Electronic Supplementary Information (ESI)

Enzyme-Free Fluorescent-Amplified Aptasensors Based on Target-Responsive DNA Strand Displacement via Toehold-Mediated Click Chemical Ligation

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Chemicals and Instruments:

Trizma-pH 8.3 (Sigma-Aldrich, USA), phosphate buffer powder-pH 7.4 (Wako, Japan), sodium chloride (NaCl: Wako, Japan), potassium chloride (KCl: Wako, Japan), magnesium chloride hexahydrate ($MgCl_2$•$6H_2O$: Wako, Japan), adenosine (Wako, Japan), cytidine (Wako, Japan), uridine (Wako, Japan), guanosine (Wako, Japan), human thrombin (Sigma-Aldrich, USA), human serum albumin (Wako, Japan), human factor IX (Haematologic Technologies Inc., USA), human factor Xa (Haematologic Technologies Inc., USA), acrylamide solution (Sigma-Aldrich, USA), 3-azide-1-propylamine (Bioconjugate Technology Co., USA), 2D-Silver Stain Reagent II (COSMO BIO, Japan), urea (Wako, Japan), and 5×TBE buffer (Nippon Gene, Japan) were used without further purification. Water was purified using the Milli-Q system (Millipore, USA). Oligonucleotides were purchased from Tsukuba Oligo Service, Co., Japan, and the purity and identity of oligonucleotides were confirmed by RP-HPLC and denaturing urea polyacrylamide gel electrophoresis. The DNA sequences employed are shown in Table S1. UV/vis and fluorescence spectra were recorded using a UV-2550 spectrometer (Shimadzu, Japan) equipped with a T_m analysis system TMSPC-8 and F-7000 spectrometer (Hitachi, Japan) equipped with an aqueous thermostat, respectively. To assist in the design of the DNA sequences, IDT SciTools provided free of charge on the web by Integrated DNA Technologies, Inc. (Coralville, IA) was used.

T_m Measurements:

Before T_m measurements, adenosine aptamer/ligated -DNA duplex was prepared as follows. 20 mM Tris-HCl buffer solutions (pH 8.3, 100 mM NaCl, and 5 mM $MgCl_2$) of adenosine aptamer (10 μL, 25 μM), N_3-DNA (10 μL, 25 μM), and DBCO-DNA (10 μL, 25 μM), and 20 mM Tris-HCl buffer (20 μL) were mixed, and the resulting mixture was annealed at 95 °C for 5 min, and allowed to cool to room temperature for 1 h. To confirm the formation of the ligated DNA, 7.5 μL of the mixture was added to 1×TBE buffer with 20 M urea (7.5 μL), and the resulting solution was heated at 90 °C for 5 min. 10 μL of sample was used for denaturing urea polyacrylamide gel electrophoresis assay. Five samples (adenosine aptamer/capture-DNA, adenosine aptamer/ DBCO-DNA, adenosine aptamer/N_3-DNA, adenosine aptamer/ligated DNA with triazole linkage, and adenosine aptamer/control DNA duplexes) were adjusted to 1.25 μM of each DNA duplex. T_m measurement was carried out with 100 μL of samples in 20 mM Tris-HCl buffer pH 8.3 containing 100 mM NaCl and 5 mM $MgCl_2$ using a UV/vis spectrometer equipped with a T_m analysis system. T_m analysis system was programmed to collect data every 0.5°C, and maintained a rate of 1.0 °C/min through the melting curve (Fig. S1). Data was analyzed using T_m analysis software to obtain the T_m.

Fluorescent Measurements:

The instrument settings were chosen as follows: λ_{ex} = 480 nm (slit 5 nm), emission spectra = 490 ~ 600 nm (slit 5 nm), and PMT detector voltage = 700 V. The fluorescence intensity at 520 nm was used to evaluate the performance of the proposed strategy. 20 mM Tris-HCl buffer solutions (pH 8.3, 100 mM NaCl, and 5 mM $MgCl_2$) of aptamer (150 μL, 25 μM), capture-DNA (225 μL, 25 μM), DBCO-DNA (150 μL, 25 μM), and 20 mM Tris-HCl buffer (975 μL) were mixed to form the aptamer/DBCO-DNA/capture-DNA duplex. The resulting mixture was annealed at 95 °C for 5 min, and allowed to cool to room temperature for 1 h. The solution of aptamer/DBCO-DNA/capture-DNA duplex was kept as a stock solution at 4 °C. Typical experiments were performed as follows. 20 mM Tris-HCl buffer solutions (pH 8.3, 100 mM NaCl, and 5 mM $MgCl_2$) of aptamer/DBCO-DNA/capture-DNA duplex (20 μL, [aptamer] = [DBCO-DNA] = 2.5 μM and [capture-DNA] = 3.75 μM), N_3-DNA (20 μL, 2.5 μM), and adenosine (50 μL, 10 mM), and 20 mM Tris-HCl buffer (410 μL) were mixed, and the resulting 500 μL of reaction solution contained DNA-aptamer (100 nM), capture-DNA (150 nM), DBCO-DNA (100 nM), N_3-DNA (100 nM), and adenosine (1000 μM). For detection of thrombin, 20 mM phosphate buffer pH 7.4 containing 40 mM KCl was used. The varying concentrations of adenosine or thrombin and incubated at 25 °C for 120 min to monitor the fluorescence change. The LODs given in the manuscript are corresponding to the concentrations within the final reaction mixtures.

Denaturing Urea Polyacrylamide Gel Electrophoresis:

DNAs without FAM and BHQ1 were used in this experiment. The experiment was performed in 50 μL reaction solution which contained 20 mM Tris-HCl buffer (pH 8.3, 100 mM NaCl and 5 mM $MgCl_2$), aptamer (5 μM), capture-DNA (7.5 μM), DBCO-DNA (5 μM), N_3-DNA (5 μM), and adenosine (2 mM). After incubation at 25 °C for 10 min, 25 μL of in 1×TBE buffer with 20 M urea and 300 mM 3-azide-1-propylamine was added to quench the click chemical ligation, and the resulting each sample was heated at 90 °C for 5 min. 10 μL of each sample was applied to 10 % poly(acrylamide) gel (acrylamide : bisacrylamide = 19 : 1) with 7 M of urea, and the electrophoresis was carried out at 100 V for 25 min in 1×TBE buffer. The DNA bands were visualized by sliver stain kit with manufacture's recommended procedure.

Determination of DNA Displacement Efficiency and Apparent Initial Reaction Rate Constants (k_{app}):

DNA displacement efficiency (%) for the aptamer/capture-DNA/DBCO-DNA duplexes with N_3-DNA at different adenosine concentrations (0, 100, 250, and 1000 μM) was estimated from the change in fluorescence intensity based on equation 1,

DNA displacement efficiency (%) =100 × $\{(F_{N3(+)} - F_{N3(-)}) / (F_{max} - F_{N3(-)})\}$……eq. 1

where F_{max} is the fluorescence intensity of the aptamer alone, $F_{N3(+)}$ and $F_{N3(-)}$ are the fluorescence intensity of each sample in the presence of adenosine with and without N_3-DNA, respectively.

Apparent initial second-ordered reaction rate constants (k_{app}) for the aptamer/capture-DNA/DBCO-DNA duplexes with N_3-DNA at different adenosine concentrations (0, 100, 250, and 1000 μM) was estimated also from the change in fluorescence intensity based on equation 2,

$1 / [\text{DBCO-DNA}]_t = k_{app}t + 1 / [\text{DBCO-DNA}]_0$ ……eq. 2

where t is the reaction time, $[\text{DBCO-DNA}]_0$ is the initial concentration of DBCO-DNA (100 nM), $[\text{DBCO-DNA}]_t$ is the concentration of DBCO-DNA at t. Fig. S4 shows the plot of $1/[\text{DBCO-DNA}]_t$ versus t based on eq. 2. Thus, the slope in Fig. S4 is the k_{app} as shown in Table S2.

Determination of Dissociation Constant (K_d):

The dissociation constant (K_d) of the aptamer/capture-DNA/DBCO-DNA duplexes (traditional aptasensors) against adenosine and thrombin was estimated from the change in fluorescence intensity based on equation 3,

$(F_{N3(-)}-F_0) / (F_{max}-F_{N3(-)}) = \{1/K_d\}\{[\text{adenosine or thrombin}]_0 - [\text{aptamer}]_0 \{(F_{N3(-)}-F_0) / (F_{max}-F_0)\}$……eq. 3

where F_{max} is the fluorescence intensity of the aptamer alone, $F_{N3(-)}$ is the fluorescence intensity of each sample without N_3-DNA in the presence of adenosine or thrombin, F_0 is the background fluorescence intensity of the aptamer/capture-DNA/ DBCO-DNA duplex alone, $[\text{adenosine or thrombin}]_0$ is the initial concentration of adenosine or thrombin, $[\text{aptamer}]_0$ is the initial concentration of aptamer (100 nM). Fig. S5 shows the plot of $(F_{N3(-)} - F_0)/(F_{max} - F_{N3(-)})$ versus $[\text{adenosine or thrombin}]_o - [\text{aptamer}]_o \{(F_{N3(-)} - F_0)/(F_{max} - F_0)$ based on eq. 3. Thus, an inverse number of the slope in Fig. S5 is the K_d.(= $1/K_b$).

Table S1. DNA sequences used in this study.

Name	Sequence
adenosine aptamer	**5′-ACCTGGGGGAGTATTGCGGAGGAAGGTGTGAGXATGTGTAGTGC-3′**
capture-DNA for adenosine	**5′-BHQ1-CACACACCTTC-3′**
N_3-DNA for adenosine	**5′-N_3-CACACACCT-3′**
DBCO-DNA for adenosine and thrombin	**5′-GCACTACACATA-DBCO-3′**
control DNA	**5′-GCACTACACATACACACACCT-3′**
thrombin aptamer	**5′-GGTTGGTGTGGTTGGTGTCGXATGTGTAGTGC-3′**
capture-DNA for thrombin	**5′-BHQ1-CGACACCAACC-3′**
N_3-DNA for thrombin	**5′-N_3-CGACACCAA-3′**

The adenosine and thrombin aptamer segments are represented in underlined portions. Complementary sequences are colored (blue, pink, and green). The X indicates the FAM-labeled dT.

5′-N_3 :

3′-DBCO :

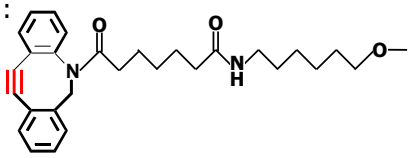

Table S2. Apparent initial second-order reaction rate constants (k_{app}) of the DNA strand displacement via toehold-mediated click chemical ligation at different concentrations of adenosine.

[adenosine] (μM)	initial k_{app} (M^{-1} sec^{-1})[a]
0	**$(6.9 \pm 0.2) \times 10^2$**
100	**$(2.3 \pm 0.1) \times 10^3$**
250	**$(3.6 \pm 0.4) \times 10^3$**
1000	**$(1.2 \pm 0.1) \times 10^4$**

a) Determined from Fig. S4.

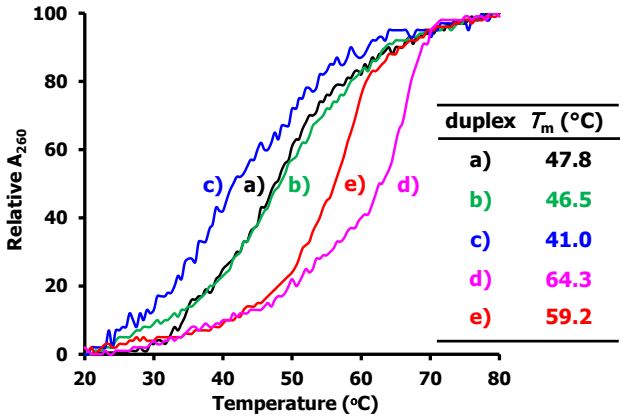

Fig. S1 Melting (T_m) curves of a) the adenosine aptamer/capture-DNA duplex, b) adenosine aptamer/DBCO-DNA duplex, c) adenosine aptamer/N_3-DNA duplex, d) adenosine aptamer/control DNA duplex, and e) adenosine aptamer/ligated-DNA with triazole linkage duplex measured by UV/vis spectrometer.

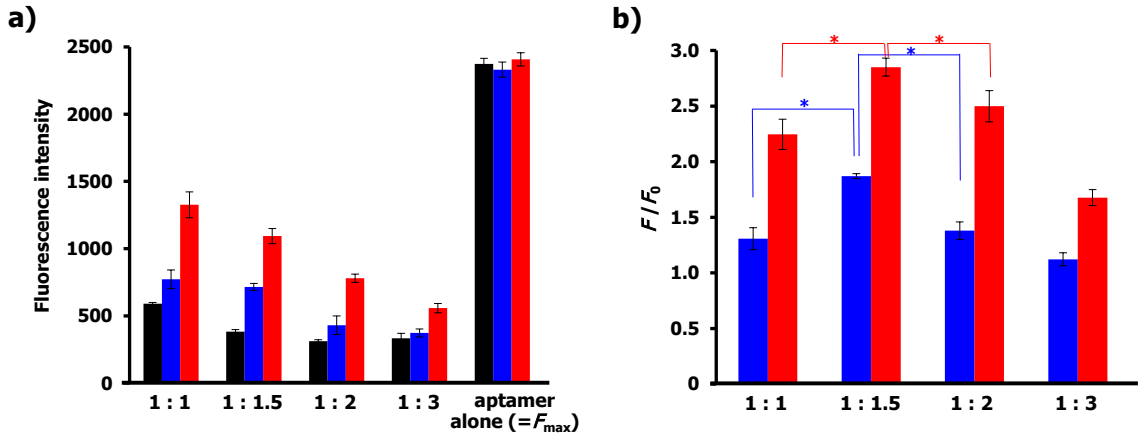

Fig. S2 a) Effect of the ratio of the adenosine aptamer (100 nM) : DBCO-DNA (100 nM) : capture-DNA (100, 150, 200, and 300 nM) on fluorescence intensity of the adenosine aptamer/capture-DNA/DBCO-DNA duplexes (traditional aptasensors) in the absence (black bars) and presence of 1000 μM (blue bars) or 2000 μM (red bars) of adenosine. b) F/F_0 ratio of the adenosine aptamer/capture-DNA/DBCO-DNA duplexes (traditional aptasensors) at difference ratio (1:1, 1:1.5, 1:2, and 1:3), where F is the fluorescence intensity of each sample in the presence of 1000 μM (blue bars) or 2000 μM (red bars) of adenosine, and F_0 is the background fluorescence intensity in the absence of adenosine. Mean values and standard deviations were obtained from three independent experiments ($^*P < 0.05$).

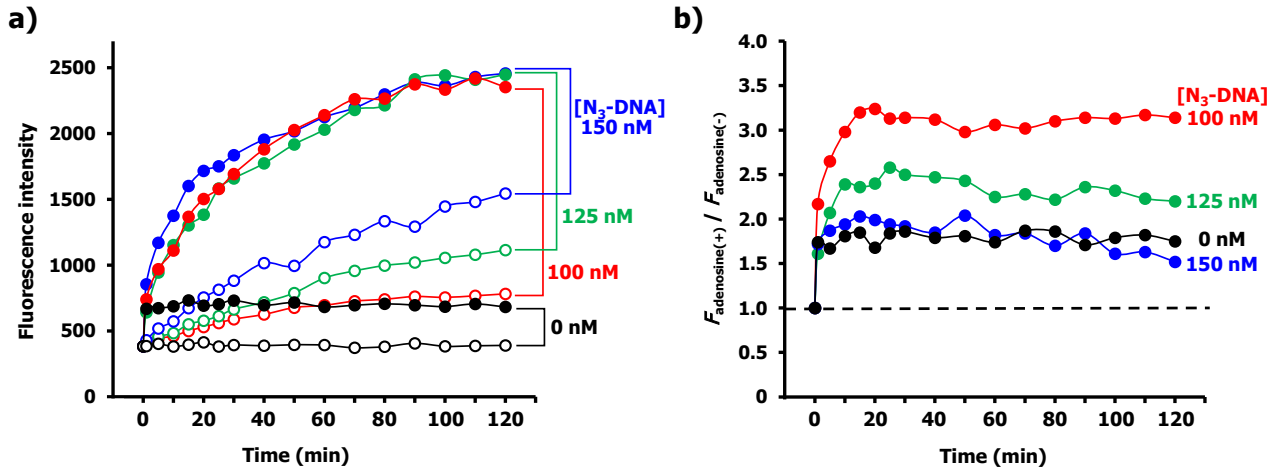

Fig. S3 a) Time-dependent fluorescence intensity of the adenosine aptamer/DBCO-DNA/capture-DNA duplexes (100 nM : 100 nM : 150 nM) in the absence (open circles) and presence (closed circles) of adenosine (1000 μM) and different amounts of N_3-DNA: 0 nM (black), 100 nM (red), 125 nM (green), and 150 nM (blue). b) Time-dependent $F_{adenosine(+)}/F_{adenosine(-)}$ ratio of the adenosine aptamer/capture-DNA/ DBCO-DNA duplexes (100 nM : 150 nM : 100 nM) different amounts of N_3-DNA: 0 nM (black), 100 nM (red), 125 nM (green), and 150 nM (blue), where $F_{adenosine(+)}$ is the fluorescence intensity of each sample in the presence of adenosine (1000 μM), and $F_{adenosine(-)}$ is the background fluorescence intensity of each sample in the absence of adenosine.

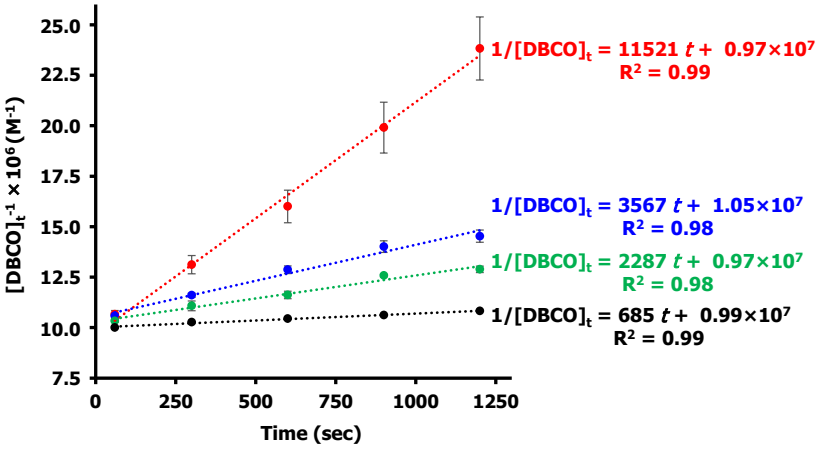

Fig. S4 Plots of 1/[DBCO-DNA]$_t$ versus t for the reaction of the aptamer /DBCO-DNA/capture-DNA duplex with N_3-DNA at different concentrations of adenosine (0 μM: black circles, 100 μM: green circles, 250 μM: blue circles, and 1000 μM: red circles). Mean values and standard deviations were obtained from three independent experiments.

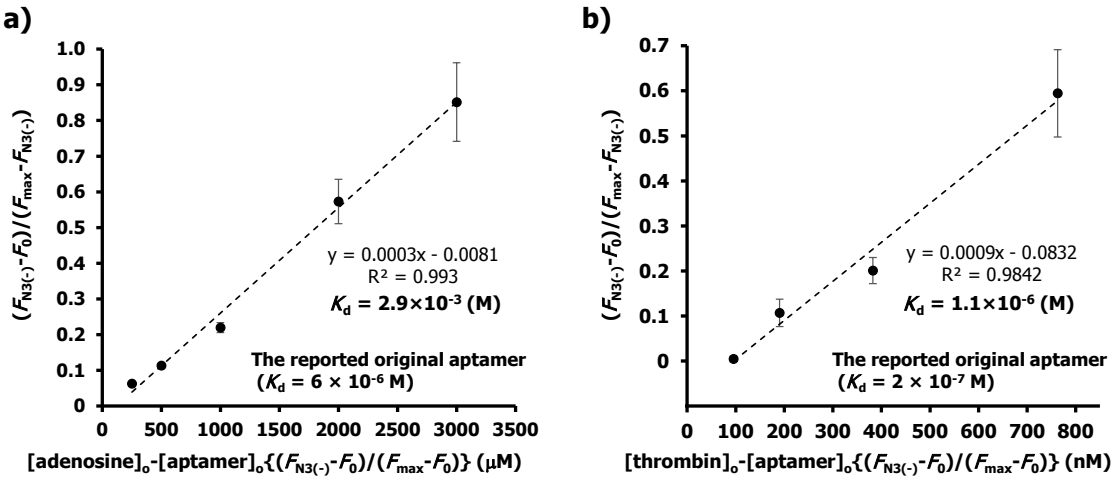

Fig. S5 Determination of dissociation constant (K_d) for the aptamer/DBCO-DNA/capture-DNA duplexes (traditional aptasensors) against a) adenosine and b) thrombin. Mean values and standard deviations were obtained from three independent experiments.

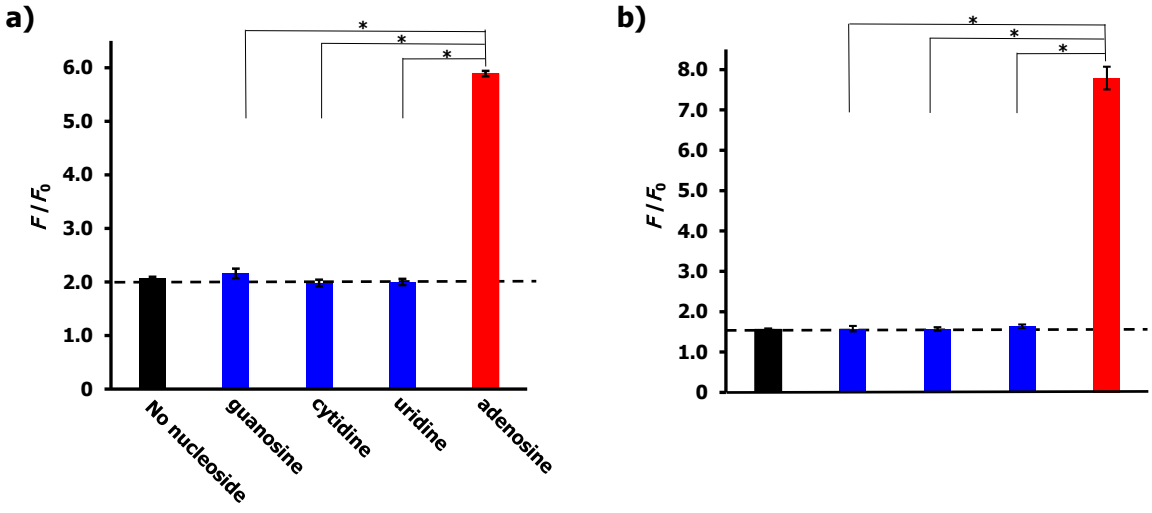

Fig. S6 F/F_0 ratio of the aptamer/DBCO-DNA/capture-DNA duplexes (100 nM : 100 nM : 150 nM) in the presence of N_3-DNA (100 nM) upon addition of a) uridine, cytidine, guanosine, or adenosine (250 μM) and b) human serum albumin, human factor IX, human factor Xa, and thrombin (100 nM) after 120 min. Mean values and standard deviations were obtained from three independent experiments ($^*P < 0.05$).