DNAzyme Footprinting: Detecting Protein-Aptamer Complexation on Surfaces by Blocking DNAzyme Cleavage Activity

Yulin Chen and Robert M. Corn*

Supporting Information

Experimental Section

Materials. 11-Amino-1-undecanethiol hydrochloride (MUAM; Dojindo), poly-L-glutamic acid (MW = 2000-15,000; pGlu; Sigma), 1-ethyl-3-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC; Pierce), N-hydroxysulfosuccinimide (NHSS; Pierce), ethanol (Gold Shield, absolute) and urea (Sigma-Aldrich) were used as received. Human thrombin (hTh, Sigma-Aldrich) was suspended in PBS as received and stored at 4 ^{o}C. Transcription kit (RiboMax) was purchased from Promega. The DNA sequences used in this paper were purchased from IDT and are listed in Table S1. A PBS buffer (100 mM Na_2HPO_4, 0.3 M NaCl, 5 mM $MgCl_2$, 1 mM EDTA, adjusted to pH 7.0) was used for all DNA fabrication, hybridization detection, and thrombin binding assays.

DNA microarray fabrication for SPRI measurements.

All SPRI chips were fabricated using a metal mask to vapor deposit (Denton DV-502A) a 45-nm gold film with a 1-nm underlayer of chromium on top of SF-10 glass slides as described elsewhere.[1,2] The steel metal mask had 17 holes with 1 mm diameter circles.[1] Amine-terminated ssDNA was attached to the gold array spots with the DNA surface attachment chemistry described elsewhere.[3] Described briefly, the slides were first immersed in a 1 mM ethanolic MUAM solution overnight, followed by a electrostatically-adsorbed monolayer polyelectrolyte (pGlu, 2 mg/mL). Next, 0.5 μL of solution containing a final concentration of 250 μM amine-terminated ssDNA and EDC/NHSS (75 mM/15 mM) was applied to each spot for more than 4 h. This carbodiimide coupling reaction simultaneously attached the pGlu to the surface and the amino-terminated DNA onto the pGlu. All DNA sequences used for immobilization are listed in Table S1. In the experiment shown in Figure S2, a DNA solution (5% of bioaffinity sequence and 95% of spacing sequence) was applied to each spot for immobilization. For transcription amplification SPRI experiments (Figure 4 and Figure S3), the microarray slides contained three components were spotted with DNA solutions: Generator element (a mixture of 2% bioaffinity sequence and 98% spacing sequence), Detector element (capture sequence), and Control element (control sequence). After DNA immobilization, the slides were rinsed with water and dried with nitrogen gas prior to use. SPRI measurements were performed using an SPRI instrument (GWC Technologies) using a protocol described in detail elsewhere.[3]

Preparation of ssDNA-modified gold nanoparticles

Gold nanoparticles (13-nm, AuNPs) were synthesized by the Turkevich method.[4] The AuNP solution has an extinction coefficient of 2×10^8 M^{-1} cm^{-1} at λ_{max} of 520 nm. DNA-modified AuNPs were prepared with the procedures similar to our previous work.[5] Briefly, 5 μL of 1 mM 3′-thiol-modified DNA (AuD, Table S1) was added to 1 mL of AuNP citrate solution (~10 nM) and kept at 37 °C for 24 h, followed by an addition of 500 μL of phosphate buffer (0.3 M NaCl, 30 mM phosphate, pH 7.4) at 37 °C for another 24 h. The unbound DNA was removed by centrifuging the nanoparticle solution at 12,000 rpm for 20 min. The supernatant was removed, and AuNPs were resuspended in 0.5X PBS. This washing process was repeated for three times, and AuNPs were resuspended in PBS buffer (pH 7.4) at the end. All AuNP binding assays measured by SPRI utilized a solution containing 1.5-2 nM AuD-AuNPs in PBS.

Human thrombin (hTh) aptamer and DNAzyme design

For the hTh aptamer, in order to create the partial duplex formation with the designed DNAzyme, we modified several bases in the stem pairing based on the aptamer sequence reported from Tasset et al.[6]; however, the bases of G-quartet were remained the same. As shown in the crystal structure,[6,7] the G-quartet of the aptamer is the major interaction part to hTh protein. The A at position 4 of the G-quartet corresponds to determining the binding site of this DNA, and is directed toward the positive charged redisues of the heparin binding exosite of hTh by the exposed side-chains of Arg126, Lys236, Lys240, and Arg93. Further crosslink between T at position 12 to Phe245 of thrombin is then formed. The DNAzyme was designed to bind to the aptamer due to the base pairing of the formation of a duplex, and the strength is adjusted and tested with a various reaction time.

DNAzyme cleavage assays

	Without hTh (6 samples)	With hTh (6 samples)
50 μM bioaffinity sequence	1 μL	1 μL
25 μM hTh	0 μL	1 μL
PBS	23 μL	22 μL

For time-based gel assays (Figure 3), 12 samples were first mixed as shown in the above table and reacted for one hour in PBS at room temperature. Then a 1 μL solution containing 3.75 mM ascorbic acid, 2.25 mM $CuCl_2$, 150 μM DNAzyme (dissolved in H_2O) was applied to the previous 12 aliquots. These samples contained a final volume of 25 μL and final concentrations of 1 μM bioaffinity sequence, 2 μM DNAzyme, 30 μM $CuCl_2$, 50 μM ascorbic acid, and 1 (or 0 M) μM hTh in PBS. Reactions were terminated by the addition of 5 μL loading buffer (8 M urea, 50 mM EDTA, 0.05% xylene cyanol,

0.02% bromophenyl blue, and 50 mM Tris acetate) at the times indicated in Figure 2. The samples were loaded into 15% polyacrylamide gel for electrophoresis (15W for 45 min) to separate the uncleaved substrate and cleaved product. The gel was cleaned and stained with toluidine blue for at least 1 h, and destained in DI water at 4 ^{o}C overnight. Figure S2 is an extension of Figure 2 and shows the gel in its entirety.

For surface cleaving assays (Figure S2, S3 and Figure 4), the DNAzyme concentration and the amount of surface-bound bioaffinity sequence were very critical, and were required to be optimized with the cleaving time. In the experiment shown in Figure S2a, after the DNA microarray was prepared by the procedure described above, 0.6 μL of buffer solutions that contained either zero or ten nanomolar hTh were then applied to the microarray elements in the alternating pattern shown in Figure S2a. After 2 h, the previous hTh solution or PBS was carefully removed, and then 0.6 μL cleaving solution (200 nM DNAzyme in cleaving buffer with or without 10 nM hTh) was applied to each spot. After cleaving for 18 hours at room temperature (23 ^{o}C), the slide was rinsed with 8 M urea and then water. Any surface-bound DNA bioaffinity sequence that did not form an thrombin-aptamer complex was selectively cleaved (see Figure S2a). The cleaving process that occurred on the surface was kept at static conditions in order to reduce the desorption of hTh from the aptamer sequence. Using an SPRI microfluidic cell, the microarray was then exposed to a solution ofAuNP that were modified with a ssDNA (AuD) sequence partially complementary to the reporter sequence. The SPR image obtained from this experiment is shown in Figure S2b. The microarray elements which were exposed to hTh exhibited a large increase in SPRI signal (11.2±1.1 Δ%R) due to the hybridization adsorption of AuNPs to the reporter sequence. In contrast, microarray elements that were not exposed to hTh showed a small SPRI signal increase (0.9±0.5 Δ%R) indicative of DNAzyme cleavage.

In the set of hTh detection measurement with the transcription enhancement (Figure 4), 2% of bioaffinity sequence was printed on the Generator element, and different concentration of hTh was applied on each chip with a volume of 500 μL for 12 hours. After hTh binding, a 10 μL of concentrated DNAzyme solution (2.5 μM of DNAzyme, 2.5 mM ascorbic acid, 1.5 mM $CuCl_2$) was added to the previous 500 μL droplet, and allowed to react for another 12-15 h at room temperature.

DNase I cleaving experiment using phosphorothioate-modified bioaffinity sequence

The bioaffinity sequence for DNase I cleaving experiment is modified with phosphorothioates for its internucleotide linkages, but the hTh aptamer sequence remains as phosphodiester bonds. Phosphorothioates (or S-oligos) are a variant of normal DNA in which one of the nonbridging oxygens is replaced by a sulfur. The phosphorothioate bonds have been proved to dramatically reduce the action of endo- and exonuclease.[8] The

modified bioaffinity sequence (Ds, IDT) was listed: 5'- /5AmMC12/TT*T T*TA* GT*C CGT GGT AGG GCA GGT TGG GGT GACT*TA* AT*A C*GA* CT*C A*CT* AT*A G*GG* AC*A G*TT* CT*A T*GA* AT*A T*AT* TT*G A*TG* GC*G A*GC* TT*T A*AT* T -3'; Note: the asterisk indicated the position incorporated with phosphorothioate internucleotide linkages.

The ssDNA (Ds) are immobilized on each Au spot of a microarray using the procedure described above. The microarray is mounted to a dual-channel flow cell on the SPRI stage. One channel (channel A) was flowed in buffer and the other channel (channel B) was flowed in 100 nM hTh for 15 min. After that, 0.02 U/μL DNase I was flowed through channel A and 0.02 U/μL DNase I plus 100 nM hTh was flowed through channel B. The cleaving reaction was processed for 15 min, and the flow cell was rinsed thoroughly with 8M urea then water. The AuD-AuNP binding experiment was then measured by SPRI. The SPRI result shows that the modified bioaffinity sequence (Ds) in both channels got cleaved (data not shown), indicating that that the DNase I enzyme cleaved the bioaffinity sequence both with and without hTh-aptamer complex formation and the cleaving process performed by DNase I might be too strong/fast to select the hTh-aptamer complex.

Transcription amplification of SPRI measurements

The on-chip transcription using a generator-detector platform was described elsewhere.[9,10] Briefly, the DNA bioaffinity sequence was immobilized on the Generator element, and the "capture sequence" (Table S1) was immobilized on the Detector element as described in the microarray fabrication section. After the hTh and DNAzyme competitive binding (details as described above), 500 μL of 1 μM complimentary template was applied onto the chip for 20 min. After that, the chip was briefly rinsed with PBS. Transcription solution (50-100 μL, RiboMax) was flowed through the microfluidic cell on the SPRI stage at 37 ^{o}C for 2 h, followed by a cooling process. SPRI measurements of AuNP adsorption was observed afterwards.

Table S1. DNA sequences.

Bioaffinity sequence: 5'-NH_2- $(CH_2)_6$- PEG_{10}- GAATTTCT AATACGTGGTAGGGCAGGTTGGGGTTATTAGCTTAATACGACTCACTATA GGGACAGTTCTATGAATATATTTGATGGCGAGCTTTAATT -3'
DNAzyme: 5'-CTACCACTGGGCCTCTTTTTTAAAGAAC-3'
Complimentary template:5'- AATTAAAGCTCGCCATCAAATATATTCATAGAACT GTCCCTATAGTGAGTCGTATTAAGCT -3'
AuD: 5'- HS-$(CH_2)_6$- AATTAAAGCTCGCCATCAAA-3'
Capture sequence: 5'- TATATTCATAGAACTGTCCC-NH_2-3'
Control sequence: 5'-NH_2- $(CH_2)_6$- T_{24}-3'
Spacing sequence: 5'-NH_2- $(CH_2)_6$- T_{10} -3'

Note:

- Transcribed RNA reporter sequence: GGG ACA GUU CUA UGA AUA UAU UUG AUG GCG AGC UUU AAU U
- "Bioaffinity sequence" contains several components indicated in different color: spacer (gray), aptamer sequence (blue), promoter sequence (green), and reporter sequence (red).

Figure S1. The complete gel image of Figure 2.

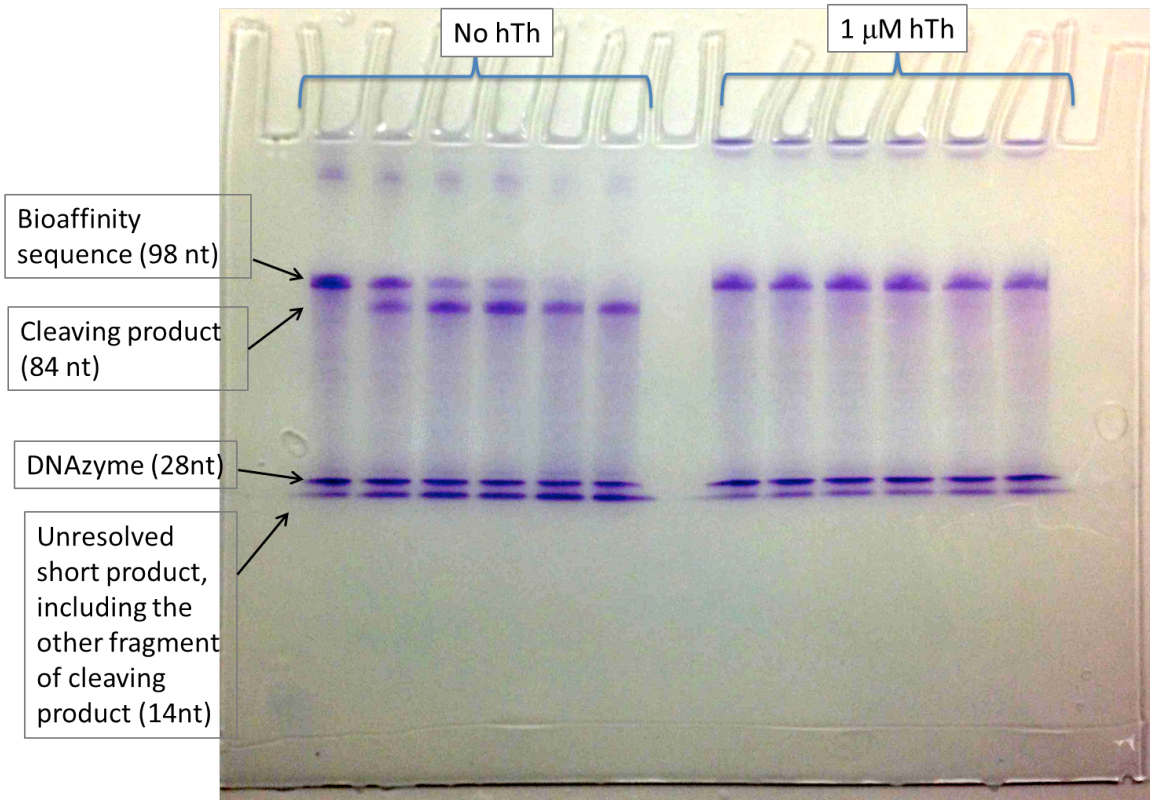


Figure S2. AuNP adsorption onto a microarray of surface-bound bioaffinity sequences. (a) Half of the microarray elements were first exposed to 10 nM hTh; a DNAzyme solution was then used to cleave any ssDNA from the microarray that was not protected by a thrombin-aptamer complex. The AuNPs can only adsorb onto microarray elements that still contain the reporter sequence (in red). (b) An SPRI difference image of the alternating AuNP adsorption pattern observed on the microarray along with a representative line profile of Δ%R .

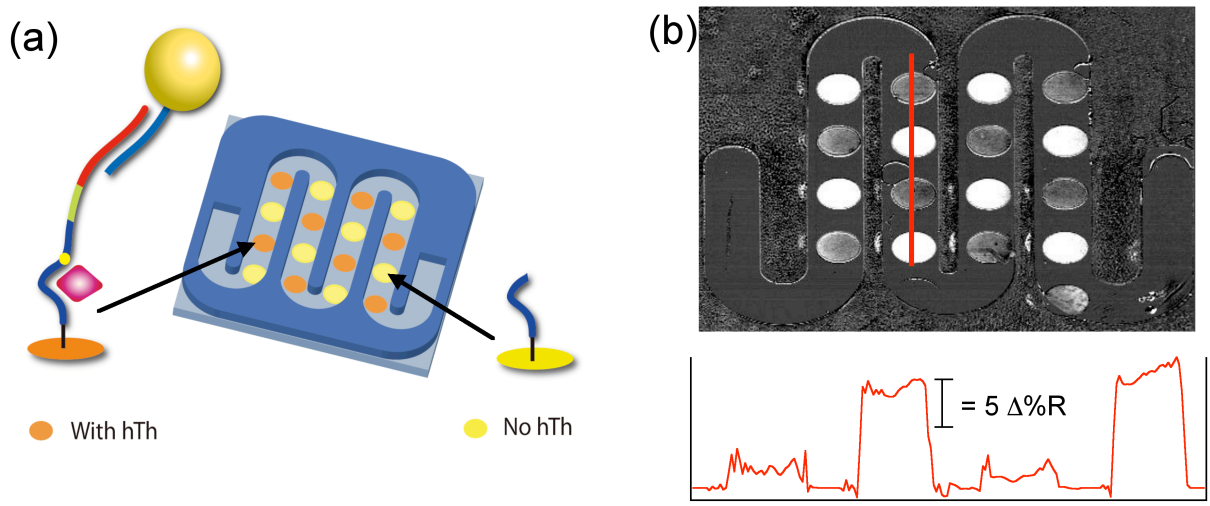


Figure S3. (a) Spatial diagram of the three components on the DNA microarray. G: Generator element; D: Detector element; C: Control element. (b) The SPRI difference image of AuNP binding from a 1 pM hTh sample. The real-time binding curve is shown in Figure 4b.

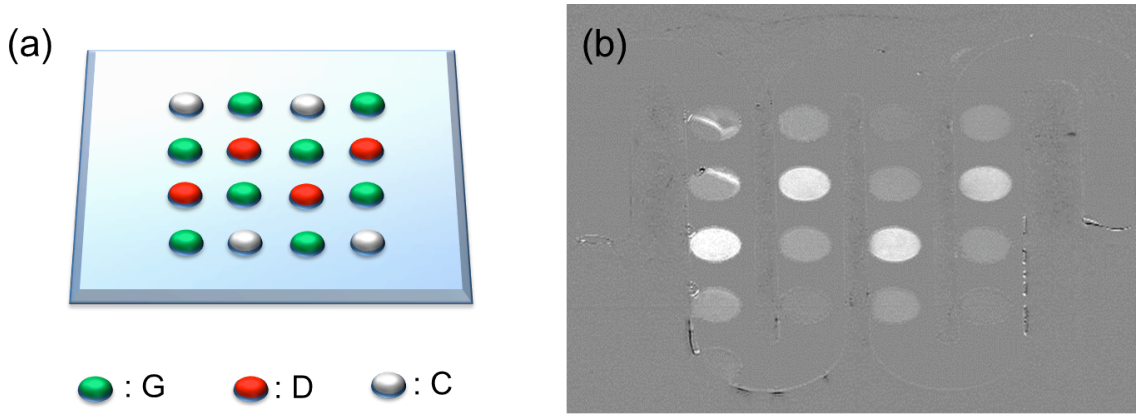
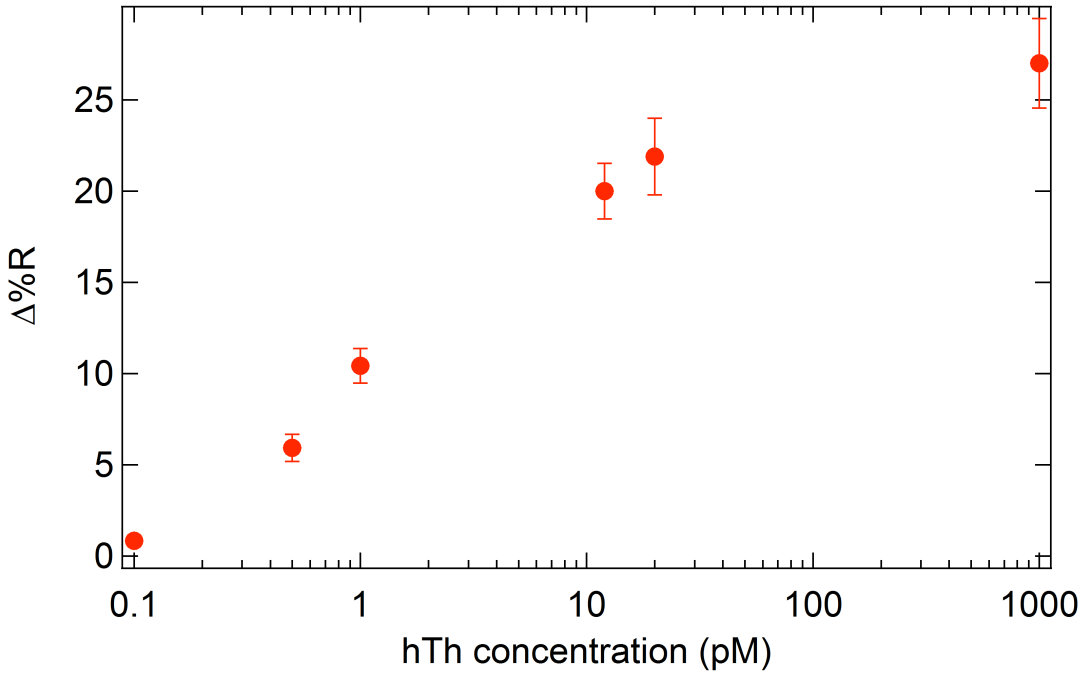


Figure S4. For RNA transcription amplification experiments, Δ%R measured at 1000 seconds for AuNP adsorption onto detector microarray elements as a function of hTh concentration. Figure 4 is the blow-up at low concentration.



References:

(1) Wark, A. W.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2005**, *77*, 3904-3907.
(2) Brockman, J. M.; Frutos, A. G.; Corn, R. M. *J. Am. Chem. Soc.* **1999**, *121*, 8044-8051.
(3) Thiel, A. J.; Frutos, A. G.; Jordan, C. E.; Corn, R. M.; Smith, L. M. *Anal. Chem.* **1997**, *69*, 4948-4956.
(4) Turkevich, J.; Stevenson, P. C.; Hillier, J. *Discuss. Faraday Soc.* **1951**, 55-&.
(5) Li, Y. A.; Wark, A. W.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2006**, *78*, 3158-3164.
(6) Tasset, D. M.; Kubik, M. F.; Steiner, W. *J. Mol. Biol.* **1997**, *272*, 688-698.
(7) Rydel, T. J.; Ravichandran, K. G.; Tulinsky, A.; Bode, W.; Huber, R.; Roitsch, C.; Fenton II, J. W. *Science* **1990**, *249*, 277-280.
(8) Spitzer, S.; Eckstein, F. *Nucleic Acids Res.* **1988**, *16*, 11691-11704.
(9) Sendroiu, I. E.; Gifford, L. K.; Luptak, A.; Corn, R. M. *J. Am. Chem. Soc.* **2011**, *133*, 4271-4273.
(10) Chen, Y.; Nakamoto, K.; Niwa, O.; Corn, R. M. *Langmuir* **2012**, *28*, 8281-8285.