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

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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 Fasxiatus 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 hypothesized to be inaccessible for serpin inhibition or auto inactivation. This results in a close reflection of actual circulating FXIa levels at the moment of blood drawing.

1. 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

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; Ki, inhibitory constant; FVIII, factor VIII.

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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 (Fig. 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 spectroscopy. 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.

2. Material and methods

2.1. Peptide synthesis of Fasxiator fragments

Solid phase peptide synthesis (0.25 mmol) of Fasxiator fragments was performed manually with *tert*-butyloxycarbonyl (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 (2 × 1 mL for 1 min) followed by addition of a pre-activated amino acid (1.1 mmol). Pre-activation of the amino acid (1.1 mmol) was done for 2 min with 1 mmol HCTU, 3 mmol iPr$_2$NEt 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.

2.2. 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 h 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.

2.3. 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 24 h. 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.

2.4. 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 × 250 mm, 12 mL/min flow rate or 22 mm × 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 from the column was followed on UV (λ = 214 nm).

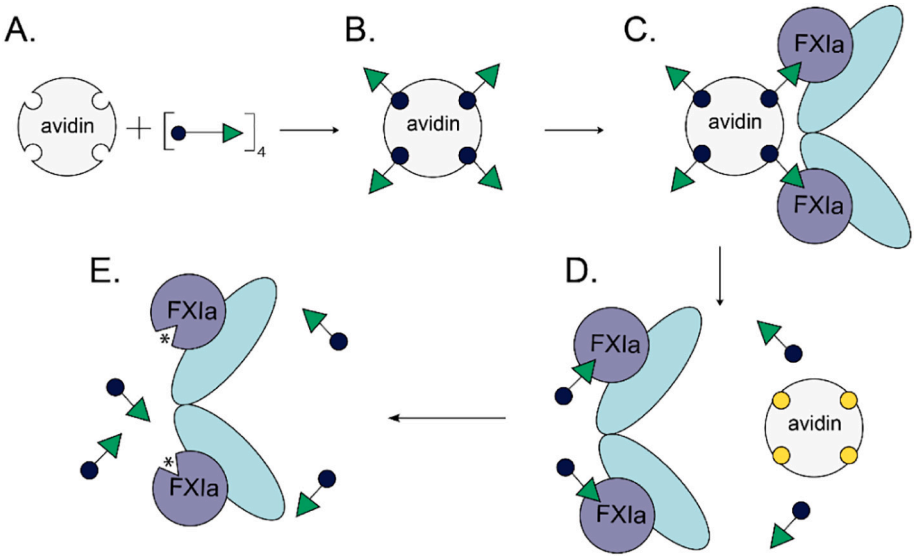

Fig. 1. Schematic FXIa catch-and-release assay. Desthiobiotin-PEG-Fasxiator is incubated with avidin (4:1 mol:mol) (A) resulting in a tetrameric protein containing 4 Fasxiator inhibitors (B). Addition of tetramer to a FXIa-containing sample will result in multivalent binding of FXIa (C). Finally, an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

Table 1
Peptide sequences of Fasxiator fragments.

Sequence					
N-term desthiobiotin-PEG_n- Fasxiator:		1	11	21	31
	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

2.5. 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 h 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.

2.6. 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 min 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 min, 100 μM biotin (stock 1 mM in H_2O) or an equal amount of H_2O was added to all wells and the measurement was continued for an additional 90 min.

2.7. Plasma collection and biotin depletion

Blood was drawn in citrate (3.2 w/v) from 47 healthy (61% males) individuals excluding oral contraceptive and antiplatelet drug users. Subsequent centrifugation (2 × 15 min. 2500 g no brake) of the blood collection tubes resulted in plasma, which was aliquoted (0.5 mL), snap frozen and stored at −80 °C. Before use, the pooled plasma was depleted from biotin by addition of 20 μL streptavidin-coated beads (MACS, streptavidin microbeads,) in 1 mL plasma for 30 min at room temperature. Subsequently, plasma was centrifuged (10,000 rpm, 10 min, 4 °C) to separate beads from plasma.

2.8. 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 min. 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 min at room temperature. Samples (200 μL) were then loaded on a biotin-coated 96-well plate (Thermo Scientific), incubated for 30 min at room temperature, followed by a wash with H_2O. 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 min 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 $\Delta Abs_{405}/\Delta$ [FXIa] [14].

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

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

2.9. Protein modelling and calculations

The dimeric conformation of FXIa, based on the crystal structure of monomeric FXIa (PDB: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.

3. Results

The natural FXIa inhibitor Fasxiator was obtained through total chemical synthesis (Fig. 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 (Fig. 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).

Fasxiator binds to the catalytic domains of FXIa's dimeric structure, which are approximately 135 Å apart as predicted by protein-protein docking (Fig. 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 (Fig. 3).

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_is 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-FXIa interactions will result in optimal dissociation of monomeric Fasxiator

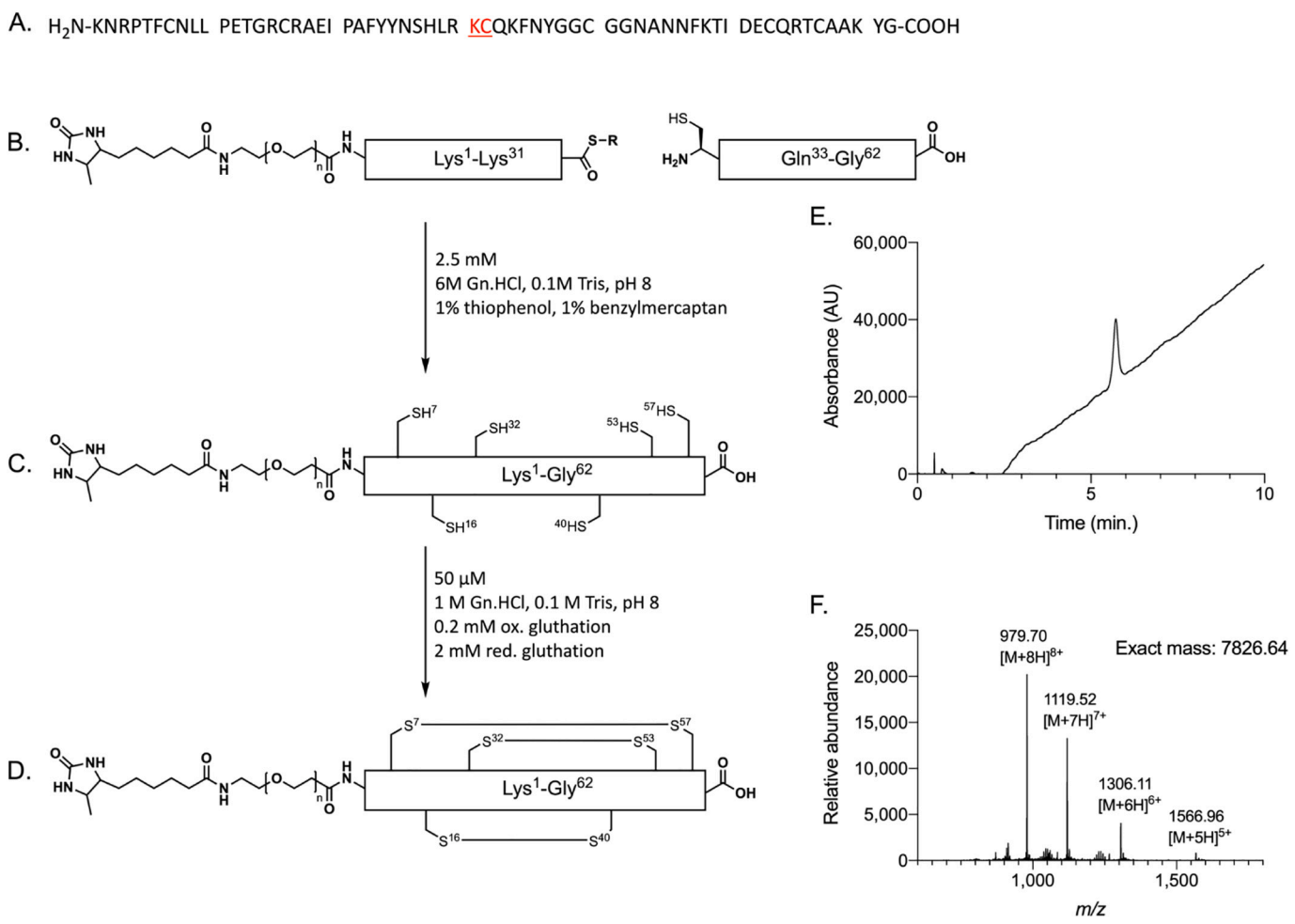

Fig. 2. Total chemical synthesis of Fasxiator. Sequence of Fasxiator, red (KC) indicates native chemical ligation site (A). Different variants of the N-terminal thioester fragment with varying PEG linker lengths (n = 4; 11; 24) and the C-terminus were synthesized using Boc solid phase peptide synthesis (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-PEG_{11}-Fasxiator with three disulfide bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from FXIa, after release of Fasxiator-FXIa from the tetrameric construct by biotin. This concept was first confirmed by monitoring FXIa activity by chromogenic substrate conversion in presence of the tetrameric construct and subsequently disrupting the construct by addition of excess biotin. (Fig. 4). Displacement of Fasxiator-FXIa from the multimeric scaffold, and the resulting decrease in affinity of monomeric Fasxiator for FXIa, resulted in release of FXIa and increased substrate conversion.

Having demonstrated the "release" part of our concept, we next studied the "catch" principles of FXIa 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 FXIa-containing solution selectively captured FXIa. 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 FXIa and addition of a range of concentrations of trimeric Fasxiator construct. To allow maximal dissociation of Fasxiator from FXIa, concentrations higher than the K_i for FXIa (0.05 nM) were used to ensure maximal binding, which should result in low enough concentrations of monomeric Fasxiator (~2 nM) after disruption of the multivalent complex (Fig. 5).

When concentrations of trimeric inhibitor were lowered, recovery of FXIa was increased with an optimum at 2 nM construct (Fig. 5A). Under these experimental conditions, a linear dose-response relationship was found with a calculated limit of detection of 7 pM. (Fig. 5B;C).

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 (Fig. 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. (Fig. 6B;C).

4. 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 135 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)

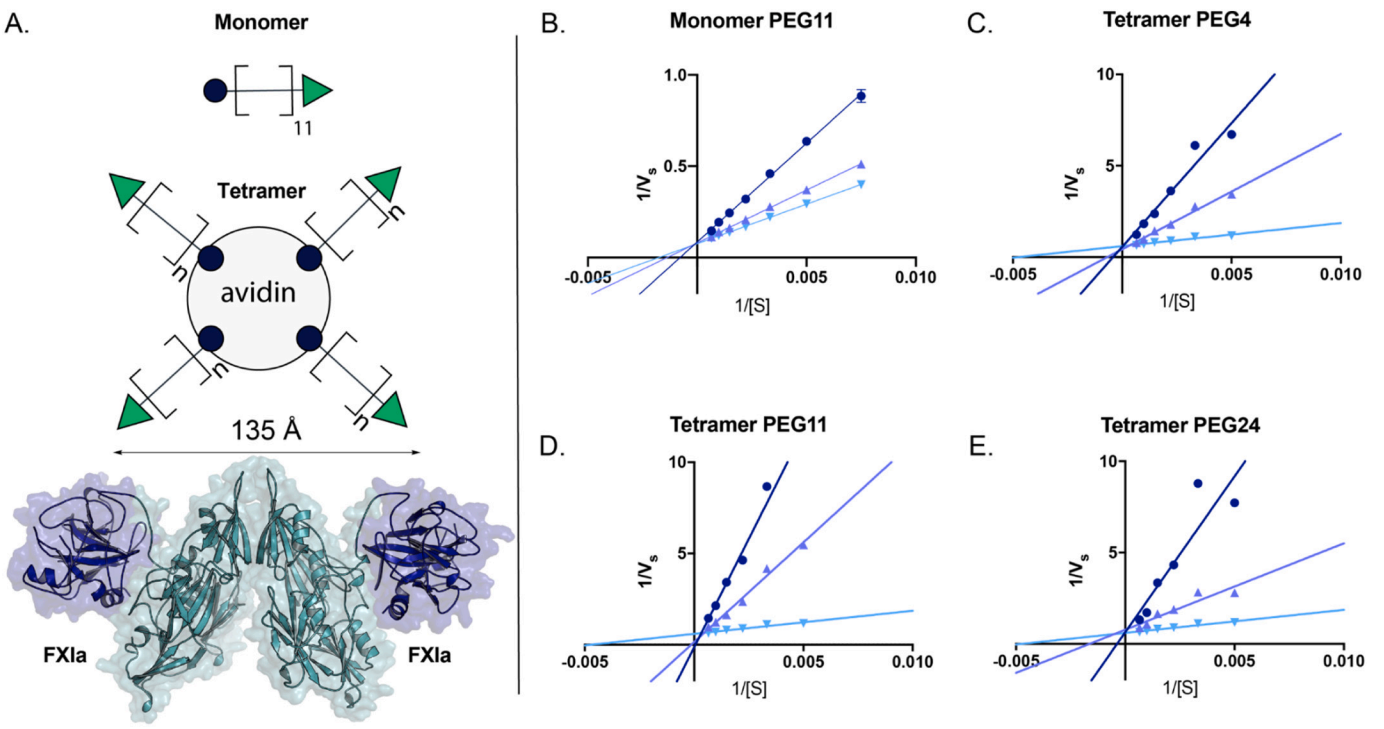

Fig. 3. Effect of linker length on FXIa-inhibition by multivalent Fasxiator complexes. Schematic representation of the monomeric and tetrameric Fasxiator constructs showing estimated distance between the two active sites (purple) in FXIa (A). Lineweaver-Burke plots of FXIa-inhibition by monomeric (B) and tetrameric Fasxiator with PEG4 (C), PEG11 (D) and PEG24 (E) linker lengths. Concentrations of inhibitor used were 0 nM (—▼—), 0.5 nM (—▲—) and 2 nM (—●—). Averages of triplicates are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

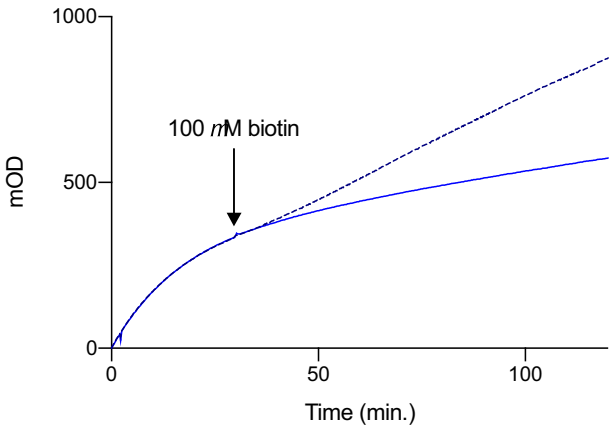

Fig. 4. inhibition of 400 pM FXIa by 1 nM tetrameric Fasxiator construct (solid line). In duplicate wells, disruption of the tetrameric inhibitor was induced after 30 min by the addition of 100 μM biotin (arrow, dashed line). Averages of triplicate measurement are shown.

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 is hypothesized to protect FXIa from inhibition by serpins and FXIa auto-inactivation, and would therefore result 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

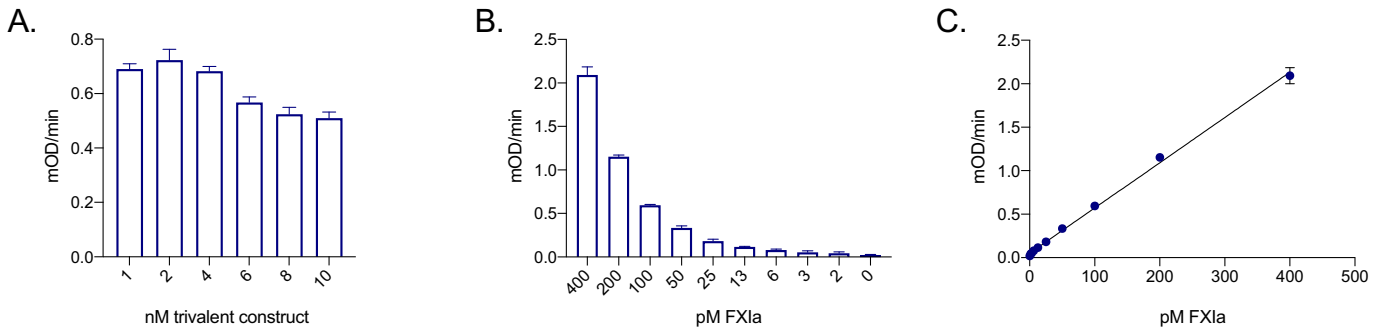

Fig. 5. FXIa catch-and-release assay in buffer model system. Optimisation of the multivalent construct concentration in buffer at 100 pM FXIa (A). Catch-and-release assay with 2 nM multivalent construct, with decreasing concentrations of FXIa (B). Linear regression of catch-and-release assay using 2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

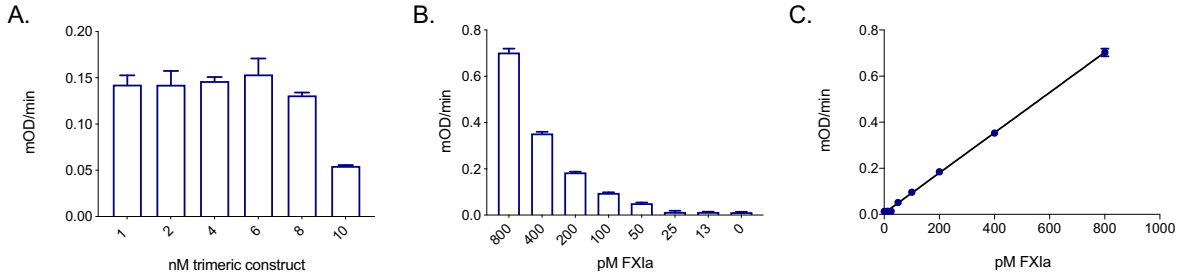

Fig. 6. FXIa catch-and-release assay in plasma model system. Optimisation of the multivalent construct concentration in plasma at 100 pM FXIa (A). Catch-and-release assay with 6 nM multivalent construct, with decreasing concentrations of FXIa (B). Linear regression of catch-and-release assay using 6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

amplify the signal.

All assays performed in this study used commercially available FXIa to validate our system. This may, however, not reflect the situation in patient samples. Factor XI is first activated to a form with only one active subunit which subsequently slowly matures into a form with two active subunits [3]. In our catch-and-release assay the multivalent inhibitor binds the fully activated FXIa dimer with a higher affinity than the partially activated FXIa dimer, therefore positively selecting for the fully activated dimer, and making detection of partially activated dimer unlikely.

A current limitation of the present 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.

Declaration of competing interest

No conflict of interest.

Acknowledgements

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