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Universal Strategy To Engineer Catalytic DNA Hairpin Assemblies for Protein Analysis

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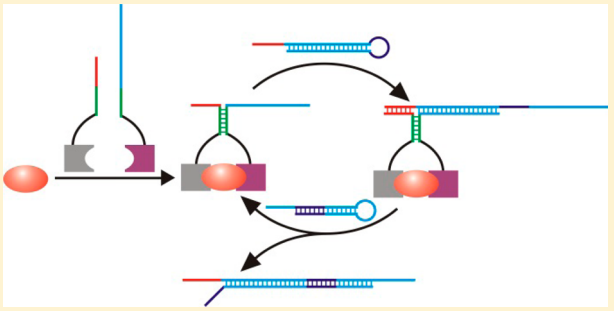
Supporting Information

ABSTRACT: Nucleic acids can be programmed into enzyme-free catalytic DNA circuits (CDCs) to carry out various functions ranging from DNA computing to signal amplifications for biosensing. Catalytic hairpin assembly (CHA), the accelerated hybridization between two DNA hairpins catalyzed by a DNA input, is one of the most widely studied and used CDCs for amplified detection of nucleic acids and small molecules. So far, it is still challenging to expand CHAs to proteins largely due to the lack of a universal strategy to construct protein-responsive CHAs. To address this challenge, we demonstrate that a rationally designed protein−DNA binding complex can be used as an effective catalyst to accelerate CHA reactions. On the basis of this principle, we developed specific CHAs for a number of important protein biomarkers, including human α-thrombin, human prostate specific antigen, and human epidermal growth factor receptor 2. Upon establishing this panel of protein-responsive CHAs, we further explore their potential applications to the detection of specific protein biomarkers from human serum samples and cancer cells.



Recent advances in dynamic DNA nanotechnology have yielded a number of catalytic DNA circuits (CDCs) that are able to execute algorithms for DNA computing and amplify detection signals for molecular diagnostics.[1−6] Catalytic hairpin assembly (CHA), the accelerated hybridization between two DNA hairpins catalyzed by a DNA input, is one of the most widely studied and used CDCs. CHA was originally developed by Pierce, Yin, and co-workers in their seminal work in 2008[7] and has been tailored by Ellington's group to be adaptable to multiple detection methods for analyzing nucleic acids and small molecules.[8] Since then, continuous efforts have been made to improve CHA as an isothermal, homogeneous, and enzyme-free signal generation strategy for amplified detection of biomolecules.[9−15] For example, CHA has been incorporated into various isothermal DNA amplification techniques to facilitate real-time signal generation and amplification.[9−13] Multiple layers of CHA reactions have been successfully stacked to achieve exponential amplification of target nucleic acids.[14] Mismatched base pairs have also been introduced into hairpin substrates to improve signal-to-background ratios of CHA for detecting DNA targets.[15] While extensive studies have been carried out to improve CHA for nucleic-acid analysis, it is still challenging to expand CHA to proteins largely due to the lack of a universal strategy to construct protein-responsive CHA. Herein, we address this challenge by using rationally designed protein−DNA binding complexes to catalyze CHA reactions.

For a typical CHA reaction (Figure 1A), a pair of DNA hairpins (H1 and H2) is designed to be complementary to one another. However, spontaneous hybridization between H1 and H2 is kinetically hindered by caging their complementary regions within the hairpin stems. In the presence of a nucleic acid input, the stem part of H1 is opened through a toehold-mediated strand displacement reaction. The newly exposed single-stranded DNA region within H1 can then hybridize to H2 through a secondary toehold-mediated DNA strand displacement. During the secondary strand displacement, the nucleic acid input will be displaced and be available for catalyzing a next round of hybridization between H1 and H2.

To design a protein-responsive CHA, it is critical to allow an input protein to open DNA hairpin H1 and be released by the second DNA hairpin H2 the same way as a nucleic acid input. To achieve this goal, we split a nucleic acid input that catalyzes CHA between H1 and H2 into two separate parts: a toehold part T and a complementary part C. As a result, the split nucleic acid input loses its catalytic activity to H1 and H2. We then extend T and C with a short complementary sequence B

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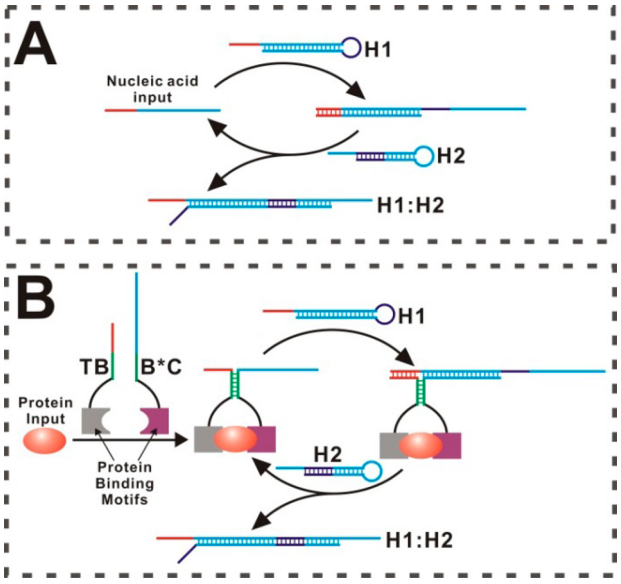

Figure 1. Schematic illustrating the principles of catalytic hairpin assembly (A) and protein-responsive catalytic hairpin assembly (B).

and B* to form DNA probes TB and B*C, respectively (Figure 1B). TB and B*C are then each conjugated with an affinity binding motif that can bind to a same protein input but at different binding epitopes. In the absence of the protein input, TB and B*C do not hybridize to each other at room temperature or elevated temperatures. However, in the presence of the specific protein input, the binding of two affinity motifs to the same protein input brings TB and B*C to close proximity, greatly increasing their local effective concentrations.[16−18] Consequently, TB and B*C hybridize to each other to form a stable TB:B*C duplex. This protein−DNA binding complex can then serve as a catalyst to accelerate the hybridization between H1 and H2 through toehold-mediated DNA strand displacements.

As a proof-of-principle, we first designed a protein-responsive CHA for a clinically relevant protein, human α-thrombin, by using two thrombin-specific aptamers as affinity ligands. We then set out to characterize thrombin-responsive CHA reaction by using native polyacrylamide gel electrophoresis (PAGE) and measuring the ratio (R) between H1:H2 band and H2 band as an indicator for hybridization yield. As shown in Figure 2, the split nucleic acid input (TB, B*C in Lane 5) loses the catalytic activity for CHA, evidenced by the background level of H1:H2

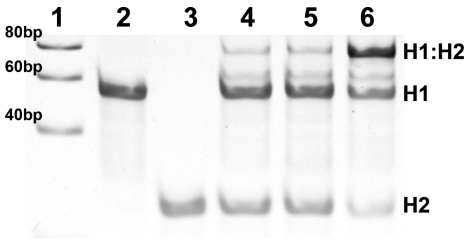

Figure 2. Native PAGE analysis of oligonucleotides from the thrombin-responsive CHA. Lane 1, low molecular weight DNA ladder; Lane 2, 2 μM H1; Lane 3, 1 μM H2; Lane 4, from analysis of a mixture containing 2 μM H1 and 1 μM H2; Lane 5, from analysis of a mixture containing 2 μM H1, 1 μM H2, 50 nM TB, and 50 nM B*C; Lane 6, from analysis of a mixture containing 2 μM H1, 1 μM H2, 50 nM TB, 50 nM B*C, and 25 nM human α-thrombin.

formation (R_{lane5} = 0.29 vs R_{lane4} = 0.22). However, in the presence of 25 nM thrombin, a strong H1:H2 DNA band was shown in PAGE gel (Lane 6) with an R value of 5.66, suggesting that the formation of thrombin-TB:B*C complex can regenerate the catalytic capability of its nucleic acid counterpart to accelerate the CHA reaction.

To quantitatively understand the thrombin-responsive CHA, we further designed a DNA strand displacement beacon FQ that was able to turn on its fluorescence upon the production of H1:H2 duplexes (Figure 3A). The use of FQ allows us to

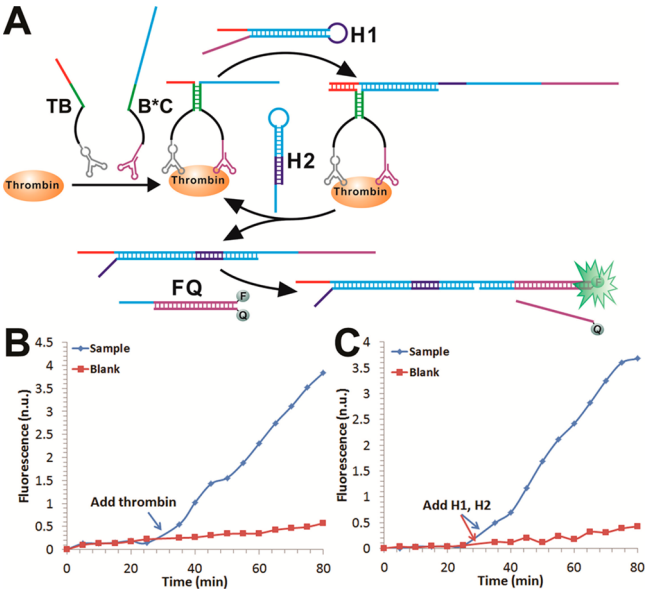

Figure 3. (A) Schematic illustrating the quantitative measurement of thrombin-responsive CHA. Thrombin-specific DNA aptamers are used as affinity binding motifs. In the presence of thrombin, the binding of two aptamers to the same thrombin assembles TB and B*C to form a protein−DNA binding complex. The resulting binding complex catalyzes the subsequent CHA reaction between H1 and H2 and turns on the fluorescence of beacon FQ through toehold-mediated DNA strand displacement. (B) Fluorescence increases as a function of time when 2 nM thrombin molecules were spiked into the reaction mixture. (C) Fluorescence increases as a function of time when H1 and H2 were spiked together into both the sample (blue arrow) that contains 2 nM target thrombin and the blank (red arrow) that contains identical reagents with the sample, but no target protein was added.

monitor the protein-responsive CHA in real-time. As shown in Figure 3B, immediately after spiking 2 nM thrombin into a solution containing 10 nM TB, 10 nM B*C, 50 nM H1, 50 nM H2, and 50 nM FQ, a distinguishable fluorescence increase is observed (sample) from the blank and keeps increasing over the period of measurement. Compared to a number of other human serum proteins, the fluorescence increase was observed only when thrombin was mixed with DNA probes (Figure S1), suggesting this CHA system is thrombin specific. Furthermore, no fluorescence increase was observed until H1 and H2 were added into the reaction mixture (Figure 3C), confirming that a thrombin-dependent fluorescence increase resulted from the CHA.

When comparing thrombin-responsive CHA to its DNA counterpart (Figure S2A), we found that thrombin-responsive CHA displayed a much lower catalytic efficiency (characterized as an amplification factor, AF, where AF = [H1:H2]/

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[Intermediate], Figure S2) after reacting over a period of 2.5 h (AF = 3.1 for thrombin-CHA vs AF = 17.9 for DNA-CHA, Figure S2B). Because the assembly of the split DNA probes into an intact catalytic DNA input is achieved through affinity bindings among thrombin and two aptamers, we suspect that the catalytic efficiency of thrombin-responsive CHA is largely limited by this affinity binding step. This hypothesis was further confirmed by designing and characterizing a streptavidin-responsive CHA that is of the same TB and B*C sequences but uses biotin as affinity ligand (Figure S3). The strong affinity binding between streptavidin and biotin ($K_d \sim 10^{-14}$ M) minimizes the influence from protein binding component, and an amplification factor of 14.8 was achieved after reacting at 37 °C for 2.5 h.

Unlike the streptavidin−biotin interaction, most protein−ligand interactions are of weaker binding affinities. To ensure the versatility of our strategy, it is critical to develop a general strategy to enhance the catalytic performance of protein-responsive CHA. To achieve this goal, we carefully examined complementary sequences of DNA probes TB and B*C for thrombin-responsive CHA. Our rationale is that increasing complementary sequences of DNA probes may create cooperative interactions between affinity bindings and DNA hybridization and thus stabilize the protein−DNA binding complex, which may eventually increase the amplification efficiency of CHA. Indeed, when increasing lengths of complementary sequences of TB and B*C from 6 to 9 nt, the free energy of the binding-induced DNA complexes were estimated to increase from −2.58 to −6.69 kcal/mol (Table S2), and AFs of the thrombin-responsive CHA increased from 2.1 to 9.4 (Figure 4A−C). Further increasing the complementary length of DNA probes to 10 nt resulted in significant high background in the blank (Figure 4B). With this optimized probe design, we were able to achieve the same level of amplification for thrombin with varying concentrations from 20 pM to 2 nM (Figure 4D). Using the same CHA system, we could also determine thrombin from 10-fold diluted human serum samples homogeneously (Figure S4). A detection limit of 100 pM was achieved.

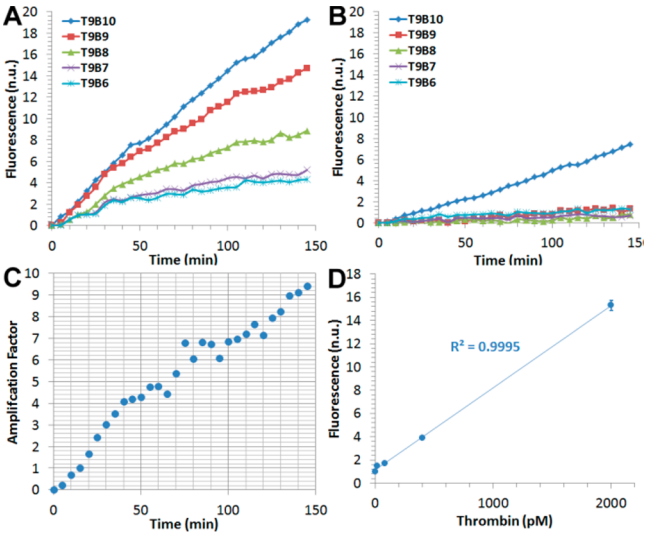

Figure 4. (A,B) Tuning the complementary lengths of DNA probes TB and B*C to achieve sufficient catalytic efficiency for thrombin-responsive CHA. Fluorescence increases as a function of time when tuning the complementary lengths from 6 to 10 nt with (A) or without (B) target thrombin. (C) Measured amplification factors (AF) as a function of time for thrombin-responsive CHA that makes use of T9B9 as a DNA probe. AF at each time point is calculated using the same equation as shown in Figure S3. (D) Increases in fluorescence as a function of concentrations of thrombin. End-point fluorescence was measured after incubating the reaction mixture for 150 min. Error bars represent one standard deviation from duplicated analyses.

Having established protein-responsive CHA using thrombin and streptavidin, we aim to further generalize this strategy by using antibodies as affinity motifs. Because the availability of two or more antibodies binding to the same target protein has been demonstrated by the widely used sandwiched immunoassays and proximity ligation assays,[19,20] CHA with the same designs can in principle be used for any protein target by simply switching a pair of target-specific antibodies. To demonstrate the versatility of our strategy, we developed two antibody-based CHA systems for two important cancer biomarkers, human prostate specific antigen (PSA) and human epidermal growth factor receptor 2 (HER2). As shown in Figure 5A, PSA or HER2-specific polyclonal antibodies are conjugated to DNA probes TB and B*C through streptavidin−biotin interactions.

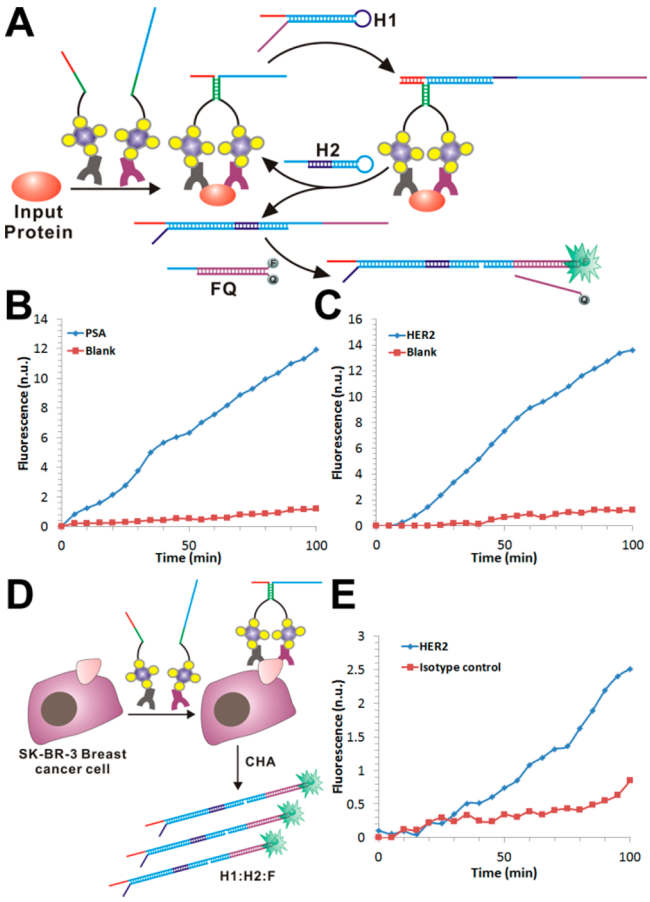

Figure 5. (A) Schematic illustrating the principle of protein-responsive CHA that is constructed using antibodies as affinity binding motifs. (B) Fluorescence increases as a function of time when mixing 2 nM PSA with PSA-responsive CHA. (C) Fluorescence increases as a function of time when mixing 2 nM HER2 with HER2-responsive CHA. (D) Principles to determine HER2 from SK-BR-3 breast cancer cells using HER2-responsive CHA. (E) Fluorescence increases as a function of time when mixing fixed SK-BR-3 cells with HER2-responsive CHA and the isotype control.

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All DNA sequences and reaction conditions were kept the same as for optimized thrombin-responsive CHA.

Figure 5B shows the fluorescence signal increase of the PSA-responsive CHA as a function of time. Within a period of 100 min, fluorescence intensities from 2 nM PSA are readily distinguishable from the blank that contained all reagents but not the target PSA. A similar kinetic profile was also observed for HER2-specific CHA, shown in Figure 5C. AFs were determined to be over 10 for both protein-responsive CHA systems, suggesting that CHA could be easily adapted to different target proteins through antibody−antigen interactions to achieve efficient signal amplifications.

Once protein-responsive CHA using antibodies is established, we explore the potential applications of this system to the determination of specific protein biomarkers directly from cancer cells. Unlike conventional immunostaining strategies that are suitable for cell imaging applications, protein-responsive CHA is able to convert an *in situ* binding event into the release of multiple fluorescently labeled H1:H2 products in homogeneous solutions through CHA (Figure 5D). Therefore, specific cell markers can potentially be determined by simply measuring fluorescence increases from cell-culture plates (e.g., 96-well microplates). To demonstrate this potential application, we carried out a preliminary experiment by using HER2-responsive CHA to determine HER2 overexpression from a human breast cancer cell line, SK-BR-3.

As shown in Figure 5E, when mixing HER2-responsive CHA with SK-BR-3 cells that were cultured and fixed in a 96-well microplate well, fluorescence signal increases as a function of time. After 30 min, fluorescence increases from HER2-responsive CHA are readily distinguishable from an isotype control that contained all reagents but replaced HER2 specific polyclonal antibodies with nonspecific goat IgGs. To further confirm that the fluorescence increases are due to HER2 specific interactions, we incubated SK-BR-3 cells that were cultured and fixed in parallel using the same biotinylated HER2-specific polyclonal antibodies (Figure S5A) and non-specific goat IgGs (Figure S5B) and stained with fluorescein isothiocyanate (FITC) labeled streptavidin. Only the specific interaction between HER2 and HER2-specific antibody produces fluorescence at the cell membranes (Figure S5A), whereas little fluorescence was observed for the isotype control group (Figure S5B). The consistency between immunostaining results and those of HER2-responsive CHA confirms that our strategy has the potential to be tailored into an assay to determine specific cellular proteins.

In conclusion, we have successfully developed a universal strategy to construct protein-responsive CHA systems. Our strategy is highly versatile and robust, is compatible with a wide range of affinity interactions, and can easily be tailored for many practical applications, such as determination of serum and cellular proteins. Our success in developing protein-responsive CHA opens up opportunities to construct various protein-responsive catalytic DNA circuits and DNA nanodevices for potential applications ranging from DNA computation to point-of-care disease diagnostics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02504.

Experimental details, Supporting Table, Supporting Figures S1 to S5 (PDF)

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Notes

The authors declare no competing financial interest.

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