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A high-performance waveguide-mode biosensor for detection of factor IX using PEG-based blocking agents to suppress non-specific binding and improve sensitivity†

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An evanescent-field-coupled waveguide-mode (EFC-WM) sensor utilizes monolithic SiO_2/Si/SiO_2 sensing plates having a multilayered structure and is used to evaluate a blocking agent comprising poly(ethylene glycol)-based block copolymers. Factor IX (FIX) protein was detected using its aptamer, *viz.* FIX was immobilized on a glutaraldehyde-modified silica surface, and then treated with a biotinylated aptamer. The quantitative analysis of FIX was carried out using streptavidin-conjugated gold nanoparticles (SA-GNPs). The blocking polymer, poly(ethylene glycol)-*b*-poly(acrylic acid) (PEG-*b*-PAAc), was found to mask unreacted amine and glutaraldehyde (Glu) moieties on the SiO_2 surface, and it completely prevented the non-specific binding of SA-GNPs. By exploiting the strong blocking effect of PEG-*b*-PAAc, we achieved high ligand–analyte interaction sensitivity (sensitive down to 100 pM). To improve the sensitivity further, we also used pentaethylenehexamine-terminated PEG (N6-PEG) on GNPs. The improvement in sensitivity was found to be 1000-fold (to 100 fM), which was substantiated by the observation of higher numbers of GNPs on the sensing surface in the results of the scanning electron microscopic examination. Based on the competition assay of free biotin premixed with SA-GNPs, it was concluded that some active biotin-binding sites on the streptavidin were blocked by N6-PEG, which improved the binding ability to the biotinylated sensing surface. An optimum number of binding sites on the SA-GNPs might improve their binding affinity. The strategy shown with dual polymers, *viz.* blocking of the sensor chip surface and coating of SA-GNPs, is recommended for developing sensors with higher sensitivity and reliability. Selective binding of the aptamer to a very small amount of FIX in the mixed sample containing FXIa and FVIIa, or albumin, makes this the optimal strategy for detecting a FIX deficiency in human blood samples.

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Introduction

The development of nanobiosensors that may be used to analyze biomolecular recognition elements is currently one of the most important targets in the field of medical diagnosis. A useful biosensing system requires some key characteristics such as specificity and sensitivity. One of the most important factors in improving the effectiveness of the sensor is the correct orientation of immobilized biomolecules on the sensing surface.[1] It is also important to minimize non-specific biofouling as much as possible to improve the signal to noise ratio (*S*/*N*). Natural polymers such as albumin, casein, and dextran are considered as candidates for suppressing the non-specific interaction on the sensor surface.[2] However, the efficiency of these natural polymers is not completely satisfactory. Natural polymers derived from animals may contain disease-causing components. The blocking performance of natural polymers is different between the lots. Synthetic polymers, in contrast, are easily reproducible and contain no contagions. Thus, high-performance surface modification techniques using synthetic polymers are highly anticipated. We have reported previously that a densely packed poly(ethylene glycol) (PEG) tethered-chain surface shows high resistance to biofouling.[3–8] In particular, a combination of long and short PEG chains greatly

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reduces non-specific adsorption of proteins onto the substrate surface.[9] It is interesting to note that sensitivity is also increased when an antibody was co-immobilized on the mixed-PEG chain surface.[4,7]

For effective immobilization of PEG-derivatives on substrate surfaces, suitable anchoring strategies must be designed using specific interactions such as hydrophobic or electrostatic interactions or covalent conjugation. We have previously reported that multi-anchoring is one of the most effective ways of stably immobilizing PEG derivatives onto the substrate surface.[10,11] Several types of PEG-based block copolymers, but not graft-type copolymers, have been used to construct highly stable, tether-like chains on the substrate surface.[7] PEG block copolymers such as PEG-*b*-polycations, PEG-*b*-polyanions, and PEG-*b*-hydrophobic polymers have been proven to be versatile materials. For example, poly(ethylene glycol)-*b*-poly(acrylic acid) (PEG-*b*-PAAc) effectively covers Y_2O_3 nanoparticles *via* an electrostatic interaction resulting from the positive charge of the yttrium oxide surface.[5] PEG-*b*-polyamine was used for the modification of gold surfaces for specific biorecognition.[6,7] The coordination of the lone pair of the amino groups to the gold surface is involved in this case.

Improvement in sensor performance is another target in the development of high-performance sensor systems. Herein, we have chosen evanescent-field-coupled waveguide-mode (EFC-WM) monolithic sensing plates having a SiO_2 surface[12] to demonstrate the capability of PEG-polymers as the blocking and specificity-enhancing agents. At present, SiO_2 is commonly used as the sensing surface in the development of high-sensitivity systems.[13–23] The great advantage of the waveguide sensor is that higher sensitivity can easily be obtained by metal-nanoparticle labeling.[22] The waveguide sensor system that we used was similar to that of a surface plasmon resonance (SPR) system, and the difference was that the mode used for measurement is a waveguide mode rather than a surface mode. Unless otherwise stated, the design and configuration of the waveguide sensor is based on the principle of Kretschmann, a well-known scheme for the light irradiation of the thin film attached to the prism.[13,21–25] Biomolecular interactions can be monitored by measuring changes in the local index of refraction upon adsorption of target molecules onto the surface of the waveguide-mode sensor.

The objective of this work is to establish a high-performance biosensor by using the EFC-WM sensor, comprising PEG-based blocking agents for both sensor surface and nanoparticles. Human coagulating factor IX (FIX) was selected as a model analyte because of its extremely low availability *in vivo* and its importance in blood coagulation analysis. A stable RNA-aptamer generated against FIX was used as the ligand[26] and demonstrated favorable properties with regard to highlighting the potential characteristics of the preferred PEG-derived polymers.

Experimental

Reagents and biomolecules

3-Aminopropyltriethoxysilane (APTES) was purchased from Sigma-Aldrich (Tokyo, Japan). Glutaraldehyde, biotin, and precast gradient polyacrylamide gels were procured from Wako Chemicals (Osaka, Japan). Gold nanoparticles (GNPs) conjugated with streptavidin (40 nm; 15 OD) were from BioAssay Works (MD, USA). The shorter oligonucleic acid (dT_{20}) conjugated with biotin at the 5′-end and the other oligonucleic acids were synthesized commercially. FIX was purchased from American Diagnostica (Stamford, CT). PEG-*b*-PAAc was from General Science Corporation (Japan). N6-PEG was kindly provided by JSR Co. Ltd. (Tokyo, Japan).

Enzymatic synthesis of the aptamer

A stable 34-mer (2′-fluoro-modified) RNA-aptamer, which was previously reported by Rusconi *et al.*,[26] was prepared and its ability to bind FIX or FIXa was verified.[26,27] The aptamer was synthesized on a synthetic DNA template by an 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′-*AGTAATACGACTCACTA TAGG*GATGGGGACTATACCGCGTAATGCTG-3′ was used to prepare the double-stranded DNA. A polymerase chain reaction (PCR) was carried out using the above DNA template and amplified using appropriate primers (5′-AGTAATACGACTCA CTATAGG-3′ [forward] and 5′-$(T)_{24}$ATGGGGAGGCAGCATTACG CGGTATA-3′ [reverse]) using 2X PrimeSTAR Max DNA Polymerase, from Takara Bio Inc. (Shiga, Japan). 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 carried out at 37 °C overnight, using a DuraScribe transcription kit (Epicentre Biotechnologies, Wisconsin, USA). The products were treated with 2 U of DNase I (RNase free) for 20 min at 37 °C, to remove the residual template DNA, and the reaction was terminated by adding an equal volume of 2× 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 to fractionate. The RNA band was visualized under UV shadow and extracted from the excised gel-piece by using the crush-and-soak method.[28] The RNAs were precipitated in ethanol, dried under vacuum, and re-dissolved in RNase-free water, and the concentration was measured spectrophotometrically at 260 nm. Using the following DNA template, 5′-*AGTAATACGACTCACTATAGG*G TACCCCTGATATGGCGCATTACGAC-3′ (T7 promoter region highlighted in italics), the complementary FIX aptamer was synthesized as described above using the same T7 forward primer and the reverse primer 5′-$(T)_{24}$TACCCCTCCGTCG TAATGCGCCATAT-3′, for the negative reaction.

Setup of the EFC-WM sensor

The evanescent-field-coupled waveguide-mode (EFC-WM) sensor consists of a multilayer sensing plate comprising a dielectric waveguide, a high-refractive index layer, and a glass substrate.[12] The sensing plate, which is illuminated using the

Kretschmann configuration, operates as a sensor that is capable of detecting modifications in the dielectric environment near the waveguide surface by measuring changes in reflectivity. The optical setup is shown in Fig. 1a. A Xe lamp was used as a light source. The light from the lamp was guided to a collimator lens and a prism was irradiated with the collimated light; the incident angle of the light was parallel to the bottom face of the prism. Then, the monolithic sensing plate placed on the bottom of a prism was illuminated and a spectrum of the reflected light was detected using a spectrophotometer (Ocean Optics, Florida, USA). The prism was made of SiO_2 glass and its bottom angle was 38°. The monolithic sensing plate consisted of a SiO_2 glass substrate, a single crystalline Si layer, and a thermally grown SiO_2 waveguide layer, where the thicknesses of the layers are 45 nm and 360 nm, respectively. The fabrication method of the sensing plate is described elsewhere.[12] The optical measurements in this study were taken around 520 nm to show a dip in reflectance, which corresponds to the optical absorption of GNPs. If GNPs are attached to the waveguide surface, the dip will be deepened by the optical absorption of the GNPs.[22]

Surface functionalization on the SiO_2-sensing plate

Sensor chips were initially treated with an alkali solution for 30 min and washed thoroughly with a copious volume of buffer (10 mM HEPES [4-{2-hydroxyethyl}-1-piperazineethanesulfonic acid]-KOH, 150 mM NaCl [pH 7.4]) to remove the free hydroxide ions, and the surface was dried. Then further modification was carried out by immersion in a 0.5% (v/v) ethanol solution of APTES for 24 h. The amine group-functionalized surface of the SiO_2 waveguide layer was rinsed with ethanol and dried in a stream of nitrogen gas. The amine-functionalized SiO_2 surface was treated with a 2.5% solution of aqueous Glu for 3 h at room temperature to form an aldehyde-activated surface. After incubation, the surface was washed thoroughly with the HEPES buffer. The Glu-activated surface was made available for the covalent attachment of FIX protein, and then the remaining free amine sites were blocked by the introduction of PEG-*b*-PAAc. The anti-FIX aptamer extended with dA_{24} at the 3′-end was permitted to react with the FIX protein on the surface. The free end of the aptamer with dA_{24} was duplexed with biotin-dT_{20}. Finally, the biotin–streptavidin interactions were monitored by immobilizing the SA-GNPs in the presence or absence of N6-PEG (Fig. 1b). The optical density (OD) of the SA-GNP solution was measured and the dilutions were prepared using 10 mM HEPES (pH 7.4) to obtain 1 OD SA-GNP solution from the original stock supplied. Each reaction was performed for 30 min on the sensor chip, after which the sensing surface was thoroughly washed 3 times with 300 μL of HEPES buffer. In order to maintain the stability of the interaction between FIX and the aptamer, 2 mM $CaCl_2$ was added to the buffering solution, as previously recommended.[26]

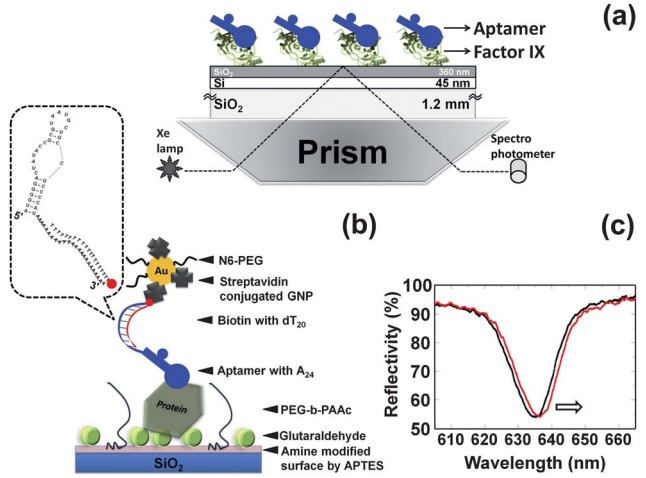

Fig. 1 (a) Schematic diagram of the waveguide-mode sensing system. A prism, a sensing plate, and other supports are shown. The light source is from a Xe lamp and light was detected using a spectrophotometer. Factor IX (FIX) protein and its aptamer are attached on the surface of the top SiO_2 layer. (b) Schematic of chemical surface modifications of the SiO_2 layer of the sensing plate for FIX and aptamer interactions. Glutaraldehyde (Glu) was attached to the amine-modified surfaces, followed by FIX protein immobilization. The aptamer ending with A_{24} was attached and then biotin with dT_{20} was allowed to react. Streptavidin conjugated gold nanoparticles (SA-GNPs) (40 nm) were used as a label. Sequences of the aptamer extended at the 3′-end are shown in the dotted box. (c) Spectrum shows the stable attachment of Glu on the amine-modified surface. Black and red spectra indicate buffer only and Glu attachment, respectively. Chips with the ability to measure the shifts around 630 nm were used. A red shift was observed by the attachment of Glu, because the transparent material, Glu, was attached on the surface.[12] There were no changes in the red spectrum after several washing steps, indicating that Glu was stably attached on the amine-modified surface.

Determination of sensitivity

To determine the sensitivity of detection of FIX on the waveguide-mode sensor, we performed experiments with aptamer and protein titrations using single (PEG-*b*-PAAc) and dual polymers (PEG-*b*-PAAc and N6-PEG). Surface modifications for all experiments were carried out as described above. Aptamer titrations were carried out after modification with PEG-*b*-PAAc of the Glu-modified surface of SiO_2. For this titration, we kept the protein (250 nM) and PEG-*b*-PAAc concentrations (4 mg mL^{-1}) constant and titrated using different concentrations of aptamer (10 pM to 200 nM). After attaching the aptamer as described above, the DNA ends with biotin were complemented. Spectral changes were monitored by using 1 OD of SA-GNPs. A similar titration was also carried out with the addition of N6-PEG (0.5 mg mL^{-1}) to the 1 OD of SA-GNPs. To optimize the sensitivity limit of FIX, we also performed the protein titration at 100 fM to 250 nM, keeping the aptamer (100 nM) and PEG-*b*-PAAc (4 mg mL^{-1}) concentrations constant. Measurements were carried out with or without addition of N6-PEG (0.5 mg mL^{-1}) on SA-GNPs.

Biotin–streptavidin competitive assay

We carried out the competition assays with the addition of different concentrations of free biotin to SA-GNPs. For this assay, the surface treatment and chemical modifications of the sensing surface were the same as described above. Biotin was used as a competitor at concentrations of 0.1 fM to 1000 fM. The biotin concentration was varied whilst maintaining a constant

SA-GNP optical density (1 OD) and titrated on a biotin-immobilized sensing surface with constant aptamer concentration.

Scanning electron microscopy (SEM)-nanoscale imaging

To increase the sensitivity of the specific biomolecular interaction described above, we mixed N6-PEG with SA-GNPs. To visually confirm the effect of the N6-PEG on the sensing performance, we carried out microscopic analyses using field emission scanning electron microscopy (SEM; JEOL, JSM-6340F) on the SA-GNP surfaces in the presence and absence of N6-PEG. Surface modifications were carried out as described in Fig. 1b. N6-PEG was premixed with SA-GNPs before being attached to the biotinylated sensing surface. The attachment of GNPs was observed using SEM. SEM measurements were carried out with 5 kV acceleration and 10 μA emission. Images were captured at a magnification of 10 000. All SEM observations were performed at a GNP-streptavidin conjugate optical density of one, as described above.

Selective and specific binding of the aptamer with FIX on the surface

For selective binding which mimics a similar situation in the blood samples, experiments with mixed clotting proteins were carried out. Different concentrations of FIX, representing concentrations lower than the saturation points from the above assays with dual polymers, were considered. For this assay we used a protein mixture (FIX, FXIa, and FVIIa); we kept FXIa and FVIIa concentrations constant and titrated the binding of FIX at concentrations of 100 fM to 1 nM. All other surface chemical modifications and detection strategies were the same as those described above. The selective binding study was also performed in the presence of 84 nM of albumin with dose-dependent FIX concentrations from 100 fM to 1 nM. These mixtures were tested against a constant aptamer concentration (100 nM).

Results and discussion

An improvement in biofouling resistance is necessary for biosensor development and biomolecular recognition. The steps of our sensing strategy are as follows: (i) the blood sample is applied to the sensor chip surface to immobilize blood proteins; (ii) the specific interaction of aptamer-A_{24} with the target protein (FIX in this case) takes place on the surface; (iii) the hybridization of biotinyl-dT_{20} to the aptamer-A_{24} takes place; (iv) contact is made with GNPs that are surface-modified with streptavidin; and (v) the absorption is measured using the EFC-WM sensor. During these steps, the issue of non-specific interactions of aptamer-A_{24} and GNPs is important with regard to suppressing background signals. In this study, we have utilized 2 kinds of synthesized polymer derived from PEG, *viz.* PEG-*b*-PAAc and N6-PEG, in order to create high-performance sensing surfaces. Our idea was to achieve the most effective blocking of both the sensor chip and the surfaces of the GNPs using PEG-*b*-PAAc and N6-PEG. With the assistance of these blocking polymers, we have analyzed human coagulating FIX. An aptamer against FIX was used to improve specific sensing. This 2′-fluoropyrimidine-modified stable aptamer, which binds FIXa with high affinity and specificity,[26] binds FIX with equal efficiency.[27] FIX used in this study has high purity and migrates as a single prominent protein under sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) (ESI, Fig. S1a†). The aptamer prepared by the enzymatic reaction (transcription) is a 58-mer and contains 34-nucleotides at its 5′-end that specifically bind with human FIX (ESI, Fig. S1b†). The sensor surface was functionalized by Glu, which has strong affinity for amine-modified SiO_2 surfaces; the spectrum shown in Fig. 1c demonstrates that stable attachment of Glu was achieved after a long incubation and several washings. The non-specific attachments of SA-GNPs were found to occur on amine- and Glu-modified surfaces. The potential blocking effect of PEG-*b*-PAAc on these surfaces was compared with that of ethanolamine, which is a common blocking agent for sensing surfaces. Further, the improvement in the sensitivity was evaluated following the additional usage of N6-PEG on GNPs.

Influence of ethanolamine or PEG-*b*-PAAc on FIX recognition of a chemically functionalized SiO_2 surface

Before optimization of the sensing of FIX by the present system, we confirmed that the system works properly by using ethanolamine and PEG-*b*-PAAc as blocking agents. After FIX solution was brought in contact with the Glu-treated sensor chip, the biotinylated aptamer was reacted in the presence of a buffering agent containing $CaCl_2$, which is necessary for these ligand–analyte interactions.[26] On this biotin-exposed surface, we immobilized the SA-GNPs, which were preferably 40 nm in size and could enhance the spectral signaling to a large degree at 520 nm (suitable for gold). A biotinylated complementary aptamer sequence was used for the control experiment. Fig. 2 clearly demonstrates the spectral changes resulting from the SA-GNP–biotin interaction on the waveguide sensor surfaces. However, in the case of the ethanolamine-treated sensing surfaces, significant spectral changes were recognized both in the specific adsorption (Fig. 2a) and in the control experiment (Fig. 2b). This result indicates that the ethanolamine-modified surfaces could not prevent the non-specific interaction between SA-GNPs and the waveguide sensor surfaces. In the case of sensing surfaces using PEG-*b*-PAAc as a blocking agent, we could clearly see the difference in the spectral changes in the specific aptamer-treated surface (Fig. 2c), and the background signal was completely suppressed (Fig. 2d). In order to significantly improve the colloidal stability and non-fouling character of SA-GNPs, we used N6-PEG as the blocking agent. Non-specific absorptions of SA-GNPs with and without N6-PEG were investigated on a PEG-*b*-PAAc-immobilized sensor chip surface using the EFC-WM sensor. Interestingly, no signal change was observed regardless of N6-PEG blocking, as shown in the ESI, Fig. S2.† Complete protection of the sensor chip surface by PEG-*b*-PAAc might prevent non-specific interactions of SA-GNPs even without N6-PEG blocking.

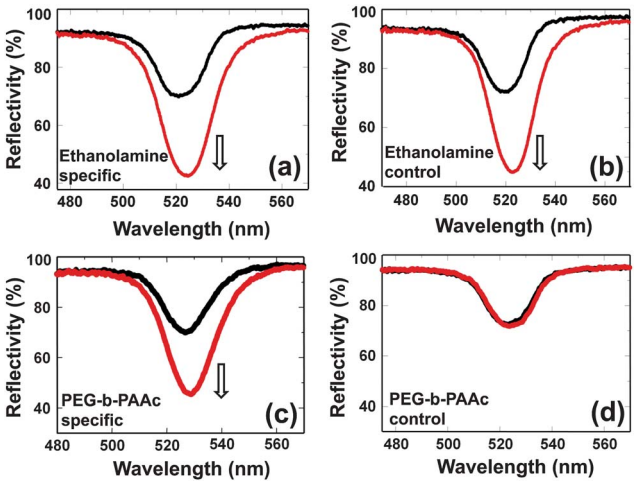

Fig. 2 Aptamer and protein interaction using ethanolamine or PEG-*b*-PAAc as the blocking material. (a) Specific reaction using ethanolamine, (b) non-specific reaction using ethanolamine, (c) specific reaction using PEG-*b*-PAAc, and (d) non-specific reaction using PEG-*b*-PAAc. Black and red curves are the spectra before and after the attachment of SA-GNPs, respectively. Arrows indicate the direction of changes in the spectrum.

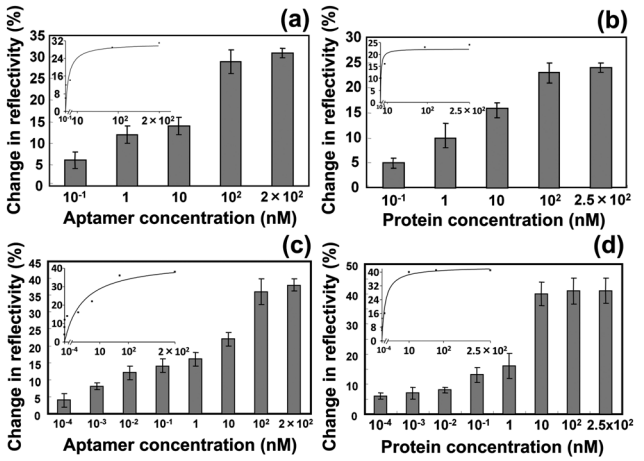

Fig. 3 Aptamer titrations against constant FIX (250 nM). Concentrations of other biomolecules were kept constant. Graphical representation of comparative analyses for the PEGs. Reflectivity changes are shown for: (a) constant FIX (250 nM) against different concentrations of aptamers in the presence of PEG-*b*-PAAc; (b) constant aptamer (100 nM) against different concentrations of FIX in the presence of PEG-*b*-PAAc; (c) constant FIX (250 nM) against different concentrations of aptamers in the presence of PEG-*b*-PAAc on the sensing plate and N6-PEG on the SA-GNP; and (d) constant aptamer (100 nM) against different concentrations of FIX in the presence of PEG-*b*-PAAc on the sensing plate and N6-PEG on the SA-GNP. Fitting curves are shown as insets.

Determination of sensitivity using single and dual PEG-polymers

The sensitivity to FIX was investigated using PEG-*b*-PAAc. Initially, the aptamer concentration necessary for the saturation level was accessed by using different aptamer concentrations against constant FIX. We found the minimal specific interaction at 100 pM of aptamer, which showed a reflectivity change of 6%. Aptamer concentrations higher than 100 nM showed constant changes in the spectrum, indicating the attainment of saturation (Fig. 3a; spectra are shown in the ESI, Fig. S3†). Using this suitable aptamer concentration, the sensitivity level to FIX was investigated. Fig. 3b shows the detection of FIX at a constant aptamer concentration of 100 nM; the FIX was detected at 100 pM as a 6% reflectivity change (spectra are shown in the ESI, Fig. S4†). We have previously reported that the densely packed PEG chains surrounding an antibody immobilized on a sensor chip surface improve its effectiveness.[1,4,8] This might be one of the reasons that our system demonstrated a 100 pM level of sensitivity. With increasing FIX concentration, the signal changes increased concomitantly. Similar titrations using saturated levels of aptamer (Fig. 3c; ESI, Fig. S5†) against FIX-dose dependency were also carried out using SA-GNPs with N6-PEG. Fig. 3d shows the sensitivity level of FIX using PEG-*b*-PAAc on the sensor chip surface and N6-PEG on the SA-GNP (spectra are shown in the ESI, Fig. S6†). As can be seen in the figure, a large increase in the detection limit was observed when we used both PEG-*b*-PAAc and N6-PEG. It is interesting to note that N6-PEG immobilized on SA-GNP surfaces influenced the sensing ability though it did not influence the suppression of non-specific binding on the sensor chip. At the sensitivity concentration determined in the earlier experiment with only PEG-*b*-PAAc (100 pM), addition of N6-PEG showed 3-fold higher spectral changes and reflectivity change at the spectral dip of 14%. Therefore, under the usage of both polymers, the sensitivity was improved and we could monitor the FIX at 100 fM with a reflectivity change of 6% (Fig. 3d; ESI, Fig. S6†). This result with the addition of N6-PEG represents a 1000-fold improvement in sensitivity. It was previously reported that the K_d values were 600 by a filter binding assay and 400 pM by a commercially established SPR analysis, when the same aptamer and FIX were employed. In both assays, nanomolar levels of proteins were used and statistical calculations were performed for the K_d values.[26,27] In the routine clotting assay it is reported that clotting was delayed in the presence of an anti-factor IX aptamer at low nanomolar levels.[27]

Biotin–streptavidin competition revealing the sensitivity enhancement

To explore the reason behind the sensitivity improvement in the presence of N6-PEG and to determine the genuine interactions between biotinylated aptamers and SA-GNPs, we studied dose-dependent biotin competition on the sensor surface (Fig. 4a), *viz.* different concentrations of free biotin were mixed with SA-GNPs prior to application to the sensing chip. At low concentrations (0.1 fM of biotin), the presence of free biotin did not affect the results. However, it was interesting to note that with an increase in the biotin concentration there was a significant change in the spectral signal. These changes continued until the free biotin concentration reached 5 fM. At concentrations beyond 5 fM, there was a decline in the spectral changes of the waveguide sensor (ESI, Fig. S7†). The maximum sensitivity was seen at a free biotin concentration of around 1.5–2.5 fM, as shown in Fig. 4b. This implies that the binding affinity of SA-GNPs with respect to the surface biotin increases

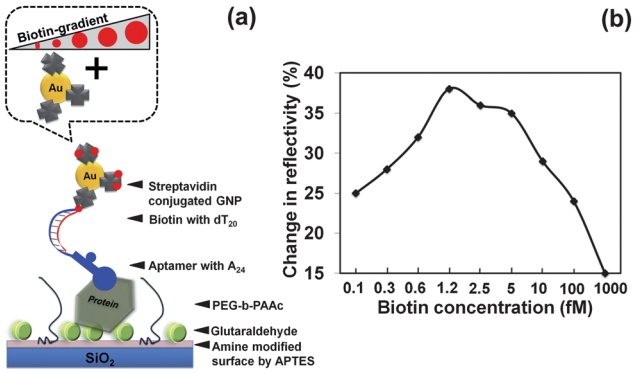

Fig. 4 Competition assays using biotin. Different concentrations of biotin were added to SA-GNPs before reacting with pre-immobilized biotinylated aptamers on the sensing surface. (a) Schematic illustrates the experimental procedure. (b) Graphic representation of changes in the reflectivity, under competition.

with increasing free biotin concentration up to 1.2 fM, suggesting that the competitive biotin molecules at a concentration of less than 1.2 fM might occupy some of the SA-GNP active binding sites. Although streptavidin has 4 active binding sites for biotin, several sites were occupied by the free biotin under the above competitive situation, which might increase the affinity. This kind of competition has been reported recently by Duan *et al.*[29] who studied the dissociation of streptavidin–biotin complexes by addition of biotin in a dose-dependent manner. A similar effect might be observed in the case of N6-PEG, *viz.* it could mask some of the biotin-active binding sites on the streptavidin. The results of the free biotin and SA-GNP competition assays are illustrated in Fig. 4b. The crowding property of PEG might allow more SA-GNPs to properly align with the pre-immobilized biotinylated aptamers on the sensing plate.

Nanoscale imaging of captured GNPs on the sensing plate

To substantiate the above results demonstrating a 1000-fold improvement in sensitivity with N6-PEG, scanning electron microscope (SEM) analysis of the sensor chip surface was carried out. GNPs were clearly observed on the sensing plate used in the above experiments (experiments with single and double polymers) after attaching SA-GNPs. Measurements were taken at different places and the number of attached GNPs was counted. The observations of the number of GNPs attached strongly corroborate the results obtained experimentally by the waveguide-mode sensor, *viz.* the number of GNPs from the sensing plate treated with only PEG-*b*-PAAc was about three times lower than from that treated with dual polymers (Fig. 5a and b).

Selective binding of the aptamer to FIX in the presence of other proteins

We have confirmed above the high sensitivity of the present sensing system to FIX. To provide further information on the sensitivity of the present system, sensing experiments were carried out in the presence of other proteins. The human blood clotting cascade involves clotting factors other than FIX, as well

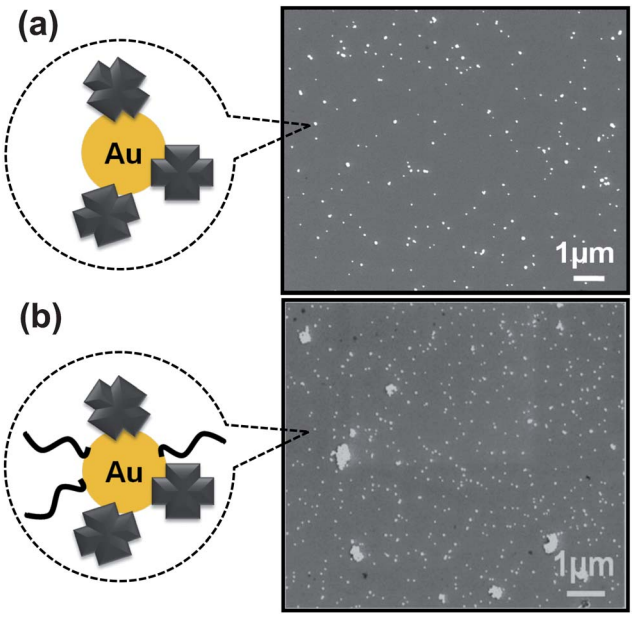

Fig. 5 SEM images of the surfaces of the sensing plates, on which FIX and the biotinylated aptamer were immobilized and PEG-*b*-PAAc was attached as a blocking material. (a) SA-GNPs were attached on them. (b) N6-PEG-coated SA-GNPs were attached on them.

as major additional components such as albumin. Therefore, we studied the reaction with the complex mixture of FIX containing FXIa and FVIIa, or albumin. FXIa is involved in the cleavage of FIX in the intrinsic clotting pathway, and FVIIa plays an important role in the cleavage of FIX during the extrinsic clotting pathway.[27] These components of human plasma are present in different quantities: FIX, 3–5 μg mL^{-1};[30] FVIIa, 3.6 ng mL^{-1};[31] FXI, 3–6 μg mL^{-1};[32] and albumin, 45 mg mL^{-1}.[33] If we can detect FIX selectively among these activation factors, it is anticipated that we will be able to precisely determine the blood activation level. Albumin is one of the major components of blood protein (45 mg mL^{-1}; 50–70% of blood protein). It is therefore important to confirm that FIX can be detected in such a large excess of albumin. In the mixture containing human clotting proteins (FIX, FXIa, and FVIIa), at levels similar to those in human blood, we could find the specific interaction of the anti-FIX aptamer with dose-dependent FIX (ESI, Fig. S8†). Titrations were performed with concentrations lower than the saturation levels (5 pg mL^{-1} to 50 ng mL^{-1}) from the above experiments using PEG-*b*-PAAc and N6-PEG. We observed a similar sensitivity (5 pg mL^{-1}) to that shown in the above experiments with a pure sample containing only FIX (Fig. 6). This clearly indicates that the other clotting factors do not interfere with the detection of FIX using the EFC-WM sensor. The detection experiments were also carried out in the presence of albumin. As stated above, the concentration of albumin is quite high (670 μM; 45 mg mL^{-1}) compared to FIX (~3 to 5 μg mL^{-1}) in human blood; a ratio of ~8400 : 1. Considering the diluted blood sample, we used 5.6 mg mL^{-1} of albumin and 0.5 ng mL^{-1} of FIX, which represents the same ratio (8400 : 1). In the presence of 5.6 mg mL^{-1} of albumin, FIX detection

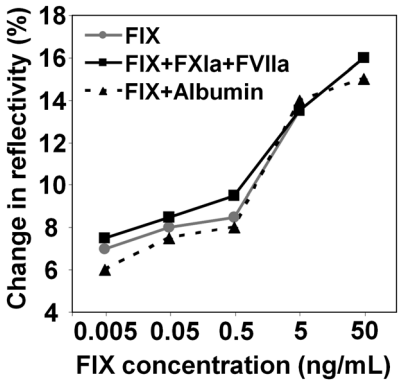

Fig. 6 Selective binding of the aptamer with FIX in the presence of FXIa and FVIIa or albumin. Titrations of different FIX concentrations against constant aptamer and other components are shown by graphical representation. FIX concentrations lower than saturation levels of previous experiments were used.

suitable for normal blood sample analyses was carried out by the present waveguide-mode sensor system. As can be seen in Fig. 6, 0.5 ng mL^{-1} of FIX in 5.6 mg mL^{-1} of albumin can be detected. In addition, the detection signal was clearly observed up to a level of 5 pg mL^{-1} (see ESI, Fig. S9†), which is 2 orders of magnitude lower than that of the normal level of FIX in blood. It is also confirmed that the detection limit is the same as that without albumin, as shown in Fig. 6. At present, an aptamer generated against FIXa is undergoing phase 2 clinical trials. Owing to its high specificity against FIXa in blood samples, this aptamer is injected into patients.[34] The anti-FIX aptamer was also shown to work well with human plasma containing FIX and FIX-deficient plasma spiked with FIXa,[27] both studies corroborate our results for the detection of FIX in mixed protein samples. The detection system demonstrated in the present investigation, which is resistant to biofouling, would be useful in determining the abundance of FIX in blood samples containing multiple proteins.

Conclusions

We have designed a new biosensing system for detecting human coagulation FIX by using its aptamer and GNPs and a waveguide sensor. The Glu-modified sensor chip surface was brought into contact with the FIX sample and was subsequently blocked using PEG-*b*-PAAc. This blocking treatment completely prevents non-specific binding on the sensor surface. It is interesting to note that coating of SA-GNPs by N6-PEG increases the sensitivity by 1000 times. The higher sensitivity arises from the crowding effect of N6-PEG on the SA-GNPs, which occupy a certain number of active biotin-binding sites on the streptavidin. Based on these results, it is concluded that PEG-*b*-PAAc and N6-PEG may be the ideal blocking agents for use in the development of an aptamer-based sensing system (aptasensor) with higher specificity. An FIX detection limit of 0.1 pM was attained even in the presence of other coagulation factors or a realistic albumin concentration. The strategy developed in this study by using an anti-FIX aptamer to detect minute amounts of FIX in a mixed sample (which represents blood serum) is highly recommended for finding clotting deficiencies in human samples.

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