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Spectroscopic and Electrochemical Detection of Thrombin/5'-SH or 3'-SH Aptamer Immobilized on (porous) Gold Substrates

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Thrombin is a serine protease that catalyzes the conversion of soluble fibrinogen to insoluble fibrin, and thus induces physiological and pathological blood coagulation. Therefore, it is important to detect thrombin in blood serum for purposes of diagnosis. To achieve this goal, it has been suggested that a 15-mer aptamer strongly binds with thrombin to form a G-quartet structure of the aptamer. Generally, 5'-end thiol-functionalized aptamer has been used as an *anti*-thrombin binder. Herein, we evaluate the possibility of utilizing a 3'-SH aptasensor for thrombin detection using SPR spectroscopy, and compare the enhancement of the electrochemical signal of the thrombin-aptamer bound on a porous gold substrate. Although the two aptamers have similar configurations, in SPR analysis, the 3'-SH aptamer was a effective aptasensor as well as 5'-SH aptamer. Results from electrochemical analysis showed that the porous gold substrate acted as a good substrate for an aptasensor and demonstrated 5-fold enhancement of current change, as compared to gold thin film.

Key Words : Aptamer, Thrombin, SPR sensor, CV, porous gold

Introduction

Thrombin, also known as coagulation factor II, is a multifunctional serine protease that catalyzes the conversion of soluble fibrinogen (factor I) into long, sticky threads of insoluble fibrin (factor Ia).[1] Factor Ia induced by factor II forms a mesh that traps platelets, blood cells, and plasma to enable physiological and pathological blood coagulation.[2] During the coagulation process, the concentration of thrombin in blood varies from nM to μM levels, and thus it is important to detect thrombin in blood serum for clinical and diagnostic applications.[3-7]

The 15-mer ssDNA aptamer, which can strongly and selectively bind with thrombin, was first selected from in vitro studies conducted by Bock and co-workers.[8] This aptamer can form a G-quartet structure to bind the fibrinogen recognition sites on thrombin.[9,10] The 29-mer aptamer, which binds to the heparin binding sites of thrombin, was selected later.[11,12] An aptamer is an artificial oligonucleotide (DNA or RNA) that can bind various targets, such as metal ions, small molecules, proteins, and cells, with high selectivity and specificity.[13] Additionally, aptamers have advantages for immobilization, such as ease of labeling, good stability, simple preparation, and being inexpensive. Therefore, recently, aptamers integrated with well-developed DNA or RNA sensor technologies have been widely used as recyclable aptasensors.

The 15-mer aptamer has been generally used for incorporation into biosensors, and the 5'-end of the aptamer was modified with -SH or -NH_2 groups for immobilization on a sensing substrate, such as gold film. The 5'-SH aptamer based biosensor showed high sensitivity for label-free protein detection of thrombin, hemoglobin, and BSA (bovine serum albumin) by electrochemical analysis.[14] During modification of aptamer ends (5' and 3'), the 3'-end as well as the 5'-end can be modified with thiol groups. It is well-known that 3'-end and 5'-end of aptamer has OH and P group, respectively. Therefore, yield of thiol-functionalization on aptamer ends was not same; yield of 5'-SH aptamer is larger than that of 3'-SH aptamer. In addition, because PCR (polymerase chain reaction) needs bare 3'-end site, 5'-end aptamer was usually used to modify the end-functionalization. Recently, although yield of 3'-SH aptamer is low, both the 3'-SH and the 5'-SH modified 15-mer aptamers were synthesized at Bioneer Inc. (Korea), and we therefore attempted to compare the spectroscopic and electrochemical sensing properties of 3'-SH with those of 5'-SH when used for binding to thrombin.

Various aptasensors based on colorimetry, electrochemistry, surface plasmon resonance (SPR) spectroscopy, fluorescence, and microbalance have been developed for thrombin detection.[8-18] Among these sensing technologies, electrochemical aptasensors have attracted particular attention because they provide a simple, sensitive, and selective platform for thrombin detection. A SPR spectroscopic aptasensor allows use of a microsecond time-resolved change of SPR reflectance.

In this study, we investigated the possibility of using the 3'-SH aptasensor for thrombin detection using SPR spectroscopy, and attempted to compare the enhancement of the electrochemical signal of the thrombin-aptamer when it binds on a porous gold substrate. For the initial application study, 5'-SH and 3'-SH aptamers, designed to specifically bind to the fibrinogen recognition sites of thrombin, were immobilized on the gold thin film of a SPR chip. The reflectance change in SPR signals was easily and directly measured by the refractive index close to the chip surface. In the second application study, an electrochemical analysis for thrombin

detection by a 3'-SH aptamer immobilized on the porous gold substrate was compared to that on a gold thin film. We expected that the porous gold substrate with high a surface area would demonstrate a higher current change, compared to the change shown using the gold thin film.

Experimental

All aptamers, HPLC-purified, were purchased from Bioneer Inc. (Daejeon, Korea) and their sequences are as follows: 5'-SH modified aptamer 5'SH-$(CH_2)_6$-GGTTGGTGTGGTTGG-3', and 3'-SH modified aptamer 5'-GGTTGGTGTGGTTGG-$(CH_2)_3$-SH-3'. For 3'-end aptamer, modification of $(CH_2)_6$-SH was not easy and thus Bioneer Inc. provided 3'-SH-$(CH_2)_3$ modified aptamer. Human thrombin (T6884) was purchased from Sigma-Aldrich and stored in phosphate buffer solution (PBS).

For electrochemical analysis, 5'-SH and 3'-SH modified aptamers (10 μM in PBS buffer) were initially immobilized on the gold thin film of a SPR chip (K-MAC, Korea) based on the covalent chemistry. A block type gold chip was composed of 2 nm Cr as an adhesive layer and a 50 nm Au film was coated on the BaCD4 prism. After 24 h, the SPR chip was sequentially rinsed with PBS, DI water, and ethanol to remove the non-covalently bond aptamer. The 3'-SH aptamer was also immobilized on the porous gold substrate to enhance the electrochemical signal.

Application of free-standing porous gold was followed by templating using aluminum alkoxide, which acted as both a pore generator and a reinforcement agent for the reverse network of porous gold. As described in a previous report,[19,20] porous gold powder was easily obtained and pressed to form a pellet-type electrode. The Immobilization process for the 3'-SH aptamer followed the method used for the SPR chip.

Spectroscopic analysis for thrombin detection was carried out with the SPR spectrometer (SPRLAB^{TM}, K-MAC, Korea). An aptamer solution of 10 μM was injected by syringe pump onto the bare gold chip to immobilize the aptamer on gold film. After washing with PBS buffer, a thrombin solution of 1 μM was injected on the aptamer immobilized gold chip. As shown in Figure 1, the change in the incident angle in response to the addition of aptamer and thrombin was recorded, and the time-resolved reflectance change was also measured at fixed mode. A 3D structure of thrombin (Fig. 1) was obtained from a reference.[21]

Cyclic voltammograms (CVs) for aptamers immobilized on gold film and porous gold substrate were determined with a potentiostate (WEIS-500, WonA Tech, Korea) in 20 mM PBS buffer containing of 5 mM ferricyanide, 100 mM NaCl, and 50 mM KCl. Pt substrate and Ag/AgCl were used as counter and reference electrodes, respectively. After addition of 1 μM thrombin, the change of current at the redox potential point of the CVs was compared with that of bare and aptamer immobilized (porous) gold substrate.

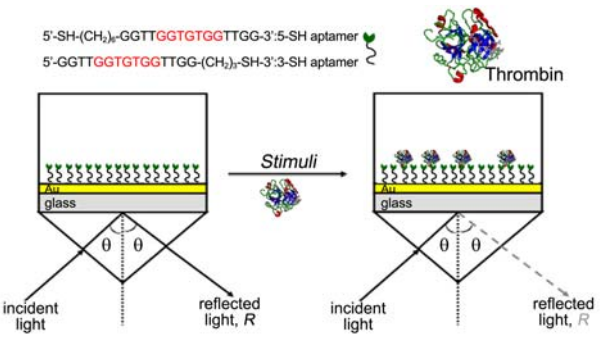

Figure 1. Schematic diagram of SPR sensing for detection of thrombin binding with 3'-SH or 5'-SH aptamers. A 3D structure of thrombin (PDB 2C93) from human plasma was obtained from reference.[21]

Results and Discussion

SPR analysis for thrombin bounded with 5'-SH and 3'-SH aptamers was measured as shown in Figure 2. The SPR angle increased as a function of both the thickness and the dielectric constant of the organic layers (aptamer and thrombin) on the gold film, because a smaller change in refractive index or layer thickness at the sensor surface would cause a clear wavelength shift of the resonant wavelength in SPR-reflected spectra. Angle-resolved SPR contour plots (Fig. 2) for the free gold chip and the thrombin/aptamer/gold film showed that the angle change ($\Delta\theta$) between the substrate and thrombin/aptamer was 0.45 for the 3'-SH aptamer and 0.37 for the 5'-SH aptamer. While the angle change between bare gold and the thrombin/aptamer was large, that between the 3'-SH and the 5'-SH aptamer was small. It was noted that the thickness of the immobilized aptamer (either 3'-SH or 5'-SH aptamer) formed by the self-assembled method was almost the same, and thus the final dielectric constant and thickness of the 3'-SH and 5'-SH aptamers were similar after binding of thrombin.

Because this study was focused on the detection possibility of thrombin/3'-SH aptamer not find the limit of detection (LOD) of thrombin using 3'-SH aptamer, a single concentration of aptamer and thrombin was selected in SPR analysis. Therefore, the measured change in SPR reflectance

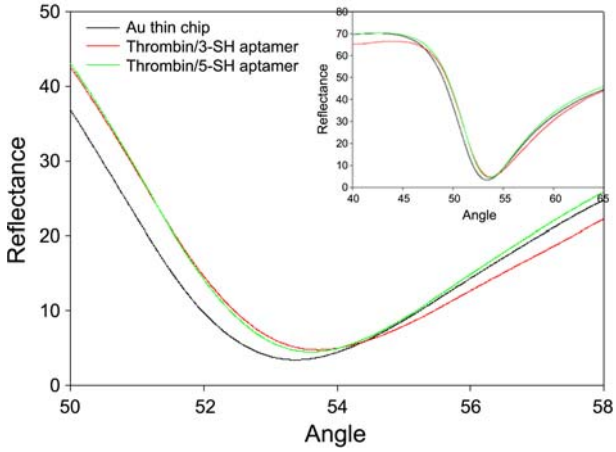

Figure 2. Angle-resolved SPR spectra of 3'-SH and 5'-SH aptamers on Au thin film after binding with thrombin.

(ΔR) was caused by a change in the optical properties of the gold chip resulting from binding between the aptamer and thrombin. As shown in Figure 3, SPR sensograms were obtained by successive addition of aptamers, PBS buffer, and thrombin. When the thrombin solution with successive PBS buffer was applied on the bare gold (inset figure in Fig. 3), ΔR returned to the initial value of SPR reflectance. It was noted that thrombin does not have affinity binding sites for bare gold. After the aptamer was injected twice onto the gold chip, the un-bound aptamer on the gold chip was removed by washing with PBS buffer. For in-situ SPR measurements, aptamer binding on the gold chip was affected by the change in the dielectric constant relative to the refractive index of bare gold. The reflectance change of the 5'-SH aptamer was much larger than that of the 3'-SH aptamer, but the ΔR value for the 5'-SH aptamer was smaller than that for the 3'-SH-aptamer after washing with PBS buffer. Therefore, after 1200 sec, ΔR was changed by *ca.* 3 for the 3'-SH and 4 for the 5'-SH aptamer, respectively. After loading thrombin on both aptamer/gold chips, ΔR was increased due to the change of film thickness caused by binding of thrombin on the aptamer. The change was maintained after PBS washing, because thrombin has a strong affinity for aptamers. Angle-resolve SPR spectra and time-resolve SPR spectra showed

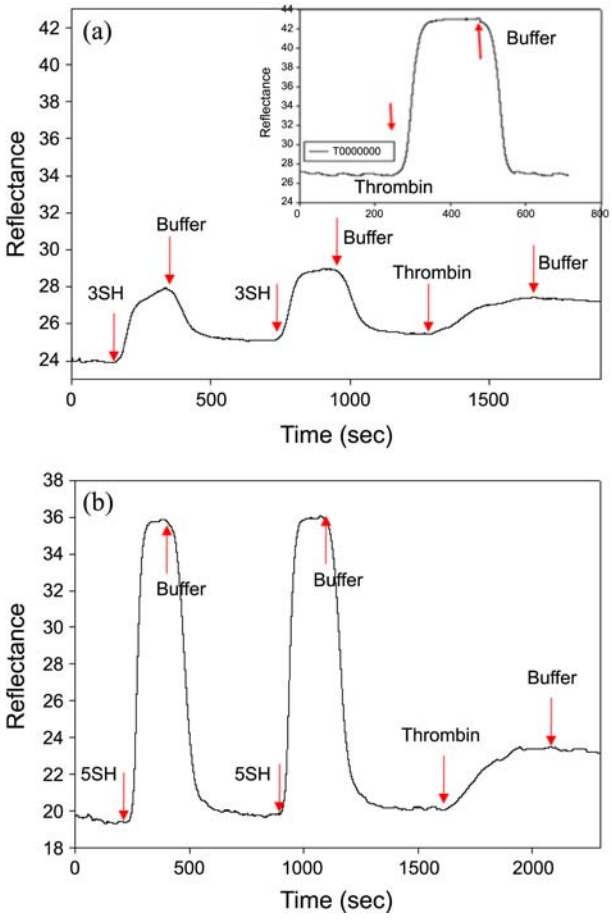

Figure 3. SPR sensorgrams of successive addition of (a) 3'-SH and (b) 5'-SH aptamers at fixed mode. Inset figure in (a) is the change of reflectance with addition of thrombin on bare gold thin film.

the 3'-SH aptamer could be used as a thrombin binder in a manner similar to the 5'-SH aptamer.

It is known that the 15-mer aptamer folds into a chair-form quadruplex with the adjacent 5' and 3' ends in the corner of the quadruplex, and has 2 staked G-quartets linked with TT and TGT loops.[13] Thrombin molecules are thought to bind adjacent to the TT loops. The binding sites of the 15-mer aptamer are known as T_3, T_4, T_{12}, and T_{13}, as shown in Figure 4. Hianik has stated that a single one nucleotide (T_{12}) is responsible for binding to thrombin through Phe245.[22] Thus, the 3'-SH aptamer has an inverse configuration, but the same oligonucleotides sequence as the 5'-SH aptamer on the gold film. Therefore, it may have the same affinity for thrombin, and should reveal a similar angle and reflectance change in SPR analysis. However, small differences were revealed in SPR analysis.

An aptamer generally achieves equilibrium between random (Fig. 4(a)) and quadruplex (Fig. 4(b)) configuratoins when diluted in an aqueous solution. H-bonds are established between quanines, which are responsible for thrombin binding. In the report by Baldrich *et al.*, the authors studied whether a 6C-spacer arm in the aptamer allowed increased flexibility of thrombin to form the 3D quadruplex structure.[23] As expected, addition of a linker correlated with improved target binding, presumably due to the increased spatial differentiation between the aptamer and the gold surface and decreased steric hindrance. These effects resulted in improved folding and thrombin recognition. This finding is in agreement with our observation that the addition of several functional groups to the 5'-end of the *anti*-thrombin aptamer would allow the aptamer to form an alternate conformation that could destroy the G-quartet structure, while addition to the 3'-end improves stability.[23-25]

The 5'-end SH aptamer was obtained mainly by thiol modification of the 15-mer aptamer with 3'- and 5'-ends, and was used as an *anti*-thrombin aptamer. However, based on SPR analysis, the 3'-SH aptamer can bind thrombin to the same degree as the 5'-SH aptamer.

In electrochemical analysis, ferricyanide, $K_3[Fe(CN)_6]$ was used as a redox probe. The thrombin bound aptamer blocked electron transfer of $Fe(CN)_6^{3-}$ solution to the gold surface,[14,16]

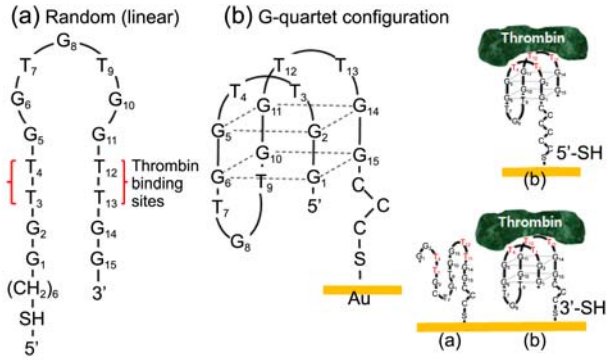

Figure 4. 2D structure of 5'-SH aptamer and 3D binding structure of thrombin/3'-SH aptamer/Au film. Configuration variation for (a) random and (b) G-quartet structure.

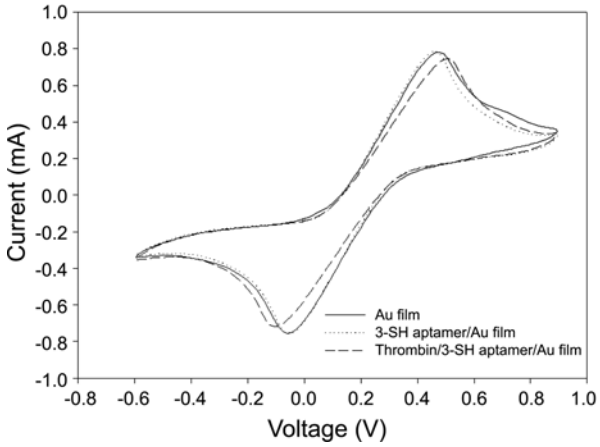

Figure 5. Cyclic voltammograms (CVs) for bare gold film, 3'-SH aptamer, and thrombin linked aptamer in phosphate buffer solution containing 5 mM ferricyanide, 100 mM NaCl, and 50 mM KCl.

thus the affinities of thrombin and the 3'-SH aptamer could be measured by electrochemical analysis. As shown in cyclic voltammograms (Fig. 5), with successive loadings of thrombin and aptamer on the gold substrate, the current values at redox potentials of *ca.* −0.1 and +0.5 V were decreased. The current value of the 3'-SH aptamer on gold film was similar to that of the 5'-SH aptamer (Fig. 6). To enhance the current change for the 3'-SH aptamer, a porous gold substrate was selected instead of a gold film. Porous gold (PAu) showed a window pore size of 200-400 nm and a framework thickness of 100-300 nm.[19] PAu has many window pores formed due to the overlapping of the branched gold networks, which is the foundation of the interconnected pore system. Formation of submicron-sized window pores was induced by the removal of the alumina framework during the etching step. Therefore, we expected to improve the signal enhancement, as compared to gold thin film. As shown in Figure 6, the current value of the 3'-SH aptamer on porous gold was 5-fold higher than that on gold thin film. The sensitivities of the current intensity, $(I_o\text{-}I)/I_o$, where I_o is the current value for bare gold, for 3'-SH and 5'-SH on gold thin film were 4.23 and 4.60, respectively. The sensitivity for the 3'-SH aptamer on porous gold was 4.34, indicating that the

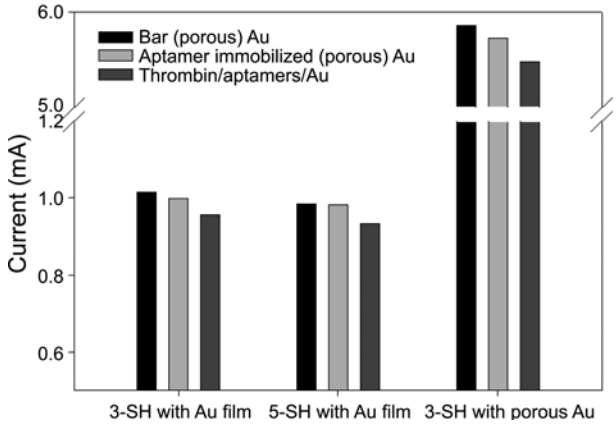

Figure 6. Current change at redox potential point of CVs for 5'-SH or 3'-SH aptamer-thrombin (porous) gold substrate.

sensitivity did not change with the type of gold substrate.

Although functionalization yield to obtain 3'-SH aptamer is lower as compared to 5'-SH aptamer, their sensing properties is almost same or higher than 5'-SH aptamer. Therefore, 3'-SH aptamer could be used as *anti*-thrombin aptasensor.

Conclusion

Herein, we evaluated whether a 3'-SH aptamer has same affinity for thrombin as a 5'-SH aptamer. Thiol functionalized aptamers were easily immobilized on gold substrate, and for binding with thrombin, the 15-mer aptamer folded into a chair-form quadruplex with the adjacent 5' and 3' ends in the corner of the quadruplex, and 2 staked G-quartets were linked with TT and TGT loops. Therefore, the two aptamers should have similar affinities for thrombin; and in SPR analysis, both the 3'-SH aptamer and the 5'-SH aptamer acted as effective aptasensors. In electrochemical analysis, both aptamers demonstrated similar current changes. Specifically, although the configuration of the 3'-SH aptamer was similar to that of the 5'-SH aptamer, the addition of several functional groups to the 5'-end of the *anti*-thrombin aptamer would allow for suppressing the formation of the G-quartet structure. To enhance the current signal in CVs, porous gold substrate, which has a high pore size and surface area compared to gold thin film, was introduced and showed a 5-fold larger current signal without change of sensitivity. Further studies should be conducted to investigate the LOD when using porous gold to develop highly sensitive aptasensors.

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