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RESEARCH PAPER

Biomimetic biosensor to distinguish between inhibitory and non-inhibitory factor VIII antibodies

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Abstract Patients with hereditary or acquired haemophilia A may develop inhibitory factor VIII (FVIII) antibodies. These disrupt FVIII activity predominantly by preventing the formation of the tenase complex, leading to a serious bleeding disorder. Antibodies without inhibiting activity, however, can also be found when screening patients with haemophilia A under FVIII supplementation. Therefore, the detection of only these allo- or autoantibodies from plasma is not sufficient. Rather, the characterization of the antibody-induced effects on the coagulation cascade should be considered due to its great diagnostic importance. Currently, inhibitory activities are detected by the functional Bethesda assay, which directly measures the delay in clotting time by the patient plasma. However, this assay does not provide information on the cause of the inhibition. Here, we report the development of a surface plasmon resonance (SPR) biosensor that has the potential to integrate both quantitative and functional information on patient antibody characteristics in one measurement. Recombinant FVIII protein was immobilized on the sensor surface to detect antibodies from patient plasma. The interaction of the FIX- and FXa-clotting proteins with the formed anti-FVIII/FVIII complex could be detected subsequently within the same SPR measurement cycle. Inhibitory antibodies led to the prevention of these interactions. Thus, discrimination between the clinically relevant inhibitory and non-inhibitory antibodies was enabled. In a group of 16 patients with inhibitory antibodies (both ELISA- and Bethesda-positive), 5 patients with non-inhibitory antibodies (ELISA-positive but Bethesda-negative) and 12 healthy controls, diagnostic sensitivity and specificity data of 100 % for the FIX interaction were achieved using this biomimetic biosensor approach. The new method allows for detection and quantification, as well as for evaluation of inhibitory activity of allo- and autoantibodies, using small sample volume and short analysis time.

Keywords Factor VIII · Antibodies · Haemophilia A · Surface plasmon resonance

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Abbreviations

Anti-FVIII	Antibody against FVIII
AUC	Area under the curve
BDD	B-domain-deleted
BU	Bethesda units
CV	Coefficient of variation
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
FC	Flow cell
FIX	Factor IX
FL	Full length
FVIII	Factor VIII
FXa	Activated factor Xa

NHS	*N*-Hydroxysuccinimide
ROC	Receiver operating characteristic
RU	Resonance units
SPR	Surface plasmon resonance
vWF	von Willebrand factor

Introduction

Haemophilia A is a X-linked inherited coagulopathy with a prevalence of 1 in 5000 males worldwide [1]. Patients with this disease suffer from bleeding episodes in the joints, muscles and soft tissue caused by a deficiency of the blood coagulation factor (F) VIII (in severe cases <1 % activity of FVIII). Treatment is based on exogenous replacement of FVIII by concentrates throughout life isolated from plasma or produced recombinantly. However, in both cases, such treatment regimes lead to a development of specific alloantibodies in 25 % of patients [2]. These inhibitory antibodies frequently compromise the function of the FVIII therapy [3, 4], which is the most serious complication in haemophilia treatment. In addition to alloantibodies, autoantibodies directed against the intrinsic FVIII can also be found in patients with no pre-existing history of haemophilia. The approximate incidence of the so-called acquired haemophilia is 0.2 to 1 per million per year [5]. Patients who develop inhibitory autoantibodies demonstrate excessive bleeding episodes, despite having no prior history of a coagulation disorder [6].

FVIII is a 330-kDa glycoprotein produced in hepatocytes and endothelial cells. It is proteolyzed into a heavy chain consisting of domains A1, A2 and a B domain with various lengths as well as a light chain comprising domains A3, C1 and C2 [7, 8]. These two subunits are complexed by a metal ion. While circulating in blood as the inactive form, FVIII is covered by the von Willebrand factor (vWF) [9]. Specifically, vWF is a large multimeric protein that protects FVIII from fast proteolytic degradation and prevents premature coagulation. Upon activation and dissociation from vWF, FVIII acts as cofactor to FIX in the intrinsic pathway (besides the other physiologically relevant factors phospholipids and calcium ions). Together, these two factors are responsible for the proteolytic FX activation (Xase complex). The resulting FXa is essential for thrombin generation [10]. As shown later, this interaction is used for distinguishing between inhibitory and non-inhibitory antibodies.

Inhibitor plasma levels are routinely analysed by use of the functional Bethesda [11] or Nijmegen assay [12], which measures clotting activity. The amount of antibody in patient plasma that inactivates 50 % of normal plasma FVIII after 120 min incubation at 37 °C is defined as 1.0 Bethesda unit (BU/mL). Patients with >0.6 BU/mL are considered to be Bethesda-positive. The Bethesda assay requires large sample volumes, and hands on time and the inter-laboratory variation coefficients (CV) of FVIII inhibitor tests remain very high (40–60 %) [13]. As such, further method development is required to provide appropriate patient care and monitoring.

The FVIII Antibody Screen ELISA from Gen-Probe is a qualitative solid phase assay designed to detect IgG antibodies reactive with rFVIII in human plasma. The assay is characterized by a high diagnostic sensitivity at a low-to-moderate specificity. In cases with a positive ELISA signal and a negative Bethesda result, the existence of non-inhibitory antibodies can be concluded. These antibodies bind to non-functional epitopes and do not cause clotting problems. The existence of these antibodies is described in different publications [14, 15].

A novel approach for antibody detection in patient plasma is the application of surface plasmon resonance (SPR) technology. The efficiency of this technology with respect to autoantibody detection has been proven in a variety of papers by our group [16–18]. SPR is a quantum optical phenomenon arising in thin metal films under conditions of total internal reflection of incoming monochromatic and *p*-polarized light at the interface of two transparent media of differing refractive indices. By bioconjugating this surface, the SPR biosensor allows label-free, real-time and quantitative detection of biospecific interactions such as antigen–antibody, protein–protein or protein–DNA.

In this paper, we describe a SPR biosensor for FVIII allo- and autoantibody detection and functional characterization from plasma samples of affected patients. This is enabled by monitoring the ability of surface-bound FVIII to interact with FIX and FXa after incubation with patient antibodies isolated from human plasma. A scheme of this two-step assay design is illustrated in Fig. 1. Using the new assay, Bethesda-positive samples were distinguished from two control groups with 100 % diagnostic sensitivity and 100 % specificity when applying the FIX interaction. Therefore, the new biomimetic approach combines the information of both the ELISA and the Bethesda assays and can therefore improve therapeutic management of FVIII inhibitor patients.

Material and methods

Reagents, proteins and antibodies

All reagents, unless otherwise indicated, were from Sigma-Aldrich and of analytical purity. Recombinant full-length (FL)-FVIII was purchased from Bayer (Leverkusen, Germany). Recombinant FIX was purchased from Pfizer (New York, NY, USA) and plasma FXa from Enzo Life Sciences (Lausen, Switzerland). Polyclonal goat anti-human-FVIII IgG (stock concentration 10 mg/mL) was purchased from Cedarlane, monoclonal mouse anti-human-FVIII IgG (stock solution 0.5 mg/mL) from GeneTex (Irvine, CA, USA). The FVIII

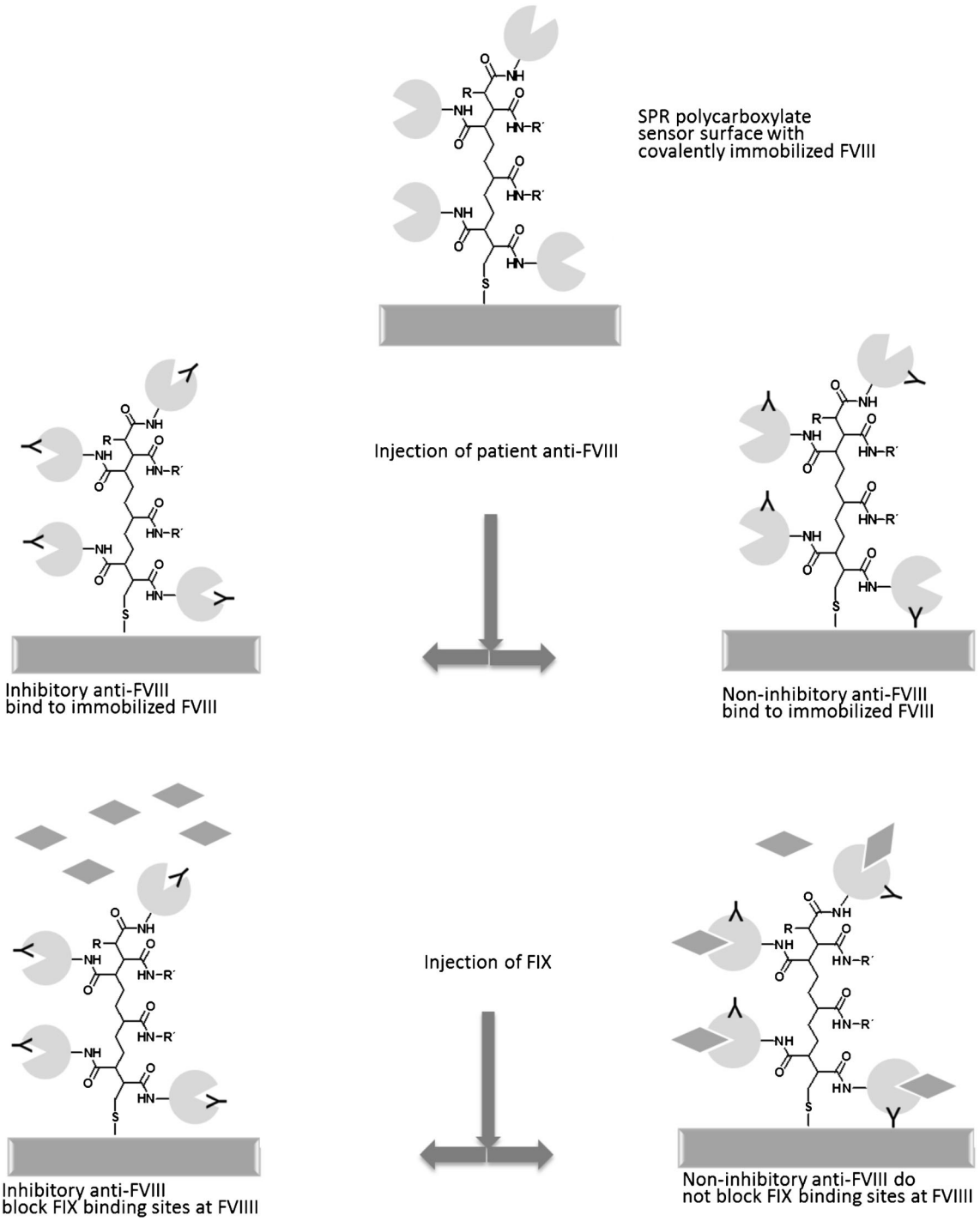

Fig. 1 Scheme of the FVIII biosensor format. The surface-bound FVIII first binds anti-FVIII, isolated from patient samples. In the second step, the biospecific chip surface may interact with FIX and FXa

Antibody Screen assay from Gen-Probe (Bedford, MA, USA) was carried out in accordance to the manufacturer's instructions.

Samples of citrate plasma from apparently healthy donors were collected in-house (n=17). Five of these samples were found to be positive in an ELISA measuring FVIII antibodies. These were defined as non-inhibitory, antibody-positive patients. Bethesda-positive plasmas were obtained from consecutively recruited patients admitted from two different sites: (i) Haemophilia Centre, Ludwig Maximilians-Universität München (n=9 autoantibodies, n=5 alloantibodies) and (ii) Haemophilia Centre, Goethe Universität Frankfurt am Main (n=4 autoantibodies, n=3 alloantibodies). FVIII inhibitor levels were measured according to the Bethesda method [11]

with the Nijmegen modification [12] at the laboratories of the two haemophilia centres and expressed in Bethesda units/mL (BU/mL). Plasma samples were collected and stored as aliquots at −80 °C. The retrospective trial conformed with the principles of the Helsinki Declaration as reflected prior approval of the Ethics Committee of the University of Munich. This approval was also valid for the patients from Frankfurt as both centres are members of the German Haemophilia network.

Purification of human antibodies from plasma samples

Protein A/G recombinant fusion protein, covalently immobilized on Sepharose from Thermo Fisher (Waltham, MA, USA), was used for affinity purification (NAb™spin columns 0.2 mL). The A/G fusion protein binds all immunoglobulin subtypes [19, 20]. Fifty microliters of plasma were incubated and processed according to manufacturer instructions. The buffer was exchanged to HEPES running buffer by using spin columns (Millipore, Darmstadt, Germany) with a 30-kDA cutoff. The samples were centrifuged 5×5 min with 13,000*g*. Final volume of each sample was adjusted with HEPES buffer to 300 μL. The total protein content was finally measured for a quality control with a Bradford solution from Bio-Rad (Hercules, CA, USA) [21].

SPR measurements

SPR biosensor measurements were performed using BIAcoreX and BIAcoreX100 (GE Healthcare, Freiburg, Germany) devices. All measurements were performed at 25 °C at a flow rate of 10 μL/min on HC 1500 nm chips (Xantec, Münster, Germany), consisting of a polycarboxylate-coated gold surface. All buffers for the biosensor measurements were sterile-filtered using 0.2-μm FPCA-S filters (Whatman, Dassel, Germany) and extensively helium-degassed before use.

As antigen, 100 μL of intact FVIII protein (100 IU/mL) was immobilized onto the polycarboxylate surface with the original Biacore amine coupling kit according to manufacturer's instructions at a flow rate of 10 μL/min. This FL FVIII protein proved as the best-suited antigen to be immobilized onto the biosensor surface. Other FVIII preparations, such as B-domain deleted (BDD) FVIII (Cedarlane or Advate and Refacto) or other FL FVIII compounds, were tested and found to be inferior in terms of binding antigenicity and on-chip stability (data not shown).

The sensor surface was conditioned by a threefold injection of 30 μL of high ionic strength borate buffer (0.1 mM sodium borate, 1 M NaCl, pH 9.0), followed by activation with NHS/EDC for 50 s. Subsequent antigen injection was repeated until an immobilization level of at least 15,000 RU was found. The reference cell was activated with EDC/NHS and blocked with 50 μL 1 M ethanolamine.

HEPES (10 mM), pH 6.9, supplemented with 300 mM NaCl, 5 mM $CaCl_2$ and 0.2 % Tween 80 was used as running and dilution buffer. In all measurements, antibodies extracted from original plasma samples were diluted 1:6 (v/v). In each case, 45 μL was injected over both cells (FC1 and FC2), followed immediately by injection of FIX or FXa for 200 s contact time. Both proteins were solubilized according to manufacturer's instructions and diluted 1:10 in running buffer. The surface was then regenerated by one injection of 1 s 10 mM NaOH, 1 M NaCl buffer, pH 12.0. As quality control, FVIII chips were tested for viability by measurements with monoclonal and polyclonal FVIII antibodies before use.

All sensorgrams are presented as the difference curve between the measuring cell FC2 and the nonspecific signals from the reference cell FC1. SPR biosensors detect the refractive index change in an approximately 200 nm distance to the chip surface, the signal being quantitatively expressed as resonance units (RU). Maximum binding in the association phase was specified as the difference in RU at the baseline and at the end point of injection of the respective sample.

We extensively checked for any non-specific binding phenomena that might occur. We optimized the buffer, as well as the running conditions. Figure S1 in the Electronic Supplementary Material depicts that no unspecific binding to immobilized FVIII was seen when using two polyclonal antibodies with different antigen specificity (anti-human IgG and anti-human von Willebrand factor). The optimized system shows a high binding signal when using a polyclonal anti-FVIII.

Statistical analysis

For statistical analysis, SPSS software, version 20.0 (IBM, Armonk, NY, USA) and Origin, version 8.5 software (Origin Lab, Northampton, MA, USA) were used. Receiver operating characteristic (ROC) curves were plotted, and areas under the curves (AUC) as a measure of the differentiation ability ill vs. healthy according to Bethesda measurements were determined. Optimal cutoff values were set to yield a maximum Youden Index (J=sensitivity+specificity−1).

Results and discussion

Patient characterization

Patient samples were tested using the Bethesda assay at the site where the sample was collected. Healthy controls were assumed to be Bethesda-negative. In addition, an ELISA and the different SPR assays were performed. The ELISA has no strict cutoff value. The cutoff between positive and

negative samples is set by the reactivity of the lot-specific kit control (KC). Samples with average optical density (OD) values greater than the average OD value of the KC are considered positive. Samples with average OD values equal to or less than the average of the KC are considered negative. The results are summarized in Table 1.

An example sensorgram of the two-step assay is shown in Fig. 2. As described in the introduction, positive ELISA and negative Bethesda results account for the existence of non-inhibitory antibodies in plasma samples, whereas inhibitory antibodies show positive results in both test formats.

Factor VIII immobilization

The appropriate anti-FVIII detection with this SPR biosensor is largely dependent on the conservation of the antigenicity when immobilizing FVIII onto a solid surface. With amide coupling, we could achieve antigen immobilization levels of

Table 1 ELISA and SPR measurements in the plasma samples of the 16 patients with inhibitors (Bethesda- and ELISA-positive), the 5 patients with non-inhibitory anti-FVIII (ELISA-positive, but no bleeding disorder) and the 12 healthy controls (ELISA-negative and no bleeding disorder). In contrast to the ELISA measurements, the FIX-binding signal shows a clear distinction between inhibitory and non-inhibitory antibodies

Sample #	Bethesda assay (>0.6) [BU/mL]	ELISA [OD]	SPR–FIX binding [RU]	SPR–FXa binding [RU]	SPR–anti-FVIII binding to FVIII [RU]
Patients with inhibitory antibodies (Bethesda +, ELISA +, SPR +)					
1	37	2.00	−53.2	−433	410
2	3	1.42	−634	−242	1586
3	2	1.17	−56.5	−47	1066
4	3	0.86	−428	−400	1932
5	153.6	2.48	−420	−574	5458
6	48.1	2.49	−88.7	−545	3646
7	7	0.83	−589	−58	1590
8	16	2.21	−437	−398	1803
9	8.5	1.79	−489	−417	1288
10	3	0.94	−86.6	−629	922
11	45	2.33	−61.9	−266	1706
12	23	1.98	−139	−806	1392
13	0.6	0.80	−590	−291	1778
14	1	0.34	−68.8	−288	1564
15	0.6	0.34	−51.2	−292	1660
16	3	0.88	−147	−545	3397
Patients with non-inhibitory antibodies (Bethesda ND, ELISA +, SPR −)					
1	ND	1.28	386	116	767
2	ND	0.57	534	−152	1633
3	ND	0.88	386	−172	1788
4	ND	2.24	803	184	1207
5	ND	1.10	1148	369	612
Controls (Bethesda ND, ELISA−, SPR−)					
1	ND	0.22	239	189	416
2	ND	0.22	169	−5.8	374
3	ND	0.43	916	213	2036
4	ND	0.16	177	187	360
5	ND	0.38	569	239	688
6	ND	0.19	827	186	1222
7	ND	0.22	105	92	583
8	ND	0.2	961	163	2540
9	ND	0.23	158	296	493
10	ND	0.25	137	−47	118
11	ND	0.21	158	−66	277
12	ND	0.26	150	−6.8	159

15,000 to 20,000 RU. Measurements of FIX (see Fig. 2) and FXa interactions with immobilized FVIII after application of patient samples require antibody purification in order to deplete the naturally occurring FVIII interaction partners in plasma, such as vWF. These interactions were found to compromise assay performance due to high levels of non-specific binding (data not shown) and saturation of the chip-immobilized FVIII protein to be monitored in the next step. The finding that anti-FVIII cannot interact with FVIII in the presence of vWF was already seen by Grancha et al. [22]. Antigenicity—even after several measurement cycles—could be confirmed by applying FVIII monoclonal and polyclonal antibodies and the undisturbed binding of FIX and FXa (data not shown). As depicted in Figure S2 in the Electronic Supplementary Material, the regeneration protocol proved to be effective (attenuations of the maximum RU of less than 10 % over approx. 60 measuring cycles).

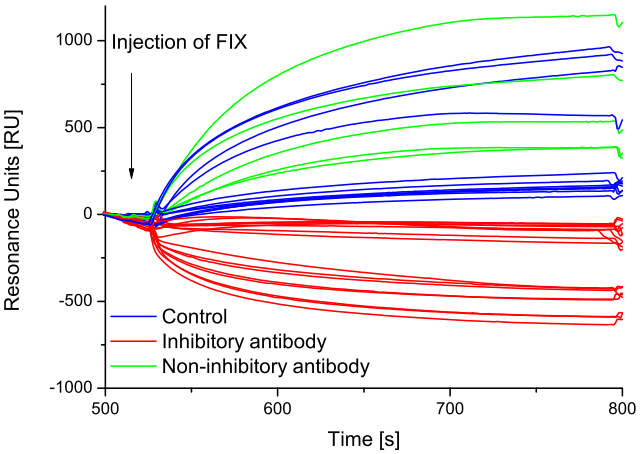

Fig. 3 Sensorgrams of FIX binding to the anti-FVIII/FVIII complex (all samples) after incubation with: inhibitory antibodies (Bethesda-positive patients) depicted in *red*, non-inhibitory antibodies (ELISA-positive, but Bethesda-negative patients) in *green* and healthy controls in *blue*

Purification of human antibodies from plasma samples

Measurements with diluted plasma samples from healthy controls unselectively showed a strong binding to the antigen-coated chip. A differentiation between samples from either patients with anti-FVIII or healthy subjects was not feasible. We could identify that vWF, present in high concentrations in human plasma, is responsible for the binding signals (data not shown). Physiologically, vWF covers approximately 99 % of FVIII to protect the clotting factor from degradation. vWF from untreated plasma samples completely complexes the immobilized FVIII on the biosensor surface. This signal overlaps with the specific signal being generated from anti-FVIII (see Figure S3 in the Electronic Supplementary Material). Thus, an immunoglobulin-separating step was necessary prior to the biosensor measurements of plasma samples.

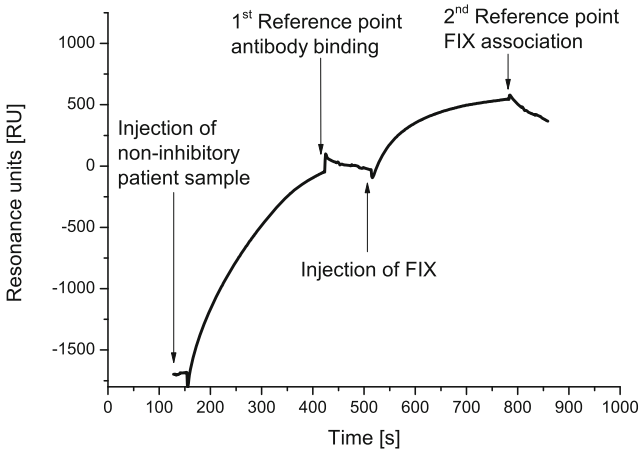

Fig. 2 Example of a SPR sensorgram showing binding of (non-inhibitory) anti-FVIII to FVIII, immobilized to the sensor surface, and subsequent binding of FIX to the anti-FVIII/FVIII complex

Inhibitory activity of patient anti-FVIII

We evaluated the binding behavior of FIX and FXa to the immobilized FVIII after injection of purified antibody samples from patients and healthy controls. Figures 3 and 4 portray the subsequent binding of these secondary interaction partners; negative values are due to the dissociation of the first bound analyte. As shown in Fig. 3, injection of FIX yielded a clear differentiation for patients with inhibitory antibodies from patients with non-inhibitory antibodies or healthy controls. Figure 4 shows the same assay concept using FXa instead of FIX. The diagnostic utility of the FXa biosensor assay is apparently inferior to the FIX assay. These data, concerning the tenase complex, are in accordance with clinical

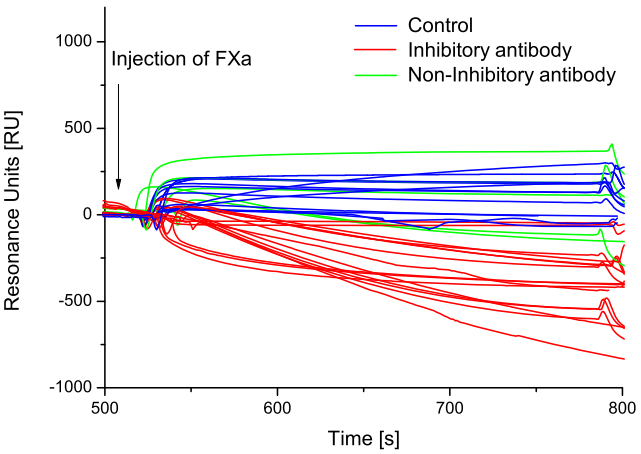

Fig. 4 Sensorgrams of FXa binding to the anti-FVIII/FVIII complex (all samples) after incubation with: inhibitory antibodies (Bethesda-positive patients) in *red*, non-inhibitory antibodies (ELISA-positive, but Bethesda-negative patients) in *green* and healthy controls in *blue*

observations that antibodies against the FIX-binding epitopes lead to more serious bleeding episodes.

Each sensor was used for one working day. In total, six biosensor chips were applied for measurements with the coagulation factors IX and Xa. Repetitive measurements of antibody binding using the biosensor methods showed high imprecision CV values (up to 26 % inter-chip and 9 % intra-chip) in preliminary experiments. The chip-to-chip variability is presumably due to different surface coating densities. Isolated high binding outliers in the control group (see Table 1) could be tolerated due to the high binding of FIX in these samples, indicating that unspecific binding in the first part of the measuring cycle does not interfere with the FIX interaction. Even though the chips are not fully comparable with respect to signal intensities and curve shapes, a clear cutoff was found for the patient and control groups tested, yielding both diagnostic sensitivity and specificity of 100 % for the FIX assay and a sensitivity of 88.2 % and specificity of 100 % for the FXa assay.

In Fig. 5a, b, the box plots of the binding signals of FIX and FXa to the immobilized FVIII in the presence of samples of the three patient groups are depicted. The figures impressively illustrate the significant RU suppression in the presence of inhibitors in the patient samples. The biomimetic two-step approach reveals that the significant discrimination of the absolute binding values of the second binding partner (FIX or FXa) yields the diagnostic information.

Figure S4 in the Electronic Supplementary Material additionally shows the ROC analysis for the two SPR and the ELISA methods in order to calculate diagnostic sensitivities and specificities for the respective assay methods. While the AUC is 1.0 for the FIX assay, it is 0.98 for the FXa assay and 0.85 for the ELISA. The optimum cutoff levels of the FIX and FXa assays are 26.9 RU and −207 RU, respectively. The ELISA only showed 87.5 % sensitivity and 76.6 % specificity when using the Bethesda assay as reference method. This is due to the fact that non-inhibitory antibodies cause false-positive results.

The development of FVIII-neutralizing inhibitors in haemophiliacs is the most harmful complication of FVIII supplementation, but the idiopathic development of anti-FVIII autoantibodies may also lead to unexpected and serious bleeding episodes. The presented analytical study describes the establishment and validation of a semi-quantitative SPR biosensor for FVIII allo- and autoantibody detection. The key novelty of the biosensor is the discrimination between inhibitory and non-inhibitory antibodies, being facilitated by measuring the interaction of FIX and FXa in the two-step SPR protocol. It is known that allo- and autoantibodies interfere with the FIX- and FIXa-binding site and inhibit the cofactor function of FVIII [23, 24]. An alternative inhibition mechanism is blocking of the FXa-binding site [25]. Therefore, we investigated these interactions in detail. Compared to ELISA methods, the SPR biosensor approach allows for a high degree of flexibility in assay design and is also suitable to analyse biomolecular interactions with fast kinetics in a label-free manner. For this reason, the biosensor was employed to mimic the physiological processes. This yields information similar to the one obtained in Bethesda assays, but combined with information about the antibodies' target.

In the described biosensor system, a polycarboxylate surface was used to covalently immobilize FVIII onto a gold chip. The polycarboxylate residues exhibit multiple binding sites for the protein, resulting in a high surface density of FVII I and, therefore, a possibly decreased non-specific binding behaviour [26]. Our approach to detect anti-FVIII finds its equivalence in a recent publication of Lewis et al. [27]. The authors aimed to quantify the IgG subtype distribution of anti-FVIII in patient samples. They applied a SPR sensor with an immobilized mouse anti-FVIII surface which subsequently captures synthetic FVIII proteins as antigenic structure. Thus, phenotypes of allo- and autoimmune antibody responses to

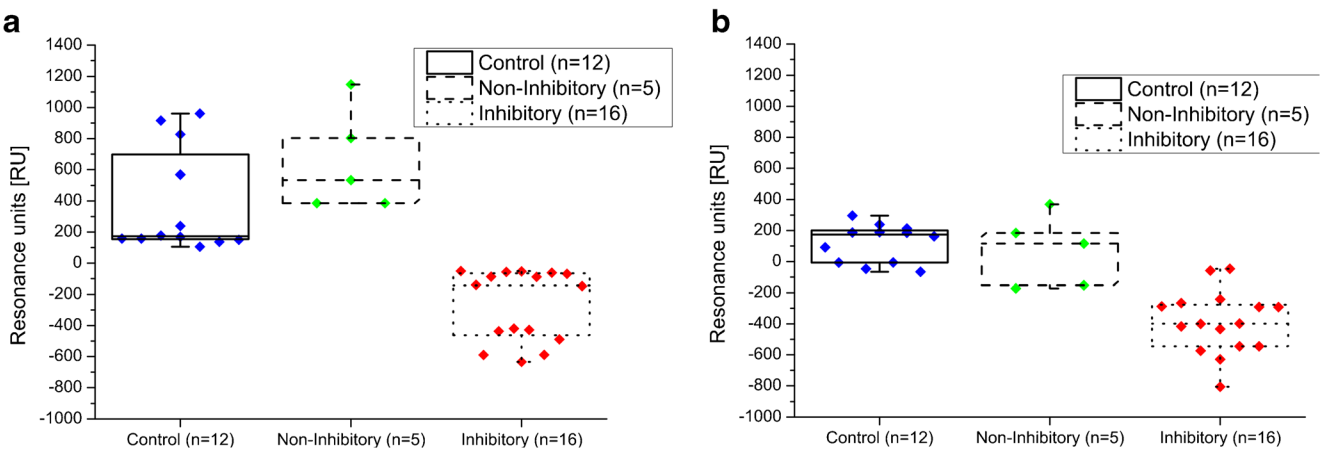

Fig. 5 **a** Box plot of FIX-binding signal (2nd reference point) of the three patient groups. **b** Box plot of FXa binding signal (2nd reference point) of the three patient groups

FVIII could be characterized in patient plasma samples. In contrast to this approach, our FVIII-coated biosensor surface allows repetitive analyses of inhibitory and non-inhibitory anti-FVIII in a precise and robust manner. These are important performance features for an assay format, being applied for a diagnostic screening in the haematological ambulance. Our SPR biosensor analysis also requires only a small sample volume of <200 μL and features a reasonable short analysis time of less than 1 h.

Conclusion

As stated by Fay et al., inhibitory antibodies block the epitopes necessary for the interaction of FVIII and/or FIX [28]. As suggested by Matsumoto et al., the presence of FVIII immune complexes can sterically hinder the formation of the Xase complex [29]. The presented biosensor can be used to exploit this fact in a diagnostic test. Our data suggest that inhibition of the interaction of FVIII with FIX is a good diagnostic marker, whereas inhibition of FXa binding to FVIII does not necessarily occur. However, this hypothesis needs further verification using a larger group of patients. This trial is currently under way.

The interaction between FVIII and the other clotting factors takes place even without phospholipids. This is due to the fact that FVIII is covalently bound and adequately sterically oriented to the biosensor surface. In further experiments, we will extend the sensor protocol with the binding of vWF, after antibody injection, because many antibodies bind to the C2 region of FVIII and hinder the binding of vWF and phospholipids to the factor [30]. Another application of the biomimetic biosensor could be the prognostic analysis of antibody appearance or disappearance in individual patients over time, as first described by Fulcher et al. [31].

The presented two-step assay format integrates the information from the ELISA and additionally gives an indication on the inhibitory properties of the bound antibodies within one single measurement by use of the high informational content of the SPR sensorgram.

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