A nanobody-based method for tracking factor XII activation in plasma

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Summary

The physiological role of the plasma protein factor XII (FXII), as well as its involvement in human pathology, is poorly understood. While FXII is implicated in thrombotic pathology as a coagulation factor, it can contribute to inflammatory conditions without triggering coagulation. We recently generated nanobodies against the catalytic domain of activated FXII (FXIIa). Here, we describe two of these nanobodies, A10 and B7, both of which do not recognise FXII. Nanobody A10 recognises the catalytic domain of purified α-FXIIa (80 kDa), but not that of purified β-FXIIa (28 kDa), whereas nanobody B7 recognises both. This suggests minute differences in the catalytic domain between these isoforms of FXIIa. The detection of FXIIa by these nanobodies in plasma can be compromised through inactivation by serine protease inhibitors. This effect can be efficiently countered through the addition of the small-molecular protease inhibitor PPACK. Finally, we show that our nanobody-based assays in vitro distinguish various activation products of FXII that differ with the type of activator present: whereas procoagulant activators solely trigger the formation of a species that is captured by B7, proinflammatory activators first generate a species that is recognised by B7, which is later converted into a species that is recognised by A10. These findings suggest that a progressive proteolytic of FXIIa results in the generation a non-procoagulant form of FXIIa, whereas retention of intermediate forms triggers coagulation. Moreover, our findings indicate the development of nanobodies against activated enzymes offers improved opportunities to investigate their contribution to health and disease.

Keywords

Plasma contact system, factor XII, bradykinin, nanobody, thrombosis, kallikrein-kinin system

Introduction

When a negatively charged surface, such as glass, meets blood plasma, the contact system becomes activated. This enzyme system consists of factor XII (FXII), plasma prekallikrein (PPK) and high-molecular-weight kininogen (HK) (1-4). HK is a cofactor for PPK and coagulation factor XI (FXI) in the circulation. In a first step, FXII binds to the negatively charged surface and undergoes conformational changes (5). As a result, small amounts of active FXII (FXIIa) are formed. This molecule retains the 80 kDa molecular size of its parent protein and is termed α-FXIIa. Simultaneously, HK binds to the same surface and presents PPK to FXIIa for activation. The resulting active plasma kallikrein (PK) can reciprocally activate FXII, leading to rapid activation of the contact system (6). PK also cleaves its own cofactor HK, releasing the nonapeptide bradykinin (BK). This peptide evokes vascular leakage and tissue swelling, through interaction with the kinin B2 receptor on endothelial cells (7). Surface-bound HK also presents FXI to FXIIa for activation (8). This triggers the intrinsic pathway of coagulation and results in fibrin formation. During this process, further cleavage of FXIIa by PK fragments it into β-FXIIa (28 kDa; alternatively termed FXIIIa). Besides the loss of its 50 kDa heavy chain, β-FXIIa also loses its ability to bind to surfaces and to activate FXI. However, this enzyme can still activate PPK in solution.

For decades, it was thought that contact system activation was exclusively induced by non-physiological materials. However, in recent years, several in vivo activators of the plasma contact system have been reported, such as platelet- and bacterial polyphosphates (9), extracellular RNA and –DNA (10, 11), mast-cell released heparin (12), amyloid β peptide (13) and other misfolded protein aggregates (14, 15). Surprisingly, not all of these compounds trigger the same responses; while polyphosphates and extracellular nucleic acids drive both coagulation and PK formation, the others exclusively induce PK formation. Based on these observations, it is attractive to think that different forms of FXIIa are formed in response to different triggers.

At present, a number of methods are available for studying FXII activation in plasma. Firstly, the activation of FXII in plasma ex vivo can be tracked by the cleavage of chromogenic substrates (9). In vivo studies can be performed by: a) quantifying the amount of FXII antigen/activity (16, 17); b) analysing the amount of FXIIa in complex with its naturally occurring inhibitors, such as C1 esterase.
inhibitor (C1inh) and α1-antitrypsin (α1-AT [18]); or c) quantify-
ing the amount of free FXIIa by ELISA (19). This latter assay
makes use of a high-affinity monoclonal antibody that was raised
against β-FXIIa to capture FXIIa in solution. Polyclonal antibodies
are subsequently used to detect captured FXIIa (20). It was
demonstrated that antibody 2/215 only captures free FXIIa, as cap-
turing was compromised in the presence of C1inh. At present, this
assay (previously commercially offered by Axis-Shield diagnostics)
has been discontinued. It is uncertain whether this assay can be
used to discriminate between functional isoforms of FXIIa. Simi-
larly, it is also uncertain if certain types of FXIIa-inhibitor com-
plex(es) represent specific functions of FXIIa. These arguments
motivated us to generate a set of new assays to interrogate the mo-
lecular functions of FXIIa. Hereto, we selected nanobodies that
specifically recognise FXIIa. Heavy-chain-only antibodies are pro-
duced naturally by camelids, such as llamas. They do not contain a
light chain and their single N-terminal domain (V\text{H}) mediates
antigen binding. Since these V\text{H} do not require domain pairing,
they can be cloned separately and produced, after which they are
called nanobodies (21). Nanobodies are small enough to be effi-
ciently produced in bacteria and are therefore useful for phase se-
lection, genetic modification, and large-scale production (re-
viewed in [22]).

Using this technology, we have developed specific and sensitive
ELISA-based methods that can recognise different forms of FXIIa.
Detection of FXIIa was greatly improved by the addition of the
small peptide-based inhibitor PPACK (Phe-Pro-Arg-chloro-
methylketone) to our plasma samples. With our novel nanobody-
based ELISA, as well as our PPACK-based sample preparation
procedure, we could detect FXIIa formation upon plasma contact
system activation by various activators. Interestingly, our assays
provided evidence that different forms of FXIIa formed over time,
corresponding to the type of activator used to trigger contact acti-
vation.

Materials and methods

Reagents

FXII was purchased from Calbiochem (Darmstadt, Germany); α-FXIIa, β-FXIIa and corn trypsin inhibitor (CTI) were purchased from Enzyme Research Laboratories (ERL; South Bend, IN, USA). RNase H, SfiI, and BstElI were acquired from Thermo Scientific Inc (Waltham, MA, USA) and Ficoll 400 from Merck Millipore (Darmstadt, Germany). ELISA Blocking reagent was obtained from Roche (Cat.No.#11112589001, Woerden, the Netherlands). Maxisorp and Polysorp 96-wells flat bottom microtitre plates were purchased from Nunc A/S (Roskilde, Denmark); chromogenic as-
says were performed in a vinyl 96-wells flat bottom microtitre plate from Cor-
ing (Costar 2595; Corning, NY, USA). Coagulation assays were performed in KC10A MicroPC cups, purchased from Trinity Bio-
techn (Wicklow, Ireland). Polybrene (hexadimethrine bromide), α1-Antitrypsin, dextran sulfate (av. Mr = 500,000), triethylamine (TEA), ellagic acid, skimmed milk powder and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-
Aldrich (St. Louis, MO, USA). Chromogenic substrate L-2120
(H-D-Pro-Phe-Arg-pNA), for FXIIa and plasma kallikrein, was
 purchased from Bachem (Bubendorf, Switzerland). Plasma puri-
 fied C1inh was purchased from Alpha Diagnostics (San Antonio,
TX, USA). Kaolin (Light) was acquired from BDH Ltd. (Poole,
UK), and Phe-Pro-Arg-chloromethylketone (PPACK) from Hae-
mologic Technologies Inc. (Essex Junction, VT, USA). Goat
polyclonal anti-FXII was purchased from Cedarlane Ltd. (Burling-
ton, ON, Canada), and preadsorbed donkey-anti sheep/goat
(Star88P) from AbD Serotec (Kidlington, UK). TMB substrate
(3,3’-5,5’-Tetramethyl-benzidine) was obtained from Tebu Bio
(Heerhugowaard, the Netherlands). Ampicillin (Amp) was ac-
quired from Carl Roth GmbH (Karlsruhe, Germany). Citratted
human normal pooled plasma was prepared from the whole blood
of approximately 170 healthy volunteers, according to standard-
ised procedures to serve as reference material in routine clinical
diagnostic determinations. Prekallikrein-deficient plasma was ob-
tained from George King Biomedical (Overland Park, KS, USA).
HiLoad 26/600 Superdex 200 pg columns for nanobody purifi-
cation was obtained from GE Healthcare (Diegem, Belgium).
Phospholipid vesicles were prepared in a PS:PC:PE ratio of
20:40:40, as published earlier (23).

Llama immunisation and bacteriophage library
preparation

A pair of Llama glama received four subcutaneous injections with
α-FXIIa and β-FXIIa over a four-week period. Venous blood was
collected, from which peripheral blood lymphocytes were isolated
by Ficol gradient centrifugation. Subsequently, total RNA was iso-
lated and transcribed into cDNA, after which residual RNA was
removed with RNase H. Using an oligo-dT framework 1-specific
primer containing a 5’ SfiI restriction site and the cDNA as tem-
plate, two PCR products (1.6 and 1.3 kb) were generated, coding
for full-length IgG or IgG heavy chain, respectively. The isolated
1.3 kb product was treated with both SfiI and BstElI, resulting in a
300-400 bp fragment. This fragment was purified and ligated into
the phagemid vector pUR8100, conferring Amp-resistance for se-
lection, and encoding a C-terminal Myc- and His6 tag for detection
and purification of the nanobodies (24). The resulting li-
gation product was transformed into the Escherichia coli (E. coli)
TG1 strain (25). A transformation efficiency of 108 was achieved
for both libraries.

Nanobody selection against activated FXII

β-FXIIa (6 µg/ml in Phosphate Buffered Saline, (PBS; 21 mM
Na2HPO4, 2.8 mM NaH4PO4, 140 mM NaCl, pH=7.4)) was im-
mobilised overnight at 4°C on a 96-well polystyrol plate. Phages
were produced from both libraries and isolated via polyethylene
glycol (PEG; Mr=6000) precipitation (20% PEG in 2.5M NaCl).
Both the plate and phages were blocked with 4% skimmed milk
(m/v) in PBS. Subsequently, the phages were then allowed to bind
to the immobilised β-FXIIa. After extensive washing with PBS,
TWEEN20 (0.05% v/v, PBST), bound phages were eluted by the ad-
dition of triethylamine (TEA; 0.1 M). The high pH of the TEA fraction was neutralised by addition of Tris (1 M, pH=7.5) and phages were allowed to infect exponentially growing E. coli TG1. Infected bacteria were plated on Yeast Tryptone-agar plates containing glucose (2% m/v) and ampicillin (100 µg/ml) and grown overnight at 37°C.

Nanobody production and purification

Nanobody production by infected E. coli TG1 was induced by the addition of IPTG (0.1 M final concentration) and purified by immobilised metal affinity chromatography (IMAC) as published (26). Nanobodies were dialysed against Tris Buffered Saline (TBS; 50 mM Tris, 150 mM NaCl, pH=7.4) and further purified by gel filtration over Superdex columns. Protein concentration was spectrophotometrically determined via OD_{280nm}. Absorption coefficients of the nanobodies were determined based on their amino acid sequences using the online available ProtParam tool (http://web.expasy.org/protparam/). The purified nanobodies were assessed for impurity and degradation by coomassie blue staining after SDS-PAGE.

Detection of immobilised (activated) FXII by nanobodies

FXII, α-FXIIa (3 µg/ml) and β-FXIIa (6 µg/ml) were immobilised for 2 hours (h) in PBS, containing PPACK (12.5 µM). This inhibitor was present in all solutions throughout the experiment to prevent autoactivation and --degradation and all nano- and antibodies were diluted in blocking reagent. Subsequently, the wells were rinsed with PBS and blocked for 1 h. Nanobodies were diluted to 4 µg/ml and allowed to bind to the coated proteins for 1 h. Next, the wells were rinsed with PBST and incubated for 1 h with a monoclonal anti-myc antibody (9E10; 1 µg/ml) or goat polyclonal anti-FXII (1:2,000). R&-PO was used for the detection of bound anti-myc, and STAR88P (1:20,000) for the detection of bound anti-FXII. Finally, the wells were rinsed with PBST and stained with 100 µl TMB. The substrate reaction was stopped by the addition of 50 µl H$_2$SO$_4$ (0.3 M). Absorbance was determined at 450 nm. The entire assay was performed at room temperature (RT).

Nanobody capture ELISA for FXIIa

Nanobodies (4 µg/ml in PBS) were immobilised overnight at 4°C onto maxisorp plates. Wells were rinsed with PBS and blocked for 1 h at RT, after which samples were added to the plate and incubated for 2 h at RT while shaking. Next, the wells were rinsed with PBST and incubated with goat polyclonal anti-FXII (1:2,000) for 1 h at RT. After washing with PBST, the wells were incubated with STAR88P (1:20,000) for 1 h at RT. Finally, the wells were rinsed with PBST and stained with 100 µl TMB substrate. Substrate development was stopped by the addition of 50 µl H$_2$SO$_4$ (0.3M). Absorbance was determined at 450 nm.

The effect of protease inhibitors on the capture of purified FXIIa

FXII, α-FXIIa, or β-FXIIa (12.5 nM) was diluted in blocking reagent and incubated on ice in the presence or absence of PPACK (200 µM) for 30 minutes (min). Next, samples were spiked with Clino (250 µg/ml), α-1-AT (1.5 mg/ml), CTI (200 µg/ml) or vehicle (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO$_4$, 5 mM KCl, pH=7.4). Complexes were allowed to form for 30 min at 37°C. To stop complex formation, samples were diluted eight times in blocking reagent containing PPACK (200 µM) and incubated for 10 min on iced. Finally, the samples were analysed for the presence of FXIIa in the nanobody capture ELISA as described above.

Coagulation assays

Plasma coagulation times were recorded on a KC10 coagulometer (Amelung, Lemgo, Germany). Hereto, 50 µl prewarmed plasma was incubated in rolling KC10 cups with 50 µl of TBS containing concentration series of kaolin or dextran sulfate and a fixed concentration of phospholipid vesicles (final in-assay vesicle concentration; 10 µM). After 2 min, the mixture was recalculated with 50 µl of CaCl$_2$ (25 mM). In competition experiments, 50 µl plasma was preincubated with 10 µl of dextran sulfate in TBS for 1 min. Next, 40 µl of kaolin and phospholipid vesicles in TBS were added and incubated for 2 min (final in-assay concentrations were 150 µg/ml and 10 µM, respectively). Finally, coagulation was triggered by recalcification with 50 µl of CaCl$_2$ (25 mM). Plasma clotting was followed for 15 min. When no clotting occurred within this period, values were plotted at 900 seconds (s) and labelled ‘not detectable’ (ND).

Chromogenic contact activation assays

 Twenty µl of FXIIa/kallikrein substrate (H-D-Pro-Phe-Arg-pNA; final concentration 0.5 mM) and 74 µl of citrated plasma were distributed to each well of a 96-wells microtitre plate. Next, the plasma was activated by the addition of 6 µl of contact activator (final concentrations; kaolin: 150 µg/ml; ellagic acid: 12.5 µg/ml; dextran sulfate: 30 µg/ml). HBS served as vehicle control. Substrate cleavage was monitored spectrophotometrically (405 nm) at 37°C, over time. The inhibitory capacity of nanobodies on plasma contact activation was tested by mixing 20 µl H-D-Pro-Phe-Arg-pNA (final concentration; 0.5 mM), 50 µl of citrated plasma, and 24 µl nanobody (final concentration: 10 µg/ml). Next, this mixture was activated by kaolin, and amidolytic activity was monitored as described above. The direct effects of nanobodies on the amidolytic activity of purified FXIIa were determined by mixing α-FXIIa or β-FXIIa (375 nM) with 10 µg/ml (588 nM) of all nanobodies and monitored for cleavage of H-D-Pro-Phe-Arg-pNA (0.5 mM) as described above.
Recovery of FXIIa from plasma

α-FXIIa or β-FXIIa (12.5 nM) was added to normal pooled plasma containing a concentration series of PPACK (0-200 µM), and incubated at RT for 30 min. As a control, α-FXIIa or β-FXIIa was pre-incubated for 30 min with PPACK (200 µM) before addition to plasma or blocking reagent, each containing PPACK (200 µM). The samples were diluted eight times in blocking reagent, containing PPACK (200 µM) and incubated on ice for 10 min. The samples were then analysed for the presence of FXIIa in the nanobody capture ELISA described above. In another series of experiments, plasma was activated at 37°C with several contact activating materials in a time series (final concentrations; kaolin; 150 µg/ml, ellagic acid; 12.5 µg/ml, dextran sulfate; 30 µg/ml). Samples were taken and diluted eight times in blocking reagent, containing PPACK (200 µM) and polybrene (0.1%, m/v).

Results

The generation of nanobodies against FXIIa

In recent years, the development of nanobodies has resulted in several exciting discoveries, potentially offering novel therapeutic options for the treatment of cancer and human immunodeficiency virus (HIV) (27-29). Besides their therapeutic potential, these small camelid antibodies are capable of detecting discrete conformational differences in proteins, and can be selected specifically for these properties through phage display technology. In the past, nanobody-based assays were developed by us to assess the activation state of other haemostatic proteins, and study their role in pathology (30). In the current project, we chose a similar approach for the development of a bioassay to detect FXIIa. We started out by constructing an immune library, derived from peripheral blood mononuclear cells extracted from the blood of two llamas that had been immunised with both α-FXIIa and β-FXIIa. These libraries each contain a nanobody repertoire of >10^6 transformants. Phages were selected from these libraries for their ability to bind to immobilised β-FXIIa. This immobilised β-FXIIa was catalytically active, as it converted the chromogenic substrate H-D-Pro-Phe-Arg-pNA, commonly used to assay the catalytic activity of FXIIa. After the first round of selection, 96 single clones per library were screened for the ability to detect immobilised FXII, α-FXIIa, or β-FXIIa. Clones that recognised FXII zymogen, as well as genetic duplicates, were eliminated from further selections. Nanobodies were produced from the remaining clones, and tested for their binding to immobilised FXIIa. Next, we selected two clones that could bind to immobilised FXIIa, when in the presence of the small-molecular serine protease inhibitor Phe-Pro-Arg-chloromethylketone (PPACK), without recognising FXII zymogen. PPACK was added to restrict autoactivation and self-digestion of immobilised FXIIa. The selected clones were named A10 and B7; both of them could detect immobilised α-FXIIa, but only B7 could bind to β-FXIIa (Figure 1A). Biophysical characteristics of both nanobodies are reported in Table 1. Next, we investigated the functional properties of these nanobodies: while neither of these nanobodies or a control nanobody against activated FXI (FXIa) inhibited the cleavage of H-D-Pro-Phe-Arg-pNA by purified FXIIa (see Suppl. Figure 1, available online at www.thrombosis-online.com), both of them inhibited the cleavage of the same substrate when plasma was activated by kaolin (Figure 1B). The FXIa-control nanobody did not inhibit the generation of this kallikrein-like activity in plasma. This experiment indicates that nanobodies A10 and B7 bind in close vicinity of the active site of FXIIa, preventing interaction with macromolecular substrates. The remaining substrate cleavage that is detected in the presence of these nanobodies may be caused by direct amidolytic activity of kaolin-bound FXIIa, generated independently of prekallikrein activation (31).

We next investigated whether these nanobodies were able to capture purified FXIIa from solution. Here, the nanobodies were immobilised in microtitre plates and incubated with FXII zymogen, α-FXIIa, or β-FXIIa in equimolar amounts, in the presence or absence of PPACK. Captured FXIIa was subsequently detected with a polyclonal antibody. In line with our previous experiment (Figure 1A), nanobody A10 could only capture α-FXIIa (Figure 1C). Nanobody B7, however, was able to capture both α- and β-FXIIa (Figure 1D). This suggests that these nanobodies recognise different epitopes within α-FXIIa. It should be noted that the differences in signals between α- and β-FXIIa in Figure 1D can be attributed, in part, to the size differences between these two forms of FXIIa. As a result, a lower number of binding sites for the polyclonal detection antibody is available in captured β-FXIIa. Next we assessed the sensitivity of our assay setup in dose-response experiments with purified FXII zymogen, α-FXIIa, or β-FXIIa. We found that nanobody A10 recognised α-FXIIa with a detection limit of 0.1 nM (8 ng/ml; Figure 1E). However, no significant signals were obtained with β-FXIIa or FXII zymogen. Nanobody B7 could detect α-FXIIa at 25 PM (2 ng/ml) or β-FXIIa at 100 PM (3 ng/ml; Figure 1F). FXII zymogen showed no binding for any of the concentrations tested. These experiments show that nanobodies selected against FXIIa can be used to sensitively detect minute amounts of FXIIa in solution. Moreover, nanobodies A10 and B7 display different selectivities towards minor structural differences between the active sites of the isoforms of FXIIa in solution. We next went on to determine whether our nanobodies could also detect FXIIa in a plasma environment.

PPACK protects FXIIa from macromolecular inhibitors and improves detection in plasma

In plasma, serine protease inhibitors rapidly inactivate FXIIa. C1inh is seen as the major inhibitor, followed by α1-Antitrypsin (α1-AT) and antithrombin III (AT). However, AT functions mainly as an inhibitor when immobilised heparin is present (32). We first investigated whether our nanobody-based capture ELISA was influenced by the presence of these inhibitors. When we pre-incubated α-FXIIa and β-FXIIa (12.5 nM) with plasma concentrations of C1inh (250 µg/ml), capturing by nanobody A10 (in the case of α-FXIIa, since β-FXIIa is not efficiently captured) and na-
nobody B7 (in the case of both α-FXIIa and β-FXIIa) was severely compromised (Figure 2 A and B). When the active site was blocked by PPACK prior to exposure to C1inh, detection was completely restored. In contrast, the preincubation of α-FXIIa and β-FXIIa with α1-AT (1.5 mg/ml; reflecting plasma levels in the general population [33, 34]) did not affect capturing by either nanobody. Finally, when FXIIa was preincubated with CTI (200 µg/ml), a specific non-physiological inhibitor of FXIIa (35), capturing of α-FXIIa by nanobody A10 was severely reduced, although not to the extent as was the case for C1inh. However, the capturing of both α- and β-FXIIa by nanobody B7 remained largely intact in the presence of CTI. In the presence of PPACK,
capturing of FXIIa by both nanobodies was restored to normal levels. We next aligned these findings with functional experiments: while the amidolytic activity of both α-FXIIa (►Figure 2C) and β-FXIIa (►Figure 2D) was completely blocked in the presence of C1inh and CTI, it remained completely unaffected in the presence of α1-AT. In our experiments, which were all performed in the absence of heparin, AT was unable to inhibit FXIIa amidolytic activity and did not block the capturing of FXIIa by our nanobodies (not shown). Together, these experiments indicate that both C1inh and CTI, but not α1-AT, effectively form inhibited complexes with both forms of FXIIa in solution. The different effects of C1inh and CTI on FXIIa capturing by our nanobodies might be explained by their different molecular weights; whereas C1inh is approximately 104 kDa, CTI is only 12 kDa. As a result, it is possible that C1inh shields off a larger part of the FXIIa active site than CTI does, thereby disturbing the binding of A10 but not B7. Although differences in affinity between both nanobodies may help to explain these findings, it is unlikely that either of them displaces the inhibitors from FXIIa, as these bind to FXIIa in a covalent manner.

Since our data showed that FXIIa is protected from macro-molecular inhibitors and remains available for nanobody capturing, we next investigated the effects of PPACK on the recovery of FXIIa from citrated plasma. Hereto, we added a concentration series of PPACK to plasma, after which we spiked it with FXIIa (12.5 nM) and incubated it for 30 min at RT. ►Figure 2E shows that no α-FXIIa was captured by nanobody A10 from normal pooled plasma (NPP), nor from plasma that had been spiked with α-FXIIa in the absence of PPACK (indicated as 0 μM PPACK), compared to FXIIa that had been preblocked prior to spiking (pre-blocked). With increasing PPACK concentrations, the recovery of α-FXIIa from plasma progressively increased to the level of pre-blocked α-FXIIa. In similar fashion, the recovery of α- and β-FXIIa by nanobody B7 was strongly reduced in the absence of PPACK, but increased sharply as the PPACK levels became elevated (►Figure 2F). In both series of recovery experiments, optimal recovery was achieved in the presence of PPACK (200 μM; higher concentrations up to 6.4 mM showed no improvement [not shown]). Interestingly, while the recovery of FXIIa in plasma with nanobody B7 closely approximated the recovery of FXIIa in the absence of plasma (“Input” = purified FXIIa in blocking reagent), the maximal recovery of α-FXIIa from plasma by nanobody A10 was about 40% of the input levels in absence of plasma. This reduced recovery could not be explained by trace amounts of uninhibited plasma kallikrein or unperturbed PK activation (leading to reciprocal digestion of FXIIa), as the recovered amounts of α-FXIIa by A10 in congenitally PK-deficient plasma remained at 40% (indicated with “PK Def” on the figure axis). Hence, the possibility arises that plasma contains an undefined non-enzymatic high-affinity ligand for FXIIa that binds to the presence PPACK. Taken together, these experiments indicate that the presence of PPACK is a prerequisite for the recovery of FXIIa from plasma by nanobodies.

### Table 1: Biophysical characteristics of nanobody A10 and B7.

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<thead>
<tr>
<th>Characteristic</th>
<th>A10</th>
<th>B7</th>
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<td>Amino Acids (kDa)</td>
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<tr>
<td>Mw (kDa)</td>
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<tr>
<td>Isoelectric point (°)</td>
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<td>Extinction coefficient (°)</td>
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### Tracking surface-induced plasma contact activation with nanobodies

Kaolin, ellagic acid and long-chain dextran sulfate (DXS; average Mr =500.000) are three non-physiological triggers for contact system activation in vitro. All three evoke autoactivation of FXII and activation of the kallikrein-kinin system, but for unclear reasons, only kaolin and ellagic acid trigger coagulation. We first aimed to confirm this striking phenomenon in coagulation assays. Hereto, we used an activated partial thromboplastin time (aPTT)-based assay. In this assay, recalcified plasma coagulated after approximately 300 s in the presence of phospholipid vesicles (10 μM) and in absence of an added contact surface. A 2-min exposure of plasma to kaolin, prior to recalcification, induced a robust shortening of clotting times in a concentration-dependent manner (►Figure 3A). When the same experiment was performed with a wide concentration series of DXS instead of kaolin, no reduction in clotting times occurred. In contrast to kaolin, concentrations of DXS over 8.13 μg/ml prolonged the background recalcification time beyond 15 min (►Figure 3B). We next investigated whether the observed pro- and anticoagulant effects of kaolin and DXS in plasma compete for each other. When plasma was preincubated for 1 min with the same range of DXS, kaolin-induced clotting became dose-dependently prolonged (►Figure 3C; kaolin-induced clotting already prolonged from 42 ± 2 to 53 ± 3 s in the presence of 1 μg/ml of DXS). These experiments show that, although both kaolin and DXS are well-known activators of FXII and the contact system, only kaolin triggers coagulation, while DXS opposes it. Interestingly, the application of DXS as anticoagulant was already investigated and patented in the 1950s ([36] + US Patent 2715091), motivated by its structural comparability to heparin. Indeed, intravenous administration of DXS prolongs the aPTT in human subjects ([37], but has known toxic bleeding-related side effects ([38]). Surprisingly, unlike heparin, DXS is not able to activate AT ([39], and has instead been reported to directly inhibit purified thrombin. In contrast to these anticoagulant properties of DXS, in vitro experiments using purified factors (and in the absence of PK), have shown that it can support the activation of FXI by thrombin ([40] as well as by FXIIa ([14]).
Figure 2: Effect of plasma protease inhibitors, corn trypsin inhibitor and PPACK on the recovery of active FXII by nanobodies from plasma. A-B) C1-esterase inhibitor (C1inh; 250 μg/ml) and corn trypsin inhibitor (CTI; 200 μg/ml), but not α1-antitrypsin (α1-AT; 1.5 mg/ml) abolish the recovery of FXIIa (12.5 nM) by nanobodies (panel A and B represent capturing by nanobodies A10 and B7, respectively). The presence of PPACK (200 μM) attenuates loss of recovery. C, D) Amidolytic activity of purified α- and β-FXIIa (37.5 nM) in the presence of C1inh (250 μg/ml), CTI (200 μg/ml), and α1-antitrypsin (1.5 mg/ml). (panel C and D represent α- and β-FXIIa, respectively). E, F) Stabilisation of FXIIa in plasma is dose-dependent on PPACK concentration and independent of the presence of plasma prekallikrein (input = FXIIa signal in buffer that was used to spike plasma; preblocked = plasma spiked with FXIIa that had been preincubated with PPACK prior to spiking; PK Def = preblocked FXIIa in congenital PK deficient plasma) (panel E and F represent capturing by nanobodies A10 and B7, respectively). Panels A, B, E and F represent the mean ± SD of three individual experiments, each performed in duplicates. Panels C and D represent the mean ± SD of a representative experiment, performed in triplicate.

Taken together, it presently remains elusive why DXS acts as an anticoagulant in a plasma environment. However, we expect that activation of the kallikrein-kinin system does take place in DXS-treated plasma. We therefore hypothesised that FXII may respond to DXS in a manner that is different from its response to procoagulant surfaces, thereby limiting its contribution to coagulation. We next investigated whether our novel nanobody-based assays could help to provide evidence for this hypothesised mechanism.
Hereto, we first triggered activation of citrated plasma by kaolin, ellagic acid and DXS and confirmed their PK-activating potential by cleavage of the chromogenic substrate H-D-Pro-Phe-Arg-pNA (Figure 4A, D and G, left column). In a similar fashion, we activated plasma, now taking samples in a time series for immediate dilution in a mixture of PPACK and polybrene. In this way, the generated FXIIa was catalytically inhibited, protected from macromolecular inhibitors and eluted from its activating surface. We next analysed the obtained samples in our nanobody capture ELISAs. Kaolin evoked the swift generation of a form of FXIIa that could be captured by nanobody B7 (Figure 4B; 30 min pattern shown in Suppl. Figure 2A, available online at www.thrombosis-online.com), but not by nanobody A10 (Figure 4C; 30 min pattern shown in Suppl. Figure 2B, available online at www.thrombosis-online.com). In similar fashion, ellagic acid evoked the generation of a form of FXIIa that was recognised by B7 (Figure 4E; 30 min pattern shown in Suppl. Figure 2C, available online at www.thrombosis-online.com), but not A10 (Figure 4F; 30 min pattern shown in Suppl. Figure 2D, available online at www.thrombosis-online.com). In both cases, maximal levels of FXIIa were reached within 1 min, after which they gradually reduced by approximately 50% over a 10-min time period. We presume that this decreased recognition results in part from the binding of plasma inhibitors to the generated FXIIa (i.e. C1inh). Interestingly, kaolin- and ellagic acid-triggered FXIIa remained detectable by B7, up to 30 min (Suppl. Figure 2A and C). In contrast, when plasma was activated by DXS, a species of FXIIa formed that was recognised by nanobody B7 (Figure 4H; 30 min pattern shown in Suppl. Figure 2E, available online at www.thrombosis-online.com). The swift generation of FXIIa peaked within 2.5 min, but had largely disappeared within 10 min and was completely gone after 30 min (Suppl. Figure 2E, available online at www.thrombosis-online.com). Unlike activation by kaolin and ellagic acid, activation of plasma with DXS evoked the gradual formation of a species of FXIIa, detectable by nanobody A10 (Figure 4I; 30 min pattern shown in Suppl. Figure 2F, available online at www.thrombosis-online.com). Notably, the generation of this species coincided with the accelerated decline (compared to other activators) of the species detected by nanobody B7 in the same DXS-activated plasma samples. Our data suggests that the rapid decline of DXS-triggered FXIIa, detected by nanobody B7, represents inactivation through plasma inhibitors as well as enzymatic conversion into a secondary species of FXIIa that is in turn recognised by nanobody A10, but no longer by B7. Based on these observations, it is probable that nanobody A10 recognises a full-length, non-procoagulant form of FXIIa, which is generated by DXS. Moreover, this species does not seem to be spontaneously neutralised by macromolecular plasma inhibitors such as C1inh, that compromise capturing by nanobody A10.

**Discussion**

In the present studies, we have generated and characterised two nanobodies, selected against the catalytic domain of FXIIa. With these nanobodies, we developed an ELISA-based method to detect FXIIa in plasma and track the fate of FXIIa during plasma contact activation.

Based on their inhibitory effects on plasma contact activation (Figure 1B), both nanobody B7 and A10 are likely to recognise epitopes in close vicinity to the active site, that are formed upon
activation (i.e., exosites) and needed for PPK activation. They do not directly bind inside the active site or distort its conformation, as direct amidolytic activity of purified FXIIa was not inhibited in their presence. Our data indicate that nanobodies A10 and B7 recognise separate epitopes, as only nanobody A10 displays a decreased antigen capturing in the presence of CTI (Figure 2A and B). This is also reflected in their ability to capture FXIIa: nanobody B7 recognises both $\alpha$-FXIIa and $\beta$-FXIIa in solution, whereas nanobody A10 only recognises $\alpha$-FXIIa in solution (Figure 1C and D). This is surprising, as both nanobodies were raised against immobilised $\beta$-FXIIa. Since nanobody B7 recognises both $\alpha$-FXIIa and $\beta$-FXIIa in solution, we presume that this nanobody recognises a common feature in the FXIIa catalytic domain that is generally presented upon activation. But how can we explain that nanobody A10 is only able to capture $\alpha$-FXIIa? During the selections on immobilised $\beta$-FXIIa, phages are able to bind via multiple copies of the nanobody (estimated at three), allowing for selection through high avidity, rather than high-affinity. This can result in the seemingly undesirable selection of a nanobody, which is a poor binder of $\beta$-FXIIa. However, in the case of nanobody A10, the specific structural requirements for binding are heavily represented in $\alpha$-FXIIa, hereby making it a potentially useful tool for investigation of the existence of functional differences in the FXIIa catalytic domain.

Figure 4: Detection of factor XII activation during contact activation in plasma by nanobodies. Contact activation was triggered in citrated plasma by the addition of kaolin (150 $\mu$g/ml), ellagic acid (12.5 $\mu$g/ml) or DXS (30 $\mu$g/ml) and monitored by chromogenic assay (left column, panels A, D, G; data represent mean ± SD of a representative experiment). Alternatively, samples were taken from plasma that had been activated as described above and diluted in blocking reagent with PPACK (200 $\mu$M) after sampling. Subsequently, FXII activation was determined by nanobody B7 capture ELISA (middle column, panels B, E, H), or nanobody A10 capture ELISA (right column, panels C, F, I). Data represent the mean ± SD of three individual experiments.
In the current project, we found that the binding of our nanobodies to the FXIIa catalytic domain could be fully inhibited by C1inh, similar to the previously reported monoclonal antibody 2/215 (19). We here show that FXIIa capturing by nanobodies is unaffected by C1inh when PPACK is present (Figure 2A and B). As PPACK irreversibly blocks the FXIIa active site and prevents C1inh binding, but still allows for nanobody capturing, the negative influence of C1inh on our capture assay is effectively overruled. Using our assay, we were able to fully recover spiked FXIIa from plasma with nanobody B7 (Figure 2F). However, nanobody A10 maximally returned 40% of the plasma-spiked α-FXIIa in the same experiments (Figure 2E). Interestingly, nanobody A10 lost an equal amount of α-FXIIa capturing capacity (60%), when bound to CTI (Figure 2A), whereas no capturing was possible in the presence of C1inh.

This suggests that CTI modifies the structure of the FXIIa catalytic domain in a way that reduces the binding affinity of A10 for its exosite. In line with this, our data suggest that plasma contains a factor that behaves similarly to CTI, reducing the binding of A10 by 60%, but, in contrast, cannot be reversed by PPACK. Since C1inh, α1-AT, or AT irreversibly bind to the FXIIa active site serine, this hypothetical factor should bind through a putative exosite, present in α-FXIIa, close to the epitope for A10 binding.

Based on our plasma activation assays, we propose a model for the activation of FXII on various surfaces, which may help to explain their different characteristics. In a primary activation step, all surfaces trigger a conformational change in FXII during (auto)activation. This can be recognised by nanobody B7 and is represented in purified α-FXIIa and β-FXIIa (Figure 1F). In a second step, plasma inhibitors gradually inactivate (surface-bound) FXIIa. Indeed, the maximal amount of FXIIa obtained during activation by kaolin or ellagic acid, is reduced by >50% over time, as detected by nanobody B7 (Figure 4B, E). This is in good agreement with our purified experiments, shown in Figure 2B, where the recognition of FXIIa by B7 remains partially intact in the presence of C1inh. We therefore propose that the remaining capturing signals after 10 min of activation by these activators are predominantly α-FXIIa-C1inh complexes. For procoagulant activators of the contact system, the story ends here: the third step of the process only takes place on material surfaces, such as DXS, that do not evoke coagulation (Figure 3; [36]). In our experiments, we found that plasma activation by DXS leads to a sharp increase in a FXIIa species, recognised by nanobody B7, which is completely lost over time (Figure 4H). This is suggestive for the formation of β-FXIIa-C1inh complexes, which are not efficiently recognised by nanobody B7 (Figure 2B). However, according to this explanation, we would not be able to detect them in our nanobody A10 ELISA either, as this does not recognise free β-FXIIa, nor β-FXIIa in complex with C1inh (Figure 2A, E).

In sharp contrast, when we analysed DXS-activated plasma samples in our A10 capture ELISA, we observed a gradual generation of a FXIIa species (Figure 4I) that did not occur in the presence of the procoagulant activators kaolin and ellagic acid (Figure 4C, F). This suggests that the captured molecule is a proteolytic product of FXIIa, most likely converted from the original pool of FXIIa as detected by nanobody B7 capture ELISA. Moreover, this experiment indicates that C1inh does not efficiently inhibit this species (which would fully prevent capturing by A10; Figure 2A). Since it is difficult to imagine that this species remains uninhibited within a 30-min timespan, it could again hint towards the modulation of free α-FXIIa by a previously undescribed factor that binds to FXIIa, reducing its recognition by A10 (as described above and shown in Figure 2E). In conclusion, the outcomes of our experiments indicate that the activation of FXII on DXS results in the formation of two successively generated forms of FXIIa, whereas procoagulant activators retain the first form of FXIIa until inhibition occurs. It is possible that the multiple kallikrein cleavage sites in FXII play a role in the conversion between different forms of FXIIa (41).

The role of FXII in coagulation was long thought to be insignificant in vivo, as FXII-deficient patients display no overt bleeding tendency (42). However, recent studies in FXII knockout mice show that these mice are protected against arterial thrombosis (43). Moreover, antisense oligonucleotide-directed targeting of FXII reduced the susceptibility to venous thrombosis (44), in which FXII has been recently implicated (11). Besides its role in coagulation, FXII and the plasma contact system are intimately involved in the pathogenesis of (hereditary) angioedema (HAE [MIM #106100]), and implicated in various other inflammation-related conditions, including hypersensitivity reactions (12), rheumatoid arthritis (45), and sepsis-related hypotension (46).

At present, the role of FXII in human pathophysiology is uncertain, as reports have shown evidence of pathological, neutral and protective roles in disease (16-18, 47-50). We hope that, with the development of our nanobody-based assays against FXIIa, it will be possible to distinguish procoagulant responses (B7+/A10-) from proinflammatory responses (B7+/A10+) in patient plasmas, allowing for further investigations into the role of FXII in pathophysiology. By untangling the activation mechanism of FXII and delineating the potential functionally different isoforms of this protein, we can provide new insights into the roles of this volatile enzyme in health and disease.

Acknowledgments
The work of SM is supported by Technology Foundation STW (project 10714). A Veni Fellowship (016-126-159), provided by the Netherlands Organisation for Scientific Research (NWO) supports the work of CM. The authors want to thank Dr. Mohamed El Khattabi and Prof. Dr. Theo Verrips from the Department of Biomolecular Imaging at Utrecht University, for their help in the construction of the nanobody immune libraries. We want to thank Suzanne E. Atkins for critically reading the manuscript. The help of Joyce van Schaffelaar during the selections is greatly appreciated.

Conflicts of interest
None declared.

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