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Co-immobilized poly(ethylene glycol)-*block*-polyamines promote sensitivity and restrict biofouling on gold sensor surface for detecting Factor IX in human plasma

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In order to detect an extremely low amount of human coagulation factor IX (FIX), polyethylene glycol **(**PEG)/aptamer co-immobilized surface was constructed using original PEG-polyamine surface modification agents on surface plasmon resonance (SPR) sensor chip. Initially, a gold (Au) sensor chip of SPR was modified using poly(ethylene glycol)-*b*-poly[2-(N,N-dimethylamino)ethyl methacrylate] (PEG-*b*-PAMA) followed by treatment with SH-dT_{20} and was duplexed with anti-FIX aptamer extended using A_{24}. Further, the co-immobilization of pentaethylenehexamine-terminated poly(ethylene glycol) (N6-PEG) on the sensing surface completely quenched the bio-fouling. On this dual tethered PEG-surface, we determined that the dissociation constant for FIX-aptamer interaction was 37±10 pM, and the sensitivity of detection could reach upto 800 fM on using aptamer-FIX-antibody sandwich pattern detected by gold nanoparticle-conjugated anti-mouse antibody. We could detect FIX in the presence of abundant albumin. Further, to mimic the actual detection of FIX in clinical samples, we demonstrated our experimental evidence with human blood plasma instead of FIX. Higher-sensitivity was attained due to dual polymers immobilized on Au surface, and this can emerge as a common strategy for any aptamer-protein interactions. Selective binding of the aptamer in human blood plasma shown here indicates the suitability of the present strategy for detection in clinically relevant samples.

Key words: Factor IX, Surface Plasmon Resonance, PEG-*b*-PAMA, N6-PEG, gold nanoaprticle, aptamer

Introduction

Biomolecular recognition is considered as an important feature to understand the events of a disease and gene regulations. The first biosensor with signal recognition elements and transducers was reported in 1962, which is considered as the basis for sensor development.[1] After this initial report, various sensors have been proposed and developed using versatile signal detection methods.[2] Among different sensing systems, which include surface plasmon resonance (SPR) and other sensors mimics, SPR dominates as a major in label-free or labeled method of detection.[3] However, a method of detection without chemical tags is highly essential for scalable biosensor technology with real-time monitoring and high sensitivity. Even though, the sensitivity varies with different systems, sensitivity is the prime determining factor for the quality of successful sensors.[4] Different surface functionalization chemistries with suitable probes have been proposed to improve the sensing system. From the very early stage of sensor development, antibodies have been used as probes, and in 1967, the first immobilized antibody-solid surface was proposed by Catt and Niall.[5] Later, an advanced method

such as ELISA was introduced[6], followed by several antibody-sensing strategies on solid surfaces were established.[7] Even though antibodies have been potential molecules for sensor development since the past several decades, in 1990, an alternate molecule to substitute the antibodies was developed, which was called 'aptamer' or 'chemical antibody.' A strategy called Systemic Evolution of Ligands by Exponential Enrichment (SELEX) was introduced by three independent researchers to generate the aptamers, and to find the selective molecules from the randomized molecules.[8-10] Aptamers have the potential characteristics and behave similar to antibodies. A combination of antibody- and aptamer-sensing strategy have been proposed, because these molecules could complement each other.[11]

Aptamers or antibodies immobilized on solid surfaces have a lower antigen capturing efficiency compared to in-solution based detections due to random orientations and steric hindrances by interaction with the solid surface.[12-14] To fabricate an efficient solid-surface sensor, proper immobilization of probe or receptor molecule on the surface of sensor is a crucial step, and correctly oriented higher-density immobilization would lead to an improved level of detection.[14] Right orientation of biomolecules on the solid surface was accomplished by co-immobilization of inert materials such as polymers.[15] Poly(ethylene glycol) (PEG) is one of the popular polymers, which is chemically inert, provides terminal hydroxyl groups that can be used as anchors for functional groups, is nontoxic, and disperable in water due to hydrogen bonding between water molecules and ether oxygen molecules of the PEG chain. Surface modification with PEG (so-called 'PEGylation') is shown to have non-fouling effects due to the hydrophilic and flexible nature of this polymer.[16-19] PEG-block polymers (PEG-*b*-polymers) attracted the attention of the researchers in the past, especially in the fields of biotechnology, pharmaceuticals, and medicine for the purposes of bio-recognition, cosmetics, drug delivery systems, and microsystems.[20,21] PEG-*b*-polymer-modified surfaces are highly efficient in reducing protein adsorption from a mixture of samples, such as blood, thereby improving biocompatibility.[22,23] Natural polymers such as BSA and casein may not help in these aspects as they

contain immunoglobulin (IgG) that may interfere with antigen binding and cross-react with secondary antibodies.[24] PEG-based polymers are also considered as an efficient component in the development of biointerfaces and for applications as non-ionic surfactants, lubricants, and adhesives.[15,25] The length and density of the constructed polymers play a role in the improvement of sensitivity and specificity of a given sensor. However, increased chain-length tend to be less dense due to steric exclusion of PEG-chains on the immobilized surface.[26,27] Instead, a mixture of long and short PEG-chains overcome the density issues, due to the occupying properties of short chains between the interface of two long chains and were found to reduce non-specific adsorption onto the sensing surface.[19,28-32]

On the other hand, among different reported solid-sensing surfaces, gold (Au) is one of the preferred metals for sensing due to easy water dispersal, compatibility with surface functionalization, biological non-reactivity, and ability to be tailored with uniform and different nano-sizes.[33-37] In this report, a combination of Au surface and PEG-polymers were used to develop the higher sensing system with the assistance of a SPR-based Biacore system. The excitation of surface plasmon by monochromatic waves on the metal-coated surface surrounded by biological fluid environment and the excitation of surface plasmon by light is known as a SPR. Absorption of molecules on the receptor layer of biosensor changes the refractive index of the layer, thereby reporting the event of binding (Supplementary Figure S1a-c). With the SPR-based sensors, PEG-assisted surface has been shown to have higher-sensitivity.[38-40] A specially synthesized PEG-polymers, such as poly(ethylene glycol)-b-poly[2-(N,N-dimethylamino)ethyl methacrylate] (PEG-*b*-PAMA) and pentaethylenehexamine-terminated poly(ethylene glycol) (N6-PEG), were demonstrated for different sensing surface with higher non-fouling and increased sensivity.[41-43] One of the most important factors in improving the effectiveness of the sensor is the correct orientation of immobilized biomolecules on the given sensing surface.[15] The above PEG-*b*-polymers (PEG-*b*-PAMA and N6-PEG) have an unique participation in making properly oriented bio-molecular immobilization with higher numbers.[11,41,42] In the present study, the

strategy with sandwich pattern of aptamer-Factor IX (FIX)-antibody was used to demonstrate the applications of with the above described PEG-*b*-polymers, PEG-*b*-PAMA and N6-PEG.

The biological importance of FIX is reflected in Hemophilia B (Christmas disease), where the FIX concentration in the plasma reaches abnormal levels from the normal level of around 5 µg/mL (87 nM). The human plasma has clotting factors other than FIX, as well as major additional components such as albumin (45 mg/mL). It is therefore important to conform that FIX can be detected amidst such high levels of albumin. If we can detect FIX selectively among a mixture of components, it is anticipated that we will be able to precisely determine the active level of FIX. In this study, we demonstrated the detection of FIX using the following sensing strategy: (i) initially Au sensor chip of SPR was modified with PEG-*b*-PAMA followed by SH-dT_{20}; (ii) aptamer-A_{24} was complemented with SH-dT_{20}; (iii) unreacted Au surface was blocked by N6-PEG; (iv) FIX was allowed to interact with aptamer; (v) anti-FIX mouse IgG was incubated on the FIX-modified surface; or (vi) anti-mouse IgG antibody-conjugated gold nanoparticle (GNP) was allowed to interact with anti-FIX antibody; and (vii) all the interactions were monitored by SPR.

Experimental

Reagents and biomolecules

All oligoes were commercially synthesized by Tsukuba Oligo company. TaKaRa Ex Taq, DuraScribe® T7 Transcription Kit, propionic acid, factor IX, Monoclonal Anti-FIX antibody produced in mouse, human serum, and bovine serum albumin (BSA) were purchased from sigma Aldrich, USA. Anti-Mouse IgG (H+L)-40 nm GNP conjugate was from Cytodiagnostics, Canada. Sensor chip was from GE healthcare, Japan. N6-PEG (Block master) was kindly provided by JSR (Tokyo, Japan). Synthesis of PEG-*b*-PAMA is outlined by Miyamoto et al.[37] and Horiguchi et al.[43] All the reagents were stored according to the suppliers' recommendations.

Enzymatic synthesis of aptamer

A stable 33-mer (2′-fluoro-modified) RNA-aptamer, which was previously reported by Rusconi et al.,[44] was followed and its ability to bind to FIX or FIXa was verified.[44,45] The aptamer was synthesized on a synthetic DNA template by enzymatic reaction (in vitro transcription) using T7 RNA polymerase. The T7 promoter region (letters are highlighted in italics) was maintained at the 5′-end of the aptamer to generate the RNA molecules. The template, 5′-*AGTAATACGACTCACTATAGG*GATGGGGACTATACCGCGTAATGCTG-3′ was used to prepare the double-stranded DNA. A polymerase chain reaction (PCR) was performed using the above DNA template and amplified using appropriate primers (5′-AGTAATACGACTCACTATAGG-3′ [forward] and 5′-$(T)_{24}$ATGGGGAGGCAGCATTACGCGGTATA-3′ [reverse]) and the TaKaRa Ex Taq mix. Twenty PCR cycles were completed at 94°C for 70 s, 55°C for 50 s, and 72°C for 70 s. After proper PCR amplification, the PCR product was precipitated in ethanol and used for RNA preparation by *in vitro* T7 transcription. Transcription was performed at 37°C overnight, using a DuraScribe transcription kit (Epicentre Biotechnologies, USA), and the reaction was terminated by adding an equal volume of 2x urea buffer (7 M urea, 50 mM EDTA [ethylenediaminetetraacetic acid], 90 mM tris-borate containing 0.05% bromophenol blue). Afterwards, the reaction mixture was heated at 90°C for 2 min and loaded onto a 12% polyacrylamide gel containing 7 M urea for fractionation. The RNA band was visualized using UV light, and the band was extracted from the excised gel-piece. The RNAs were precipitated in ethanol and dried under vacuum, re-dissolved in ddH_2O, and the concentration was measured spectrophotometrically at 260 nm. Using the following DNA template, 5′-*AGTAATACGACTCACTATAGG*GTACCCCTGATATGGCGCATTACGAC-3′ (T7 promoter region highlighted in italics), the mis-match FIX aptamer was synthesized as described above using the same T7 forward primer and the reverse primer 5′-$(T)_{24}$TACCCCTCCGTCGTAATGCGCCATAT-3′ for a negative reaction.

Interactive analyses of aptamer and FIX

Initially, the Au-sensing surface of SPR was modified using PEG-*b*-PAMA followed by SH-dT_{20} and was duplexed with anti-factor aptamer extended with A_{24}. Different concentrations of FIX (0.8 pM to 80 nM) were injected to determine the dissociation constant and sensitivity. On the FIX-immobilized surface, FIX antibody followed by GNP conjugated anti-mouse IgG (mouse-IgG-GNP) were passed. A four line Biacore system (Biacore 3000) was used for all the experiments. Among these, one flow channel was always used for the control experiment. Flow rates were as follows: PEG-*b*-PAMA (10 µL/min, 300 µl); SH-dT_{20} and Aptamer-A_{24} (2 µL/min, 20 µL); N6-PEG (10 µL/min, 300 µL); and FIX, FIX antibody, and mouse-IgG-GNP, (10 µL/min, 50 µL). Immobilized aptamers were regenerated using 10 mM NaOH (60 µL/min,5 µl). All the measurements were carried out at 25°C.

To find the saturated concentration of each biomolecule for the strategy explained above, we titrated the optimal biomolecule concentration. First we injected different concentrations of PEG-*b*-PAMA (2 mg/mL to 50 mg/mL) on the Au surfaces. Then the different concentrations (1 µM to 5 µM) of SH-dT_{20} were injected on the optimized PEG-*b*-PAMA immobilized surfaces. Similarly, we also titrated the aptamer concentration from 500 nM to 1000 nM on the optimized SH-dT_{20}-modified surfaces. FIX antibody titration (25 nM to 200 nM) was carried out on the FIX (200 nM)-immobilized surfaces.

Interactive analyses of antibody and FIX

Au surface modified with COOH by using 3-mercaptopropionic acid was used to immobilize the FIX antibody. The COOH-modified surface was activated by 100 mM *N*-hydroxysuccinimide (NHS) and *N*-ethtyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to get an active ester surface. Then, 200 nM of the FIX antibody was passed directly on this surface. The remaining free COOH-surfaces were then

blocked using 10 mg/mL of N6-PEG or 1 M ethanolamine. Different concentrations of FIX (30 nM to 240 nM) were injected on the FIX antibody-immobilized surfaces.

Dissociation constant of FIX-aptamer and FIX-antibody

To find the dissociation (KD) value of FIX and aptamer, we injected different concentrations of FIX (15 nM to 120 nM) on the constant (500 nM) aptamer-immobilized surfaces. To check the FIX-antibody dissociation constant, we injected different concentrations of FIX (30 nM to 240 nM) on the constant FIX antibody (200 nM)-immobilized surfaces. KD value was calculated using the BIA Evaluation software by association and dissociation of FIX on aptamer- and antibody-immobilized surfaces. The rate of association was measured from the forward reaction, and the dissociation rate was measured from the reverse reaction. The equilibrium dissociation rate constant *(*KD) was calculated using the formula, *k*d/*k*a (KD, equilibrium dissociation constant; *k*d, dissociation rate; and *k*a, association rate).

Sandwich assay

To detect FIX, here we performed two kinds of sandwich assay, which include aptamer-protein-antibody (200 nM antibody was passed on the aptamer-protein immobilized surfaces) and antibody-protein-aptamer (500 nM aptamer was passed on the antibody-protein immobilized surfaces). Different binding sites of the aptamer and antibody on FIX were confirmed by performing SPR and the gel-shift assay. For SPR, on the aptamer-FIX (120 nM)-immobilized surface, different concentrations (25 to 200 nM) of antibody were passed (10 µL/min, 50 µL). For the gel-shift assay, we used the native polyacrylamide gel electrophoresis (Native-PAGE). Before loading on the gel, the aptamer (0.3 µg) was denatured at 90°C for 2 min and cooled to room temperature. Then, the pre-mix was made with different ratios of FIX (1:3, 1:6 and 1:9) in 10 mM Hepes-KOH (pH 7.4) buffer containing 150 mM NaCl and 2 mM $CaCl_2$. The $CaCl_2$ was added after the denaturation step. Similarly, aptamer:FIX:antibody pre-mix was made

with different ratios (1:3:6, 1:3:12, and 1:3:24). After incubation at room temperature for 10 min, all the mixes were resolved by performing 10% native-PAGE. The control lane was loaded with only the denatured aptamer. The gel was run at 150 V for 1 h and stained with ethidium bromide. Then, the gel was destained with ddH_2O, and the image was photographed.

Detection limit of FIX in the presence of albumin or human plasma

To check the detection limit of FIX, we titrated FIX at different concentrations (from 0.8 pM to 80 nM) on the FIX aptamer (500 nM)-immobilized surfaces, followed by reaction with constant FIX antibody (200 nM), and mouse-IgG-GNP (0.1 OD). Control experiments were carried out with mis-match FIX aptamer. For selective binding, which mimics a similar situation in the blood samples, experiments with mixed albumin was carried out. Different concentrations of FIX (from 0.8 pM to 8000 pM) were mixed with constant higher concentration of albumin (45 mg/mL) and passed on the FIX aptamer-immobilized surface. Then, constant FIX antibody (200 nM) and mouse-IgG-GNP (0.1 OD) were injected. To detect the FIX in the human plasma, instead of FIX, we injected different dilutions of human plasma (from 1:10 to 1:1280) by using half serial dilutions. Similarly, spiking of FIX in 1:160 dilution of human blood plasma was performed followed by analyses with SPR. All the other surface chemical modifications and detection strategies were similar to those described previously.

Results and discussion

The objective of this work is to establish high-performance PEG hybridized sensing on Au surfaces and we demonstrated here with one of the well-established SPR-based sensors, Biacore. In order to prepare desirable sensor chip surface, poly(ethylene glycol)-block-polyamines (PEG-*b*-polyamines), such as pentaethylenehexamine-terminated PEG (N6-PEG) and PEG-b-poly[2-(N,N-dimethylamino)ethyl methacrylate] (PEG-*b*-PAMA) were employed. As we reported previously[41], PEG-b-PAMA with

suitable polyamine chain length on gold sensor chip surface controls alignment of oligonucleotide. We would like to investigate this surface modification technique for aptamer surface. N6-PEG is anticipated for suppression of the bio-fouling as blocking agent. Human coagulating factor IX protein (FIX) was chosen as a model analyte to interact anti-FIX aptamer, because FIX has extremely low availability *in vivo* and important to detect defects in human blood coagulation system. With the complementation of aptamer and antibody, a sandwich pattern is designed with aptamer-FIX-antibody and higher sensitivity was attained with Gold nanoparticle (GNP) conjugated antibody.

PEG-*b*-PAMA assisted the immobilization of aptamer on Au surface

Prior to verifying the interaction of aptamer and factor IX (FIX), preliminary assessment was performed by immobilizing PEG-*b*-PAMA on Au surface at acidic (4.0), neutral (7.4), and alkaline (9.0) pH values. The responses obtained by SPR were similar at all the investigated pH conditions, which indicates the stability of PEG-*b*-PAMA under these conditions. Similar results under varied pH conditions was reported by Yoshimoto et al.[41] As the manufacturer recommended neutral pH (7.4) as ideal for the SPR system, further experiments were performed under this condition. At this pH, a higher response was obtained (1200 RU) with 6 mg/mL of PEG-*b*-PAMA-reconstructed surface, and the response increased further to only a few hundreds upon increasing the concentration to 12, 25, and 50 mg/mL, suggesting that the saturation stage had been attained. Au surface can be stabilized through co-ordination of non-protonated amine and the adsorption of PEG-*b*-PAMA on the Au surface via multipoint coordination by the tertiary amino groups.[41,46,] Once the PEG-*b*-PAMA is immobilized on the Au surface, it becomes very strong even under neutral conditons.[41] After attaching the PEG-*b*-PAMA on the Au surface, we analyzed the immobilization of thiolated-DNA oligo (SH-dT_{20}) in order to make the duplex with the target aptamer (Supplementary Figure S2). Interestingly, we could observe a clear response (600 RU) at 1 μM concentration. The response increased further when the concentration of SH-dT_{20} was increased to 2 μM (900); at the concentration of 3 μM, higher immobilization of SH-dT_{20} on PEG-*b*-PAMA surface was detected (980 RU), which reached the saturation level. On the contrary, lower amount of SH-dT_{20} was immobilized on bare Au surface even at this concentration (620 RU). PEG-*b*-PAMA assisted about 350 RU increment compared with the surface absence of PEG-*b*-PAMA (Figure 1). We have previously confirmed that the immobilization of sulfanyl-ended oligonucleotide increased significantly on the PEG-b-PAMA immobilized gold sensor surface[41], which is ascribed to the electrostatic interaction of the negatively charged oligonucleotide and the positively charged gold surface. Because PAMA is polyamine homologues, it has positive charge. PEG-PAMA

modified surface possesses positive charge on the very periphery of the gold surface, attractive force between negatively charged SH-dT_{20} and the surface might increase the modification efficiency regardless that PEG tethered chains tends to retard access of SH-dT_{20} of the surface. This might be one of the reasons for increase in SH-dT_{20} modifications as shown in Fig.1 Upon attaching aptamer-A_{24}, the obtained values are ~700 and ~1000 RU for the sensing surface without and with PEG-*b*-PAMA, respectively, indicates the increment of ~300 RU (nearly equivalent to the value obtained from SH-dT_{20} attachment) with the assistance of PEG-*b*-PAMA. Cationic charge of the PAMA segment on the periphery of the Au surface attracted anionic SH-dT_{20} to result in increasing amount than that of bare Au surface, which possessed negative charge.[46] Previously, it was reported that PEG-*b*-PAMA-co-immobilized Au surface with SH-ssDNA inhibits the interaction of nucleobase of oligo and Au, and leads to upright confirmation of immobilized ssDNA. This confirmation further supports the proper duplex formation as described previously.[47] Moreover, PEG-*b*-PAMA-co-immobilized Au-nanoparticle with siRNA were shown to have higher interference efficiency have reported the role of PEG-*b*-PAMA on Au surface for the immobilization of SH-DNA and indicated that it would be stronger even under salt conditions.[47,48] Using this ideal situation, a stable anti-factor IX aptamer (2' fluoro-modified) extended with A_{24} (20 bases for complementation and 4 bases for spacer) was immobilized. With the injection of FIX aptamer-A_{24}, we could notice a proper duplex of the aptamer on the SH-dT_{20} with nicely displayed sensogram to 900 RU, at the concentration of 500 nM. The aptamer-A_{24} interaction with the SH-dT_{20} detected higher response on the PEG-*b*-PAMA-modified surfaces compared to the surface free from PEG-*b*-PAMA (Figure 1). When the aptamer concentration was increased to 1 μM, the response was similar, indicating the saturation level of aptamer at 500 nM concentration (Supplementary Figure S3).

Co-immobilization of PEG-*b*-PAMA and N6-PEG restricts biofouling on Au surface for detection of FIX

After the construction of PEG-*b*-PAMA/aptmaer-A_{24} surface, the efficiency of blocking treatment was analyzed using conventional BSA and our original N6-PEG, because the remaining Au-surface may disturb effective sensing. Mouse-IgG-GNP was employed for non-specific binding on these surfaces. When the PEG-*b*-PAMA/aptamer-A_{24} surface was treated with 45 mg/mL of BSA, the signal (i.e. RU ~200) was definitely observed (Supplementary Figure S4), while no remarkable signal was observed by N6-PEG blocking, indicating a complete coverage by N6-PEG. N6-PEG (6 kDa) has 6 amino groups at one end of the PEG chain to interact with Au surface, and it has an eletrostatically attractive force.[15] This polyamine interaction with Au-surface is stable for

a long-term than a common Au-S linkage, under varied physiological conditions.[15,42] To determine the stability and replacement of SH-dT_{20} by N6-PEG, we performed two kinds of experiments. In one set-up after attachment of the aptamer-A_{24} on SH-dT_{20}, N6-PEG was passed and in the other case, the process was reversed (N6-PEG was passed on SH-dT_{20} and then aptamer-A_{24} was attached). In both the cases, we could find the same kind of responses, even with consequent injections of FIX at different concentrations (Supplementary Figure S5). With N6-PEG as a blocking agent, a similar response to FIX was observed, regardless of the order of the treatment (N6-PEG → aptamer-A_{24}; aptame-A-$_{24}$ → N6-PEG), indicating the absence of the exchange of pre-immobilized SH-dT_{20} by N6-PEG. When we passed the FIX on the PEG-*b*-PAMA- and N6-PEG-modified aptamer surfaces, a concomitant incremental enhancement in the responses was observed with increasing FIX concentrations (Figure 2a).

Dual polymers-assisted interaction: Determination of dissociation constant of aptamer and FIX

To access the dissociation constant of FIX and aptamer interaction in the presence of PEG-*b*-polyamines, we performed the experiments with different combinations of PEG-*b*- polyamines and compared the results with those for BSA-blocked Au surface. These combinations include, only BSA, only N6-PEG, only PEG-*b*-PAMA, PEG-*b*-PAMA with BSA, and PEG-*b*-PAMA with N6-PEG. Based on the responses obtained from lower concentrations (7.5 to 120 nM) of FIX, (Figure 2a) further evaluation was performed using the Biacore evaluation software, and were fitted with a 1:1 binding model (for the interaction between aptamer and FIX according to the equation A+B = AB). The lowest dissociation constant (KD) (highest sensitive) for aptamer and FIX interaction was 37 ± 10 pM with the combinations of PEG-*b*-PAMA and N6-PEG. In other cases, for the same interaction, KDs were 685 ± 15 pM (only BSA), 450 ± 8 pM (only N6-PEG), 142 ± 5 pM (only PEG-*b*-PAMA), and 81 ± 7 pM (PEG-*b*-PAMA and BSA) (Table 1). Among these, a dual PEG-b-polyamines (PEG-*b*-PAMA with N6-PEG) showed higher responses, which facilitated proper construction on the sensing surface and restricted fouling. Previously, it was proved that the KD of FIX and aptamer was 580 pM by filter

binding assay.[44] In this study, PEG-*b*-PAMA with N6-PEG polymers drastically reduced the background noise and improved the sensitivity and specificity, due to perfect blocking (by N6-PEG) and higher immobilization of aptamer in proper orientation (by PEG-*b*-PAMA). Another advantage of these kind of polymers is having lower molecular weights (~10 kDa) than other blocking agents such as BSA (66 kDa), casein (>20 kDa), and gelatin (>100 kDa). Higher molecular weight blocking causes interference with molecular-recognition site of the ligand and analyte.[15]

Determination of dissociation constant of antibody and FIX on the N6-PEG-modified COOH surface

To compare the interactions of aptamer and FIX, we evaluated the KD of FIX antibody-FIX interaction on COOH-modified Au surface. We could observe the specific binding of FIX to the antibody on the Au surface in a dose-dependent manner, when ethanolamine was used as a blocking agent. However, we could also notice the non-specific binding of FIX even in the absence of antibody (Supplementary Figure S6a). Ethanolamine is considered as one of the common blocking agent for the COOH modified surfaces, but some of the active group on the COOH surface facilitates nonspecificity with FIX, because amine in the protein easily bind on the COOH surface. To avoid these nonspecific binding, the remaining COOH surfaces could be blocked by other blocking agents. Here, we used N6-PEG instead of ethanolamine, and it was found to completely quench the nonspecific binding of FIX by the strong binding of amine groups in the N6-PEG with COOH surfaces. N6-PEG not only suppresses the bio-fouling as shown in the figure, it also increases the specific responses in a concentration-dependent manner, compared with ethanolamine-blocked surfaces (Figure 2b and Supplementary Figure S6a & b). N6-PEG is not only suitable for Au surface, but is also very effective on COOH-modified surfaces. The KD for the specific interaction of FIX and antibody were determined as 48 ± 12 nM. It is interesting to note that proper designing of aptamer or conventional antibody immobilization on the dual polymer-modified surface increased the sensing ability significantly.

Aptamer-FIX-antibody sandwich on PEGylated Au surface

Based on the findings of this study on FIX interactions with two different probes (aptamer and antibody), we designed the sandwich pattern on the aptamer-FIX-immobilized surface by interacting with anti-FIX antibody. In the past, aptamer-antigen-antibody sandwich assays have been considered to have a higher sensitivity.[11] In the sandwich assay, the key issue is the availability of two different binding sites on the target protein (FIX) for different probes (aptamer and antibody). To make sure that this issue is addressed, we performed confirmations with two kinds of experiments by analyzing SPR and gel-mobility shift. When we passed the antibody on the aptamer-FIX immobilized surface of SPR, we could see a concentration-dependent attachment of anti-FIX antibody (Figure 3a). This result confirmed the formation of a sandwich pattern of aptamer-FIX-antibody on the sensing surface. Similarly, we also evaluated the sandwich formation on the electrophoresis analysis by resolving different complexes (only aptamer, aptamer-FIX, and aptamer-FIX-antibody) on the native-PAGE. The resolved gel-pattern showed a clear shift with the aptamer-FIX complex and super-shift with the aptamer-FIX-antibody complexes in a dose-dependent manner (Figure 3b). Previously, by applying PEG/Antibody co-immobilized magnetic beads combined with a fluorescent system, a sandwich ELISA pattern was developed by our research team to detect alpha-fetoprotein.[30] In several instances, the aptamers are proposed as equal or better molecules than antibodies; however, both aptamer and antibody can complement each other in sandwich assays.[11] We also compared two different sandwich types, namely, aptamer-FIX-antibody and antibody-FIX-aptamer on the dual polymer (PEG-*b*-PAMA and N6-PEG)-immobilized Au surface. At FIX concentration of 120 nM, the response obtained was 350 RU in the case of aptamer-protein-antibody sandwich pattern. Whereas, under similar conditions, a response of 50 RU was obtained by antibody-FIX-aptamer strategy, suggesting a 7-fold lesser efficiency with the antibody-FIX-aptamer sandwich. This result indicates the higher affinity of aptamer than the conventional antibody for FIX, thus suggesting that ultimately aptamer captures higher

number of FIX molecules. Similarly, with the injection of different FIX concentrations (15 to 120 nM), the aptamer-FIX-antibody strategy showed better responses, indicating the higher affinity of aptamer with FIX and suggesting that the higher molecular size of the antibody than that of the aptamer favors the suitability of aptamer-FIX-antibody strategy (Figure 4).

Sensitive detection of FIX using mouse-IgG-GNP on PEG-*b*-PAMA- and N6-PEG-co-immobilized Au surface

Based on the above sandwich pattern, we formulated a strategy with anti-mouse IgG-GNP for higher sensitivity by using the FIX antibody (Figure 5a). Based on the KD analyses aptamer has shown higher affinity (37 pM) than antibody (48 nM). To capture the higher number of antigen (FIX), we decided to immobilize aptamer on the sensing surface. To increase the sensitivity of detection, antibody was conjugated with GNP. The idea behind is increase in the molecular size increases the sensitivity, the sizes of both antibody and GNP are larger than aptamer. With this strategy, we performed the experiments on SPR with different combinations of blocking agents (only BSA, only N6-PEG, only PEG-*b*-PAMA, PEG-*b*-PAMA with BSA, and PEG-*b*-PAMA with N6-PEG) and injected FIX. When we injected 80 nM of FIX, a dual PEG-polymer (PEG-*b*-PAMA with N6-PEG) was shown to have higher responses (Figure 5b). We could observe 3-fold higher responses in the presence PEG-*b*-PAMA and N6-PEG unlike that observed for BSA used as a blocking agent, which might be due to the higher immobilization of SH-dT_{20} and aptamer on the dual polymer-modified surface. Moreover, in the case of BSA blocking, higher non-specific attachment was noticed. With dual polymer surface construction, we determined the limit of detection of FIX as 800 fM with the response of about 200 RU. By increasing the FIX concentration, we could observe the clear increamental increase in the responses (Figure 6). These results suggest that PEG-b-polymers, have shown better performance rather than one of the common blocking agents, BSA. In addition, co-immobilization of dual polymers is good for bio-sensing, compared to single-polymer application.

The biological importance of FIX was demonstrated in Hemophilia B (Christmas disease), when the FIX concentration in plasma reaches abnormal levels compared to the normal level of around 5 µg/mL (87 nM). The plasma sample contain albumin as the major protein with other clotting factors in addition to FIX. If we can detect FIX selectively among these active major proteins, it is anticipated that we will be able to precisely determine the level of FIX. Human blood serum contain albumin at the level of ~45 mg/mL (~55%).[24,] To check the selective binding of FIX protein in the presence of albumin, we performed the experiment using FIX mixed with a high concentration of albumin (45 mg/mL). With this enhanced albumin concentration, we titrated the FIX until 80 nM which is close to the real concentration in the human plasma and we could detect FIX at 80 pM (Figure 6). Higher sensitivity was attained in the mixed sample using the sandwich pattern with anti-mouse IgG-GNP due to PEG-*b*-PAMA- and N6-PEG-immobilized Au surface. Hence, this can be used as the common strategy for any aptamer-protein interactions. Further, SH-dT_{20} and aptamer-A_{24} duplex could be regenerated by injecting 10 mM NaOH on the sensing surface (Supplementary figure S7a & b). We detected FIX with high concentrations of albumin, which mimics physiological condition, suggesting that the present strategy is suitable for detecting FIX deficiency in human blood samples.

Detection of FIX in human plasma on co-immobilized PEG-*b*-PAMA and N6-PEGmodified Au surface

Human blood plasma in addition to albumin also contains other clotting factors, including FIX involved in the human blood-clotting system and participates in the cleavage in the intrinsic and extrinsic clotting pathways.[45] To further confirm the present results, we directly used human plasma and evaluated the existing FIX using the designed strategy with GNP. We evaluated the serially diluted human blood plasma from 1:10 to 1:1280 dilution, which is equivalent to the dilutions from 8 nM to 0.062 nM of FIX. Upon injection, we could observe the clear changes in the sensogram with the

dilution of 1:160, which is equivalent to 500 pM of FIX, and at this concentration, the change in the sensorgram was 250 RU (Supplementary Figure S8). To rule out non-specific binding of serum proteins, we evaluated mismatching aptamer sequence, and no binding was noticed both by SPR and gel-shift assay analyses (Supplementary Figure S9). Similarly, the pre-mix of specific aptamer without poly-A tail (cannot make a duplex with SH-dT_{20}) and FIX caused reduction in the binding, with increasing concentration of the pre-mixed aptamer (Supplementary Figure S10a & b). To confirm the genuine interaction of aptamer and FIX in plasma, we mixed the pure FIX at different concentrations to the plasma upto 1:160 dilution, where we could not detect FIX. Upon mixing the FIX, we would observe the changes in the sensogram in a concentration-dependent manner (Figure 7a&b). The results obtained with human plasma using the designed strategy here indicates its suitability to analyse the deficiency of FIX in human blood samples.

Conclusions

In this study, using dual PEG-block polymers (PEG-*b*-PAMA and N6-PEG)-co-immobilized Au-sensing surface of SPR, a detection strategy was formulated for the detection of FIX with the assistance of FIX-aptamer and FIX-antibody in a sandwich pattern. The detection was performed with anti-mouse IgG-GNP. PEG-*b*-PAMA-treated Au surface promoted the proper orientation of thiolated DNA oligos on the Au surface with higher number of molecules. Co-immobilization of N6-PEG with PEG-*b*-PAMA completely abolished the bio-fouling, whereas BSA showed higher non-specificity. With dual polymer construction, the limit of detection was enhanced to 800 fM. Further, the selective FIX detection was shown with albumin containing sample. In addition, the utility of the present polymer-assisted detection strategy was demonstrated by the detection of FIX in human blood plasma, indicating its application in the detection of clinically relevant samples.

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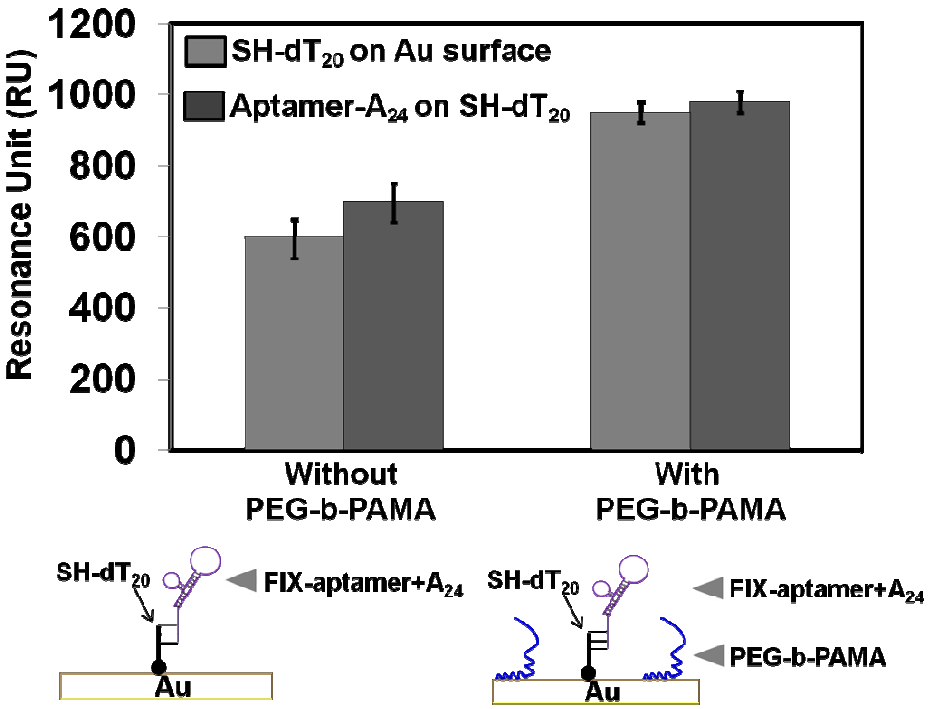

Figure 1. Evaluation of immobilization of SH-dT_{20} and aptamer-A_{24}-duplex formation on Au surface. Difference in the immobilization in the presence and absence of PEG-*b*-PAMA (6 mg/mL) was tested. About 3 μM of SH-dT_{20} was injected then duplexed with 500 nM aptamer-A_{24}. With SH-dT_{20} the obtained values are 620 and 980 RU for the sensing surface without and with PEG-*b*-PAMA, respectively. Upon attaching aptamer-A_{24}, the obtained values are ~700 and ~1000 RU for the sensing surface without and with PEG-*b*-PAMA, respectively. The schematic illustrates the strategies.

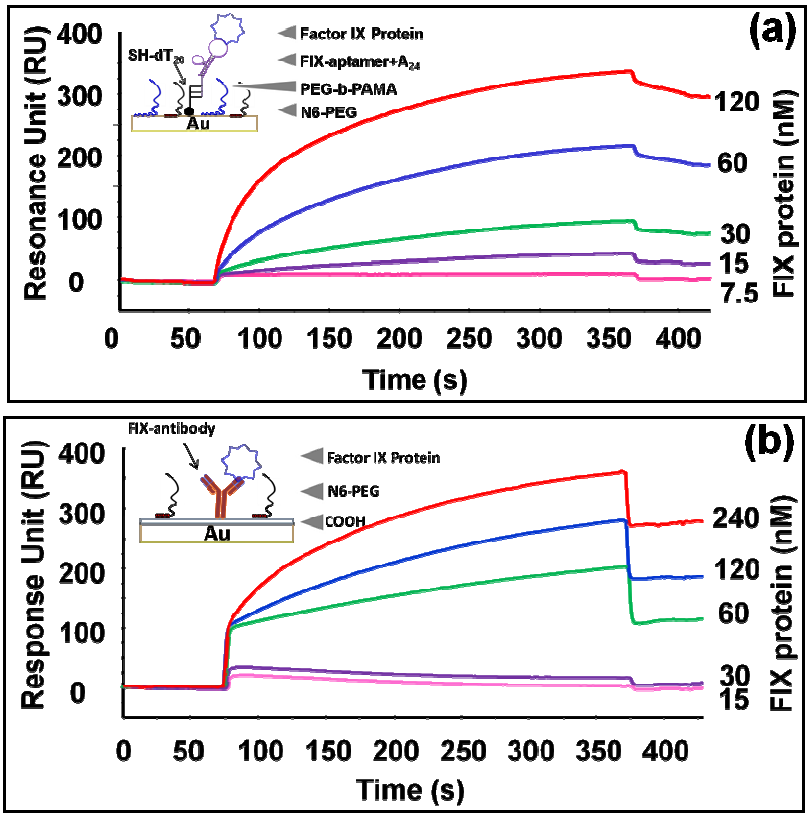

Figure 2. Determination of dissociation constant. (a) FIX-aptamer interaction. FIX at different concentrations (7.5 to 120 nM) was passed on the FIX aptamer-immobilized surface. (b) FIX-antibody interaction. FIX at different concentrations (15 to 240 nM) was passed on the FIX antibody-immobilized surface. The samples were injected at the flow rate of 10 µl/min. All the experiments were performed using 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 2 mM $CaCl_2$.

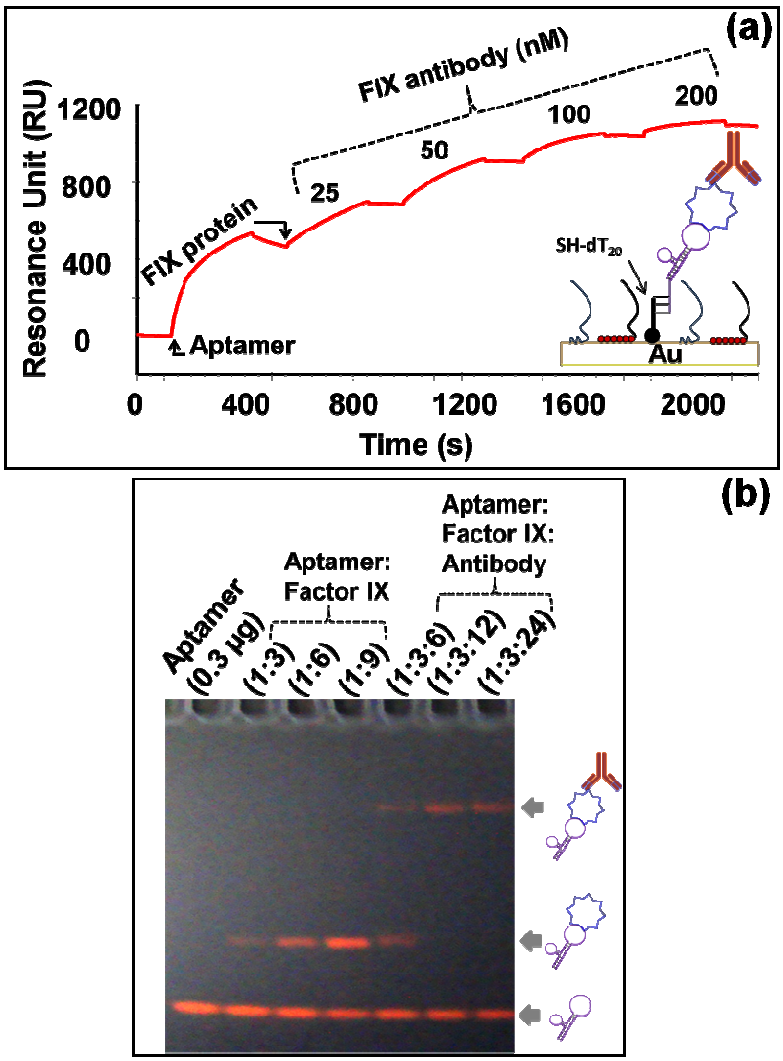

Figure 3. Analyses of sandwich formation with aptamer-FIX-antibody. (a) SPR analyses of the complex formation of aptamer-FIX-antibody on surface modified by PEG-polymers. After immobilization of the aptamer (500 nM) and blocking with N6-PEG on Au surface, FIX (200 nM) was attached. On the FIX-immobilized surface, different concentrations of FIX-antibody was passed. The inset in the figure is for a schematic representation of this complex. Samples were injected at the flow rate of 10 µL/min. (b) Native-polyacrylamide gel electrophoresis analysis of the complex of aptamer-FIX-antibody. Shift and super-shifts are shown with aptamer-FIX and aptamer-FIX-antibody complexes. All the experiments were performed with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 2 mM $CaCl_2$.

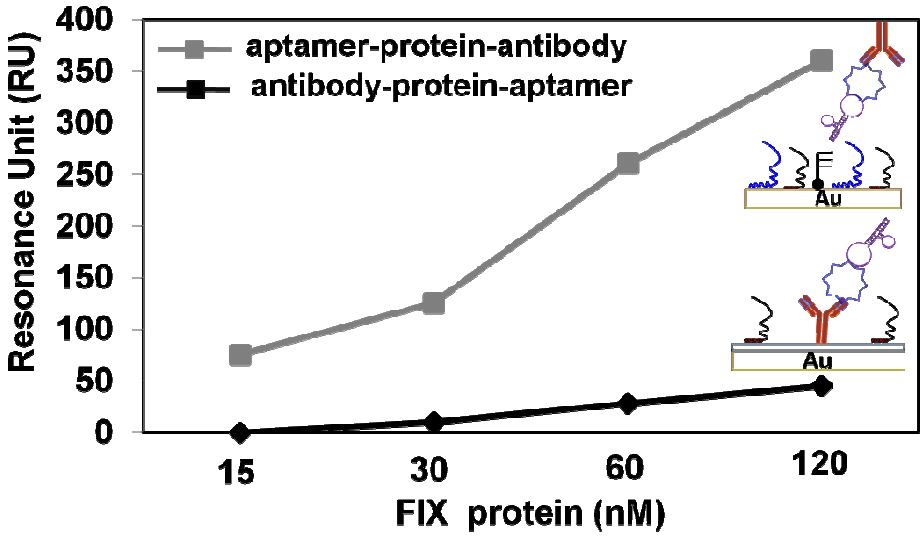

Figure 4. Analyses of sandwich patterns on PEGylated surface. Aptamer-FIX-antibody and antibody-FIX-aptamer sandwich were compared. After immobilization of aptamer (500 nM) or antibody (200 nM) and blocking with N6-PEG on Au surface, FIX (15–120 nM) was attached. On the FIX-immobilized surface, FIX-antibody or aptamer was passed. Figure inset represents the schematic of this complex. Samples were injected at the flow rate of 10 μL/min.

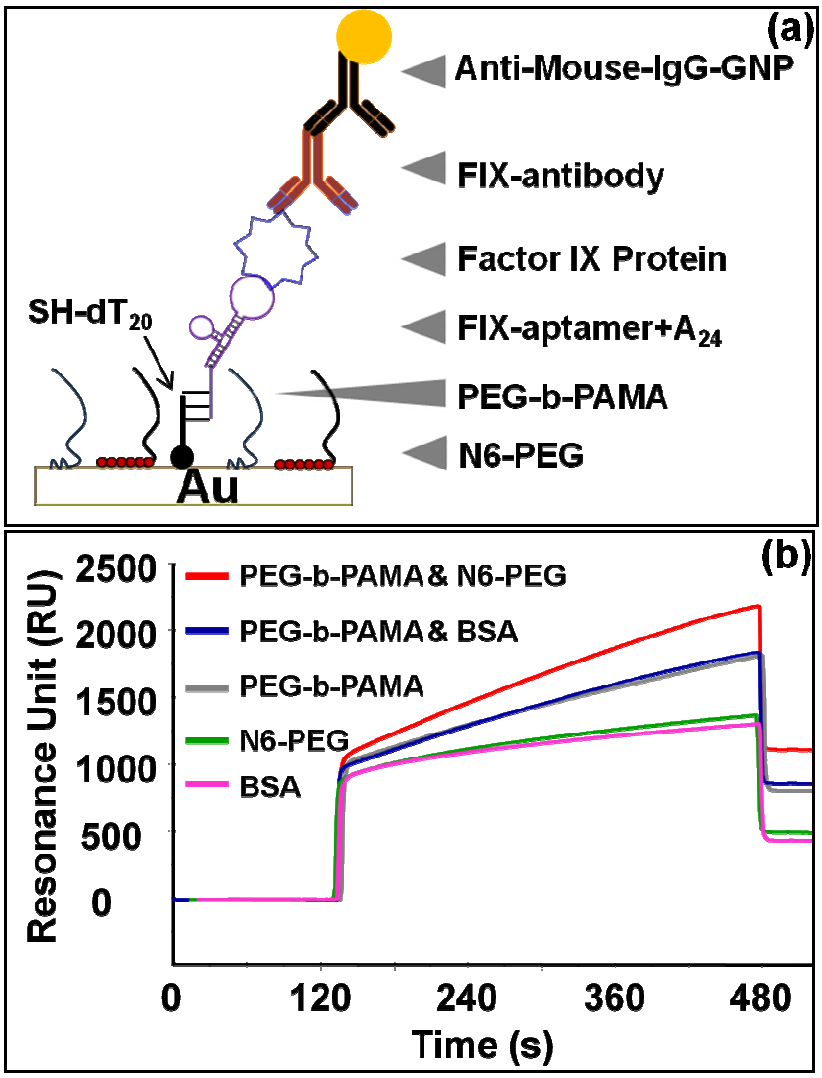

Figure 5. a) Schematic of chemical surface modifications on the Au surface of the SPR-sensing plate for aptamer and FIX interactions. (b) Detection of FIX on Au surface with different blocking agents. After immobilization of the aptamer (500 nM) and blocking with different agents on Au surface, FIX (80 nM) was attached. On the FIX-immobilized surface, 200 nM of FIX-antibody was passed. Detection was performed using anti-mouse IgG-GNP. The samples were injected at the flow rate of 10 µL/min.

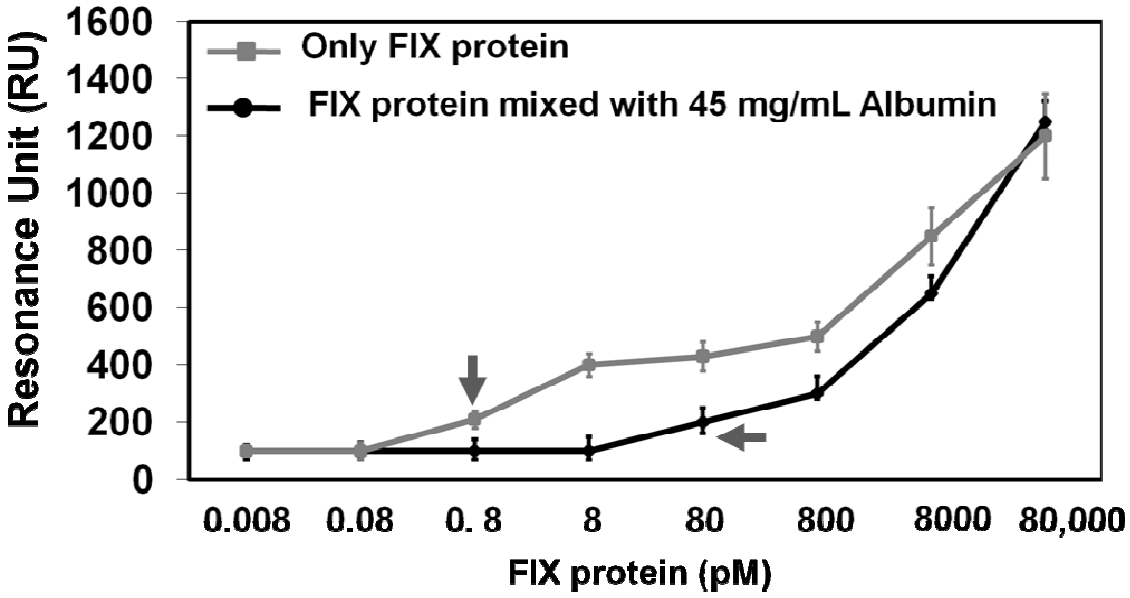

Figure 6. Detection limit of FIX in the presence or absence of albumin. FIX in the range of 0.008 to 80,000 pM were tested. All the experiments were performed with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 2 mM $CaCl_2$. Detection limits are indicated by arrows. Samples were injected at the flow rate of 10 µl/min.

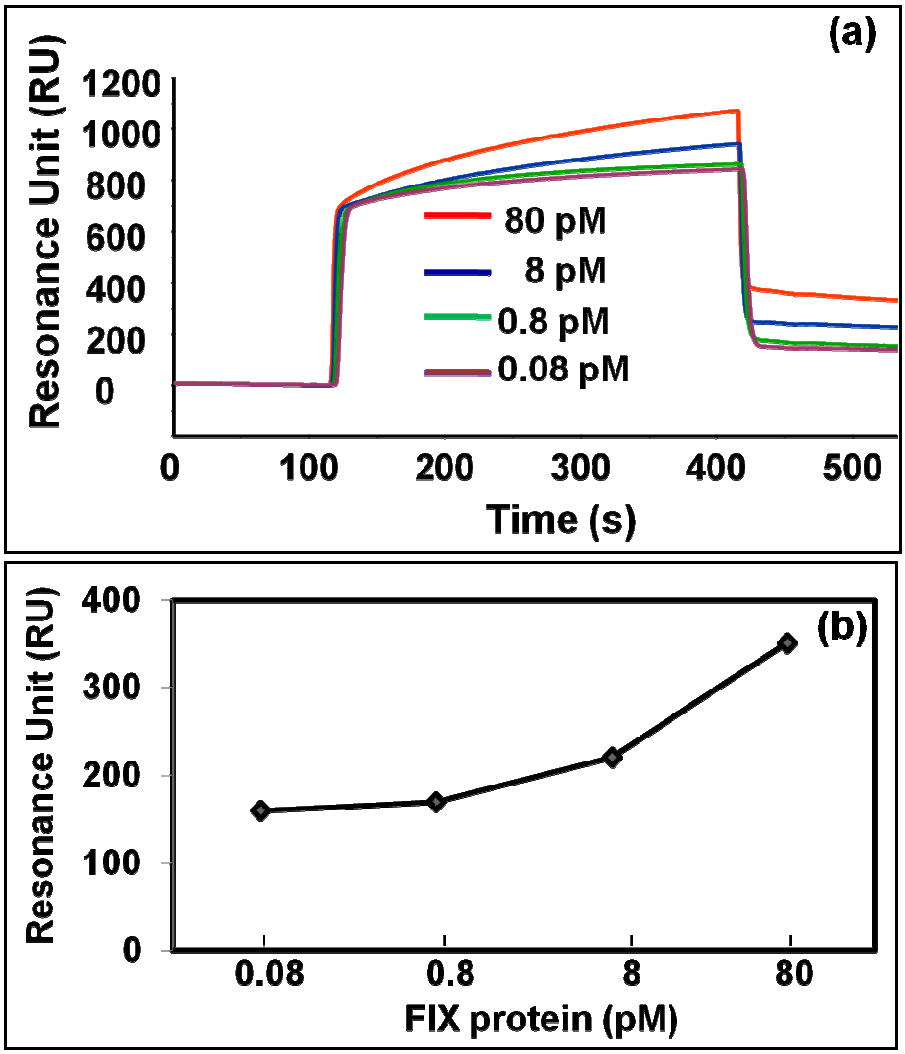

Figure 7. Spiking of FIX in human blood plasma. (a) SPR analyses. Different concentrations (0.08 to 80 pM) of FIX were spiked in 1:160-diluted human blood plasma. The samples were injected at the flow rate of 10 µL/min. All the experiments were performed with 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 2 mM $CaCl_2$. (b) Graphical representation of the results obtained from SPR analyses.

Table 1. Kinetic parameters for the interaction of aptamer and FIX in the presence of different combinations of PEG-b-polymers

Blocking agent	Association constant K_a ($M^{-1}s^{-1}$)	Dissociation constant K_d (s^{-1})	Equilibrium constant KD (M)
BSA	4.16×10^{4}	2.85×10^{-5}	$6.85 \pm 1.5 \times 10^{-10}$
N6-PEG	2.8×10^{4}	1.26×10^{-5}	$4.5 \pm 0.8 \times 10^{-10}$
PEG-b-PAMA	2.44×10^{4}	3.46×10^{-6}	$1.42 \pm 0.5 \times 10^{-10}$
PEG-b-PAMA & BSA	3.86×10^{4}	3.13×10^{-6}	$8.1 \pm 0.7 \times 10^{-11}$
PEG-b-PAMA & N6-PEG	3.4×10^{4}	1.26×10^{-6}	$3.7 \pm 1.0 \times 10^{-11}$

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