Electronic Supplementary Information

Expanding DNA nanomachines functionality through binding-induced DNA outputting for application in clinical diagnosis

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EXPERIMENTAL SECTION

Materials and Reagents

All of DNA sequences were synthesized and purified by Takara Biomedical Technology Co., Ltd. (Dalian, China). The detailed sequences and modifications are listed in Table S1, S2. 20-nm gold nanoparticle was purchased from the Ted Pella (Redding, CA). Biotin, streptavidin, HSA, transferrin, PSA, CEA, AFP, CA19-9 and GAPDH were obtained from Sigma-Aldrich (Shanghai, China). Biotinylated polyclonal anti-PSA antibody, biotinylated polyclonal anti-CEA antibody, biotinylated polyclonal anti-AFP antibody, biotinylated polyclonal anti-CA19-9 antibody were obtained from R&D Systems (Minneapolis, MN) and Abcam (Shanghai, China). All other reagents were of analytical grade. All of reagents were diluted by using RNase-free water. Estimation of Tm of Hybrid and Gibbs free energy (Δ G) was achieved by the IDT. Oligo Analyzer. Fluorescence microplate reader (Thermo Scientific™ Multiskan™ GO) for fluorescence detection

Table S1. Sequences used in BiDDCs

Oligonucleotides	Sequences(5'⟶ 3')
DNAzyme	HS-$(T)_{30}$ TAG CTA CTC TCT TCT CCG AGC CGG TCG AAA ATA GT B* Catalytic core
Substrate	HS-$(T)_{14}$CACTATrAGGAAGAGAT-6-Carboxyfluorescein (FAM)
Output	GGC CCG TAG CTA CTC TCT TC
Pd-1	GC TAC GGG CC $(T)_{20}$
Pd-2	$(T)_{20}$ GGCCCG
Sequential optimization of blocker toehold	
Toe-d6 of blocker	GAA GAG AGT AGC TAC GGG CC
Toe-d5 of blocker	GAA GAG AGT AGC TAC GGG C
Toe-d4 of blocker	GAA GAG AGT AGC TAC GGG
Toe-d3 of blocker	GAA GAG AGT AGC TAC GG
Sequential optimization of 5'-blocker	
B-14	GAA GAG AGT AGC TAC GGG C B-14 to B*
B-13	AA GAG AGT AGC TAC GGG C B-13 to B*
B-12	A GAG AGT AGC TAC GGG C B-12 to B*
B-11	GAG AGT AGC TAC GGG C B-11 to B*

Table S2. Sequences used in BiCCs

Oligonucleotides	Sequences(5'⟶ 3')
DNAzyme	HS-$(T)_{30}$ TAG CTA CTC TCT TCT CCG AGC CGG TCG AAA ATA GT B* Catalytic core
Substrate	HS-$(T)_{14}$CACTATrAGGAAGAGAT-6-Carboxyfluorescein (FAM)
Hairpin structure(HP)	CAGTACCTAG CTACT CTCTTC TrAG AATGTC$(T)_{13}$GGTACTG Hybrid with C1 Hybrid with C2
Pc1	Biotin-$(T)_{19}$GCCCGCACCCATGTGAAGAG D1 C1
Pc2	GACATTAGCGATCGGGC$(T)_{19}$-Biotin C2 D2
Sequential optimization of blocker toehold in BiCCs	
Toe-c7 of blocker	GAA GAG AGT AGC TA GGTACTG
Toe-c6 of blocker	GAA GAG AGT AGC TA GGTACT
Toe-c5 of blocker	GAA GAG AGT AGC TA GGTAC
Toe-c4 of blocker	GAA GAG AGT AGC TA GGTA

Construction of the DNA nanomachines

Prior to the functionalizing AuNP, the blocked DNAzyme was prepared by using blocker strand and DNAzyme (Table S1). With a molar ratio of 3:1, the blocker strand and the DNAzyme strand were mixed in 1×PBS buffer (pH 7.4). The mixture was then heated to 85 °C and gradually cooled down to room temperature (20 °C). Consequently, the blocked DNAzyme strand then ready to be conjugated to the AuNPs. 20 nm AuNPs, blocked DNAzyme and FAM-labelled substrate were incubated at a molar ratio of 1:100:10,000. This mixture with 0.05% Tween 20 was incubated at 20 °C for 15 h. Afterward, NaCl was added in increments of 0.05 M for the first two times and added increments of 0.1 M for six more times. After incubation for extra 24 h, this mixture was washed four times by centrifuge of 16,000 g for 20 min to remove the free DNA. The AuNPs were resuspended in 1×PBS buffer (pH 7.4) contained 0.05% Tween at a concentration of 2.3 nM.

Preparation of O-Pd1 and Pd2 in binding-induced DNA displacement conversion system (BiDDCs) and Pc1and Pc2 in binding-induced cleavage conversion system (BiCCs)

To recognize PSA, biotinylated polyclonal anti-PSA antibodies were linked to O-Pd1 and Pd2.DNA probe (O-Pd1) was constructed at a final concentration of 4 μM by mixing 20 μL 50 μM probe DNA (Pd1) with 13.3 μL 50 μM output DNA(O) in 25 mM Tris-acetate (pH 7.4) with 10 mM MgCl2, 200 mM NaCl and 0.05% Tween-20. This solution was heated to 85 °C and gradually cooled down to room temperature (ca.20 °C). Afterward, 20 μL of 4 μM biotinylated O-Pd1 or 20 μL of 4 μM biotinylated Pd2 was mixed with 20 μL of 4 μM streptavidin in 1×PBS (pH 7.4) with 0.1% BSA. After 2 h incubation of those two mixtures at 37 °C, 40 μL of 2 μM biotinylated anti-PSA antibodies was added to each mixture and incubated at 37 °C for another 1 h. Thus,

PSA-antibody-conjugated O-Pd1, Pd2 were formed and further diluted to 100 nM in 25 mM Tris-acetate (pH 7.4) with 10 mM $MgCl_2$, 200 mM NaCl ,0.05% Tween-20, 0.1% BSA and 10 μM biotin. The excess biotin was utilized to occupy the free biotin-binding sites of streptavidin. Similar process for recognition of CEA, AFP and CA19-9 expect that the biotinylated polyclonal anti-CEA antibodies, biotinylated polyclonal anti- AFP antibodies and biotinylated polyclonal anti- CA19-9 antibodies was used, respectively.

In BiCCs, the procedure of Pc1and Pc2 (Table S2) preparation was similar with that of O-Pd1 and Pd2. Briefly, 20 μL of 4 μM biotinylated Pc1 or 20 μL of 4 μM biotinylated Pc2 was mixed with 20 μL of 4 μM streptavidin in 1×PBS (pH 7.4) with 0.1% BSA. After 2 h incubation of those two mixtures at 37 ◦C, 40 μL of 2 μM biotinylated anti-PSA/CEA antibodies was added to each mixture and incubated at 37 ◦C for another 1 h. Thus, PSA/CEA -antibody-conjugated O-Pd1, Pd2 are formed and further diluted to 100 nM in 25 mM Tris-acetate (pH 7.4) with 10 mM $MgCl_2$, 200 mM NaCl ,0.05% Tween-20, 0.1% BSA and 10 μM biotin. The probe solutions were stored at 4 ◦C prior to use.

Test the feasibility of binding-induced DNA displacement conversion system (BiDDCs)

The reaction solution composed of 20 nM O-Pd1, 20 nM Pd2, 0.23 nM DNA nanomachines, 1 nM PSA and buffer (25 mM pH 8.0 Tris-acetate, 30 mM MgCl2, 200 mM NaCl and 0.05% Tween-20). This mixture was incubated at 20 ◦C and monitored in real-time by using Thermo Scientific™ Multiskan™ GO. For FAM, 485 nm for excitation and 515 nm were set for emission detection.

Test the feasibility of binding-induced cleavage conversion system (BiCCs)

The reaction mixture contained 20 nM O-Pd1, 20 nM Pd2, 0.23 nM DNA nanomachines, 200 nM HP, 1 nM PSA and buffer (25 mM Tris-acetate with pH 8.0, 10 mM $MgCl_2$, 200 mM NaCl and 0.05% Tween-20). This mixture was incubated at 20 ◦C

and monitor it in real-time by using Thermo Scientific™ Multiskan™ GO with 485 nm for excitation and 515 nm for emission.

Comparison of kinetic between BiDDCs and BiCCs.

The solution of BiDDCs included 20 nM O-Pd1, 20 nM Pd2, 0.23 nM DNA nanomachines, 1 nM PSA. The solution of BiCCs contained 20 nM Pc1, 20 nM Pc2, 0.23 nM DNA nanomachines, 200 nM HP, 1 nM PSA. The both of reaction buffer is that 25 mM Tris-acetate with pH 8.0, 30 mM $MgCl_2$, 200 mM NaCl and 0.05% Tween-20 were mixed. Those two mixture were transferred into the Thermo Scientific™ Multiskan™ GO and the signal was monitored in real-time. 485 nm for excitation and 515 nm were set for emission detection.

Quantification of conversion efficiency and evaluation of general applicability to BiCCs

Varying concentrations of PSA or CEA were placed in 100 μL reaction buffer, including 25 mM Tris-acetate with pH 8.0, 30 mM MgCl2, 200 mM NaCl and 0.05% Tween-20 , contained 20 nM Pc1, 20 nM Pc2, 0.23 nM DNA nanomachines, 200 nM HP. The mixture were transferred into the Thermo Scientific™ Multiskan™ GO and the signal was monitored in real-time for 1h.

Evaluation of specificity of BiCCs and its performance in diluted serum

1 nM of PSA and 5% serum or 50% serum were placed in 100 μL reaction buffer as previous experiments, respectively. The mixture was tested at 1 h end points. 485 nm for excitation and 515 nm were set for emission detection.

Evaluation of length of toehold effect in BiDDCs

To evaluate the length of toehold effect, four different toeholds (Toe-d3, d4, d5 and d6) were designed with addition or depletion of nucleotides, respectively (Table S1).

The PSA test solutions contained 1 nM PSA, 20 nM O-Pd1, 20 nM Pd2, and 0.23 nM DNA nanomachines with varied length of toehold. All other buffer is the same as in the previous study. The mixture was tested at 1 h end points. Error bars represent one standard deviation from duplicated analyses.

Investigating stability of duplex of blocker strand and DNAzyme in BiDDCs.

To investigate the stability of duplex of blocker strand and DNAzyme, we designed four blocker strands (B-11, B-12, B-13 B-14) by changing the length complementary sequence with DNAzyme of DNA nanomachines. The PSA test solutions contained 1 nM PSA, 20 nM O-Pd1, 20 nM Pd2, and 0.23 nM DNA nanomachines with varied blocker strand. All other buffer is the same as the previous study. Error bars represent one standard deviation from duplicated analyses.

Effect of ratio of substrate to DNAzyme on the DNA nanomachines operation.

To test the effect of ratio of substrate to DNAzyme on the DNA nanomachines operation. Different molar ratio between substrate and DNAzyme, including 50:1, 100:1 and 200:1, was set for determination of real-time value for 3 h. The reaction buffer contained 0.23 nM of the activated DNA nanomachines without blocker strand, 25 mM Tris-acetate with pH 8.0, 30 mM $MgCl_2$, 200 mM NaCl and 0.05% Tween-20 . Error bars represent one standard deviation from duplicated analyses.

Evaluation of length of toehold effect in BiCCs.

Because of the output in BiCCs was redesigned, the length of toehold (Toe-c) of blocker needs to be reevaluated. Four toeholds domains (Toe-c4, c5, c6 and c7, TableS2) with different lengths were investigated. The PSA (1 nM) test solutions contained 20 nM Pc1, 20 nM Pc2, 0.23 nM DNA nanomachines, 200 nM HP, with varied length of toehold. All other buffer is the same as in the previous study. The mixture was tested at 1 h end points. Error bars represent one standard deviation from duplicated analyses.

Effect of Mg^{2+} concentration on the folding of split DNAzyme for DNA nanomachine operation in BiCCs.

In this experiment, different concentrations, including 10 mM-40 mM Mg^{2+}, were prepared. As previous experiments, 1 nM of PSA was chosen as the target. All other buffer is the same as in the previous study. The mixture was tested at 1 h end points. Error bars represent one standard deviation from duplicated analyses.

To verify DNA nanomachines prefer to operate as inner-AuNP cleavage instead of cross-AuNP cleavage

To confirm the DNA nanomachines prefer to as inner-AuNP cleavage, we designed following control: activated DNA nanomachines was prepared as in the previous study except the addition of a blocker strand. Control AuNP-1 (c-AuNP-1) was functionalized with only the substrates as activated DNA nanomachines. Control AuNP-2(c-AuNP-2) was built with same amount of DNAzyme as activated DNA nanomachines, and the same amount of poly T DNA was introduced to replace the substrates as activated DNA nanomachines. 0.23 nM of activated DNA nanomachines, c-AuNP-1 and c-AuNP-2 were used for determination of real-time value for 3 h. All other buffer is the same as in the previous study. Error bars represent one standard deviation from duplicated analyses.

Detection of liver cancer biomarker derived from clinical samples using BiCCs

The human serum of clinical samples were obtained from Biliary Surgical Department, West China Hospital, Sichuan University. All collection procedures of human serum were approved by Ethics Committee of West China Hospital. First, 5 ml venous blood samples of each of the clinical samples were transferred into a collection tube. Subsequently, collected blood samples were centrifuged at room temperature for 10 min (3000 r/min). The obtained serum samples were aliquoted into 250 uL cryotubes and stored in −80 ◦C to use. 5 μL serum from clinical samples were placed in 100 μL reaction buffer, including 25 mM Tris-acetate with pH 8.0, 30 mM $MgCl_2$, 200 mM

NaCl and 0.05% Tween-20, contained 20 nM Pc1 with AFP antibody/ CEA antibody/CA19-9 antibody, 20 nM Pc2 with AFP antibody/ CEA antibody/CA19-9 antibody, 0.23 nM DNA nanomachines, 200 nM HP. The mixture was transferred into the Thermo Scientific™ Multiskan™ GO to monitor the signal in real-time for 1 h.

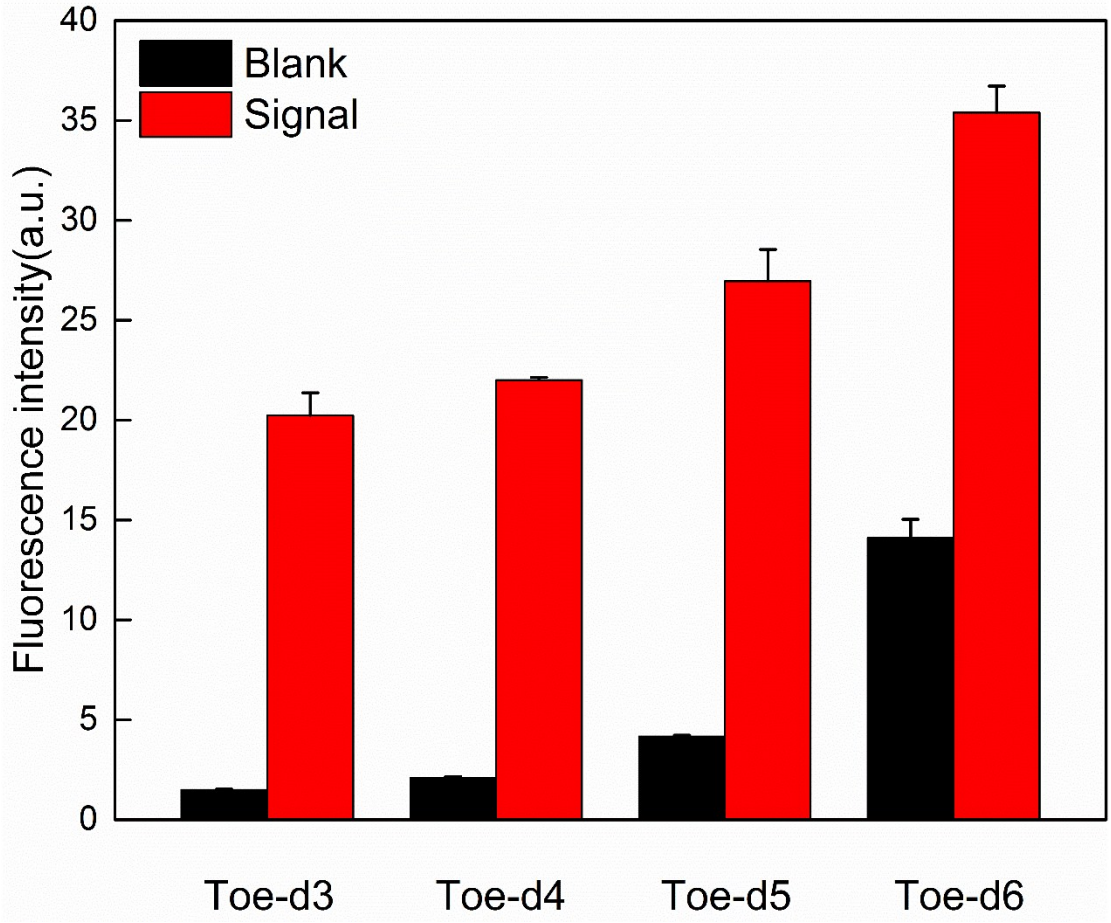

Figure S1 Evaluation of length of toehold effect in BiDDCs. Four toeholds, including Toe-3, 4, 5 and 6, are designed to compare (Table S1). 1 nM PSA was chosen as the recognition target for initiation of conversion system. After 1 hr incubation, end-point of fluorescence values of each group was tested.

Table S3. The tested melting temperatures (Tm) and free energy (ΔG) values of

	Toe-d6 of blocker	**Toe-d5 of blocker**	**Toe-d4 of blocker**	**Toe-d3 of blocker**
Output DNA(Od)	ΔG:-38.37 kcal/mole Tm: 62.2 ^{0}C	ΔG:-35.3 kcal/mole Tm: 61 ^{0}C	ΔG:-32.16 kcal/mole Tm: 58.2 ^{0}C	ΔG:-29.09 kcal/mole Tm: 54 ^{0}C
Pd2	ΔG:-15.96 kcal/mole Tm: 19.2 ^{0}C	ΔG:-12.89 kcal/mole Tm: 19.2 ^{0}C	ΔG: -9.75 kcal/mole Tm: 0 ^{0}C	ΔG: -6.68 kcal/mole Tm: 0 ^{0}C
B* of DNAzyme in BiDDCs	ΔG: -21.07 kcal/mole Tm: 43.8 ^{0}C	ΔG:-21.07 kcal/mole Tm: 43.8^{0}C	ΔG: -21.07 kcal/mole Tm: 43.8 ^{0}C	ΔG: -21.07 kcal/mole Tm: 43.8 ^{0}C

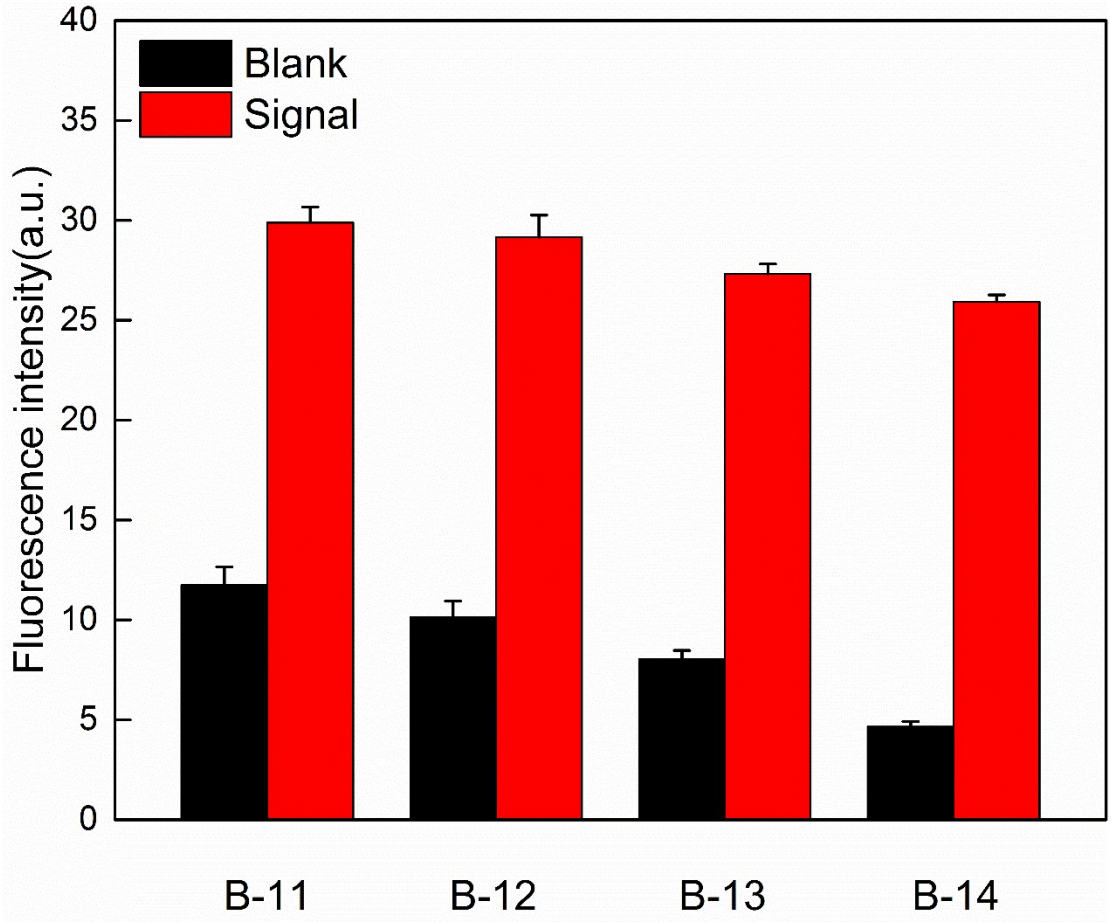

Figure S2 Investigating stability of duplex of blocker strand and DNAzyme in BiDDCs. Four blocker strand with different length was employed (Table S1). End-point of fluorescence values for 1 h incubation was tested using 1 nM PSA.

Table S4. The melting temperatures (Tm) and Free energy (ΔG) values of tested

	B-11	B-12	B-13	B-14
B* of DNAzyme in BiDDTs	Δ G: -15.95 kcal/mole Tm: 37.6 ^{0}C	Δ G: -17.55 kcal/mole Tm: 41.6 ^{0}C	Δ G: -19.49 kcal/mole Tm: 44.3 ^{0}C	Δ G: -21.07 kcal/mole Tm: 47 ^{0}C

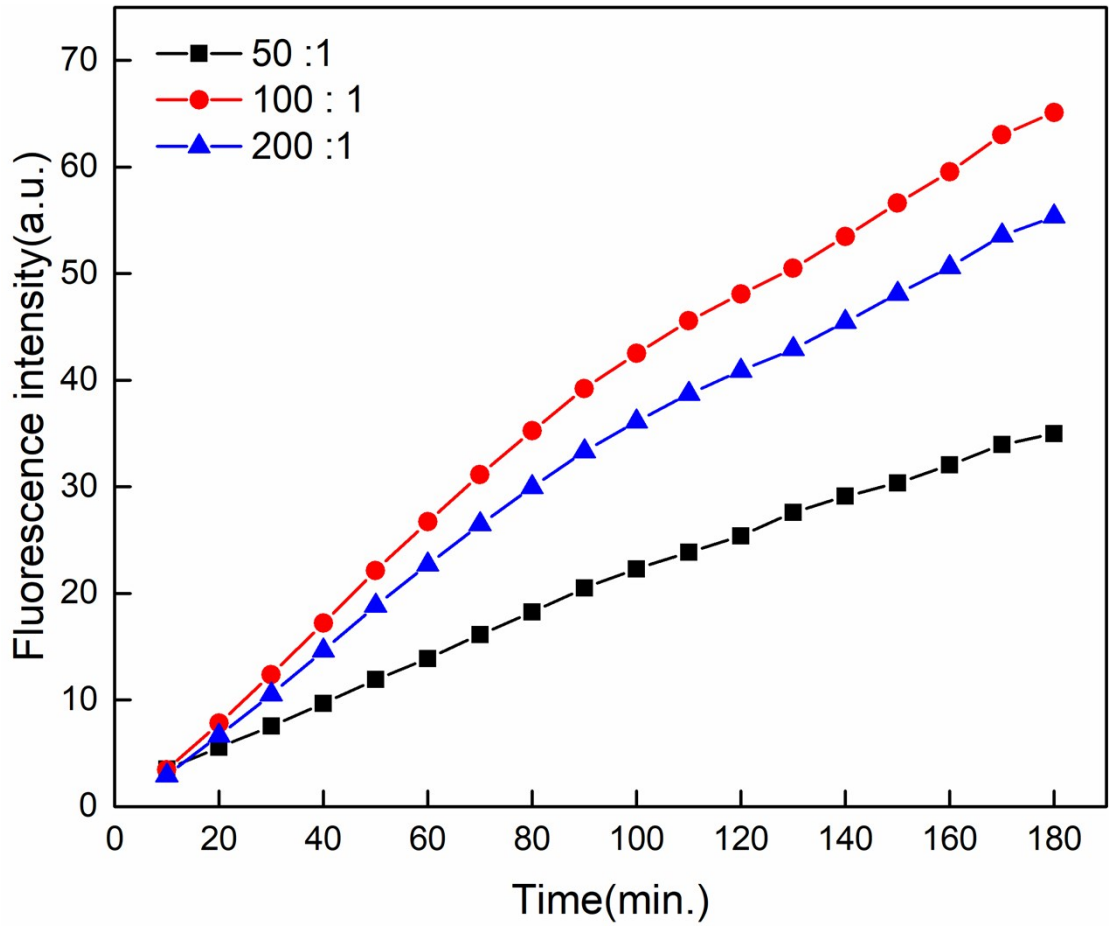

Figure S3 Effect of ratio of substrate to DNAzyme on the DNA nanomachines operation. Different molar ratio between substrate and DNAzyme, including 50:1, 100:1 and 200:1, was applied to investigate. Activated DNA nanomachine without blocker strand was employed in this experiment. The signal have been monitored for 1h.

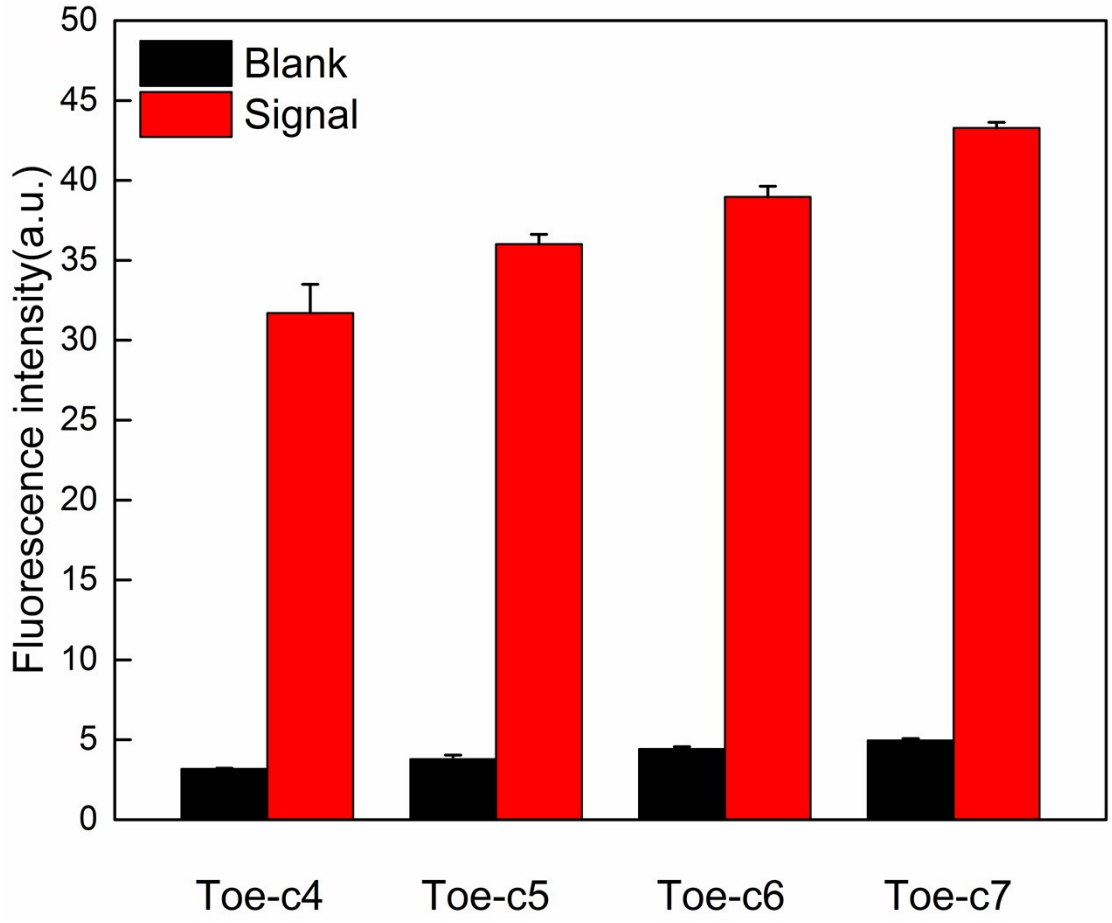

Figure S4 Evaluation of length of toehold effect in BiCCs. Four toeholds, including Toe-c4, c5, c6 and c7, are designed (Table S2). 1 nM PSA was chosen as the recognition target for initiation of BiCCs. After 1 h incubation, end-point of fluorescence values of each group was tested.

Table S5. The tested melting temperatures (Tm) and Free energy (ΔG) values

	Toe-c4 of blocker	Toe-c5 of blocker	Toe-c6 of blocker	Toe-c7 of blocker
Output of HP	ΔG:-28.04 kcal/mole Tm:53.8 ^{0}C	ΔG:-29.38 kcal/mole Tm: 55.4 ^{0}C	ΔG: -30.98 kcal/mole Tm: 57.2 ^{0}C	ΔG: -32.93 kcal/mole Tm: 58.4 ^{0}C
B* of DNAzyme in BiCCs	ΔG:-21.07 kcal/mole Tm: 46.7 ^{0}C	ΔG:-21.07 kcal/mole Tm: 46.7 ^{0}C	ΔG:-21.07 kcal/mole Tm: 46.7 ^{0}C	ΔG:-21.07 kcal/mole Tm: 46.7 ^{0}C

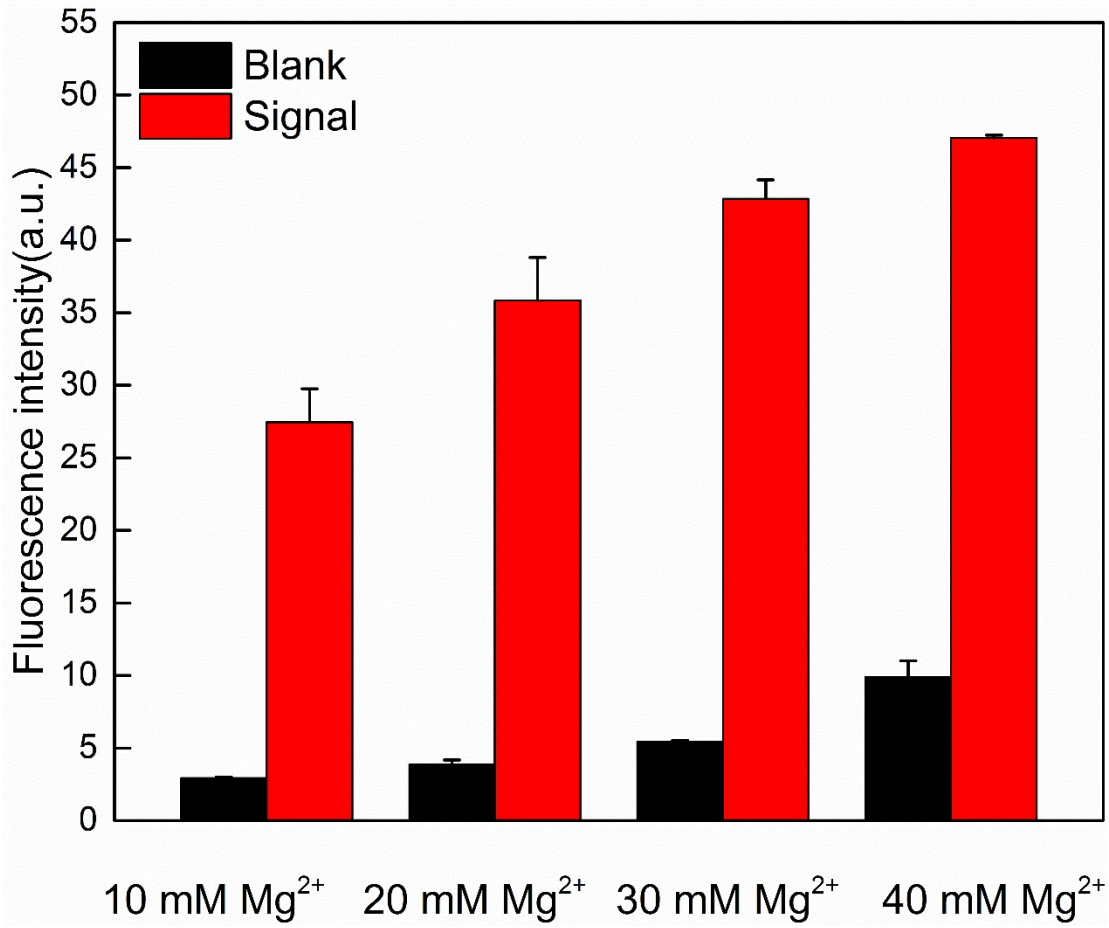

Figure S5 Effect of Mg^{2+} concentration on the folding of split DNAzyme for DNA nanomachine operation in BiCCs. The response of nanomachine operation to 1 nM PSA in the presence of 10–40 mM Mg^{2+}.

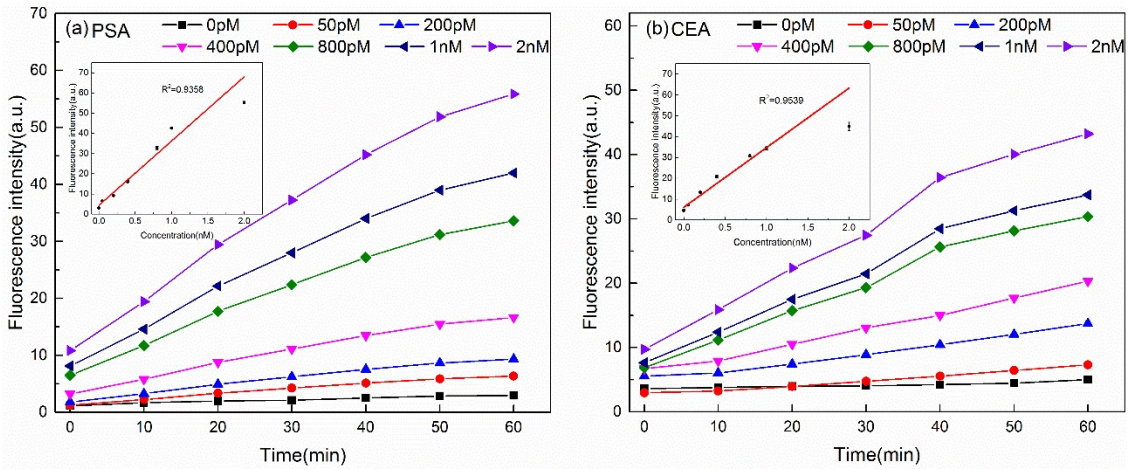

Figure S6 Quantification of conversion efficiency and evaluation of general applicability to BiCCs. **(a)** Real-time monitoring of fluorescence increase of BiCCs in the presence of PSA with varied concentration over a period of 1 h. **(b)** Real-time monitoring of fluorescence increase of BiCCs in the presence of CEA with varied concentration over a period of 1 h. Inset figures show calibration curves using the values at 1 h end points. Error bars represent one standard deviation from triplicate analyses.

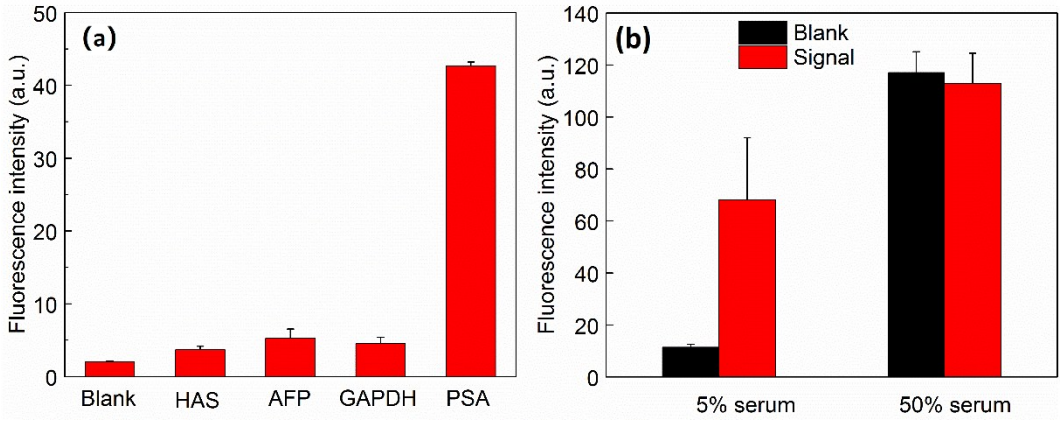

Figure S7 Evaluation of the specificity of the BiCCs and its performance in diluted serum. (a) End-point fluorescence values after 1 h of incubation using 1 nM of PSA and 100 nM of referents. (b) End-point fluorescence values after 1 h of incubation using 1 nM of PSA spiked into 5% and 50% serum, respectively.

Table. S6 Comparison of liver cancer biomarker in healthy people and liver cancer patients

Method	Test group	AFP (ng/ml)	CEA (ng/ml)	CA19-9 (U/L)
BiCCs	Healthy people (n=10)	7.11±0.93	3.21±1.25	8.27±2.60
	liver cancer patients (n=10)	153±75*	55.2±11.4*	62.2±38.5*
ELISA	Healthy people (n=10)	5.92±0.32	2.63±0.98	10.4±3.69
	liver cancer patients (n=10)	171±83.2*	69.6±28.5*	80.3±41.7*

Significance effect at the 0.05 level ($p<0.05$)