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Development of a novel aptamer-based sensing system using atomic force microscopy

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Atomic force microscopy (AFM) can dynamically detect the adhesion or affinity force between a sample surface and a cantilever. This feature is useful as a detection method using aptamers — single-strand DNA that recognizes its target with very high affinity. The present study proposes a novel DNA aptamer-based sensing system using AFM. In this study, thrombin was chosen as the target molecule, and a DNA aptamer-based AFM sensing system based on competition was developed. The affinity force between the gold chip and the cantilever decreased as the concentration of thrombin increased. Moreover, a low detection limit of 0.2 nM was achieved. Therefore, the AFM sensing system used would be appropriate for the measurement of various chemical compounds.

[**Key words:** DNA aptamer; Atomic force microscopy; Molecular interaction; Force curve measurement; Competitive assay]

Aptamers are rare functional nucleic acid motifs derived from libraries of nucleic acids by iterative rounds of selection and amplification, using a process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (1–5). They have been applied in biochemical analysis as alternative recognition elements due to their relatively low cost, high binding affinity and good stability (6). DNA aptamers against proteins such as thrombin and low-molecular compounds such as ethanolamine and ATP have been previously reported (7–10). Many aptamer-based protein biosensors have been developed using electrochemical, fluorescent, colorimetric, and other methods (11,12). Most of these methods require time-consuming steps for labeling analysis. Hence, a biosensor fabrication strategy that avoids complicated labeling steps is desirable. Herein, therefore, is the proposal for a new aptamer-based sensing system using atomic force microscopy (AFM).

AFM scans an imperceptible sample surface using a probe called a cantilever, detecting the weak force between the sample surface and the probe, and makes a topographic image of the sample surface. AFM can also dynamically detect the adhesion or affinity force between the sample surface and the cantilever. This notable feature is useful for measurement of the affinity force of biomolecular interactions using the sample and biomolecules immobilized on the cantilever (13–16). It is thought that this system may be used as a biosensing system using DNA aptamers.

The aptamer-based AFM sensing system works in a competitive manner (Fig. 1). First, a DNA aptamer is immobilized on a gold chip surface. The target molecules are added to the DNA aptamer immobilized on the chip for a reaction between the DNA aptamer and the target molecules. After the reaction, the gold chip is applied to AFM with the target molecules on a modified cantilever. Since DNA aptamers that are already bound with target molecules are not able to bind to the target on the cantilever, it is assumed that the lower affinity force between the gold chip and the cantilever is due to the higher thrombin concentration added to the gold chip. For the present study, as a model of the AFM sensing strategy, thrombin was chosen as the target molecule of the DNA aptamer.

MATERIALS AND METHODS

Materials Thrombin from human plasma (T6884) and streptavidin from *Streptomyces avidinii* were purchased from Sigma (MO, USA). The 3,3′-Dithiobis [sulfosuccinimidylpropionate] (DTSSP) was purchased from Pierce (MO, USA). The other chemicals used were analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan). The sequence of thrombin binding aptamers and control DNA were as follows: TBA1: 5′-biotin-GGTTGGTGTGGTTGG-3′ (9), TBA2: 5′-biotin-AGTCCGTGG-TAGGGCAGGTGGGGGTGACT-3′ (10), control DNA: 5′-biotin-CAGCTCAGAAGCTT-GATCCTGTG-3′.

Preparation of the thrombin-modified cantilever To remove the organic compounds that adhered to the cantilever, it was treated with ultraviolet (UV) light for 2 h. The probe was then exposed to 100 μl of 4 mg/ml DTSSP solution in 20 mM acetate (pH 4.8) at room temperature for 30 min. After the reaction, the probe was dipped in 20 ml of ultrapure water to remove unreacted DTSSP. The succinimide-immobilized probe was then doused at room temperature for 1 h with 100 μl of 0.02–200 nM thrombin solution in PBS (100 mM phosphate, 150 mM NaCl, pH 7.5). After immobilization of thrombin, the cantilever was dipped in 100 μl of 1 mg/ml bovine serum albumin (BSA) solution in TBA buffer (20 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, 1 mM CaCl, 1 mM $MgCl_2$, pH 7.4) for blocking the unreacted succinimide and activated gold surface, followed by washing with 20 ml of washing buffer (50 mM Tris–HCl, 300 mM NaCl, 30 mM KCl, 5 mM $MgCl_2$, pH 7.6).

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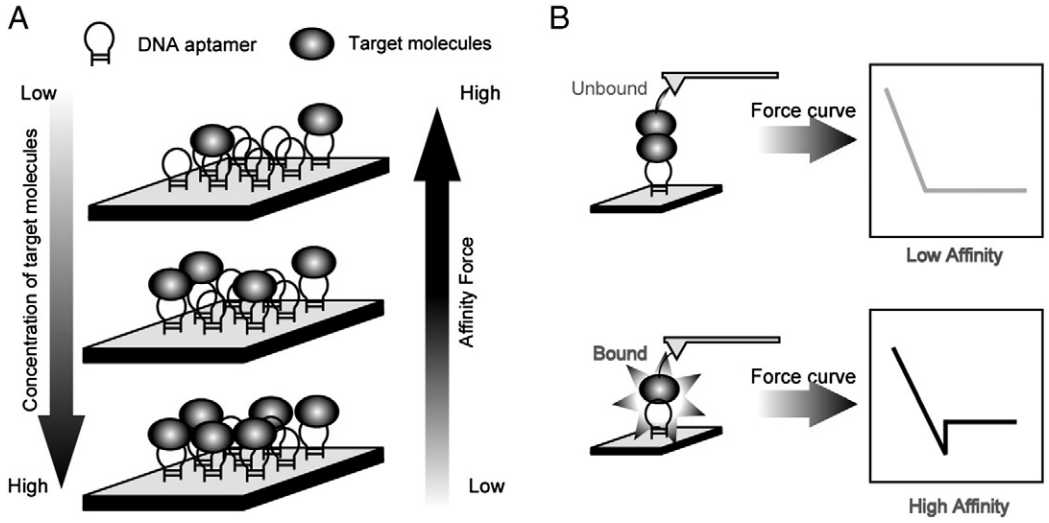

FIG. 1. The schema of aptamer-based AFM sensing based on competition. (A) The scheme of aptamer immobilized gold chip surface. The higher the thrombin concentration added gold chip becomes the less the aptamer that binds to thrombin modified on cantilever. (B) The force measurement between gold chip surface and cantilever. The high affinity force between aptamer that is not binding to thrombin and thrombin on cantilever is analyzed and the low affinity force between aptamer that binds to thrombin and thrombin on cantilever is measured.

Preparation of a gold chip modified by a thrombin-binding aptamer A gold chip was covered with 200 μl of 4 mg/ml DTSSP solution in 20 mM acetate at room temperature for 30 min. The chip was then washed with 20 ml of ultrapure water. After washing, the succinimide-immobilized gold chip was covered with 100 μl of 1 mg/ml streptavidin solution in PBS at room temperature for 1 h. Finally, the gold chip was washed with 20 ml of washing buffer. After the immobilization of streptavidin on the gold chip, 100 μl of 1 mg/ml BSA was dropped onto gold chip for blocking activated gold surface. 100 μl of 10 μM biotinated thrombin-binding aptamer was dropped onto the gold chip and incubated at room temperature for 1 h. Finally, the ssDNA-immobilized gold chip was put in 20 ml of washing buffer containing 0.01% Tween 20 to remove unbound biotinated ssDNA.

Measurement of thrombin concentration by AFM Each different concentration of thrombin solution in 200 μl TBA buffer was dropped onto the TBA-immobilized gold chip and incubated for 1 h at room temperature. After the reaction, the gold chip was washed with washing buffer containing 0.01% Tween 20 to remove the unreacted thrombin. Force curve mapping was then performed with the cantilever and gold chip described above in the liquid cell of a SPA400-Nanonavi AFM unit (SII Nanotechnology Inc., Chiba, Japan). The force curve measurements were performed in TBA buffer. Force curves were recorded at a velocity of 17 μm/s. Topographic images were captured at a resolution of 64×64 pixels with a scan size of 1 μm×1 μm. The adhesion forces between TBA and thrombin were analyzed from 4096 data points of the force curves for promoting the correctness and repeatability, and a histogram of adhesion force was charted.

RESULTS

Affinity assay of TBA 1 and TBA 2 using AFM The affinity of TBA 1 and TBA 2 to thrombin was compared to determine which aptamer was more appropriate as a sensing device. The affinity forces of both aptamers that bind to thrombin were stronger than those of the control DNA. Therefore, both TBA 1 and TBA 2 had an affinity to thrombin (Fig. 2). Moreover, the affinity force of TBA 2 to thrombin was stronger than that of TBA 1, so TBA 2 was used as the sensing device in the present study.

Optimization of thrombin concentration when modified on a cantilever An investigation into the optimal concentration of immobilized thrombin for its high-sensitivity detection was performed. Different immobilized concentrations of thrombin, ranging from 0.02 nM to 200 nM, were prepared on the cantilever. The result was that the average affinity force between thrombin and the aptamer gradually decreased with decreasing concentration of thrombin (Fig. 3). The adhesion force between BSA and TBA was measured as a control. The adhesion force of BSA to TBA 2 was less than that of 0.02 nM thrombin when modified to TBA 2 (Fig. 3).

The measurement of affinity force between thrombin aptamers and free thrombin by AFM The affinity force between thrombin and TBA at various thrombin concentrations was measured by AFM. Thrombin (200 pmol) was immobilized on a cantilever. Different concentrations of thrombin, from 0 nM to 2000 nM, were added to a TBA 2-immobilized gold chip. The result was that the average affinity force between the thrombin-modified cantilever and the TBA-immobilized gold chip decreased as the concentration of added thrombin solution decreased (Fig. 4).

DISCUSSION

The self-assembled monolayer (SAM) method is useful for the immobilization of biomolecules on gold chips. However, the gold surface used in the present study had 5–10 nm of roughness. It is possible that the immobilized DNA was hidden by the roughness of the gold surface. Therefore, to prevent the DNA from being hidden in the roughness of the gold, it was immobilized on the gold chip with the streptavidin–biotin interaction system.

In this strategy, the detection sensitivity is dependent on the binding affinity of the DNA aptamer to thrombin. Therefore, the affinity force of TBA1 and TBA2 to thrombin was compared to decide which one was appropriate as a sensing device. It is common knowledge that TBA2 has higher affinity than TBA1. However, it has been pointed out that it is important to destroy the bond between aptamers and target analyzed by AFM is depending on the number of hydrogen-bond which constructs the structure of DNA aptamer (13). Therefore, the binding force of TBA1 and 2 to thrombin was confirmed using AFM respectively. The results showed that the force of TBA2 affinity to thrombin was higher than that of TBA1 (Fig. 2). The dissociation constant of TBA2 to thrombin was previously reported as 0.5 nM (9). However, the dissociation constant of TBA1 to thrombin was 200 nM (10). The affinity force between the DNA aptamer and thrombin is believed to be reflected in these dissociation constants. Moreover, the number of bases of TBA1 and TBA2 was 15 mer and 29 mer, respectively. The structure of TBA2 had more hydroxyl bonds than the structure of TBA1. Therefore, the structure of TBA2 appeared to be tighter than that of TBA1. Considering the degree of affinity to thrombin, TBA2 would be more suitable as a biosensing device than TBA1. TBA2 was, therefore, chosen as the sensing device for the AFM sensing system.