Development of a highly specific amine-terminated aptamer functionalized surface plasmon resonance biosensor for blood protein detection

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Abstract: This paper presents a generally applicable approach for the highly specific detection of blood proteins. Thrombin and thrombin-binding aptamers are chosen for demonstration purposes. The sensor was prepared by immobilizing amine-terminated aptamers onto a gold modified surface using a two-step self-assembled monolayer (SAM) immobilization technique and the physical detection is performed using Surface Plasmon Resonance (SPR). The developed sensor has an optimal detectable range of 5–1000 nM and the results show the sensor has good reversibility, sensitivity and selectivity. Furthermore, the sensor shows the potential of being improved and standardized for direct detection of other blood proteins for clinical applications.

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1. Introduction

The direct detection of blood proteins can benefit a number of scientific and clinical applications, such as in monitoring the ratio of specific protein glycation in diabetes [1], biomarkers for drug research and environmental monitoring [2], cancer diagnostics and treatment [3], etc. The current clinical and laboratory measurement techniques for blood proteins are boronate affinity immunoassay, high-performance liquid chromatography (HPLC) and capillary based systems [4–6], which are time consuming and costly. More efficient and fast response measurement methods could greatly benefit and enhance related application areas, especially for developing the next generation of portable handheld diagnostic devices capable of real-time analysis. Several optics-based diagnostic techniques, such as near-infrared spectroscopy [7,8], polarimetry [9], optical coherence tomography [10], Surface Plasmon Resonance (SPR) [11], Raman [12] and fluorescence spectroscopy [13] have recently been investigated for monitoring blood components. Changes in optical properties resulting from variations in blood components/chemistry provide a convenient method for the use of optical diagnostic techniques to provide for fast and *in vivo* measurements. In this paper, an approach is presented for the detection of specific blood proteins based on custom SPR aptamer modified sensors that can have broad applications.

Aptamers are artificial oligonucleotides which can serve as antibody mimics because of their high affinity and selectivity for various target compounds ranging from small molecules, such as drugs and dyes, to complex biological molecules such as enzymes, peptides, and proteins. Custom aptamers can be identified from random oligonucleotide libraries for specific target compounds by an *in vitro* iterative process called Systematic Evolution of Ligands by Exponential Amplification (SELEX) [14,15]. Aptamers can form a 3D structure serving as receptors specific to their target molecules similar to antibodies. Aptamers also have a number of advantages over antibodies such as a tolerance to wide ranges of pH and salt concentrations, heat stability, ease of synthesis, and cost efficiency. The specificity and affinity of aptamers are comparable, if not higher, to antibodies. Aptamers are also capable of being reversibly denatured for the release of target molecules, which makes them perfect receptors for biosensing applications.

SPR is a member of a family of spectroscopic techniques based on evanescent wave optics. It is commonly used for determination of refractive index, dielectric constant, and layer thickness with high sensitivity. By itself, however, SPR is not a selective sensing technique and requires adaptation for specific target identification and quantification. Therefore, a number of SPR-based biosensors have been recently reported on for a variety of environmental and biological applications [16].

Some groups have successfully developed aptamer-based biosensors for the detection of proteins [17–24] and related SPR based sensors have also been reported [25,26]. Use of a self-assembled monolayer (SAM) as a linker and coadsorbent thiol-modified aptamers together to form a aptamer-SAM matrix on a gold surface has demonstrated its potential as a reliable and easy approach [27]. However, the cost of the thiol-modified aptamers is much higher than the non-modified or amine-modified aptamers. The uniformity and density of the SAM are also not guaranteed from sample to sample. We report on a 3-mercaptopropionic acid (MPA) SAM based coupling approach for a more stable and repeatable modification of the sensor surface. Electrochemical Impedance Spectroscopy (EIS) was utilized to monitor the formation of the SAMs on the gold surfaces. Aptamer binding capacity was then determined by a magnetic beads (MBs) coupling method. The developed sensor has an optimal detectable range of 5-1000 nM with good reversibility, sensitivity and selectivity. Furthermore, the sensor shows

the potential of being improved and standardized for the direct detection of other blood proteins for clinical applications.

2. Materials and Experimental

2.1. Materials

The identified aptamers were synthesized by Integrated DNA Technologies (Coralville, IA), including a 15bp aptamer (APT1): 5′-NH_2-$(CH_2)_6$-GGTTGGTGTGGTTGG-3′, and a 34bp aptamer (APT2): 5′-NH_2-$(CH_2)_6$-CTATCAGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′.

Tosylactivated MBs were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma Aldrich (Carlsbad, CA) at the highest purity available. Aptamer solutions were prepared with 1M pH 8 phosphate buffer and 3-mercaptopropionic acid (MPA) solution was prepared in ethanol. Protein sample solutions were prepared using a 0.1M pH 7.2 PBS buffer solution with 5 mM KCl and 1 mM $MgCl_2$. The phosphoric acid (PPA) used in this study was 100 mM. All other solutions were prepared in deionized (DI) water.

2.2. Instrumentation

SPR measurements were performed using a commercial grade SensiQ Discovery system (ICx Technologies, Arlington, VA) at 25°C. This sensor is based on a Kretschmann configuration, in which the light from a light-emitting diode (LED) integrated with a prism is firstly p-polarized and then internally reflected from a gold surface. The angle of light reflection and the relative intensity was measured with a photodiode array. When the sample solution is applied to the sensing surface, the SPR profile minimum (also known as the SPR angle) will shift as a function of the refractive index of the loaded sample, giving a real time refractive index reading (although, by itself the sensor is not specific/selective for any given target). In this study, the SPR response profile was recorded by the SensiQ software and then processed within MATLAB®.

2.3. Experimental

Electrochemical impedance spectroscopy (EIS) measurements were carried out using a Gamry Reference 600 potentiostat (Warminster, PA) in 5 mM $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ solution with KCl as a supporting electrolyte. All the experiments were carried out at room temperature with the solutions purged with nitrogen gas for 15 minutes and the nitrogen blanket was maintained during the experiments. The experiments were performed at 25°C. Impedance spectra were collected in the frequency range from 0.1 Hz to 100 kHz with a potential amplitude of 5 mV_{rms} at 10 points per decade. EIS results were analyzed by fitting the experimental impedance data to electrical equivalent circuit models. Parameters of the electrical-equivalent circuits were obtained by fitting the impedance function to the measured Bode and Nyquist plots with a complex nonlinear least square (CNLS) program built into the Gamry EIS 300 electrochemical impedance spectroscope.

Aptamer coupling to the MBs was performed as followed: 10 nmol of amine modified aptamer was coupled to 10 mg washed MBs in a shaker incubator at 37°C for 18 hours. The unoccupied binding sites were blocked by Bovine Serum Albumin (BSA). The MBs were washed thoroughly and then 10 nmol of thrombin was mixed with the aptamer-coupled MBs for 2 hours in a shaker at room temperature. The control group was prepared by exactly the same method except for the absence of aptamers. The total and unbounded proteins were measured with a carboxyl functionalized SPR sensor provided by SensiQ.

To use an aptamer-based SPR sensor for detecting blood proteins, thrombin and anti-thrombin aptamer were chosen for demonstration purposes. Gold slides were prepared by physical vapor deposition (PVD) forming a 1 nm layer of titanium and a 50 nm layer of gold onto pre-cleaned microscope cover slides. These were then washed by copious amounts of DI water and ethanol. They were dried in nitrogen gas before usage. To functionalize the gold

slides, they were immersed in the 10 mM MPA solution for 30 min and then washed with ethanol and DI water. After the slides were dried, then they were immersed in a solution of N-hydroxysuccinimide (NHS) and N-(3-dimethylamnopropyl)-N-ethylcarbodiimide hydrochloride (EDC) (NHS 0.2M, EDC 0.05M) for 30 min. The slides were then washed with DI water and then immersed in the 5 μM aptamer solution. Finally, the slides were rinsed with the PBS buffer to flush off non-specifically adsorbed proteins. Then the slides were ready for measurement. This two-step surface functionalization procedure is illustrated in Fig. 1.

Non-coated (i.e. no gold) SensiQ base sensors were custom modified with the developed gold based SPR sensing surfaces. Specifically, freshly prepared aptamer- immobilized gold substrates were coupled to the stripped sensors with index matching optical oil. This was followed by then loading of 100μL 1 M ethanolamine (EA) at a flow rate of 20 μL/min to block the non-occupied MPA sites activated by the EDC/NHS, followed by an injection of 100 μL of 100 mM phosphoric acid (PPA) at 50 μL/min to remove the non-specific binding. The running buffer was 0.1 M pH 7.2 PBS. The sensor was first normalized with the buffer for 10 min, then the thrombin sample (25 μL) at concentrations of 5 nM, 25 nM, 50 nM, 250 nM 500 nM, 1000 nM, 2000 nM were loaded at 5 μL/min. Samples with BSA were all prepared with 400 nM BSA. All data was recorded at 290s, 300s, and 310s after the sample injection and averaged. Sensor regeneration was performed by the injection of 100 μL PPA at 50 μL/min followed by washing with the running buffer.
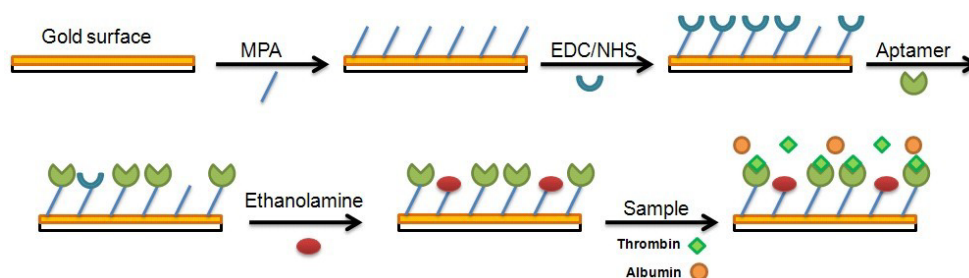

Fig. 1. Schematic diagram of the sensing surface functionalization procedure.

3. Results and Discussion

3.1. EIS measurement

Electrochemical impedance spectroscopy (EIS) has been proven as a powerful tool for surface characterization. The successful immobilization of each functionalized layer was confirmed through EIS measurements. Figure 2 shows the Nyquist plots of impedance spectra at different electrodes. The bare gold electrode represented a very small circle at high frequencies, suggesting a very low electron transfer resistance to the redox probe dissolved in the electrolyte solution (curve A). When the MPA was immobilized on the electrode and treated with EA and PPA, the electron transfer resistance (R_{et}) increased to 125 Ω, (curve B). Then, when 5 μM of the APT1 aptamer was added and bound with the SAM, R_{et} increased to 600 Ω (curve C). Note that the reactive sites on the gold electrode were blocked by EA (ethanolamine) to prevent non-specific adsorption of aptamers onto the gold surface, thus making sure that the aptamers were attached only to the SAM. The R_{et} increase is attributed to the electrostatic repulsion between the immobilized aptamer and the redox probe, causing a barrier for the interfacial electron transfer. These results indicate successful immobilization of the SAM layer onto the gold surface and stable bonding of the aptamer to the SAM.

3.2. MB based maximum binding capacity study

It is assumed that all aptamers were coupled to the MBs completely since twice the amount of the MBs, as instructed by the MB manufacturer's protocol sheet, were used along with the
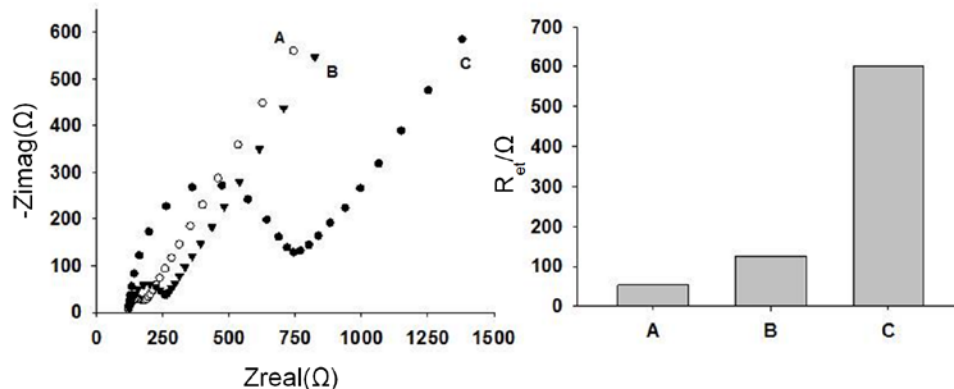
Fig. 2. Nyquist plots of impedance spectra obtained in 100 mM PB solution (pH 7.2) containing 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$: (A) Bare Au; (B) Au/MPA/EDC-NHS/EA/PPA; (C) Au/MPA/EDC-NHS/EA/PPA/APT1. The right plot shows the (R_{et}) of each layers. Impedance spectra were collected in the frequency range from 0.1 Hz to 100 kHz with a potential amplitude of 5 mV rms at 10 points per decade.

longest allowed reaction time. After the modified MBs were thoroughly washed, thrombin was added and the concentration change was measured using a carboxyl modified SPR sensor. It is also assumed that the refractive index is controlled only by the concentration change of the added thrombin.

Other experimental variables such as protein degeneration and temperature had minor influences on SPR results and thus were not considered. As shown in Fig. 3, the concentration change of thrombin was insignificant for the control group (less than 3%) which was not functionalized by the aptamer. This indicates that the concentration change in the two experimental groups was mainly due to the binding between the aptamer and thrombin. For the APT1 and APT2 groups, the mixture of aptamer functionalized MBs and thrombin solution was allowed to react for 18 hours and the reaction was considered to be completed based on the MB manufacturer's specifications (see section 2.3 for the experimental details). Thus, the final concentration reflected the maximum mol/mol binding capacity of aptamer to thrombin. The results showed the binding ratio of APT1 (57.1%) has a slightly better capacity than APT2 (55.2%). Both aptamers had more than 50% mol/mol binding ratio to thrombin,
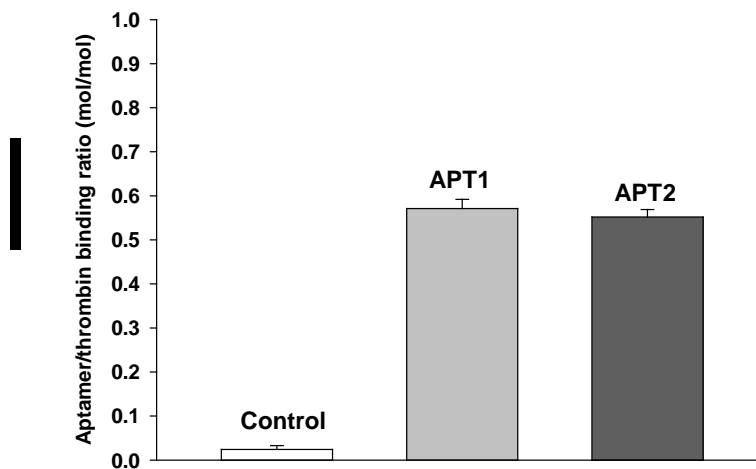

Fig. 3. Aptamer/thrombin binding ratio in mol by the MBs coupling method.

indicating that they are good receptor candidates for thrombin sensing applications. In practice, not all the aptamers may bind to the MBs and therefore the actual binding capacity of two thrombin binding aptamers toward thrombin may be slightly greater. It should be mentioned that MBs binding to aptamers could form a MB-based biosensor by involving fluorescence labeling [28], however, the sample preparation procedure of this method is complicated. Here we only utilized the MBs to determine the maximum binding capacity of the aptamers used in this study. This could facilitate choosing the best aptamer from multiple candidates in future optimization studies.

The Control group consists of MBs without aptamer functionalization and all binding sites blocked by BSA. The APT1 and APT2 groups consist of MBs functionalized by the respective aptamers with the unoccupied binding sites blocked by BSA. The error bars represent the standard deviation of the values determined from three samples.

3.3. SPR results

Two different aptamers were immobilized on gold surfaces and the binding performance of each one was compared. For reference, samples of different thrombin concentrations (5 nM, 25 nM, 50 nM, 250 nM, 1000 nM, 2000 nM) were individually loaded onto a bare Au, an APT1, and APT2 sensor, respectively. A secondary experiment was then performed using the same thrombin concentrations, however, with a 400nM BSA confounding component added to each thrombin sample for comparison. As shown for the "Thrombin only" experiment in Fig. 4, the SPR shifts were very low for the bare Au sensor surface even for the relatively high thrombin concentrations; which was expected. In contrast, for the aptamer modified sensors the SPR shifts were significantly enhanced and the optimal detection range was 5 nM to 1000 nM (linear range). As shown in the "Thrombin + 400 nM BSA" experiment in Fig. 4., the prior experiment was replicated, however, with a large 400nM BSA confounding concentration component added to each thrombin sample concentration. As compared to the thrombin only group, the responses are nearly identical indicating the developed APT1 and APT2 sensors are highly specific to only thrombin; which was expected. This is further illustrated in Fig. 5, which shows the SPR shift for the 500 nM thrombin concentration with and without 400 nM of BSA. Adding BSA to the sample had minimal effect on the SPR response for the aptamer modified sensors, indicating a good selectivity of the sensor toward thrombin. This is in contrast to the bare Au sensor, which experienced a significant change between the thrombin samples with and without BSA. The APT1 modified sensor did have a slightly higher shift than the APT2 sensor for all the thrombin concentrations. The slope of the fitting line for APT1 is also slightly larger than APT2 in the linear response range (Fig. 6), again demonstrating a slightly better sensitivity. These two aptamers bind to different sites of thrombin, thus the affinity to the target should be different in both the interfacial binding environment and in solution [29]. In the MBs binding tests, we demonstrated that APT1 had a slightly higher binding capacity than APT2, which corresponds to the SPR results in terms of sensitivity of the functionalized sensor. A potential cause of this could be due to the smaller aptamer having a greater probability to access the binding sites of the target protein. Another possibility is that larger aptamers are thought to have more complicated secondary structures that require an extra spatial flexibility to form bonding with target molecules [30].

The MPA layer is known to have excellent coverage rate on gold and is widely used for antibody immunization for biosensing purposes [29]. MPA is cost effective and the related SAMs are easy to prepare. Our results show that the amine-modified aptamer can be easily immobilized onto the MPA layer and the sensor performance was comparable to antibody-based sensors. Imprecision guidelines for blood proteins quantification has been proposed by several reports and the common finding is that an imprecision of 3% for quantification methods is adequate for clinical purposes [28,31,32]. In our tests, three sensing slides were prepared for each aptamer and also the control group. The sensor to sensor performance was
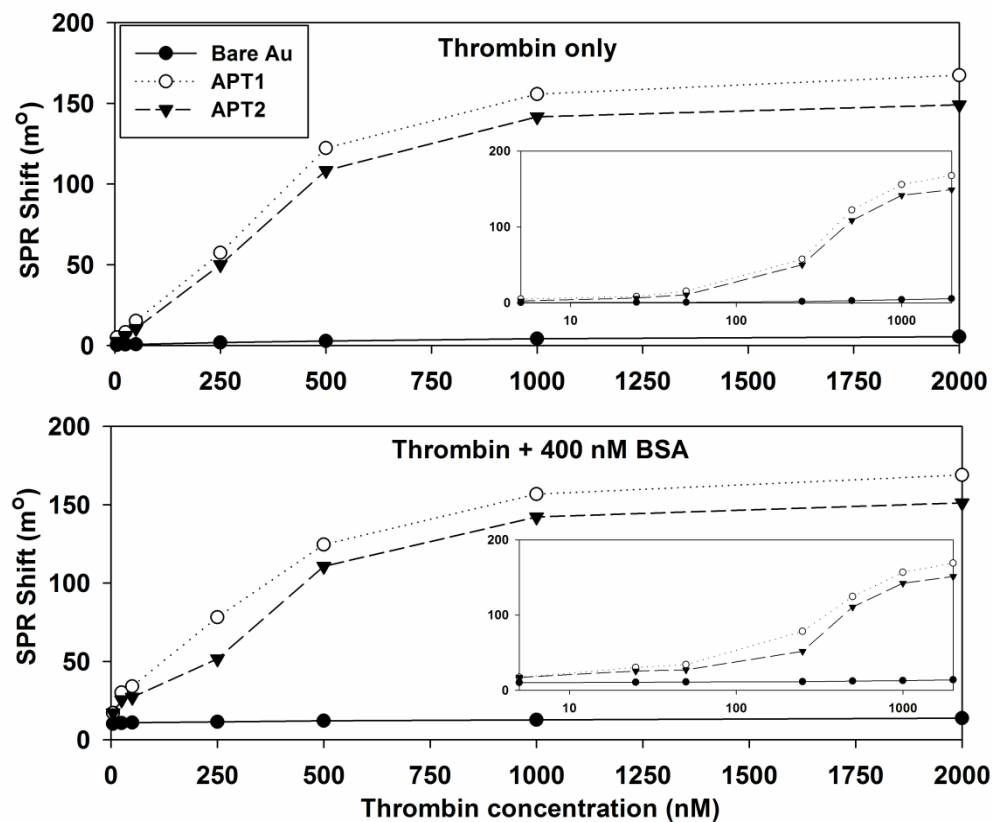
Fig. 4. SPR response of bare Au and aptamer-modified sensors. All data points were averaged from 3 experimental data readings. Samples were thrombin only (top plot) and thrombin with 400 nM BSA (bottom plot). The inlay plots are same data plotted on logarithmic scale to allow for better visualization at lower concentrations.
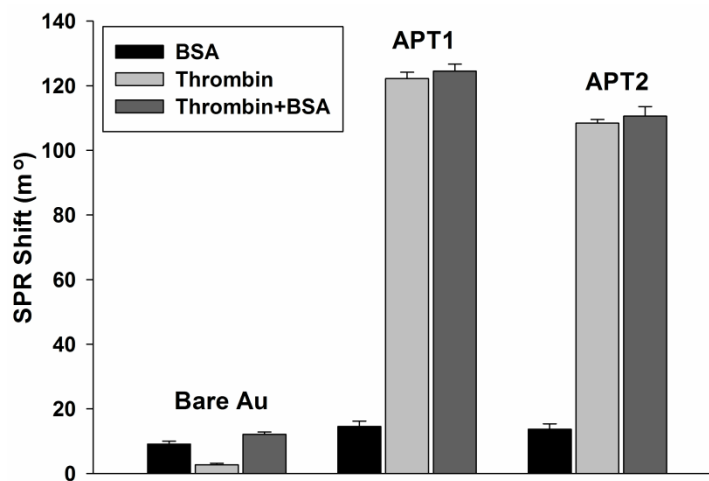

Fig. 5. SPR responses of different sensing surfaces for 400 nM BSA (BSA group), 500 nM thrombin (Thrombin group), and 500 nM thrombin with 400 nM BSA (Thrombin + BSA group). The error bars represent the standard deviation of the values determined from three freshly prepared samples.

consistent when using the freshly prepared samples, yielding relatively small errors for each measurement and averaging less than 2% standard deviation of the total signal (error bar showed in Fig. 5). Adding BSA did introduce a slightly larger error and by lowering the flow rate and increasing the sample loading time, the error can be reduced although deemed not significant enough to be considered. The majority of the error is thought to be caused by temperature variance; thereby placing the sensor in a temperature controlled environment should help increase the accuracy. Recently, aptamer immobilization has been done on a variety of surfaces for sensing purposes such as magnetic nanoparticles [33], gold nanoparticles [34], and carbon nanotubes [35]. Our sensing surface in this study had an optimal dynamic range from 5 nM to 1000 nM, which is comparable to or greater than the largest reported dynamic ranges for thrombin aptamer-based sensing techniques. The thrombin concentration range in the human blood is reported to be within the low nanomolar to low micromolar range [36], suggesting that the reported method could be well suited for potential *in vivo* thrombin quantitative detection.
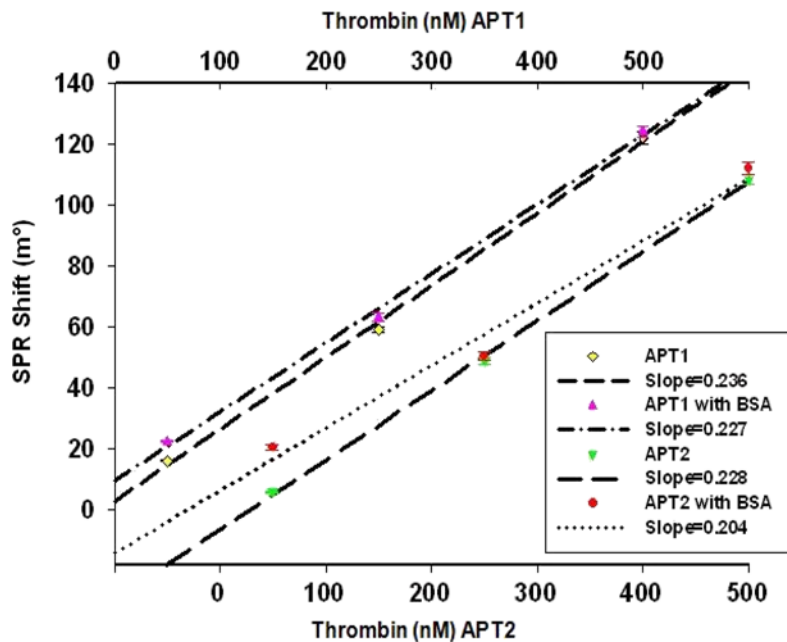

Fig. 6. SPR responses of different sensing surfaces for 50 nM, 250 nM, 500 nM thrombin with and without 400 nM BSA, upper axis (APT1), lower axis (APT2); The zero position of lower axis has been shifted intentionally to better distinguish between data points that would be overlapping.

To test the reversibility of the sensor, fixed sample concentrations were repeatedly loaded to the sensor 10 times. The sensor regeneration was done by PPA, as previously described. The average SPR response with error bars for standard deviation using thrombin concentrations of 50 nM, 250 nM and 500 nM are shown in Fig. 6. All data were obtained from freshly prepared sensing slides. According to our experience, the SPR response would decrease about 0.5% for each loading for a same sample concentration. All the sensing slides maintained more than 95% of the original SPR shift response after the 10th loading. It was also noticed that the second sample loading usually had the greatest response change as compared to the following loadings. We expect that with a longer PPA injection time, the sensor recovery rate can be increased, depending on the experimental requirements. The appearance of BSA did lower the sensitivity of the sensor (e.g. in Fig. 6, the appearance of BSA did reduce the slope slightly in the response curve), although it did not affect the

reversibility of the sensor. Figure 6 demonstrates that sensor maintained a linear response with and without the appearance of BSA in the 50 nM to 500 nM sample range.

3.4. Further optimization and development of the sensor

Better performance of the sensor may be possibly achieved by using a mixed length spacer layer [37], such as 11-mercaptoundecanoic acid (MUA) combined with MPA to increase the sensitivity and specificity [38]. Such a configuration could potentially help form and maintain the specific shape of the immobilized aptamers. Furthermore, inserting a hydrophilic group such as ethylene oxide onto the 5′-end of the aptamer may reduce nonspecific protein binding [27]. However, using our two-step immobilization method, in which a SAM was first immobilized followed by the aptamers, this should be a more cost effective and controllable method compared to adding all of the modifications to the aptamer monomer at once. Consequently, by specifically designing the SAM, e.g. optimizing the hydrocarbon length and introducing co-adsorbents [39], we can still use the simple amine-modified aptamer as the receptor to improve the sensitivity and selectivity.

Since the surface density of immobilized aptamer affects the efficiency of target capture, and the surface coverage was mainly determined by the aptamer immobilization concentrations [29], here we used relatively high aptamer concentrations (i.e. 5 μM) to guarantee the density and the uniformity of the surface. However, closely packed aptamers might prevent the target molecules from approaching their respective binding sites efficiently, and at meantime, they may inhibit themselves from folding into secondary structures. Therefore, by optimizing the aptamer immobilization concentrations, the performance of the sensor may be improved. For the two step immobilization method, spacing the aptamers could be also done by adjusting the MPA SAM density, or by co-incubating ethanolamine and the aptamer at various molar ratios.

For the detection of different blood proteins, the key factor is to find the aptamer that specifically and directly binds to the target protein of interest [40], which can be achieved using a SELEX procedure. Then, the developed aptamer can then be amine-terminated and immobilized onto the gold surface based on the same methods described in this paper to form a target specific sensor for almost any protein.

4. Conclusions

Aptamers can be generated through SELEX to target specific molecules with advantages over antibodies. We have demonstrated a two-step procedure for the immobilization of a SAM and amine-terminated aptamer onto a gold SPR sensing surface. The advantages of the SPR sensor, such as low sample consumption, the lack of labeling requirement, high sensitivity, and fast response have been demonstrated. Also, the advantages of the two-step immobilization method has demonstrable cost efficiency, good reversibility uniform density, and shows promise for the eventual development of a robust and specific blood protein detection platform.

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