt, and K. von Figura, *Conversion of cysteine to formylglycine: a protein modification in the endoplasmic reticulum*. Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(22): p. 11963-8.
31. Carrico, I.S., B.L. Carlson, and C.R. Bertozzi, *Introducing genetically encoded aldehydes into proteins*. Nature chemical biology, 2007. **3**(6): p. 321-2.
32. Appel, M.J. and C.R. Bertozzi, *Formylglycine, a Post-Translationally Generated Residue with Unique Catalytic Capabilities and Biotechnology Applications*. Acs Chemical Biology, 2015. **10**(1): p. 72-84.
33. Wang, L., J. Xie, and P.G. Schultz, *Expanding the genetic code*. Annual review of biophysics and biomolecular structure, 2006. **35**: p. 225-49.
34. Wang, L., J. Xie, and P.G. Schultz, *Expanding the genetic code*. Annu Rev Biophys Biomol Struct, 2006. **35**: p. 225-49.
35. Chin, J.W., *Expanding and reprogramming the genetic code*. Nature, 2017. **550**(7674): p. 53-60.
36. Liu, C.C. and P.G. Schultz, *Adding new chemistries to the genetic code*. Annu Rev Biochem, 2010. **79**: p. 413-44.
37. Wang, L., et al., *Addition of the keto functional group to the genetic code of Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(1): p. 56-61.
38. Chin, J.W., et al., *An expanded eukaryotic genetic code*. Science, 2003. **301**(5635): p. 964-7.
39. Zhang, Z., et al., *A new strategy for the site-specific modification of proteins in vivo*. Biochemistry, 2003. **42**(22): p. 6735-46.
40. Fleissner, M.R., et al., *Site-directed spin labeling of a genetically encoded unnatural amino acid*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(51): p. 21637-42.
41. Kim, C.H., et al., *Synthesis of bispecific antibodies using genetically encoded unnatural amino acids*. Journal of the American Chemical Society, 2012. **134**(24): p. 9918-21.
42. Rashidian, M., et al., *A highly efficient catalyst for oxime ligation and hydrazone-oxime exchange suitable for bioconjugation*. Bioconjugate chemistry, 2013. **24**(3): p. 333-42.

43. Agten, S.M., et al., *Chemoselective oxime reactions in proteins and peptides by using an optimized oxime strategy: the demise of levulinic acid*. *Chembiochem : a European journal of chemical biology*, 2013. **14**(18): p. 2431-4.
44. Zeng, H., J. Xie, and P.G. Schultz, *Genetic introduction of a diketone-containing amino acid into proteins*. *Bioorganic & medicinal chemistry letters*, 2006. **16**(20): p. 5356-9.
45. Baldwin, J.E., et al., *Proline 4-Hydroxylase - Stereochemical Course of the Reaction*. *Tetrahedron Letters*, 1993. **34**(46): p. 7489-7492.
46. Choudhary, A., et al., *4-ketoproline: An electrophilic proline analog for bioconjugation*. *Biopolymers*, 2015. **104**(2): p. 110-5.
47. Haney, C.M., M.T. Loch, and W.S. Horne, *Promoting peptide alpha-helix formation with dynamic covalent oxime side-chain cross-links*. *Chem Commun (Camb)*, 2011. **47**(39): p. 10915-7.
48. Lempens, E.H., et al., *Dendrimer display of tumor-homing peptides*. *Bioconjugate chemistry*, 2011. **22**(3): p. 397-405.
49. Roberts, K.D., et al., *Preparation of cyclic peptide libraries using intramolecular oxime formation*. *Journal of peptide science : an official publication of the European Peptide Society*, 2004. **10**(11): p. 659-65.
50. Sohma, Y. and S.B. Kent, *Biomimetic synthesis of lispro insulin via a chemically synthesized "mini-proinsulin" prepared by oxime-forming ligation*. *Journal of the American Chemical Society*, 2009. **131**(44): p. 16313-8.
51. Lau, Y.H., et al., *Peptide stapling techniques based on different macrocyclisation chemistries*. *Chem Soc Rev*, 2015. **44**(1): p. 91-102.
52. Assem, N., et al., *Acetone-Linked Peptides: A Convergent Approach for Peptide Macrocyclization and Labeling*. *Angewandte Chemie*, 2015. **54**(30): p. 8665-8.
53. Bondalapati, S., et al., *Rapid End-Group Modification of Polysaccharides for Biomaterial Applications in Regenerative Medicine*. *Macromolecular rapid communications*, 2014. **35**(20): p. 1754-1762.
54. Thygesen, M.B., et al., *Nucleophilic catalysis of carbohydrate oxime formation by anilines*. *The Journal of organic chemistry*, 2010. **75**(5): p. 1752-5.
55. Peri, F., P. Dumy, and M. Mutter, *Chemo- and stereoselective glycosylation of hydroxylamino derivatives: A versatile approach to glycoconjugates*. *Tetrahedron*, 1998. **54**(40): p. 12269-12278.
56. Rodriguez, E.C., et al., *A strategy for the chemoselective synthesis of O-linked glycopeptides with native sugar-peptide linkages*. *Journal of the American Chemical Society*, 1997. **119**(41): p. 9905-9906.
57. Jourdan, G.W., L. Dean, and S. Roseman, *The sialic acids. XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides*. *The Journal of biological chemistry*, 1971. **246**(2): p. 430-5.
58. Bhavanandan, V.P., M. Murray, and E.A. Davidson, *Modification and Introduction of Various Radioactive Labels into the Sialic-Acid Moiety of Sialoglycoconjugates*. *Glycoconjugate Journal*, 1988. **5**(4): p. 467-484.
59. Gahmberg, C.G. and L.C. Andersson, *Selective radioactive labeling of cell surface sialoglycoproteins by periodate-tritiated borohydride*. *The Journal of biological chemistry*, 1977. **252**(16): p. 5888-94.
60. Dhal, P.K., et al., *Well-defined aminoxy terminated N-(2-hydroxypropyl) methacrylamide macromers for site specific bioconjugation of glycoproteins*. *Bioconjugate chemistry*, 2013. **24**(6): p. 865-77.
61. Zeng, Y., et al., *High-efficiency labeling of sialylated glycoproteins on living cells*. *Nature methods*, 2009. **6**(3): p. 207-9.
62. Mahal, L.K., K.J. Yarema, and C.R. Bertozzi, *Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis*. *Science*, 1997. **276**(5315): p. 1125-8.

63. Decostaire, I.P., et al., *Controlling the outcome of overacylation of N-protected aminooxyacetic acid during the synthesis of an aminooxy-peptide for chemical ligation*. Tetrahedron Letters, 2006. **47**(39): p. 7057-7060.
64. Foillard, S., et al., *1-ethoxyethylidene, a new group for the stepwise SPPS of aminooxyacetic acid containing peptides*. Journal of Organic Chemistry, 2008. **73**(3): p. 983-991.
65. Jimenez-Castells, C., et al., *Optimized synthesis of aminooxy-peptides as glycoprobe precursors for surface-based sugar-protein interaction studies*. Bioorg Med Chem Lett, 2007. **17**(18): p. 5155-8.
66. Zheng, J.S., et al., *Chemical synthesis of proteins using peptide hydrazides as thioester surrogates*. Nat Protoc, 2013. **8**(12): p. 2483-95.
67. Chelushkin, P.S., et al., *Convenient method of peptide hydrazide synthesis using a new hydrazone resin*. Tetrahedron Letters, 2015. **56**(4): p. 619-622.
68. Qvortrup, K., V.V. Komnatnyy, and T.E. Nielsen, *A Photolabile Linker for the Solid-Phase Synthesis of Peptide Hydrazides and Heterocycles*. Organic Letters, 2014. **16**(18): p. 4782-4785.
69. Bello, C., F. Kikul, and C.F.W. Becker, *Efficient generation of peptide hydrazides via direct hydrazinolysis of Peptidyl-Wang-TentaGel resins*. Journal of Peptide Science, 2015. **21**(3): p. 201-207.
70. Adams, A.L., et al., *Cysteine promoted C-terminal hydrazinolysis of native peptides and proteins*. Angew Chem Int Ed Engl, 2013. **52**(49): p. 13062-6.
71. Haney, C.M. and W.S. Horne, *Dynamic covalent side-chain cross-links via intermolecular oxime or hydrazone formation from bifunctional peptides and simple organic linkers*. J Pept Sci, 2014. **20**(2): p. 108-14.
72. Itoh, Y., et al., *Identification of SNAIL1 Peptide-Based Irreversible Lysine-Specific Demethylase 1-Selective Inactivators*. J Med Chem, 2016. **59**(4): p. 1531-44.
73. Oh, J.E. and K.H. Lee, *Synthesis of novel unnatural amino acid as a building block and its incorporation into an antimicrobial peptide*. Bioorg Med Chem, 1999. **7**(12): p. 2985-90.
74. Peluso, S. and B. Imperiali, *Asparagine surrogates for the assembly of N-linked glycopeptide mimetics by chemoselective ligation*. Tetrahedron Letters, 2001. **42**(11): p. 2085-2087.
75. Cordes, E.H. and W.P. Jencks, *On the Mechanism of Schiff Base Formation and Hydrolysis*. Journal of the American Chemical Society, 1962. **84**(5): p. 832-837.
76. Kalia, J. and R.T. Raines, *Hydrolytic stability of hydrazones and oximes*. Angewandte Chemie, 2008. **47**(39): p. 7523-6.
77. Borgia, J.A. and G.B. Fields, *Chemical synthesis of proteins*. Trends Biotechnol, 2000. **18**(6): p. 243-51.
78. Kent, S.B., *Total chemical synthesis of proteins*. Chem Soc Rev, 2009. **38**(2): p. 338-51.
79. Shao, J. and J.P. Tam, *Unprotected Peptides as Building-Blocks for the Synthesis of Peptide Dendrimers with Oxime, Hydrazone, and Thiazolidine Linkages*. Journal of the American Chemical Society, 1995. **117**(14): p. 3893-3899.
80. Shao, H., et al., *Site-specific polymer attachment to a CCL-5 (RANTES) analogue by oxime exchange*. J Am Chem Soc, 2005. **127**(5): p. 1350-1.
81. Hang, H.C. and C.R. Bertozzi, *Chemoselective approaches to glycoprotein assembly*. Accounts of Chemical Research, 2001. **34**(9): p. 727-736.
82. Kiessling, L.L. and R.A. Splain, *Chemical Approaches to Glycobiology*. Annual Review of Biochemistry, Vol 79, 2010. **79**: p. 619-653.
83. Marcaurelle, L.A., E.C. Rodriguez, and C.R. Bertozzi, *Synthesis of an oxime-linked neoglycopeptide with glycosylation-dependent activity similar to its native counterpart*. Tetrahedron Letters, 1998. **39**(46): p. 8417-8420.
84. Langenhan, J.M., et al., *Enhancing the anticancer properties of cardiac glycosides by neoglycorandomization*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(35): p. 12305-12310.

85. McBride, W.J., et al., *A novel method of ^{18}F radiolabeling for PET*. J Nucl Med, 2009. **50**(6): p. 991-8.
86. Schirmacher, R., et al., *F-18-labeling of peptides by means of an organosilicon-based fluoride acceptor*. Angewandte Chemie-International Edition, 2006. **45**(36): p. 6047-6050.
87. Li, X.-G., M. Haaparanta, and O. Solin, *Oxime formation for fluorine-18 labeling of peptides and proteins for positron emission tomography (PET) imaging: A review*. Journal of Fluorine Chemistry, 2012. **143**: p. 49-56.
88. Poethko, T., et al., *Two-step methodology for high-yield routine radiohalogenation of peptides: (^{18}F)-labeled RGD and octreotide analogs*. J Nucl Med, 2004. **45**(5): p. 892-902.
89. Battle, M.R., et al., *Monitoring tumor response to antiangiogenic sunitinib therapy with ^{18}F -fluciclatide, an ^{18}F -labeled $\alpha\text{V}\beta_3$ -integrin and $\alpha\text{V}\beta_5$ -integrin imaging agent*. J Nucl Med, 2011. **52**(3): p. 424-30.
90. Glaser, M., et al., *Radiosynthesis and biodistribution of cyclic RGD peptides conjugated with novel [^{18}F]fluorinated aldehyde-containing prosthetic groups*. Bioconjug Chem, 2008. **19**(4): p. 951-7.
91. Kenny, L.M., et al., *Phase I trial of the positron-emitting Arg-Gly-Asp (RGD) peptide radioligand ^{18}F -AH111585 in breast cancer patients*. J Nucl Med, 2008. **49**(6): p. 879-86.
92. Poethko, T., et al., *Chemoselective pre-conjugate radiohalogenation of unprotected mono- and multimeric peptides via oxime formation*. Radiochimica Acta, 2004. **92**(4-6): p. 317-327.
93. Schottelius, M., et al., *First (^{18}F)-labeled tracer suitable for routine clinical imaging of sst receptor-expressing tumors using positron emission tomography*. Clin Cancer Res, 2004. **10**(11): p. 3593-606.
94. McParland, B.J., et al., *The biodistribution and radiation dosimetry of the Arg-Gly-Asp peptide ^{18}F -AH111585 in healthy volunteers*. J Nucl Med, 2008. **49**(10): p. 1664-7.
95. Mena, E., et al., *[^{18}F]fluciclatide in the in vivo evaluation of human melanoma and renal tumors expressing $\alpha\text{V}\beta_3$ and $\alpha\text{V}\beta_5$ integrins*. Eur J Nucl Med Mol Imaging, 2014. **41**(10): p. 1879-88.
96. Sharma, R., et al., *Multicenter Reproducibility of ^{18}F -Fluciclatide PET Imaging in Subjects with Solid Tumors*. J Nucl Med, 2015. **56**(12): p. 1855-61.
97. Tegler, G., et al., *Autoradiography screening of potential positron emission tomography tracers for asymptomatic abdominal aortic aneurysms*. Ups J Med Sci, 2014. **119**(3): p. 229-35.
98. Jenkins, W.S., et al., *Cardiac $\alpha\text{V}\beta_3$ integrin expression following acute myocardial infarction in humans*. Heart, 2017. **103**(8): p. 607-615.
99. Flavell, R.R., et al., *Site-specific (^{18}F)-labeling of the protein hormone leptin using a general two-step ligation procedure*. J Am Chem Soc, 2008. **130**(28): p. 9106-12.
100. Wuest, F., et al., *Synthesis and application of [^{18}F]FDG-maleimidehexyloxime ([^{18}F]FDG-MHO): a [^{18}F]FDG-based prosthetic group for the chemoselective ^{18}F -labeling of peptides and proteins*. Bioconjug Chem, 2008. **19**(6): p. 1202-10.
101. Namavari, M., et al., *A novel method for direct site-specific radiolabeling of peptides using [^{18}F]FDG*. Bioconjug Chem, 2009. **20**(3): p. 432-6.
102. Hultsch, C., et al., *(^{18}F)-Fluoroglucosylation of peptides, exemplified on cyclo(RGDfK)*. Eur J Nucl Med Mol Imaging, 2009. **36**(9): p. 1469-74.
103. Li, X.G., et al., *[^{18}F]-5-Fluoro-5-deoxyribose, an efficient peptide bioconjugation ligand for positron emission tomography (PET) imaging*. Chemical Communications, 2012. **48**(43): p. 5247-5249.
104. Onega, M., et al., *An enzymatic route to 5-deoxy-5-[^{18}F]fluoro-D-ribose, a [^{18}F]-fluorinated sugar for PET imaging*. Chemical Communications, 2010. **46**(1): p. 139-141.
105. Venkatesan, N. and B.H. Kim, *Peptide conjugates of oligonucleotides: synthesis and applications*. Chem Rev, 2006. **106**(9): p. 3712-61.

106. Juliano, R.L., X. Ming, and O. Nakagawa, *The chemistry and biology of oligonucleotide conjugates*. Acc Chem Res, 2012. **45**(7): p. 1067-76.
107. Forget, D., et al., *Highly efficient synthesis of peptide-oligonucleotide conjugates: chemoselective oxime and thiazolidine formation*. Chemistry, 2001. **7**(18): p. 3976-84.
108. Ollivier, N., et al., *Synthesis of oligonucleotide-peptide conjugates using hydrazone chemical ligation*. Tetrahedron Letters, 2002. **43**(6): p. 997-999.
109. Villien, M., E. Defrancq, and P. Dumy, *Chemoselective oxime and thiazolidine bond formation: a versatile and efficient route to the preparation of 3'-peptide-oligonucleotide conjugates*. Nucleosides Nucleotides Nucleic Acids, 2004. **23**(10): p. 1657-66.
110. Zatsepin, T.S., et al., *Synthesis of peptide-oligonucleotide conjugates with single and multiple peptides attached to 2'-aldehydes through thiazolidine, oxime, and hydrazine linkages*. Bioconjugate Chemistry, 2002. **13**(4): p. 822-830.
111. Meyer, A., et al., *Oligonucleotide sequential bis-conjugation via click-oxime and click-Huisgen procedures*. J Org Chem, 2010. **75**(11): p. 3927-30.
112. Nguyen R, H.I., *Optimizing the reversibility of hydrazone formation for dynamic combinatorial chemistry*. Chem Commun (Camb). 2003. **8**: p. 942-3.
113. Belowich, M.E. and J.F. Stoddart, *Dynamic imine chemistry*. Chemical Society reviews, 2012. **41**(6): p. 2003-24.
114. Dirksen, A., et al., *Nucleophilic catalysis of hydrazone formation and transimination: implications for dynamic covalent chemistry*. Journal of the American Chemical Society, 2006. **128**(49): p. 15602-3.
115. Vanderesse, R., et al., *Alpha-aminoxycarboxylic acids as building blocks for the oxime and hydroxylamine pseudopeptide links. Application to the synthesis of human elastase inhibitors*. J Pept Sci, 2003. **9**(5): p. 282-99.
116. Liu, F., et al., *Hydrazone- and hydrazide-containing N-substituted glycines as peptoid surrogates for expedited library synthesis: application to the preparation of Tsg101-directed HIV-1 budding antagonists*. Org Lett, 2006. **8**(22): p. 5165-8.
117. Polyakov, V.A.N., M. I.; Nazarpak-Kandlousy, N.; Ryabov, A. D.; Eliseev, A. V, *Imine exchange in O-aryl and O-alkyl oximes as a base reaction for aqueous 'dynamic' combinatorial libraries. A kinetic and thermodynamic study*. J. Phys. Org. Chem., 1999. **12**: p. 357-363.
118. Simpson, M.G., et al., *Dynamic combinatorial chemistry with hydrazones: libraries incorporating heterocyclic and steroidal motifs*. Org Biomol Chem, 2010. **8**(5): p. 1181-7.
119. Bouchard, H., C. Viskov, and C. Garcia-Echeverria, *Antibody-drug conjugates-a new wave of cancer drugs*. Bioorg Med Chem Lett, 2014. **24**(23): p. 5357-63.
120. Doronina, S.O., et al., *Development of potent monoclonal antibody auristatin conjugates for cancer therapy*. Nat Biotechnol, 2003. **21**(7): p. 778-84.
121. King, T.P., S.W. Zhao, and T. Lam, *Preparation of protein conjugates via intermolecular hydrazone linkage*. Biochemistry, 1986. **25**(19): p. 5774-9.
122. Lai, P.S., et al., *Doxorubicin delivery by polyamidoamine dendrimer conjugation and photochemical internalization for cancer therapy*. J Control Release, 2007. **122**(1): p. 39-46.
123. Bae, Y., et al., *Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy*. Bioconjug Chem, 2005. **16**(1): p. 122-30.
124. Cousins, G.R.L., S.A. Poulsen, and J.K.M. Sanders, *Dynamic combinatorial libraries of pseudo-peptide hydrazone macrocycles*. Chemical Communications, 1999(16): p. 1575-1576.
125. Cousins, G.R.L., et al., *Identification and Isolation of a Receptor for N-Methyl Alkylammonium Salts: Molecular Amplification in a Pseudo-peptide Dynamic Combinatorial Library*. Angew Chem Int Ed Engl, 2001. **40**(2): p. 423-428.
126. Furlan, R.L., et al., *A new cyclic pseudopeptide receptor for Li+ from a dynamic combinatorial library*. J Am Chem Soc, 2001. **123**(36): p. 8876-7.

127. Lam, R.T., et al., *Amplification of acetylcholine-binding catenanes from dynamic combinatorial libraries*. Science, 2005. **308**(5722): p. 667-9.
128. Stockigt, J., et al., *The Pictet-Spengler reaction in nature and in organic chemistry*. Angew Chem Int Ed Engl, 2011. **50**(37): p. 8538-64.
129. Sasaki, T., et al., *N-terminal labeling of proteins by the Pictet-Spengler reaction*. Bioorg Med Chem Lett, 2008. **18**(16): p. 4550-3.
130. Agarwal, P., et al., *A Pictet-Spengler ligation for protein chemical modification*. Proc Natl Acad Sci U S A, 2013. **110**(1): p. 46-51.
131. Agarwal, P., et al., *Hydrazino-Pictet-Spengler ligation as a biocompatible method for the generation of stable protein conjugates*. Bioconjug Chem, 2013. **24**(6): p. 846-51.
132. Cordes, E.H.J., W. P., *General Acid Catalysis of Semicarbazone Formation*. J. Am. Chem. Soc, 1962. **84**: p. 4319-4328.
133. Cordes, E.H. and W.P. Jencks, *Nucleophilic Catalysis of Semicarbazone Formation by Anilines*. Journal of the American Chemical Society, 1962. **84**(5): p. 826-831.
134. Dirksen, A., T.M. Hackeng, and P.E. Dawson, *Nucleophilic catalysis of oxime ligation*. Angewandte Chemie, 2006. **45**(45): p. 7581-4.
135. Mahmoodi, M.M., et al., *Application of meta- and para-Phenylenediamine as Enhanced Oxime Ligation Catalysts for Protein Labeling, PEGylation, Immobilization, and Release*. Curr Protoc Protein Sci, 2015. **79**: p. 15 4 1-28.
136. Wendeler, M., et al., *Enhanced catalysis of oxime-based bioconjugations by substituted anilines*. Bioconjugate chemistry, 2014. **25**(1): p. 93-101.
137. Crisalli, P. and E.T. Kool, *Water-soluble organocatalysts for hydrazone and oxime formation*. The Journal of organic chemistry, 2013. **78**(3): p. 1184-9.
138. Crisalli, P. and E.T. Kool, *Importance of ortho proton donors in catalysis of hydrazone formation*. Org Lett, 2013. **15**(7): p. 1646-9.
139. Larsen, D., et al., *New organocatalyst scaffolds with high activity in promoting hydrazone and oxime formation at neutral pH*. Org Lett, 2015. **17**(2): p. 274-7.
140. Blanden, A.R., et al., *4-aminophenylalanine as a biocompatible nucleophilic catalyst for hydrazone ligations at low temperature and neutral pH*. Bioconjugate chemistry, 2011. **22**(10): p. 1954-61.
141. Yuen, L.H., et al., *Dark Hydrazone Fluorescence Labeling Agents Enable Imaging of Cellular Aldehydic Load*. Acs Chemical Biology, 2016. **11**(8): p. 2312-2319.
142. Agten, S.M., D.P. Suylen, and T.M. Hackeng, *Oxime Catalysis by Freezing*. Bioconjugate chemistry, 2016. **27**(1): p. 42-6.

Chapter 3

Design and synthesis of a multivalent
catch-and-release assay to measure circulating
FXIa.

Stan H.E. van der Beelen, Stijn M. Agten, Dennis P.L. Suylen,

Kanin Wichapong, Johana Hrdinova, Barend M.E. Mees,

Henri M.H. Spronk, Tilman M. Hackeng.

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Abstract

Background: Decreased blood coagulation factor (F)XIa levels have been shown to protect from thrombosis without bleeding side effects, but less is known on effects of increased FXIa levels. Studies are hampered by lack of a reliable and robust method for FXIa quantification in blood. We aim to develop a new assay employing a unique multivalent catch-and-release system. The system selectively isolates and protects homodimeric FXIa from plasma and releases free FXIa allowing subsequent quantification.

Methods: A dynamic multivalent construct was synthesized by complexing four identical FXIa inhibitors from the snake *Bungarus Faszatus* to avidin through desthiobiotin-PEG-linkers, allowing dissociation of FXIa by excess biotin. PEG-linker lengths were optimised for FXIa inhibitory activity and analysed by Michaelis-Menten kinetics. Finally, the catch-and-release assay was validated in buffer and plasma model systems.

Results: Monovalent and multivalent inhibitor constructs were successfully obtained by total chemical synthesis. Multimerisation of Fasxiator resulted in a 30-fold increase in affinity for FXIa from 1.6 nM to 0.05 nM. With use of this system, FXIa could be quantified down to a concentration of 7 pM in buffer and 20 pM in plasma.

Conclusion: In this proof-of-concept study, we have shown that the catch-and-release approach is a promising technique to quantify FXIa in plasma or buffer. By binding FXIa to the multivalent construct directly after blood drawing, FXIa is inaccessible for serpin inhibition or auto inactivation. This results in a close reflection of actual circulating FXIa levels at the moment of blood drawing.

Keywords: Factor XIa, thrombosis, Catch-and-Release system, FXIa, FXIa quantification.

Introduction

Factor XI (FXI) is a zymogen in the intrinsic pathway of the coagulation cascade and exists as a homodimer in plasma at a concentration of approximately 30 nM.(1) FXI can be activated by factor XIIa or thrombin to form activated FXI (FXIa) which subsequently progresses the coagulation cascade by activation of FIX, ultimately leading to formation of thrombin and the fibrin clot.(2,3) Activation of FXI by thrombin is one of the positive feedback loops in the coagulation cascade, and results in amplification of thrombin generation leading to fibrin formation.(4) FXIa is proposed to be constitutively present in human plasma at low concentrations (<10 pM) as part of the intricate balance between pro- and anticoagulant processes.(5,6)

It has been shown that deviation from normal FXIa levels is clinically relevant, with decreased levels shown to be protective against thrombosis while increased levels may be linked to acute myocardial infarction and acute coronary syndrome. (5–7)

Free FXIa levels are regulated by four natural serine protease inhibitors (serpins: C1-inhibitor, α 1-antitrypsin, antithrombin and α 2-antiplasmin), which irreversibly inactivate FXIa's catalytic domain and thereby complicate FXIa activity quantification.(8)

Currently two methods have been used to quantify FXIa in blood. The first method determines FXIa-inhibitor complex levels by an enzyme-linked immunosorbent assay (ELISA) as an indirect measure of the free FXIa concentration.(8,9) However, as relative levels of serpins are subject to natural variation, all four FXIa-inhibitor complexes should be measured for a full quantification of complexed FXIa in blood. The second method quantifies FXIa by taking advantage of its enzymatic activity using calibrated automated thrombography (CAT).(5,6) However, due to FXIa auto-inactivation and inhibition by serpins, blood samples should be processed and measured as soon as possible with a standardized time between blood drawing and measurements. Ideally, FXIa levels should be “frozen” at the time of blood drawing and analysed when convenient.

The current paper reports a platform that catches free FXIa directly after blood drawing and allows quantification independent from FXIa inhibition or auto-inactivation. We aim to quantify FXIa using a multivalent catch-and-release system (Figure 1). The assay is based on a small Kunitz-type inhibitor (Fasxiator) produced by the snake *Bungarus fasciatus*, which is proven to be selective for FXIa with an affinity of 1 nM.(10) We hypothesise that binding

affinity can be further increased by multimerisation of Fasxiator and utilising the fact that FXIa is a homodimer. In this proof-of-concept study, we use a multivalent system to isolate FXIa from plasma, followed by disruption of the multivalent system allowing dissociation of FXIa from the inhibitor. Finally, the enzymatic activity of free FXIa can be measured by chromogenic substrate measurements. Binding of FXIa in plasma to the multivalent construct protects the enzyme from auto-inactivation and inhibition by serpins. The FXIa measurements from the current approach will therefore reflect as close as possible the actual FXIa levels in the circulation.

Figure 1, Schematic FXIa catch-and-release assay. Desthiobiotin-PEG-Fasxiator is incubated with avidin (4:1 mol:mol) (A) resulting in a tetrameric protein containing 4 Fasxiator inhibitors (B). Addition of tetramer to a FXIa-containing sample will result in multivalent binding of FXIa (C). Finally, an excess of biotin will disrupt the multivalent construct (D) and release FXIa and loss of Fasxiator-binding due to loss of multivalency (E).

Material and Methods

Peptide synthesis of Fasxiator fragments

Solid phase peptide synthesis (0.25 mmol) of Fasxiator fragments was performed manually with *tert*-butoxycarbonyl (Boc) chemistry on PAM resin preloaded with the first amino acid (0.56 mmol/g) essentially as described (Table 1).(11) Each synthetic cycle consisted of the removal of the Boc protecting group by trifluoroacetic acid (2x 1 mL for 1 minute) followed by addition of a preactivated amino acid (1.1 mmol). Preactivation of the amino acid (1.1 mmol) was done for 2 minutes with 1 mmol HCTU, 3 mmol *i*Pr₂NEt in 2 ml DMF. Each step

was followed by a DMF wash. After chain assembly, a Boc-PEG_{xx}-COOH (x = 4, 11 or 24)(Broadpharm, San Diego) and desthiobiotin were coupled via similar protocol. After completion of the peptide chain, peptides were cleaved from resin using anhydrous HF, isolated using RP-HPLC-purification, lyophilized, and stored at -20 °C. All final products were analysed by UPLC and mass spectrometry.

Table 1: Peptide sequences of Fasxiator fragments

	Sequence			
N-term	1	11	21	31
desthiobiotin- PEG _n - Fasxiator:	Db - [PEG] _n -KNRPTFCNLL PETGRCRAEI PAFYYNSHLR K-MPAL			
C-term Fasxiator:	41	51	61	
	NH ₂ -CQKFNYGG CGGNANNFKT IDECQRTCAA KYG-COOH			

[PEG]_n: n = 4, 11 or 24.); Db: Desthiobiotin; MPAL: mercaptopropionic acid leucine

Peptide fragment ligation using native chemical ligation

Equimolar amounts C- and N-terminal fragments of Fasxiator were dissolved at a concentration of 2.5 mM each in 0.1 M Tris pH 8, containing 6 M guanidine.HCl. After addition of benzylmercaptan (2 vol%) and thiophenol (2 vol%) the mixture was left to react at 37 °C for 18 hours with periodic mixing. (12,13) Ligation products were isolated by RP-HPLC-purification, lyophilized, and stored at -20 °C. All final products were analysed by UPLC and mass spectrometry.

Oxidative folding of Fasxiator

The purified ligation product was dissolved (50 µM) in 0.1 M Tris pH 8, containing 1 M guanidine.HCl. After addition of 2 mM reduced glutathione and 0.2 mM oxidized glutathione, the peptide was left to fold at 4 °C with continuous stirring for 24h. Subsequent HPLC-purification of the reaction mixture resulted in the isolation of the folded product. Finally, the pure fractions were pooled and lyophilised. All final products were analysed by UPLC and mass spectrometry.

Purification of peptides using high-performance liquid chromatography

Purification of the peptides was performed on a Waters delta prep system (Waters Prep LC controller, Waters 2487 dual absorbance wavelength detector). Crude peptides were loaded on Vydac C18 columns (10 mm x 250 mm, 12 mL/min flow rate or 22 mm x 250 mm, 20 mL/min flow rate) and separated using a linear gradient of B (90% acetonitrile, 10% H₂O, 0.1% trifluoroacetic acid) in A (100% H₂O, 0.1% trifluoroacetic acid). Elution of the peptides from the column was followed on UV (λ =214nm).

Inhibitor analysis using Michaelis-Menten kinetics

Substrate (Biophen CS-21(66)) was titrated into fixed concentrations of FXIa (100 pM) and Fasxiator (2 nM; 1 nM; 0.5 nM) in Cuvette buffer (50 mM Tris pH 8.0, 175 mM NaCl, 20 mM EDTA, 0.5 mg/mL Ovalbumin) at 37 °C. To evaluate the effect of tetramerisation, avidin was added to the well in a molar ratio of 4:1 (Fasxiator:avidin). Chromogenic substrate conversion was measured (405 nm) with periodic mixing at 37 °C for 1 hour using a plate reader (Biotek EL808). Michaelis-Menten curves were obtained by plotting the slope of the curve (Vs) against the concentration of substrate. Finally, Lineweaver-Burke plots were obtained by reciprocal conversion of the Michaelis-Menten curves.

Dissociation of FXIa from tetrameric inhibitor complex

Desthiobiotin-PEG11-Fasxiator was complexed with avidin to form a tetrameric complex. 10 nM avidin was incubated with 40 nM desbiotin-PEG11-Fasxiator for 30 minutes at room temperature in TN-buffer (50 mM Tris pH 8.0, 175 mM NaCl). Subsequently, 1 nM of construct and 0.5 mM chromogenic substrate (Biophen CS-21(66)) were loaded in Cuvette buffer on a 96-well plate. Finally, 400 pM FXIa was added to wells and substrate conversion was measured at 405 nm (Biotek EL808). After 30 minutes, 100 μ M biotin (stock 1 mM in H₂O) or an equal amount of H₂O was added to all wells and the measurement was continued for an additional 90 minutes.

Depletion of biotin from plasma

Pooled normal citrated plasma was depleted from biotin by addition of 20 μ L streptavidin-coated beads (MACS, streptavidin microbeads,) in 1 mL plasma for 30 minutes at room

temperature. Subsequently, plasma was centrifuged (10,000 rpm, 10 min, 4 °C) to separate beads from plasma.

Catch-and-release assay

First, avidin was incubated with desthiobiotin-PEG11-Fasxiator in TN-buffer in a 1:3 ratio (avidin: Fasxiator) at room temperature for 30 minutes. Next, biotin-depleted plasma or TN-buffer was spiked with a known concentration of FXIa and subsequently the multivalent construct was added and incubated for 60 minutes at room temperature. Samples (200 µL) were then loaded on a biotin-coated 96-well plate (Thermo Scientific), incubated for 30 minutes at room temperature, followed by a wash with H₂O. Finally, 200 µL Cuvette buffer was added to the well containing 100 µM biotin and 0.5 mM chromogenic substrate (Biophen CS-21(66)). The readout (405 nm) was performed in a plate-reader (Biotek El808) for 120 minutes at 37 °C with periodic mixing. Subsequently, the Limit of detection (LoD) was calculated using the limit of blank (LoB) and formulas i and ii, respectively, with sensitivity calculated from $\Delta\text{Abs}_{405}/\Delta [\text{FXIa}]$.(14)

$$\text{LoB} = \text{Mean blank} + 1.645 * \text{standard deviation of the blank} \quad i$$

$$\text{LoD} = \frac{\text{LoB} + 1.645 * \text{standard deviation of lowest concentration sample}}{\text{Sensitivity}} \quad ii$$

Protein modelling and calculations

The dimeric conformation of FXIa, based on the crystal structure of monomeric FXIa (:5I25), was generated after protein-protein docking using HADDOCK webserver.(15,16) The binding pose with the lowest HADDOCK score after molecular dynamics simulations, implying the most reliable conformation, was selected as the representative dimeric conformation of FXIa. Lengths of multivalent avidin-PEG-Fasxiator constructs were calculated in Pymol using crystal model structures of Fasxiator (PDB: 1JC6), Avidin (PDB: 2AVI) and a PEG-repeat length of 5.4 Å.

Results

The natural FXIa inhibitor Fasxiator was obtained through total chemical synthesis (Figure 2). The inhibitor was synthesized in two parts via Boc solid phase peptide synthesis which were subsequently joined to yield the full-length linear peptide chain using native chemical ligation.⁽¹³⁾ The linear peptide was then folded using oxidative conditions to yield triple disulfide-bonded Fasxiator with an observed monoisotopic mass of 7826.64 corresponding well with the theoretical monoisotopic mass of 7826.55. To enable multimerisation, the inhibitor was *N*-terminally modified with a desthiobiotin moiety by different length polyethylene glycol (PEG) chains (PEG4; PEG11; PEG24). Multivalent constructs were obtained by binding four desthiobiotin-Fasxiator molecules to a single tetrameric avidin (Figure 1).⁽¹⁷⁾ Binding affinity of desthiobiotin for avidin (K_D : 10^{-11} M) allowed displacement of desthiobiotin-PEG-Fasxiator by biotin.⁽¹⁸⁾ Earlier attempts to displace a biotin-conjugated PEG11-Fasxiator variant from avidin (K_D : 10^{-15} M) with excess free biotin were unsuccessful (data not shown).

Figure 2, Total chemical synthesis of Fasxiator. Sequence of Fasxiator (A). Different variants of the *N*-terminal thioester fragment with varying PEG linker lengths ($n = 4; 11; 24$) and the *C*-terminus were synthesised using Boc solid phase peptide synthesis (B). Native Chemical Ligation resulting in reduced Fasxiator polypeptide chain (C). Spontaneous oxidative folding results in the desired product Fasxiator (D). UPLC-trace (E) and QTOF-mass spectrum (F) of final purified desthiobiotin-PEG₁₁-Fasxiator with three disulfide bonds.

Fasxiator binds to the active sites of FXIa's dimeric catalytic domains, which are approximately 135 Å apart as predicted by protein-protein docking (Figure 3A). We determined the total expanded lengths of the avidin desthiobiotin-PEG-Fasxiator complexes to be approximately 80, 140 and 240 Å for the PEG4, PEG11 and PEG24 variants, respectively. To study the binding affinity of the various mono- and multivalent Fasxiator complexes to FXIa, Michaelis-Menten kinetics were performed. Enzyme activity as a function of substrate concentration was measured in the presence of varying concentrations of monomeric or tetrameric inhibitors (Figure 3).

Figure 3, Effect of linker length on FXIa-inhibition by multivalent Fasxiator complexes. Schematic representation of the monomeric and tetrameric Fasxiator constructs showing estimated distance between the two active sites (purple) in FXIa (A). Lineweaver-Burk plots of FXIa-inhibition by monomeric (B) and tetrameric Fasxiator with PEG4 (C), PEG11 (D) and PEG24 (E) linker lengths. Concentrations of inhibitor used were 0 nM ($\triangleleft$), 0.5 nM ($\triangleleft$) and 2 nM ($\bullet$). Averages of triplicates are shown.

A K_i of 1.6 nM was derived for monomeric Fasxiator-FXIa inhibition, in line with previous results.⁽¹⁰⁾ As expected, tetramerisation improved binding affinity of the inhibitor and showed K_i s of 0.08, 0.05 and 0.2 nM for PEG4, PEG11 and PEG24 tetrameric constructs, respectively. All Lineweaver-Burk plots show a pattern of competitive inhibition.

Based on these results we continued with the desthiobiotin-PEG11-Fasxiator construct that showed a 30-fold increase (1.6 to 0.05 nM) in binding affinity. Subsequently, the release of FXIa from this high affinity complex was studied by addition of free excess biotin. The 30-fold

difference in affinity between monovalent and multivalent Fasxiator-FXla interactions will result in optimal dissociation of monomeric Fasxiator from FXla, after release of Fasxiator-FXla from the tetrameric construct by biotin. This concept was first confirmed by monitoring FXla activity by chromogenic substrate conversion in presence of the tetrameric construct and subsequently disrupting the construct by addition of excess biotin. (Figure 4). Displacement of Fasxiator-FXla from the multimeric scaffold, and the resulting decrease in affinity of monomeric Fasxiator for FXla, resulted in release of FXla and increased substrate conversion.

Figure 4, inhibition of 400 pM FXla by 1 nM tetrameric Fasxiator construct (solid line). In duplicate wells, disruption of the tetrameric inhibitor was induced after 30 min by the addition of 100 μ M biotin (arrow, dashed line). Averages of triplicate measurement are shown.

Having demonstrated the “release” part of our concept, we next studied the “catch” principles of FXla isolation. For this purpose, avidin was loaded with 3 copies of desthiobiotin-PEG11-Fasxiator resulting in a single free biotin-binding pocket. Addition of this trimeric construct to FXla-containing solution selectively captured FXla. Subsequently, the single free avidin pocket was utilised to isolate the complex by loading it on biotin-coated plates.

We first tested this hypothesis in a buffer system spiked with a known amount of FXla and addition of a range of concentrations of trimeric Fasxiator construct. To allow maximal dissociation of Fasxiator from FXla, concentrations higher than the K_i for FXla (0.05 nM) were used to ensure maximal binding and, which should result in low enough concentrations of monomeric Fasxiator (~2 nM) after disruption of the multivalent complex (Figure 5).

When concentrations of trimeric inhibitor were lowered, recovery of FXla was increased with an optimum at 2 nM construct (Figure 5A). Under these experimental conditions, a linear

dose-response relationship was found with a calculated limit of detection of 7 pM. (Figure 5B;C).

Figure 5, FXIa catch-and-release assay in buffer model system. Optimisation of the multivalent construct concentration in buffer at 100 pM FXIa (A). Catch-and-release assay with 2 nM multivalent construct, with decreasing concentrations of FXIa (B). Linear regression of catch-and-release assay using 2 nM multivalent construct ($R^2=0.98$) (C). Averages of triplicates $\pm$ SD are shown.

Finally, our catch-and-release assay was validated in human blood plasma. We pre-treated plasma with streptavidin-coated beads as plasma contains naturally occurring biotin (1.6-4.9 nM).(19) After biotin removal, we optimised the concentration of our multivalent inhibitor in the plasma system (Figure 6A). In contrast to the assay performed in a buffer system, the optimal concentration of multivalent construct for FXIa quantification in plasma was found to be 6 nM. Under these conditions also a linear dose-response relation was found, with a calculated limit of detection of 20 pM. (Figure 6B;C).

Figure 6, FXIa catch-and-release assay in plasma model system. Optimisation of the multivalent construct concentration in plasma at 100 pM FXIa (A). Catch-and-release assay with 6 nM multivalent construct, with decreasing concentrations of FXIa (B). Linear regression of catch-and-release assay using 6 nM multivalent construct ($R^2=0.99$) (C). Averages of triplicates $\pm$ SD are shown.

Discussion

In the current study, we chemically synthesized and bioconjugated Fasxiator with different length PEG-desthiobiotinylated linkers (PEG4; PEG11; PEG24). To most effectively inhibit FXIa homodimers an optimal distance between two Fasxiator monomers was calculated to be 140 Å agreeing well with the avidin-(desthiobiotin-PEG11-Fasxiator)₄ construct. Shortening of the linker length (PEG4) resulted in a 1.5-fold decrease in affinity compared to the optimal PEG11-linker. This is most likely explained by the flexible nature of the single disulfide-bonded FXIa dimer that allows the catalytic domains of the FXIa dimer to come in closer proximity. Increasing the linker length (PEG24) resulted in a 4-fold decrease in affinity compared to the PEG11-construct. This could be caused by increased degrees of freedom for the Fasxiator proteins within the PEG24-construct, maintaining multivalent aspects but reducing the avidity of the inhibitors for FXIa.

After dissociation, monomeric Fasxiator remains in solution which obstructs full expression of FXIa activity. Our catch-and-release assay is therefore governed by a delicate balance between high enough concentrations of multivalent construct to effectively capture circulating FXIa and a low enough concentration of free monomeric Fasxiator after disruption of the multivalent construct to allow maximal detection of FXIa activity. Testing different multivalent construct concentrations in buffer and plasma resulted in slightly different optimal concentrations of 2 nM in buffer and 6 nM of multivalent Fasxiator construct in plasma. The difference in concentration was explained by potential non-specific binding of the multivalent construct to plasma proteins or to other enzyme active sites, although the latter is less likely since affinities for other coagulation enzymes were found to be three orders of magnitude lower.⁽¹⁰⁾ An alternative explanation for the lower efficacy of our catch-and-release system in plasma is the loss of FXIa to plasma inhibitors in our model system after addition of FXIa to plasma.⁽⁸⁾ However, in future applications this limitation would not apply to the assay since the multivalent inhibitor construct is envisioned to be present in the blood drawing tube to capture circulating FXIa levels as a net result of *in vivo* generation and inhibition of FXIa. This is in contrast to the addition of FXIa to our plasma model system in which only inhibition, and no generation, of FXIa can take place.

Indirect FXIa measurements by complex-ELISA can be compromised by various reasons. Although the relative distribution of FXIa between the four main inhibitors will not change,

absolute abundance of various inhibitors may vary among individuals.(8) Therefore, to approximate pre-existing FXIa levels, all FXIa-inhibitor complex ELISAs should be performed. In addition, the ratio FXIa/FXIa-complexes at the time of blood drawing may decrease in time, also dependent on storage conditions.

Capturing FXIa through its active sites thereby protecting FXIa from inhibition and FXIa auto-inactivation also results in a more reliable assay compared to the CAT-assay. Similar to the challenge with complex ELISAs, FXIa is inactivated in time, obstructing detection by CAT. To quantify FXIa by CAT, samples should be measured as soon as possible after blood drawing with a standardized time between blood drawing and quantification. However, a side by side comparison between the limit of detection of the CAT-assay and the catch-and-release assay, reveals there is still a 50-fold difference (0.39 pM Vs 20 pM).(5,6) The difference can be explained by the fact that the CAT-assay makes use of the intrinsic route of the coagulation cascade in which multiple serine proteases are involved that amplify the signal.

A current limitation of the catch-and-release assay is the limit of detection of the chromogenic substrate method that does not allow further dilution of the sample after dissociation of the multivalent complex, which could decrease the inhibitory capacity of monomeric Fasxiator. In future work, this could be overcome by implementing the natural amplification cascade using purified zymogens (FIX/FVIII/FX/prothrombin), phospholipids and calcium, which would result in a lower limit of detection and higher sensitivity.

In future optimisation studies, the biotin-mediated release model system, which requires biotin depletion before analysis, will be replaced by an alternative releasable linker allowing addition of the inhibitory construct to the blood collection tube. After optimisation, this assay can be used to measure large patient populations to assess *in vivo* implications of elevated circulating FXIa levels, independent of FXIa-inhibition or auto-inactivation.

References

1. He R, Chen D, He S. Factor XI: Hemostasis, Thrombosis, and Antithrombosis. *Thromb Res.* 2012 May 1;129(5):541–50.
2. Geng Y, Verhamme IM, Smith SB, Sun M, Matafonov A, Cheng Q, et al. The dimeric structure of factor XI and zymogen activation. *Blood.* 2013 May 9;121(19):3962–9.
3. Gailani D, Geng Y, Verhamme I, Sun M, Bajaj SP, Messer A, et al. The mechanism underlying activation of factor IX by factor XIa. *Thromb Res.* 2014 May 1;133:S48–51.
4. Yun TH, Baglia FA, Myles T, Navaneetham D, López JA, Walsh PN, et al. Thrombin Activation of Factor XI on Activated Platelets Requires the Interaction of Factor XI and Platelet Glycoprotein Ib α with Thrombin Anion-binding Exosites I and II, Respectively. *J Biol Chem.* 2003 Nov 28;278(48):48112–9.
5. Loeffen R, Oerle R van, Groot PG de, Waltenberger J, Crijns HJ, Spronk HMH, et al. Increased factor XIa levels in patients with a first acute myocardial infarction: The introduction of a new thrombin generation based factor XIa assay. *Thromb Res.* 2014 Dec;134(6):1328–34.
6. Loeffen R, Oerle R van, Leers MPG, Kragten JA, Crijns H, Spronk HMH, et al. Factor XIa and Thrombin Generation Are Elevated in Patients with Acute Coronary Syndrome and Predict Recurrent Cardiovascular Events. *PLOS ONE.* 2016 Jul 15;11(7):e0158355.
7. Zhang H, Löwenberg EC, Crosby JR, MacLeod AR, Zhao C, Gao D, et al. Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk. *Blood.* 2010 Nov 25;116(22):4684–92.
8. Willemin WA, Minnema M, Meijers JC, Roem D, Eerenberg AJ, Nuijens JH, et al. Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role for C1-inhibitor. *Blood.* 1995 Mar 15;85(6):1517–26.
9. Murakami T, Komiyama Y, Masuda M, Karakawa M, Iwasaka T, Takahashi H. Evaluation of factor XIa-alpha 1-antitrypsin in plasma, a contact phase-activated coagulation factor-inhibitor complex, in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol.* 1995 Aug;15(8):1107–13.
10. Chen W, Carvalho LPD, Chan MY, Kini RM, Kang TS. Fasxiator, a novel factor XIa inhibitor from snake venom, and its site-specific mutagenesis to improve potency and selectivity. *J Thromb Haemost.* 2015;13(2):248–61.
11. Schnölzer M, Alewood P, Jones A, Alewood D, Kent SB. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res.* 1992 Oct;40(3–4):180–93.
12. Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. *Science.* 1994 Nov 4;266(5186):776–9.
13. Hackeng TM, Griffin JH, Dawson PE. Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. *Proc Natl Acad Sci U S A.* 1999 Aug 31;96(18):10068–73.
14. Armbruster DA, Pry T. Limit of Blank, Limit of Detection and Limit of Quantitation. *Clin Biochem Rev.* 2008 Aug;29(Suppl 1):S49–52.
15. van Zundert GCP, Rodrigues JPGLM, Trellet M, Schmitz C, Kastiris PL, Karaca E, et al. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *J Mol Biol.* 2016 Feb 22;428(4):720–5.

16. Wong SS, Østergaard S, Hall G, Li C, Williams PM, Stennicke H, et al. A novel DFP tripeptide motif interacts with the coagulation factor XI apple 2 domain. *Blood*. 2016 Jun 9;127(23):2915–23.
17. Green NM. Avidin. 3. The nature of the biotin-binding site. *Biochem J*. 1963 Dec;89(3):599–609.
18. Green NM, Konieczny L, Toms EJ, Valentine RC. The use of bifunctional biotinyl compounds to determine the arrangement of subunits in avidin. *Biochem J*. 1971 Dec;125(3):781–91.
19. Clevidence BA, Marshall MW, Canary JJ. Biotin levels in plasma and urine of healthy adults consuming physiological doses of biotin. *Nutr Res*. 1988 Oct 1;8(10):1109–18.

List of abbreviations

FXI: Factor XI

FIX: Factor IX

ELISA: Enzyme-linked immunosorbent assay

CAT: Calibrated Automated thrombography.

Boc: *Tert*-butyloxycarbonyl

PAM: 4-(Hydroxymethyl)phenylacetamidomethyl

HCTU: O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate

DMF: Dimethylformamide

PEG: Polyethylene glycol

RP-HPLC: Reverse phase high-performance liquid chromatography

EDTA: Ethylenediaminetetraacetic acid

TN: Tris-sodium chloride

rpm: Rounds per minute

LoB: Limit of Blank

LoD: Limit of Detection

PDB: Protein data bank

K_i: Inhibitory constant

FVIII: Factor VIII

Addendum**Letter: in response to a recent article by van der Beelen et al.**

Shannon Prior, Saulius Butenas

Dear Sir/Madam,

We read with interest the paper of van der Beelen et al. (1) published in the January 18th online issue of Thrombosis Research. However, omissions and unsupported statements within the paper related to existing factor (F)XIa assays in plasma require some comments.

In 2008, a paper was published introducing a simple and sensitive assay for the quantitation of functional FXIa and tissue factor (TF) in plasma from stable angina and stroke patients (2). This assay was based on the response of plasma clotting time to the addition of specific monoclonal inhibitory antibodies to FXIa and TF, respectively, which allowed for the quantitation of endogenous levels of these proteins. The detectability limit in that assay was 10 pM for FXIa and the results of that study showed that the occurrence of FXIa in patient plasma correlated with disease severity. In other studies, this assay was used for the analysis of plasma from patients with cardiovascular diseases (3), chronic obstructive pulmonary disease (4), inflammatory bowel disease (5) and trauma (6).

In an effort to accumulate more data on the effect of FXIa on thrombin generation, we employed anti-FXIa antibody methodology for FXIa quantitation in plasma using a previously described assay (7). This approach provided us with several parameters of thrombin generation that allowed for the quantitation of functional FXIa with an increased sensitivity (detectability limit decreased to 0.25 pM). Once established, this assay was used for the quantitation of FXIa in trauma (8) and burn (9) patients, among others, and the results from these studies also showed a correlation between functional FXIa levels in patient plasma and disease severity. Most recently, this FXIa assay based on thrombin generation was modified to allow for the quantification of FXIa in whole blood (10). Overall, 15 papers were published employing these simple, sensitive and easy-to-use FXIa quantitation assays in plasma and

blood of various patient populations. None of these publications were referenced by Beelen and coauthors (1).

References

1. S.H.E. van der Beelen, S.M. Agten, D.P.L. Suylen, K. Wichapong, J. Hrdinova, E. Mees, H.M.H. Sprong, et al., Design and synthesis of a multivalent catch-and-release assay to measure circulating FXIa, *Thromb. Res.* 200 (2021) 16–22.
2. S. Butenas, A. Undas, M.T. Gissel, K. Szuldrzynski, K. Zmudka, K.G. Mann, Factor Xla and tissue factor activity in patients with coronary artery disease, *Thromb. Haemost.* 99 (2008) 142–149.
3. A. Undas, A. Slowik, M. Gissel, K.G. Mann, S. Butenas, Circulating activated factor XI and active tissue factor as predictors of worse prognosis in patients following ischemic cerebrovascular events, *Thromb. Res.* 128 (5) (2011) e62–e66.
4. M. Jankowski, A. Undas, P. Kaczmarek, S. Butenas, Activated factor XI and tissue factor in chronic obstructive pulmonary disease: links with inflammation and thrombin generation, *Thromb. Res.* 127 (3) (2011) 242–246.
5. A. Undas, D. Owczarek, M. Gissel, K. Salapa, K.G. Mann, S. Butenas, Activated factor XI and tissue factor in inflammatory bowel disease, *Inflamm. Bowel Dis.* 16 (9) (2010) 1447–1448.
6. S.M. Prior, M.S. Park, K.G. Mann, S. Butenas, Endogenous procoagulant activity in trauma patients and its relationship to trauma severity, *TH Open* 3 (1) (2019) e10–e19.
7. H.C. Hemker, P. Giesen, R. Al Dieri, V. Regnault, E. de Smedt, R. Wagenvoort, T. Lecompte, et al., Calibrated automated thrombin generation measurement in clotting plasma, *Pathophysiol. Haemost. Thromb.* 33 (1) (2003) 4–15.
8. S.M. Prior, M.J. Cohen, A.S. Conroy, M.F. Nelson, L.Z. Kornblith, B.M. Howard, S. Butenas, Correlation between factor (F)Xla, FIXa and tissue factor and trauma severity, *J. Trauma Acute Care Surg.* 82 (6) (2017) 1073–1079.
9. J.W. Shupp, S.M. Prior, D.Y. Jo, L.T. Moffatt, K.G. Mann, S. Butenas, Analysis of factor Xla, factor IXa and tissue factor activity in burn patients, *Burns.* 44 (2) (2018) 436–444.
10. S.M. Prior, K.G. Mann, K. Freeman, S. Butenas, Continuous thrombin generation in whole blood: new applications for assessing activators and inhibitors of coagulation, *Anal. Biochem.* 551 (2018) 19–25.

Letter: In response to a recent letter by Prior et al.

S.H.E.van der Beelen^a S.M.Agten^a D.P.L.Suylen^a K.Wichapong^a J.Hrdinova^a B.M.E.Mees^b
H.M.H.Spronk^{ac} T.M.Hackeng^a

^a Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University Medical Centre, Maastricht, the Netherlands

^b Department of Vascular Surgery, Cardiovascular Research Institute Maastricht, Maastricht University Medical Centre, Maastricht, the Netherlands

^c Internal Medicine, Cardiovascular Research Institute Maastricht, Maastricht University Medical Centre, Maastricht, the Netherlands

Dear Sir/Madam,

Within this letter, kindly find below our response to the letter submitted by Prior and Butenas (1). First and foremost, we would like to apologise for raising the suggestion that we had overlooked previous contributions to the development of FXIa assays. We would like to clarify our work and point of view in relation to the comments made by Prior and Butenas.

In their letter Prior and Butenas highlight several studies in which factor XIa (FXIa) and tissue factor (TF) were quantified using a newly developed assay based on prolongation of plasma clotting time upon addition of monoclonal antibodies against FXIa or TF (2). This assay was later improved upon, and the same principle could be used to quantify FXIa in plasma and whole blood *via* a thrombin generation assay (3), (4), (5). These studies have repeatedly shown correlations between procoagulant phenotype and increased FXIa concentration in circulation. We acknowledge the importance of these assays and their clinical impact, which emphasizes the importance of FXIa quantification in general.

The sample preparation of these assays, however, is similar to studies done by Loeffen et al., and is therefore susceptible to time-dependent FXIa-inactivation (6), (7). Stability experiments of FXIa in plasma showed a significant decrease in activity after 30 min (11%), up to 38% after 120 min (6). The instability of FXIa in plasma requires swift processing and analysis of samples for accuracy. This makes quantification of a large group of patients (>1000) logistically challenging. The main focus of our proof-of-concept study is the development of an FXIa-assay that is independent of serpin inhibition and auto-inactivation.

In our assay, a multivalent inhibitor is used to bind, protect and isolate FXIa from fresh blood samples (8).

Ultimately, optimization of this assay will allow direct addition of the multivalent construct to the blood collection tube with subsequent instantaneous binding and protection of FXIa from serpin inhibition and auto-inactivation during blood drawing. In the end a FXIa determination procedure is achieved that is independent of blood work-up and storage time.

It is evident that we and Prior and Butenas share similar goals. It is likely that this will result in a better understanding of FXIa and its role in cardiovascular diseases. However, the instability of FXIa in plasma due to serpin-inhibition and auto-inactivation creates the need for a different and more dependable quantification strategy.

References

1. Prior S, Butenas S. Letter: in response to a recent article by van der Beelen et al.1. *Thromb Res*. 2021 Jun 1;202:104.
2. Butenas S, Undas A, Gissel MT, Szuldrzynski K, Zmudka K, Mann KG. Factor XIa and tissue factor activity in patients with coronary artery disease. *Thromb Haemost*. 2008 Jan;99(1):142–9.
3. Prior SM, Cohen MJ, Conroy AS, Nelson MF, Kornblith LZ, Howard BM, et al. Correlation between factor (F)XIa, FIXa and tissue factor and trauma severity. *J Trauma Acute Care Surg*. 2017 Jun;82(6):1073–9.
4. Shupp JW, Prior SM, Jo DY, Moffatt LT, Mann KG, Butenas S. Analysis of factor XIa, factor IXa and tissue factor activity in burn patients. *Burns J Int Soc Burn Inj*. 2018 Mar;44(2):436–44.
5. Prior SM, Mann KG, Freeman K, Butenas S. Continuous thrombin generation in whole blood: New applications for assessing activators and inhibitors of coagulation. *Anal Biochem*. 2018 Jun 15;551:19–25.
6. Loeffen R, Oerle R van, Groot PG de, Waltenberger J, Crijns HJ, Spronk HMH, et al. Increased factor XIa levels in patients with a first acute myocardial infarction: The introduction of a new thrombin generation based factor XIa assay. *Thromb Res*. 2014 Dec;134(6):1328–34.
7. Loeffen R, Oerle R van, Leers MPG, Kragten JA, Crijns H, Spronk HMH, et al. Factor XIa and Thrombin Generation Are Elevated in Patients with Acute Coronary Syndrome and Predict Recurrent Cardiovascular Events. *PLOS ONE*. 2016 Jul 15;11(7):e0158355.
8. van der Beelen SHE, Agten SM, Suylen DPL, Wichapong K, Hrdinova J, Mees BME, et al. Design and synthesis of a multivalent catch-and-release assay to measure circulating FXIa. *Thromb Res*. 2021 Jan 18;200:16–22.

Chapter 4

Evaluation of FXIa quantification assays: a direct
comparison of Limit of Detection and sensitivity

Stan H. E. van der Beelen, Stijn M. Agten, Tilman M. Hackeng

Abstract

Background: Protein quantification assays are import tools to study the role of specific proteins in a disease or cellular process. Recently, elevated concentrations of FXIa have been associated with a thrombotic phenotype. However, quantification of FXIa is still challenging due to its low concentrations, and its inhibition by serpins. In order to find the optimal way to quantify FXIa, we evaluate a chromogenic spectroscopy assay, an established amplification assay, and an in-house set-up ELISA for their sensitivity and Limit of detection (LoD). The in-house set-up ELISA uses a specific FXIa inhibitor from the snake *Bungarus Fasciatus* (Fasxiator) to bind the enzyme to the well, subsequently a HRP-labelled antibody is used for detection.

Methods: Assays were performed either according to the manufacturers instruction (amplification assay), or by an optimized protocol (chromogenic spectroscopy, ELISA). For the ELISA, Fasxiator was synthesized in two parts *via* solid phase peptide synthesis; subsequent native chemical ligation and oxidative folding resulted in the desired product.

Results: Total chemical synthesis of Fasxiator, and the implementation in the ELISA was successful. Side-to-side comparison demonstrated that the ELISA was least sensitive (LoD: 48.9 pM; sensitivity: 0.028 mOD/(min*pM), followed by spectroscopy assay (LoD: 1.8 pM; sensitivity: 0.07 mOD/min*pM), and the amplification assay (LoD: 0.2 pM; sensitivity: 13.5 mOD/min*pM).

Conclusion: Although all assays make use of chromogenic substrate conversion, the use of FXIa intrinsic activity results in the most sensitive assays with the lowest LoD. Additionally, using part of the intrinsic route of the coagulation cascade in the amplification assay, results in a strong increase in sensitivity (200-fold) and an additional increase in LoD (10-fold), compared to chromogenic spectroscopy.

Introduction

Low levels of circulating activated coagulation factor XI (FXIa) have been associated with a decreased thrombosis risk (1) whereas increased levels may elicit the opposite effect.(2,3) Studies into these effects are hampered by the difficulty in quantification of circulating FXIa. Quantification of proteins has long played a central role in the study of biological processes. To date, multiple assay procedures have been developed to measure protein concentrations varying from total protein content to individual protein concentrations. The former mainly make use of colorimetric methods using chemicals that bind to universal structures in proteins (e.g. Bradford; Fowlin lowry), or measure ultra violet light absorption of specific amino acids in a protein (UV-absorption at 280 nm).(4–6) These assays can be used to quantify a specific protein in purified systems, but fail to provide accurate results in more complex environments. To study the effect of a single stimulus (e.g. expression level or zymogen activation) in more complex matrices, individual protein quantification assays can be used, which make use of specific antibodies or substrates for their target protein (e.g. enzyme-linked immunosorbent assay (ELISA); Western-blot; chromogenic substrate conversion; mass spectrometry). (7–9)

These protein quantification assays can be evaluated *via* multiple parameters including: accuracy, sensitivity, specificity, and reproducibility. However, since FXIa levels in blood are estimated to be extremely low (<10pM), our main focus here is to study the sensitivity and limit of detection (LoD).(2,3) In literature, sensitivity and the LoD are often intermixed, while these parameters have a different focus. In this chapter, we refer to sensitivity (Sensitivity = $\Delta\text{signal}/\Delta[\text{FXIa}]$) as the power to distinguish between different FXIa concentrations. (10) The LoD gives a measure for the lowest amount of FXIa an assay can detect, and is calculated via formulae *i* and *ii*. (10)

$$\text{Limit of Blank (LoB)} = \text{Mean blank} + 1.645 * \text{standard deviation of the blank} \quad i$$

$$\text{LoD} = \frac{\text{LoB} + 1.645 * \text{standard deviation of lowest concentration sample}}{\text{Sensitivity}} \quad ii$$

Herein, we evaluate a chromogenic substrate assay, an amplification assay, and an ELISA to quantify FXIa in buffer systems. Both the chromogenic and the amplification assay are

established assays, while the ELISA is an in-house developed assay based on a specific FXIa inhibitor (Fasxiator). Ultimately, we aim to find a method that can either further increase the sensitivity of the previously described catch-and-release assay (chapter 2), or find a different but more sensitive method to quantify FXIa in plasma.

Material and Methods

Peptide synthesis

Solid phase peptide synthesis (0.25 mmol) was performed manually with *tert*-butyloxycarbonyl (Boc) chemistry on PAM resin preloaded with the first amino acid (0.56 mmol/g).⁽¹¹⁾ Each synthetic cycle consisted of the removal of the Boc protecting group by trifluoroacetic acid (2x 1 mL for 1 minute) followed by addition of a preactivated amino acid, PEG or biotin (1.1 mmol). Preactivation of the amino acid (1.1 mmol) was done for 2 minutes with 1 mmol HCTU, 3 mmol *i*Pr₂NEt in 2 ml DMF. Each step was followed by a DMF wash.

Table 1, Peptide sequences of Fasxiator fragments.

	Sequence
N-term biotin- PEG ₁₁ - Fasxiator:	<div>1 11 21 31</div> Biotin-[PEG] ₁₁ -KNRPTFCNLL PETGRCRAEI PAFYYNSHLR K-MPAL
C-term Fasxiator:	<div>41 51 61</div> NH ₂ -CQKFNYGG CGGNANNFKT IDECQRTCAA KYG-COOH

MPAL = 3-MercaptoPropionic Acid-Leucine

Native chemical ligation

Equimolar amounts of C- and N-terminal fragments of Fasxiator were dissolved at a concentration of 2.5 mM each in 0.1 M Tris pH 8, containing 6 M guanidine.HCl. After addition of benzylmercaptan (2 vol%) and thiophenol (2 vol%) the mixture was left to react at 37 °C for 18 hours with periodic mixing. Subsequent HPLC-purification of the reaction mixture resulted in the isolation of the ligation product. Finally, the pure fractions were pooled and lyophilized.

Oxidative folding.

The purified ligation product of Fasxiator was dissolved (50 μ M) in folding buffer (50 mM Tris pH 8, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$ 2 mM $CaCl_2$ 0.5 M arginine, 0.4 M sucrose, 0.75 M guanidine.HCl). After addition of 2 mM reduced glutathione and 0.2 mM oxidized glutathione, the peptide was left to fold at 4 °C with continuous stirring for 24 hours. Subsequent HPLC- purification of the reaction mixture resulted in the isolation of the folded product. Finally, the pure fractions were pooled and lyophilized.

HPLC

Purification of the peptides was performed on a Waters delta prep system (Waters Prep LC controller, Waters 2487 dual absorbance wavelength detector). Crude peptides were loaded on a Vydac C18 column (10 mm x 250 mm, 12 mL/min flow rate or 22 mm x 250 mm, 20 mL/min flow rate) and separated using a linear gradient of B (90% acetonitrile, 10% H_2O , 0.1% trifluoroacetic acid) in A (100% H_2O , 0.1% trifluoroacetic acid). Elution of the peptides was followed via UV ($\lambda=214$ nm).

Chromogenic substrate assay

FXIa was titrated (50 - 0.8 pM) to a fixed amount of chromogenic substrate (0.5 mM chromogenix S-2166) in Cuvette buffer (50 mM Tris-base, 175 mM NaCl, 20 mM EDTA, 0.5 mg/mL Ovalbumin) at 37 °C. Chromogenic substrate conversion was followed on a plate reader (Biotek EL808, $\lambda=405$ nm) with periodic mixing for 1 hour at 37 °C. Data was analyzed by plotting the slope of the raw data (Vs) against the concentration of FXIa.

Amplification assay

FXIa quantification with the Hyphen kit was essentially performed according to manufacturer's instructions. Briefly, 50 μ L of sample (0.03 – 2 pM FXIa in Cuvette buffer), 50 μ L of reagent R1A (FX, FVIII) and 50 μ L of reagent R1B (FIX) were mixed in a 96-well plate and incubated for 2 minutes at 37 °C. Then 50 μ L of reagent R2 (thrombin, phospholipids and calcium) was added to the well, mixed and incubated for 2 minutes at 37 °C. Finally, upon addition of 50 μ L of reagent R3 (Sxa-11 substrate), chromogenic substrate conversion was measured ($\lambda=405$ nm) under repetitive shaking conditions for 30 minutes at 37 °C.

FXIa ELISA

First, a biotin coated 96-well plate (Thermo Scientific, Pierce Biotin coated) was blocked with 3% Bovine serum albumin in H₂O for 2 hours. In the meantime, a multivalent construct was made by incubation of avidin with biotin-PEG₁₁-Fasxiator in Cuvette-buffer in a 1:3 ratio (avidin: Fasxiator) for 30 minutes at room temperature.(12) Subsequently, the multivalent construct was loaded to the wells and incubated for 30 minutes at room temperature. Different construct concentrations (1-90 nM) were tested for their effect on the LoD and sensitivity of the ELISA. Next, Cuvette buffer was spiked with different concentrations of FXIa (0.8-1000 pM). The FXIa-containing samples were then added to the wells, and incubated for 1 hour at room temperature. Subsequently, a goat-anti-FXI antibody was added, followed by a secondary Horseradish peroxidase (HRP) labelled anti-goat-antibody. Both antibodies were incubated for 1 hour at room temperature. For optimization purposes, different concentrations of the antibodies (goat-anti-FXIa 4 µg/ml - 0.25 µg/ml; rabbit-anti-goat 160 ng/ml - 5 ng/ml) were tested during incubation for their effect on the LoD and sensitivity of the ELISA. We found 2 µg/ml primary antibody and 160 ng/ml secondary antibody to be optimal. Each incubation step was followed by extensive washing of the wells with H₂O. Finally, pre-heated HRP substrate (37 °C) was added, and substrate conversion at $\lambda=630$ nm was measured on a plate reader (Biotek EL808) for 60 min at 37 °C.

Results

Three FXIa-quantification methods were evaluated for their LoD and sensitivity (Figure 1).

Figure 1, Three FXIa quantification assays. Chromogenic substrate assay in which FXIa is quantified by conversion of specific FXIa-substrate (A). Amplification assay that makes use of multiple activation steps to quantify FXIa (B). FIX is activated by the FXIa present in the sample, which subsequently activates FX into FXa. Ultimately FXa substrate conversion is measured. In-house set-up sandwich ELISA, which makes use of a multivalent FXIa-inhibitor (green) construct to bind FXIa to the plate (C). Subsequent addition of a primary and HRP-labeled secondary antibody allows quantification via HRP-substrate conversion.

All methods make use of a chromogenic substrate conversion step to determine the concentration of FXIa present in the sample. Analysis of the data can, however, be performed via two methods: kinetic analysis, which evaluates the rate of substrate conversion over a predetermined time-frame; or end-point analysis, which evaluates the total amount of converted substrate over a predetermined time-frame. We chose to analyze our results using the kinetic analysis as this was shown to lead to smaller standard deviations (Figure 2).

Figure 2, Two strategies to evaluate chromogenic substrate conversion. (A) End-point analysis, which evaluates the total amount of converted substrate over a predetermined time-frame. (B) Kinetic analysis, which evaluates the rate of substrate conversion over a predetermined time-frame.

FXIa detection via a chromogenic substrate assay

First, we evaluated FXIa quantification based on chromogenic substrate conversion by the active site of FXIa (Figure 1A). A fixed concentration of substrate (0.5 mM) was added to a FXIa containing sample (50 pM – 0.8 pM), resulting in a FXIa dose-dependent signal generation (Figure 3A). Effects of substrate depletion on data-analysis were first evaluated by performing linear regression on the initial 30 minutes of the slope. Data showed good linearity ($R^2 > 0.97$), and could thus be used without any correction. Kinetic analysis of the first 30 min was performed and a LoD of 1.8 pM and a sensitivity of 0.07 mOD/min.pM could be calculated. (Figure 3B, 3C).

Figure 3, Chromogenic substrate (S2166) conversion by FXIa. Substrate (0.5mM) conversion was measured in presence of FXIa (50 pM - 0.8 pM), (— 50pM; — 25 pM; — 12.5 pM; — 6.25 pM; — 3.1 pM; — 1.6 pM; — 0.8 pM; — blank)(A). Slopes of the raw data are plotted against their FXIa concentration, and analyzed by linear regression in an XY-plot (B). slopes of the raw data are shown in a bar plot $\pm$ SD(C).

FXIa detection via an amplification assay

Next, we studied an FXIa amplification assay, which is expected to have a higher sensitivity and lower LoD than the chromogenic substrate assay. The assay amplifies the FXIa-signal by using two sequential enzyme activations, derived from the intrinsic route of the coagulation, followed by chromogenic substrate conversion (Figure 1B). In the presence of calcium, phospholipids and thrombin, activated FXI (2 pM – 0.03 pM) can activate FIX into FIXa, which in complex with FVIIIa activates FX. The ultimately generated FXa, was quantified via a specific chromogenic substrate. To avoid internal quenching, the data was analyzed up to 1 OD (15 minutes). As for the chromogenic substrate assay, linear regression of the initial 15 minutes of the data showed that no correction was necessary for substrate depletion ($R^2 > 0.98$). Similar to the previous assay, kinetic analysis was performed using the linear region. An LoD of 0.2 pM and a sensitivity of 13.5 mOD/min.pM were calculated for the amplification assay.

Figure 4, Amplification assay. FXIa is titrated from 2 pM to 0.02 pM in the amplification kit. Raw data of the assay (— 2 pM; — 1 pM; — 0.5 pM; — 0.25 pM; — 0.1 pM; — 0.06 pM; — 0.03 pM; — blank)(A). Slopes of the raw data are plotted against their FXIa concentration, and analyzed by linear regression in an XY-plot(B). Slopes of the raw data are shown in a bar plot including their SD(C).

FXIa detection via ELISA

Finally, a modified version of a classical sandwich ELISA set-up was studied. First, a biotinylated FXIa inhibitor derived from the snake *Bungarus Fasciatus* (Fasxiator) was coated *via* avidin on a 96-well plate and was used to isolate FXIa from buffer.(13) Subsequent addition of a primary and secondary antibody, allowed quantification by addition of HRP substrate. The Fasxiator-construct concentration used for coating of the wells was optimized for FXIa binding and found to be 30 nM. Lower concentrations led to less FXIa binding, while increased concentrations did not result in more FXIa-binding. The primary and secondary antibody were used at a concentration of 2 µg/ml and 160 ng/ml, respectively. (Figure 5). Linear regression only showed a small region of linearity ($R^2 > 0.94$) between 3 and 7 minutes, most likely explained by the limited amount of H_2O_2 and its time-dependent decomposition.(14,15) Kinetic analysis was performed and a LoD of 50 pM and a sensitivity of 0.03 mOD/(min.pM) were determined. (Figure 5B, 5C),

Figure 5, ELISA assay. FXIa is titrated from 1000 pM to 1.3 pM in the ELISA assay. Raw data of the assay (— 1000 pM; — 333 pM; — 111 pM; — 37 pM; — 12 pM; — 4 pM; — 1.3 pM; — blank)(A). Slopes of the raw data (3-7 min) are plotted against their FXIa concentration, and analyzed by linear regression in an XY-plot(B). Slopes (3-7 min) of the raw data are shown in a bar plot including their SD(C).

Discussion

We have compared three FXIa quantification assays for their LoD and sensitivity. Although all assays made use of a chromogenic substrate conversion in their final step, striking differences in their LoD and sensitivity were found. FXIa quantification using an amplification assay was shown to have the lowest LoD, which was 9 times lower than the chromogenic spectroscopy assay, and 250 times lower than the ELISA. Furthermore, the sensitivity of the amplification assay (13.5 mOD/(min.pM)) was also superior to the chromogenic-assay (0.07 mOD/(min.pM)) and the ELISA (0.03 mOD/(min.pM)). Additionally, the high sensitivity and low LoD of the FXIa-amplification assay are not in line with other amplification assays such as the FIXa-assay that has a 3-fold higher LoD and a 10 fold lower sensitivity (FIXa; LoD: 2 pM; Sensitivity: 0.22 mOD/(min.pM)). This can, however, be explained by three steps of serial activation, leading to a 3rd order signal generation compared to 2nd order for the FIXa-assay. (16) We showed that both the amplification- and chromogenic substrate assay allow detection of physiological concentrations of FXIa (<10 pM). However, because other coagulation factors present in plasma will interfere with these assays, the assays cannot be used for FXIa quantification in circulation.

Despite of its high LoD, the newly developed ELISA is the only set-up which has the potential to be used in a plasma environment. We therefore performed preliminary experiments using this assay in plasma (data not shown), however, due to unspecific binding of the antibodies, FXIa could not be detected. The use of more specific antibodies could reduce unspecific binding and allow detection of FXIa in plasma. The polyclonal nature of the antibodies, however, allows binding to multiple sites of their target protein, higher concentrations of the polyclonal antibodies therefore result in a higher sensitivity and lower LoD in buffered systems. This effect also was observed during antibody optimization (primary < 4 µg/ml; secondary < 160 ng/ml), since no plateau was reached for both antibodies. Finally, other blocking strategies can be studied to avoid plasma protein from sticking to the well, thereby reducing the unspecific binding of the (secondary) antibodies in the ELISA.

Compared to our previous research, the ELISA has a higher LoD (50 pM) than the catch-and-release assay (7 pM), while the chromogenic substrate assay (1.8 pM) and amplification assay (0.2 pM) both have a lower LoD. The catch-and-release assay isolates FXIa from plasma to a buffered environment, which allows the use of a chromogenic substrate to quantify FXIa. This

chapter showed that the use of an amplification assay instead of a chromogenic substrate assay could further improve the LoD and sensitivity of our catch-and-release assay.

References

1. Zhang H, Löwenberg EC, Crosby JR, MacLeod AR, Zhao C, Gao D, et al. Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk. *Blood*. 2010 Nov 25;116(22):4684–92.
2. Loeffen R, Oerle R van, Groot PG de, Waltenberger J, Crijns HJ, Spronk HMH, et al. Increased factor XIa levels in patients with a first acute myocardial infarction: The introduction of a new thrombin generation based factor XIa assay. *Thromb Res*. 2014 Dec;134(6):1328–34.
3. Loeffen R, Oerle R van, Leers MPG, Kragten JA, Crijns H, Spronk HMH, et al. Factor XIa and Thrombin Generation Are Elevated in Patients with Acute Coronary Syndrome and Predict Recurrent Cardiovascular Events. *PLOS ONE*. 2016 Jul 15;11(7):e0158355.
4. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976 May 7;72:248–54.
5. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin Phenol Reagent. *J Biol Chem*. 1951 Nov 1;193(1):265–75.
6. Whitaker JR, Granum PE. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal Biochem*. 1980 Nov 15;109(1):156–9.
7. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J Immunol Baltim Md* 1950. 1972 Jul;109(1):129–35.
8. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979 Sep;76(9):4350–4.
9. Gallimore MJ, Friberger P. Chromogenic peptide substrate assays and their clinical applications. *Blood Rev*. 1991 Jun 1;5(2):117–27.
10. Armbruster DA, Pry T. Limit of Blank, Limit of Detection and Limit of Quantitation. *Clin Biochem Rev*. 2008 Aug;29(Suppl 1):S49–52.
11. Schnölzer M, Alewood P, Jones A, Alewood D, Kent SB. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res*. 1992 Oct;40(3–4):180–93.
12. van der Beelen SHE, Agten SM, Suylen DPL, Wichapong K, Hrdinova J, Mees BME, et al. Design and synthesis of a multivalent catch-and-release assay to measure circulating FXIa. *Thromb Res*. 2021 Jan 18;200:16–22.
13. Chen W, Carvalho LPD, Chan MY, Kini RM, Kang TS. Fasxiator, a novel factor XIa inhibitor from snake venom, and its site-specific mutagenesis to improve potency and selectivity. *J Thromb Haemost*. 2015;13(2):248–61.
14. Yazıcı E, Deveci H. Factors Affecting Decomposition of Hydrogen Peroxide. In Ankara: Mining Engineering Department, Hacettepe University; 2010. p. 609–616.
15. Crole DA, Freakley SJ, Edwards JK, Hutchings GJ. Direct synthesis of hydrogen peroxide in water at ambient temperature. *Proc Math Phys Eng Sci R Soc*. 2016 Jun;472(2190).
16. Gray E, Tubbs J, Thomas S, Oates A, Boisclair M, Kembell-Cook G, et al. Measurement of activated factor IX in factor IX concentrates: correlation with in vivo thrombogenicity. *Thromb Haemost*. 1995 Apr;73(4):675–9.

Chapter 5

Study, design and synthesis of multivalent
dynamic peptide nucleic acid constructs for a
factor Xla catch-and-release assay.

Stan H.E. van der Beelen, Stijn M. Agten, Kanin Wichapong,

Dennis P.L. Suylen, Tilman M. Hackeng

Chapter 6

Modulating Fasxiator affinity for FXIa through
development of a dynamic reversal strategy.

Stan H. J. van der Beelen, Stijn. M. Agten, Kanin Wichapong,

Tilman M. Hackeng.

Chapter 7

General discussion

Worldwide one in four people die from thrombosis-related conditions, making it the leading cause of death.(1) Current antithrombotic medication mainly focusses on inhibition of thrombin, factor Xa (FXa) or platelet aggregation. However, while these methods are effective, patients that receive anticoagulant medication need to be closely monitored to prevent spontaneous hemorrhage.(2,3) In search of new and safer anticoagulants, inhibition of FXI/FXIa has recently emerged as an alternative that prevents thrombus formation, but does not lead to an increased risk of bleeding.(4) Therefore, over the last decade FXIa has become an interesting target for the pharmaceutical industry. In addition, there is evidence that suggests that increased FXIa-levels, which are observed in acute myocardial infarction or acute coronary syndrome, lead to an increased risk of thrombus formation.(5,6) To study the efficiency of a new FXIa-based anticoagulant treatment or the effects of increased FXIa levels in circulation, a FXIa quantification assay is essential.

Currently, a single method is available which quantifies free FXIa in circulation indirectly *via* a calibrated automated thrombogram (CAT) based assay. This method has been used to study FXIa levels in a small cohort of patients suffering from myocardial infarction and acute coronary syndrome and found a significant increase in FXIa-levels. However, due to FXIa inhibition by serpins, samples had to be measured within 30 minutes after blood drawing, which complicates quantification of FXIa in larger groups, and therefore creates the need of a FXIa assay which is not affected by these inhibitors.

In this thesis, multiple FXIa quantification assays have been set-up, developed and studied; varying from established chromogenic spectroscopy assays in buffer to an innovative catch-and-release assay in plasma. The aim of this thesis was the development of an easy-to-use method to quantify FXIa in circulation that is not affected by serpins.

We evaluated two methods used to quantify isolated FXIa in buffered systems. Both methods use chromogenic spectroscopy to detect FXIa. To gain more insight in these assays, they have been studied for their sensitivity and limit of detection (LoD) (**chapter 4**). The first assay quantifies FXIa directly *via* a chromogenic substrate specific for FXIa (LoD = 1.8 pM; Sensitivity 0.07 mOD/(min.pM)) whereas the second method first amplifies the FXIa signal by making use of part of the intrinsic route of the coagulation cascade, and eventually measures chromogenic substrate conversion by factor Xa (LoD = 0.2 pM; Sensitivity 13.5 mOD/(min.pM)). A side by side comparison of these assays in similar conditions revealed a 10-fold increase in LoD, and a 200-fold increase in sensitivity for the amplification assay. While

both assays were shown to be able to detect FXIa in low picomolar range, they cannot be used in plasma samples due to the cross-reactivity of other serine proteases with the chromogenic substrate used.

A popular set-up for individual protein quantification in complex mixtures is the enzyme-linked immunosorbent assay (ELISA).(7) The use of selective- and high affinity binding antibodies, has resulted in femto-molar detection limits for their target proteins.(8) In **chapter 4** we developed a FXIa ELISA based on a commercially available polyclonal antibody. Initial experiments in buffered environments resulted in a LoD of 48.9 pM, but FXIa could not be detected in spiked plasma experiments due to a high background signal. The low signal-to-noise ratio in the plasma experiments is hypothesized to be caused by the use of the secondary antibody. The use of a different secondary antibody with less cross reactivity for the coating antibodies could resolve this problem. However, the difference in LoD of the inhouse set-up ELISA compared to the CAT-assay (0.39 pM), the abovementioned chromogenic spectroscopy (1.8 pM) or the amplification assay (0.2 pM) is substantial.(5,6) The difference can be overcome by the use of an antibody with a higher affinity for FXIa/FXI, provided that it binds an allosteric site of FXI/FXIa in order not to compete with Fasxiator. One example of such an antibody is found by Bayer pharmaceuticals as an allosteric inhibitor for FXIa.(9) The antibody binds with an affinity of 2.4 nM to an epitope on the catalytic domain of FXIa that includes Gln451. However, it has to be noted that binding of this antibody to FXIa results in substantial structural rearrangements of the active site of FXIa, which in a way could hinder the Fasxiator-FXIa interaction.

The detection limit of the ELISA in combination with the inability to quantify FXIa in plasma, forced us to look beyond traditional assay set-ups, and made us focus on the development of a new and innovative assay. The ideal FXIa quantification assay, would initially protect FXIa from serpins in plasma, but could eventually quantify FXIa *via* its intrinsic activity with a LoD comparable to the amplification assay (0.2 pM). In **chapter 3** a proof-of-concept is presented for a catch-and-release assay which has the potential to meet these requirements. The assay is based on a dynamic multivalent construct containing three identical FXIa inhibitors (Fasxiator) that bind, isolate, and eventually release FXIa into a buffered system, allowing quantification via chromogenic spectroscopy. In addition, it is hypothesized that when the multivalent inhibitor is bound to FXIa, it will protect FXIa from serpin inhibition. The proof-of-concept study showed promising results in plasma environments (LoD = 20 pM); however, to

quantify FXIa in circulation (<10 pM), the LoD has to be lowered even further. Additionally, to convert the proof-of-concept assay into a functional FXIa assay, a biotin-free dynamic construct had to be designed. In the current set up, blood samples first have to be pre-cleared from biotin, since biotin present in plasma disrupts the multivalent construct, and thereby interferes with the assay. Optimization of this construct would allow addition of the multivalent inhibitor to the blood collection tube, and thereby instantly protect FXIa from serpin-inhibition upon blood drawing.

An easy solution to lower the LoD of the catch-and-release assay was found in **chapter 4**, which shows that replacement of the chromogenic substrate into an amplification assay would increase the sensitivity (200-fold), and decrease the LoD of the assay 10-fold. Preliminary experiments of this concept resulted in a detection limit of 3 pM. A more challenging solution to lower the LoD was studied in **chapter 6**. A drawback of the current catch-and-release setup is the presence of monomeric Fاسxiator that is still able to inhibit part of the isolated FXIa after its release. In **chapter 6** we attempted to develop a reversal strategy for the Fاسxiator-FXIa interaction by covalently coupling steric bulk at strategic positions in Fاسxiator. We hypothesized that upon coupling of the steric bulk, the Fاسxiator-FXIa interaction could completely be disturbed, which would result in a more efficient release of FXIa, and thereby lowering of the LoD. The results in **chapter 6**, show a promising start of this concept by lowering the encounter complex formation, and the tight complex formation rate of the slow-tight binding inhibitor. However, optimization has to be performed to affect the already formed tight complex. In the current set-up a flexible lysine tree was coupled to Fاسxiator, located relatively far from the interaction surface. The flexibility of the lysine tree could be inefficient for the use as steric bulk, since it can be pushed aside by the incoming protein (FXIa). The use of a more rigid structure such as cysteine knot proteins, for the introduction of steric hindrance could result in more efficient manner to disrupt the FXIa-Fاسxiator interaction. Furthermore, the positioning of the steric bulk is crucial for its effectivity. Therefore, reduction of the linker length and positioning of steric bulk closer to the interaction surface of Fاسxiator would hypothetically also improve the *on/off*-concept. Additionally, the experiments were performed with pre-coupled lysine tree to Fاسxiator, which does not give any information on the ability of the lysine tree to couple to the Fاسxiator-FXIa complex nor its effect during coupling to Fاسxiator. Future studies could focus on this

mechanism, and validate whether there is efficient coupling of the lysine tree to the Fasxiator-FXIa complex *via* LC/MS.

Another approach for a reversal mechanism would be to study if antibodies could be developed that have a high affinity for Fasxiator. These antibodies could compete with FXIa for binding, and function as a reversal agent. Strategies that use more potent binders to scavenge inhibitors are already being used in the clinic for other inhibitors (e.g. andexanet alfa, idarucizumab), and show promising results.

Finally, in order to create a functional catch-and-release assay that can be used in the clinic, the multivalent construct has to be present in the blood drawing tube to directly bind free FXIa and protect it from natural serpin inhibition. However, because the release mechanism of our proof of concept study is based on an excess of biotin (present in blood at a concentration of 1-5 nM), a new dynamic multivalent construct has to be developed.⁽¹⁰⁾ Nowadays, there are many possibilities for the development of a dynamic system, but most of them rely on techniques that can affect the structural integrity of proteins (e.g. enzyme cleavable linkers; photocleavable linkers; pH/temperature cleavable linkers).⁽¹¹⁾ In **chapter 5** peptide nucleic acids are studied for their ability to be used in a dynamic multivalent construct.⁽¹²⁾ While the use of a PNA-based system exhibited the desired dynamic properties in a model setup (9 base pairs), translating this to a workable construct proved a challenge. Based on MD simulations and ITC measurements a sub-nanomolar affinity was hypothesized for a length of 18 base pairs. Synthesis of these PNA strands, however, proved to be troublesome and in addition these strands aggregated upon complexation most likely due to their neutral backbone. An optimal balance between solubility and affinity of these duplex oligomer structures should thus be found. In follow-up studies the solubility of the constructs could be increased by the addition of poly-lysine chains at the C- and N-termini of the PNA fragments.⁽¹³⁾ Furthermore, it is recommended to shorten the PNA-length to 12-15 base pairs in order to increase solubility and reversibility of the dimeric construct to its monomeric form.

As a concluding remark this thesis showed the beginning of a catch-and-release concept that has great potential for the development of a serpin-independent assay. However, in order to use current assay in the clinic, its plasma resistance and limit of detection have to be optimized. A recommended start would be to optimize the PNA-constructs for their solubility and dynamic properties. Furthermore, detecting FXIa after release *via* the amplification assay

would substantially improve the LoD of the catch-and-release assay. In future studies, it would then be interesting to add the construct to the blood-collection tube, in order to determine base levels of FXIa in healthy individuals. Ultimately the assay could be used to study patients at risk of thrombosis, and study whether increased FXIa levels in plasma could function as a biomarker for thrombosis at an early stage.

References

1. Mackman N. Triggers, targets and treatments for thrombosis. *Nature*. 2008 Feb 21;451(7181):914–8.
2. Friedman RJ, Dahl OE, Rosencranch N, Caprini JA, Kurth AA, Francis CW, et al. Dabigatran versus enoxaparin for prevention of venous thromboembolism after hip or knee arthroplasty: a pooled analysis of three trials. *Thromb Res*. 2010 Sep;126(3):175–82.
3. Cohen AT, Bauersachs R. Rivaroxaban and the EINSTEIN clinical trial programme. *Blood Coagul Fibrinolysis*. 2019 Apr;30(3):85–95.
4. Zhang H, Löwenberg EC, Crosby JR, MacLeod AR, Zhao C, Gao D, et al. Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk. *Blood*. 2010 Nov 25;116(22):4684–92.
5. Loeffen R, Oerle R van, Groot PG de, Waltenberger J, Crijns HJ, Spronk HMH, et al. Increased factor Xla levels in patients with a first acute myocardial infarction: The introduction of a new thrombin generation based factor Xla assay. *Thromb Res*. 2014 Dec;134(6):1328–34.
6. Loeffen R, Oerle R van, Leers MPG, Kragten JA, Crijns H, Spronk HMH, et al. Factor Xla and Thrombin Generation Are Elevated in Patients with Acute Coronary Syndrome and Predict Recurrent Cardiovascular Events. *PLOS ONE*. 2016 Jul 15;11(7):e0158355.
7. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J Immunol Baltim Md 1950*. 1972 Jul;109(1):129–35.
8. Zhang S, Garcia-D'Angeli A, P. Brennan J, Huo Q. Predicting detection limits of enzyme-linked immunosorbent assay (ELISA) and bioanalytical techniques in general. *Analyst*. 2014;139(2):439–45.
9. Schaefer M, Buchmueller A, Dittmer F, Straßburger J, Wilmen A. Allosteric Inhibition as a New Mode of Action for BAY 1213790, a Neutralizing Antibody Targeting the Activated Form of Coagulation Factor XI. *J Mol Biol*. 2019 Dec 6;431(24):4817–33.
10. Clevidence BA, Marshall MW, Canary JJ. Biotin levels in plasma and urine of healthy adults consuming physiological doses of biotin. *Nutr Res*. 1988 Oct 1;8(10):1109–18.
11. Leriche G, Chisholm L, Wagner A. Cleavable linkers in chemical biology. *Bioorg Med Chem*. 2012 Jan 15;20(2):571–82.
12. Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science*. 1991 Dec 6;254(5037):1497–500.
13. Bianchi E, Venturini S, Pessi A, Tramontano A, Sollazzo M. High level expression and rational mutagenesis of a designed protein, the minibody. From an insoluble to a soluble molecule. *J Mol Biol*. 1994 Feb 18;236(2):649–59.

Chapter 8

Summary

Samenvatting

Impact paragraph

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Publications

Dankwoord

Summary

This thesis focused on the development of an innovative catch-and-release assay to quantify FXIa in plasma. Developing such an assay could in future studies answer the question whether FXIa could be used as a biomarker for thrombosis, and it can help in the development of novel antithrombotic medication. As a starting point, **chapter 1** gives a brief overview of the structure and function of FXIa, the therapeutic advancements regarding FXIa-inhibition, and the current possibilities/limitations for quantification of FXIa in circulation.

Chapter 2 reviews bio-orthogonal imine chemistry in protein synthesis, of which some insights will be used in **chapter 6**. The chapter covers multiple aspects of the topic such as functionalization of the protein/peptide of interest, different types of imine chemistry (e.g. oxime ligation, hydrazone ligation, Pictet-Spengler reaction), applications in protein synthesis/labeling, and catalysis of the reaction.

In **chapter 3**, a proof of concept is presented for a catch-and-release assay that quantifies FXIa via a two-step mechanism. First, a multivalent inhibitory construct was bound to the dimeric form of FXIa, which allowed isolation of FXIa into a buffered system. Subsequently, dissociation of the multivalent construct resulted in the release of FXIa, which could be detected by chromogenic substrate conversion. For the multivalent construct a selective FXIa inhibitor (Fasxiator) was synthesized by solid phase peptide synthesis, and linked *via* desthiobiotin to avidin. Addition of an excess of biotin to this construct displaced the desthiobiotin molecules, and the resulting decrease in affinity of Fasxiator for FXIa released the free serine protease in solution. Studies in buffered and plasma environments showed the potential of the concept, with a limit of detection of 7 and 20 pM, respectively. However, plasma samples had to be depleted of biotin before use, and detection of FXIa had to be done in the presence of an inhibitor.

Chapter 4 evaluates the sensitivity and limit of detection of three different assay set-ups to quantify FXIa in a buffered system. A comparison was made between an in-house set-up ELISA, a chromogenic substrate assay, and an established amplification assay. What became evidently clear, is that direct substrate conversion by the intrinsic activity of FXIa resulted in a lower limit of detection compared to the ELISA set-up (1.8 pM versus 48.9 pM). The commercial amplification kit lowered the limit of detection even further to 0.2 pM. Furthermore, because of the nature of the ELISA set-up, it was also tested in plasma samples. However, due to unspecific binding of the antibodies FXIa could not be detected.

Chapter 5 studies whether peptide nucleic acids can be used as a basis for a dynamic multivalent construct for the catch-and-release assay. This construct was hypothesized to be unaffected by plasma components such as biotin, and therefore allows addition of the construct into the blood collection tube, to directly bind and protect FXIa from its natural inhibitors. A proof of concept study with two complementary PNA strands (9-mer) confirmed binding of both strands, and also the ability to dissociate from each other upon addition of an excess of complementary strand. Subsequently, the PNA length was optimized for its affinity *via* isothermal titration calorimetry, showing that increasing lengths were correlated to higher affinities of the complementary strands. Finally, the optimized construct was synthesized, and tested for its capacity to inhibit FXIa. Unfortunately, the increased PNA-length (18-mer) resulted in aggregation of the construct.

Chapter 6 addresses an important weakness of the catch-and-release assay, namely the fact that quantification of FXIa must be performed in the presence of the free monomeric inhibitor. By designing an *on/off* version of Fasxiator, an attempt was made to develop a reversal strategy for the FXIa-Fasxiator interaction. It was hypothesized that coupling of a sterically hindered molecule near the FXIa-Fasxiator interaction site, would decrease the binding affinity of Fasxiator to FXIa, resulting in an *off*-version of Fasxiator. Two sites near the FXIa binding-site of Fasxiator (E12 and K48) were identified and were mutated to a ketone containing amino acid, without a significant loss of affinity for FXIa. Subsequent oxime ligation of a lysine tree to these sites, resulted in the sterically hindered *off*-versions of Fasxiator. Coupling of the lysine tree showed to have an effect on the initial binding affinity, and tight complex formation. However, no effect was observed on the preformed tight complex, thus requiring further optimization of the concept.

Samenvatting

Dit proefschrift richt zich op de ontwikkeling van een innovatief “catch-and-release assay” voor de concentratiebepaling van FXIa in plasma. De ontwikkeling van een dergelijk assay zou in toekomstige studies antwoord kunnen geven op de vraag of een verhoogde concentratie van FXIa mogelijk gebruikt kan worden als biomarker voor trombose. Daarnaast zou het ook bij kunnen dragen aan de ontwikkeling van nieuwe antistollingsmiddelen. Als startpunt introduceert **hoofdstuk 1** FXIa structuur en functie, de therapeutische ontwikkelingen, en de huidige mogelijkheden/beperkingen met betrekking tot het meten van FXIa in circulatie.

In **hoofdstuk 2** wordt het gebruik van imine chemie in chemische eiwitsynthese besproken, waarvan sommige inzichten gebruikt worden in **hoofdstuk 6**. Het hoofdstuk behandelt meerdere aspecten zoals functionalisering van eiwitten/peptiden, verschillende soorten imine chemie (bijv. oxime ligatie, hydrazone ligatie, Pictet-Spengler reactie), toepassingen in eiwitsynthese, en katalyse van de reactie.

In **hoofdstuk 3** wordt een “proof of concept” gepresenteerd voor een “catch-and-release assay” die de FXIa concentratie bepaalt via een twee-staps-mechanisme. Allereerst wordt de FXIa-dimeer geïsoleerd door het te binden aan een multivalent remmer construct. Vervolgens wordt FXIa gedetecteerd met behulp van chromogeen substraat omzetting na dissociatie van het multivalente construct. Het multivalente remmer construct bestaat uit een selectieve FXIa remmer (Fasxiator), gesynthetiseerd door middel van vaste fase peptide chemie, gekoppeld aan avidine via desthiobiotine. Toevoeging van een overmaat biotine aan dit construct verdringt de desthiobiotine moleculen, leidt tot het uit elkaar vallen van het multivalente construct, en resulteert in het vrijkomen van de serine protease in oplossing. Studies in een geïsoleerd systeem en bloedplasma laten de potentie van dit concept zien met een FXIa-detectielimiet van respectievelijk 7 en 20 pM. Echter, aanwezigheid van natuurlijk biotine in plasma bemoeilijkt het testsysteem. Daarnaast wordt de detectie van FXIa gehinderd door aanwezigheid van de monomere remmer Fasxiator, die alhoewel veel minder actief dan de dimeer, nog steeds in staat is FXIa te remmen. De huidige beschikbare FXIa-testen worden bestudeerd in **hoofdstuk 4**. De sensitiviteit en de detectielimiet van drie verschillende methodes om FXIa te meten in een gebufferd systeem zijn vergeleken. Een vergelijking is gemaakt tussen een zelf ontwikkelde ELISA, een chromogeen substraat assay, en een zogenaamde versterkingsassay. Wat duidelijk wordt, is dat substraat omzetting door FXIa leidt tot een lagere detectie limiet dan de ELISA (1.8 pM ten opzichte van 48.9 pM). De

versterkingsassay verlaagt het detectielimiet nog verder tot 0.2 pM. Daarnaast is de ELISA-assay ook getest in bloedplasma, maar door onspecifieke binding van de antilichamen kon FXIa hier niet worden gedetecteerd.

Hoofdstuk 5 bestudeert of peptidenucleïne-zuren(PNA) een basis kunnen vormen voor een dynamisch multivalent construct dat gebruikt kan worden in de “catch-and-release” assay. De hypothese was dat het construct niet beïnvloed zou worden door plasma componenten zoals biotine, en daardoor toegevoegd kan worden aan de bloedafnamebuizen om zo direct FXIa te binden en te beschermen tegen zijn natuurlijke remmers. Een “proof of concept” studie met twee complementaire PNA-strengen (9-meren) liet binding van de complementaire strengen zien, maar ook de dissociatie van het complex nadat een overmaat complementaire streng werd toegevoegd. Daaropvolgend is de lengte van de PNA-steng geoptimaliseerd voor zijn affiniteit met behulp van isotherme titratiecalorimetrie. Uiteindelijk, is het geoptimaliseerde construct gesynthetiseerd en getest, maar de toename van het aantal PNA base paren zorgde voor aggregatie van het construct.

Hoofdstuk 6 behandelt een belangrijk nadeel van de “catch-and-release” assay; concentratiebepaling van FXIa gedaan moet worden in aanwezigheid van Fasxiator. Door een *aan/uit* versie van Fasxiator te ontwikkelen, is een poging gedaan de FXIa-Fasxiator interactie om te keren. De koppeling van een groot molecuul dichtbij het FXIa-Fasxiator interactie gebied, zou moeten leiden tot in een lagere affiniteit van Fasxiator voor FXIa, en daarmee zou resulteren in een uit-versie van Fasxiator. Twee posities van Fasxiator (E12 en K48) zijn gevonden die gemuteerd konden worden zonder significant verlies van affiniteit voor FXIa. De gemuteerde varianten van Fasxiator werden vervolgens gemodificeerd met een oxime reactie, wat resulteerde in de sterisch gehinderde uit-versies van Fasxiator. Het koppelen van een lysine boom als sterische hinder had een effect op de initiële

bindingsaffiniteit, en het vormen van het uiteindelijke complex. Daarentegen is er geen effect waargenomen op het voorgevormde FXIa- Fasxiator complex.

Impact paragraph

FXIa is a member of the intrinsic route of the coagulation cascade that has gained great interest over the past 10 years in the field of thrombosis and hemostasis. Inhibition of FXIa in patients that received total knee-replacement resulted in a decreased risk of thrombosis after surgery. In contrast, the admission of FXIa-contaminated intravenous gamma globulin (IVGG) packs to patients increased the risk on thrombotic events. These phenotypes originating from variations in FXIa levels suggest that FXIa is an important player in thrombus formation.

The main goal of this thesis was to develop a FXIa quantification assay that can be used in complex mixtures such as IVGG-packs and plasma. Such an assay would allow researchers to obtain more information about the effect of variation in FXIa-levels. Studies have shown that patients suffering from chronic artery disease or acute myocardial infarction have significantly increased FXIa-levels, thereby again suggesting that FXIa plays a role in thrombosis. Currently thrombosis is detected by a D-dimer test, which shows elevated concentrations when a thrombus has already been formed and subsequently been degraded, while elevated FXIa concentrations is suggested to cause thrombosis. By using elevated FXIa concentrations as a potential biomarker for thrombosis, medical doctors get a better view on patients' procoagulant status, which allows them to start antithrombotic medication at an earlier stage preventing thrombosis and reduce overall healthcare costs. Additionally, quantification of FXIa in circulation is important for the development of new anti-thrombotic medication. Nowadays, big pharma companies focus on the inhibition of FXIa as a new and safe anticoagulant therapy. Additionally, to gain more insight in the potency and safety of the drug, close monitoring of patients in clinical studies is critical. Currently, global coagulation assays are used to monitor the pro-coagulant state of these patients upon admission of the drug; however, direct quantification of FXIa activity could give more specific information on the inhibition of FXIa in circulation itself. Chapter 3 describes a proof-of-concept of an innovative catch-and-release assay to quantify FXIa. The assay is based on a two-step mechanism that first binds FXIa, and subsequently releases it in a buffered environment. In contrast to other FXIa assays, this set-up is independent of serpin inhibition, making it more reliable for clinical use, and will allow quantification of FXIa in a large group of patients. Apart from its clinical benefits, the catch-and-release mechanism is an innovative concept that could also be used to isolate other dimeric proteins from complex matrixes, ultimately allowing quantification. For example, the exchange of Fasxiator into a specific binder for a

different homodimeric protein such as 14-3-3, could result in the isolation of various homodimeric proteins from plasma. Furthermore, the development of a heterogeneous inhibitory construct could result in a quantification assay of a monomeric protein with multiple allosteric binding sites.

After a successful proof-of-concept, multiple studies were set up to optimize the catch-and-release concept. In chapter 5, peptide nucleic acids (PNA) were studied for their ability to form a dynamic multivalent construct. Unlike the construct from the proof-of-concept study, PNA's are not affected by plasma components. Therefore, development of a PNA based catch-and-release construct would allow addition of the construct directly to the blood-collection tube, where it could directly bind and protect FXIa from serpin inhibition. This concept would make the assay more straightforward to use in the clinic and less prone to external influences.

Finally, a novel reversal strategy was studied. Coupling of steric bulk to Fasxiator hindered the interaction with FXIa, and thereby resulted in an *off*-version of Fasxiator. The reversal strategy was studied to optimize the catch-and-release assay, but the development of an *off*-switch for inhibitors could be an interesting concept in multiple fields of research. Such a concept would allow the isolation of non-dimeric proteins from plasma by catching the protein *via* the *on*-state of the inhibitor, and an interesting strategy to study the effect of a specific inhibitors (e.g. novel anticoagulants) in biophysical and biological experiments.

Overall, the development of an FXIa-quantification assay will result in a better understanding, and treatment of thrombosis. Patients who are at risk of thrombosis could start anti-thrombotic medication at an earlier stage which will result in less hospital admissions reduce overall health costs, and an increase the quality of life for those at risk of thrombosis.

Curriculum Vitae

Stan Hubertus Eleonora van der Beelen was born on May 12th 1992 in Venlo, the Netherlands. He completed his high school education at Bisschoppelijk College Broekhin in Roermond, where he received his gymnasium degree in 2011. Afterwards he began his studies at the Eindhoven University of Technology to obtain his bachelor's degree in biomedical engineering, graduating on the detection of antibodies in plasma using bioluminescent sensor proteins. Thereafter, he has done a master's in biomedical engineering, at the same university working on the stabilization of the Notch4/14-3-3 interaction under the guidance of Prof. Dr. Ir. L. Brunsveld. His master was finalized with an external internship at the University of Hong Kong, collaborating with prof. dr. X.D. Li on structure-guided inhibition of the YEATS domain. Thereafter he started his Ph.D. under the guidance of prof. dr. T.M. Hackeng on the development of FXIa quantification strategies in plasma. The highlights of his doctoral work are presented in this thesis.

Publications

Publications

Agten S.M., Dijkgraaf I, van der Beelen S.H.E., Hackeng T.M. Bio-orthogonal imine chemistry in chemical protein synthesis. Total chemical protein synthesis. **2021** Jun; 327-348

van der Beelen S.H.E., Agten S.M., Suylen D.P.L., Wichapong K, Hrdinova J, Mees B.M.E., Spronk H.M.H., Hackeng T.M.. Design and synthesis of a multivalent catch-and-release assay to measure circulating FXIa. Thromb Res. **2021** Jan 18;200:16-22.

Li X, Li X.M., Jiang Y, Liu Z, Cui Y, Fung K.Y., van der Beelen S.H.E., Tian G, Wan L, Shi X, Allis C.D., Li H, Li Y, Li X.D.. Structure-guided development of YEATS domain inhibitors by targeting π - π stacking. Nat Chem Biol. **2018** Dec;14(12):1140-1149.

Arts R, den Hartog I, Zijlema S.E., Thijssen V, van der Beelen S.H.E., Merckx M. Detection of Antibodies in Blood Plasma Using Bioluminescent Sensor Proteins and a Smartphone. Anal Chem. **2016** Apr 19;88(8):4525-32.

Oral and poster presentations

Van der Beelen S.H.E., Agten S.M., Spronk H.M.H. Suylen D.P.L.,T.M. Hackeng. Quantification of free FXIa by an innovative “Catch and Release” assay, The XXVIII Congress of International Society on Thrombosis and Haemostasis (ISTH), Virtual congress 2020 (poster).

Van der Beelen S.H.E., Spronk H.M.H. Suylen D.P.L.,T.M. Hackeng. Design and synthesis of a “Catch and Release” assay to quantify FXIa in blood. Chains, Veldhoven, 2019 (poster).

Van der Beelen S.H.E., Spronk H.M.H. Suylen D.P.L.,T.M. Hackeng. Design and synthesis of a “Catch and Release” assay for circulating FXIa. European Congress on Thrombosis and Haemostasis (ECTH), Glasgow, United Kingdom, 2019 (poster).

Van der Beelen S.H.E., Spronk H.M.H. Suylen D.P.L.,T.M. Hackeng. Measuring circulating FXIa to assess elevated FXIa as a risk factor for thrombosis. European Congress on Thrombosis and Haemostasis, Marseille, France, 2018 (poster)

Van der Beelen S.H.E., Suylen D.P.L.,T.M. Hackeng. In vivo FXIa assessment: elevated FXIa as risk factor for thrombosis? Dutch peptide symposium, Maastricht, 2018 (Oral presentation).

Van der Beelen S.H.E., Suylen D.P.L.,T.M. Hackeng. Design and synthesis of a multivalent catch and release enzyme inhibitor, to measure FXIa in circulation. Figon Dutch medicin days, Ede, 2018 (Oral presentation).

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