

## Research Article

# Direct Detection of Thrombin Binding to 8-Bromodeoxyguanosine-Modified Aptamer: Effects of Modification on Affinity and Kinetics

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The affinity of an 8-bromodeoxyguanosine- (8-BrdG-) substituted thrombin-binding aptamer (TBA-Br), which has the 1st and 10th guanosine residues replaced with 8-BrdG, was estimated using reflectometric interference spectroscopy (RIfS). When comparing TBA-Br with unmodified TBA (TBA-H), it was demonstrated that the modification effectively improved the affinity of TBA; dissociation constants ( $K_D$ ) of TBA-H and TBA-Br were 45.4 nM and 1.99 nM, respectively. These values, which were obtained by direct observation of thrombin binding using RIfS, have the same order of magnitude as those obtained in our previous study utilizing conformational changes in TBA to detect thrombin binding, thus confirming the validity of the obtained  $K_D$  values. RIfS measurements also revealed that the 8-BrdG modification resulted in a lower dissociation rate constant ( $k_d$ ), which suggests that the enhancement of affinity can be attributed to the stabilization of the G-quadruplex structure on introduction of 8-BrdG.

## 1. Introduction

Chemical modification of nucleic acids is a useful approach to improve the stability of higher-order structures [1–5]. Thrombin-binding aptamer (TBA), d(GGTTGGTGTGGT-TGG), is a nucleic acid for which modification has attracted considerable attention, as the improved stability may lead to enhanced affinity for the target species, thrombin, which is potentially useful for the diagnosis and treatment of various conditions. Because the relationship between higher-order structure and affinity is well known, its modification has been widely studied. TBA is known to fold into a G-quadruplex structure consisting of two G-planes connected via a TGT loop and two TT loops, as shown in Figure 1(a) [6–11].

Strategies for modifying TBA can be classified into two categories: modification of the nucleotide backbone such as Locked Nucleic Acid (LNA) [12, 13] or 2'-fluoro-ara-binonucleic acid (2'-F-ANA) [14] and modification of nucleobases, such as alkylation or phenylation [15]. Both strategies have been shown to be effective for stabilizing higher-order

structure by stabilizing specific glycosidic bond conformations in the G-plane.

We recently demonstrated that the higher-order structure of TBA can be stabilized by introducing 8-bromodeoxyguanosine (8-BrdG, Figure 1(b)), which stabilizes a *syn* conformation of a glycosidic bond by steric hindrance between the bromo group at the C8 position and the deoxyribose moiety [16–18]. When two 8-BrdG residues were introduced in place of the 1st and 10th guanosine residues, which have a *syn* conformation, the higher-order structure of TBA was significantly stabilized, with an increase in melting point of 15.8°C, and the affinity for thrombin showed a 12.5-fold greater binding constant [19]. In a previous study, however, the binding of thrombin to TBA was detected indirectly; conformational transition of TBA from a single strand to a G-quadruplex, which is induced by the binding of thrombin, was monitored using a fluorescent dye. Therefore, direct measurement of the thrombin-binding phenomenon has been required in order to verify the estimated binding constant values and the effects of 8-BrdG on enhancing the affinity of TBA for thrombin. Furthermore, the practical

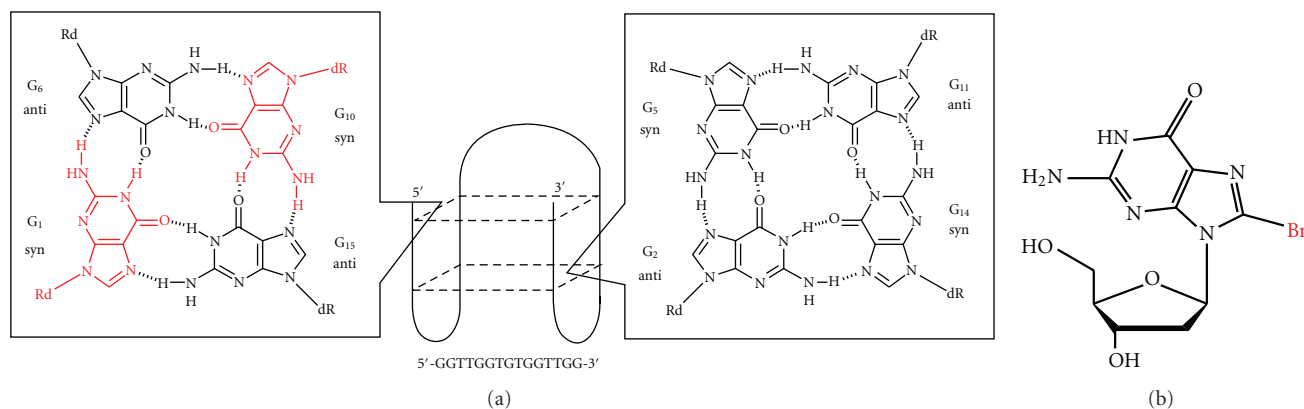


FIGURE 1: Structure of (a) G-quadruplex of thrombin binding aptamer and (b) 8-bromodeoxyguanosine. Modification sites, G1 and G10, are indicated in red.

usefulness of the 8-BrdG-modified aptamer has not been investigated.

In this study, we performed direct observation of thrombin binding to an 8-BrdG-modified TBA (TBA-Br) using reflectometric interference spectroscopy (RiFS), which can measure changes in optical thickness of a molecular layer on a sensor chip and is, therefore, expected to detect thrombin binding to the modified TBA immobilized on a chip [20–23] in order to assess the binding constants of TBA-Br and the effectiveness of TBA-Br for developing a sensitive TBA sensor.

## 2. Materials and Methods

**2.1. Materials.** Human  $\alpha$ -thrombin was purchased from Haematologic Technologies Inc. (Essex, VT). *N*-(6-Maleimidocaproyloxy)sulfosuccinimide sodium salt (Sulfo-EMCS) and 6-Hydroxy-1-hexanethiol (HTT) were purchased from Dojindo (Kumamoto, Japan). 3-Aminopropyltriethoxysilane (APTS) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All DNA amidites and 5'-thiol modifier C6 were purchased from Glen Research Corp. (Sterling, VA). 5'-Thiolated unmodified thrombin-binding aptamer (TBA-H) was obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The RiFS sensor MI-affinity LCR-01 and its consumable supplies (SiN sensor chips and PDMS flow cells) were purchased from Konica Minolta Opto, Inc. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical (Tokyo, Japan).

Base sequences of aptamers were as follows.

TBA-H:



TBA-Br:



**2.2. DNA Synthesis.** TBA-Br was synthesized using an AB 3400 DNA synthesizer (Applied Biosystems Inc., Tokyo,

Japan). Synthesized oligonucleotides were purified by 20% denaturing PAGE. Concentration of single-stranded oligonucleotides was determined by measuring the absorbance at 260 nm at a high temperature using a UV-1700 spectrometer connected with TMSPC-8 thermoprogrammer (Shimadzu Co., Ltd Kyoto, Japan). Oligonucleotides were treated by dithiothreitol prior to use.

**2.3. Chip Preparation.** The SiN Chip surface was irradiated with UV under vacuum for 1 h using UER20-172VB (Ushio Inc., Tokyo, Japan). UV-treated chips were soaked in acetone containing 1% APTS for 1 h, and after washing with acetone, chips were heated at 110°C for 30 min. Thrombin-binding aptamers were immobilized *in situ* using RiFS by injecting 1 mM Sulfo-EMCS, 50  $\mu\text{M}$  aptamer, and 5 mM HTT in PBS buffer at 30°C. An Econoflo Syringe Pump (Harvard Apparatus, Holliston, Mass, USA) was used to move PBS buffer at a flow rate of 2.0  $\mu\text{L min}^{-1}$ . Obtained sensor chips were stored in PBS buffer at 4°C.

**2.4. RiFS Measurements.** A RiFS sensor (MI-affinity LCR-01) was used for direct monitoring of the association/dissociation process. All measurements were conducted using binding buffer (20 mM Tris buffer, pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , and 1% PEG at 25°C and at a flow rate of 20  $\mu\text{L min}^{-1}$ . Kinetic analysis was carried out using software associated with the MI-affinity LCR-01. Triplicate measurements were conducted, and average data are shown in Table 1.

## 3. Results and Discussion

**3.1. Chip Preparation.** Sensor chips were prepared *in situ* using RiFS (Figure 2). A hetero bifunctional cross-linker, Sulfo-EMCS, which bears a maleimide group and succinimide group at each end, was used to immobilize a thiol-terminated aptamer onto an aminated SiN chip. HTT was used to cap the unreacted maleimide termini of the immobilized Sulfo-EMCS moieties. After injection of aptamer for immobilization, an approximately 1.2-nm shift in wavelength was

TABLE 1: Binding properties of each TBA.

	$k_a$ $M^{-1} s^{-1}$	$k_d$ $s^{-1}$	$K_D$ nM
Nonmodified TBA	$7.45 \pm 0.20$ $\times 10^4$	$3.38 \pm 0.33$ $\times 10^{-3}$	$45.4 \pm 5.6$
8-BrdG substituted TBA	$1.58 \pm 0.10$ $\times 10^5$	$3.15 \pm 0.46$ $\times 10^{-4}$	$1.99 \pm 0.29$

observed with both the unmodified (TBA-H) and modified aptamers (TBA-Br), thus confirming the same degree of immobilization for both aptamers. TBA-Br possesses two 8-BrdG residues at the 1st and 10th guanosine sites, and based on our previous study, is optimized in terms of the number and position of the 8-BrdG groups [19].

**3.2. Kinetic Measurement.** The sensor chips immobilized with TBA-H and TBA-Br were assessed by injection of 100 nM thrombin. The response was reproducible with variations of less than 8.7%. When compared to the TBA-H-immobilized chip (Figure 3(a)), the TBA-Br-immobilized chip showed a larger response (Figure 3(b)). These results suggest that TBA-Br has a higher affinity for thrombin than the TBA-H and accordingly captured a larger number of thrombin molecules.

Using the sensorgrams obtained on injection of 100 nM thrombin, kinetic analysis was performed (Table 1). TBA-Br showed a larger association rate constant ( $k_a$ ) ( $1.58 \times 10^5 M^{-1} s^{-1}$ ) than TBA-H ( $7.45 \times 10^4 M^{-1} s^{-1}$ ). However, the effect on dissociation rate constant was more significant; TBA-Br showed a dissociation rate constant an order of magnitude lower ( $k_d = 3.15 \times 10^{-4} s^{-1}$ ) than TBA-H ( $k_d = 3.38 \times 10^{-3} s^{-1}$ ). Because thrombin binding with TBA is accompanied by G-quadruplex formation of TBA, the decrease in dissociation rate constant may be attributed to the stabilization of G-quadruplex structure of TBA (TBA-Br) due to the introduction of 8-BrdG; the  $T_m$  value for TBA-H was 50.7°C while that for TBA-Br was 66.5°C [19].

Dissociation constants ( $K_D$ ) for TBA-H and TBA-Br were 45.4 nM and 1.99 nM, respectively. These values are within the same order of magnitude as those obtained by our previous study using a fluorescent dye [19]. It is also notable that the dissociation constant for TBA-H reported elsewhere is between 20 and 102.6 nM, which was obtained by other detection methods, such as SPR [24, 25], QCM [26], ITC [27], and an electrochemical sensor [28], thus supporting the suitability of the applied conditions and estimation protocol. Direct detection of binding between TBA and thrombin appeared to be successful using RIFs, and the enhancement of affinity by introducing 8-BrdG to TBA was confirmed. Interestingly, the dissociation constant ( $K_D$ ) for TBA-Br estimated by RIFs was slightly lower than that obtained using the fluorescent dye [19], whereas the dissociation constant ( $K_D$ ) for TBA-H estimated by RIFs was larger than that obtained using the fluorescent dye. Although the cause of these discrepancies is not currently understood, it is possible that even in the absence of thrombin, TBA-Br forms a G-quadruplex in the binding buffer at the temperature used

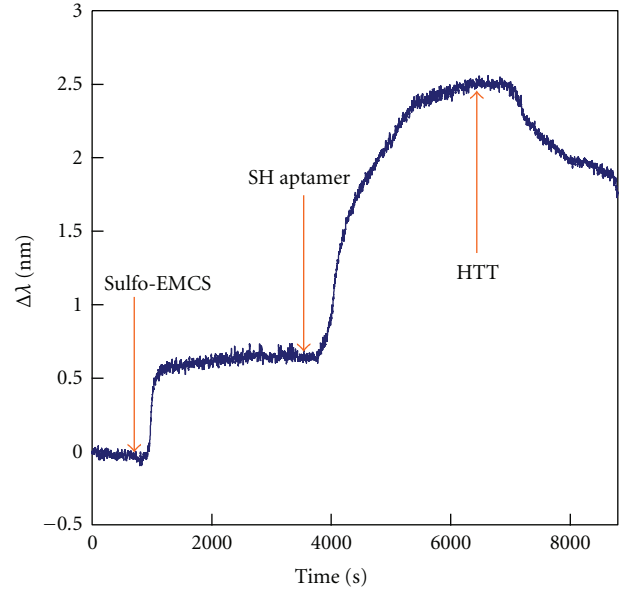


FIGURE 2: *In situ* chip preparation process monitored by reflectometric interference spectroscopy (RIFs). Chips were prepared in PBS at 30°C. Flow rate was  $2.0 \mu L \text{ min}^{-1}$ .

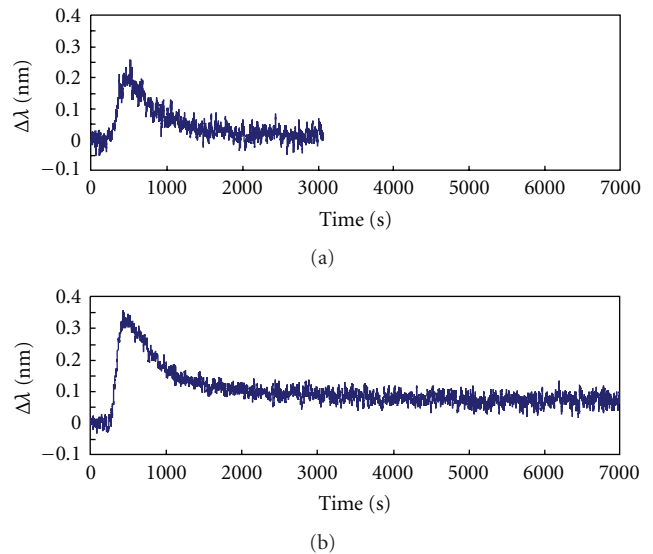


FIGURE 3: Sensorgrams on injection of 100 nM thrombin with (a) TBA-H-immobilized chip and (b) TBA-Br-immobilized chip. Measurements were conducted in binding buffer at 25°C. Flow rate was  $20 \mu L \text{ min}^{-1}$ .

for RIFs measurements due to the improved stability, which would be favorable for binding with thrombin as a result of preorganization effects.

**3.3. Effects of Aptamer Modification on Sensitivity.** Sensors that sensitively detect thrombin are potentially useful for diagnosis of thrombosis. The TBA-Br-immobilized chip is expected to offer improved sensitivity due to its high affinity for thrombin. Thus, we investigated the sensitivity of the RIFs

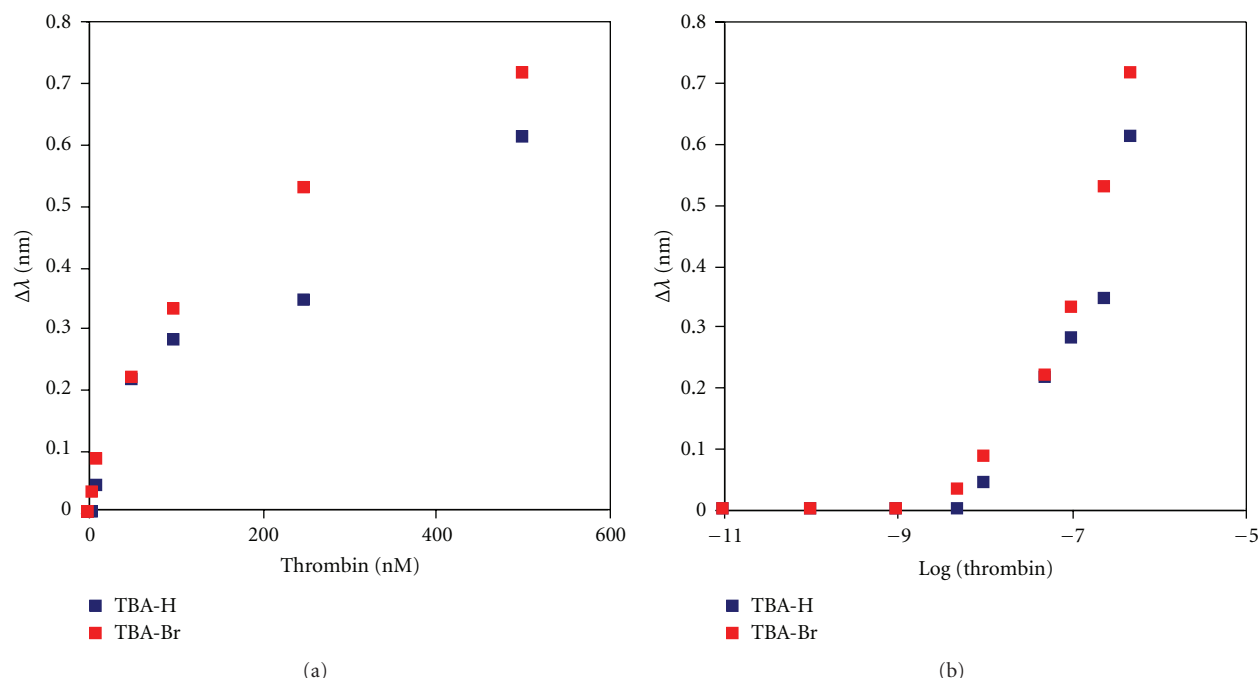


FIGURE 4: Calibration curves for thrombin on TBA-H-immobilized chip and TBA-Br-immobilized chip. (a) Change in wavelength versus thrombin concentration. (b) Change in wavelength versus log (thrombin concentration). Measurements were conducted in binding buffer at 25°C. Flow rate was 20  $\mu\text{L min}^{-1}$ .

sensor with an TBA-Br-immobilized chip. Responses to various concentrations of thrombin (from 0.01 nM to 500 nM) are plotted in Figure 4. The TBA-Br-immobilized chip showed a significant response to 1 nM thrombin with variations of less than 5%, whereas the detection limit of the TBA-H-immobilized chip was higher than 5 nM. Although the degree was moderate, it confirms that modification of TBA to improve affinity can contribute to enhancing thrombin-sensor sensitivity.

#### 4. Conclusions

In this study, we directly observed the association between TBA-Br and thrombin by RfS in order to estimate the kinetic rate and affinity constants. The estimated dissociation constants had the same order of magnitude as those obtained in a previous study using a fluorescent dye, thus suggesting that modification with 8-BrdG at appropriate residues in the G-plane increases the affinity of TBA. In addition, the decrease observed in dissociation rate constant by introduction of 8-BrdG suggests that affinity enhancement is due to the effect of 8-BrdG in stabilizing the G-quadruplex structure of TBA. A preliminary test on the sensitivity of TBA-immobilized sensor chips also demonstrated that modification to improve affinity resulted in a better detection limit although the effect is currently moderate.

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