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Proximity-induced exponential amplification reaction triggered by proteins and small molecules†

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We proposed a method to regulate nucleic acid polymerization by proximity and designed an ultrasensitive biosensor based on proximity-induced exponential amplification reaction for proximity assay of proteins (streptavidin) and small molecules (adenosine triphosphate), which allows us to detect a variety of interesting targets by simply changing the binding sites of DNA.

Cells regulate the rate of various biochemical reactions by increasing or decreasing the effective concentration of reactants, rather than simply being composed of homogeneous molecular interactions.[1] The effective molar concentration may differ by several orders of magnitude from the concentration of the bulk solution, which usually leads to a sharp change in the contact rate and binding affinity between the substances.[2,3] The rapid development of DNA assembly technology has made impressive achievements in DNA computing,[4–6] dynamical networks,[7–10] synthetic protocells,[11,12] receptor function regulation,[13] bio-chemical detection and imaging,[14–16] *etc.*

Inspired by nature, scientists have successfully developed the proximity-induced nucleic acid reaction by increasing the effective concentration. The nucleic acid amplification is an important signal amplification method[17–19] combined with nucleic acid assembly technology, proximity-induced nucleic acid reaction is widely used in non-nucleic acid target biological analysis,[1,20,21] imaging protein[22] and regulation of DNA nanostructure.[23,24] It greatly broadens the application range of nucleic acid technology, and has proven to be incredibly valuable for molecular biology research.

Among all kinds of proximity-induced nucleic acid reactions, it reported rarely that enzyme catalysed isothermal nucleic acid amplification reaction. At first, we verified the feasibility of regulating nucleic acid polymerization by proximity. On this basis, we applied the principle of proximity inducing to exponential amplification reaction (EXPAR),[25–27] which can complement the nucleic acid assembly reaction of proximity induced and promote the application range of this mechanism.

In this study, the exponential amplification reaction (EXPAR) sensor based on proximity regulation can detect two different kinds of non-nucleic acid targets, such as protein (streptavidin) and small molecule (adenosine triphosphate) which are completely different in nature, indicating that our method has extensive universality. This rapid, one-step, label-free, isothermal and ultrasensitive detection has great application potential in point-of-care analysis, studies of molecular interactions, and high-throughput screening.

In Fig. 1A, we provided a paradigm for regulation of nucleic acid polymerization using objects of interest. As a model system, we applied the principle of proximity inducing to EXPAR. Based on proximity-induced EXPAR, we designed a biosensor to demonstrate how objects of interest binding exert exquisite spatiotemporal control over EXPAR. As Fig. 1B shown, only by changing the recognition unit of DNA probe, could such a biosensor be suitable for the detection of two different kinds of non-nucleic acid targets. Fig. 1C displayed that the biosensor could detect objects of interest through the final product of G-quadruplex (it can combine with Thioflavin T (ThT) to produce specific fluorescence[28]). The detection limits were 2.9 fM for SA and 31.3 fM for ATP, which were lower compared to previous approaches (Tables S3 and S4, ESI†). The principle of proximity-induced exponential amplification reaction is shown in Fig. 1D. Two DNA strands stably assemble to initiate the EXPAR after binding the target, one as a primer and the other as a template (the red part of sequence is the recognition site of endonuclease). There are two kinds of products from EXPAR. One is the

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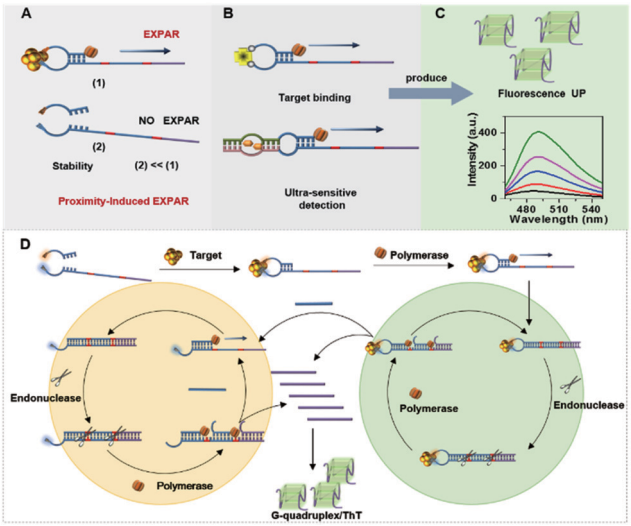

Fig. 1 Schematic illustration of (A) Proximity-induced exponential amplification reaction; (B) principle of the biosensor based on proximity-induced exponential amplification reaction; (C) detection without labelling was realized through the G-quadruplex. (D) The details of nucleic acid amplification process for ultra-sensitive detection by proximity-induced exponential amplification reaction.

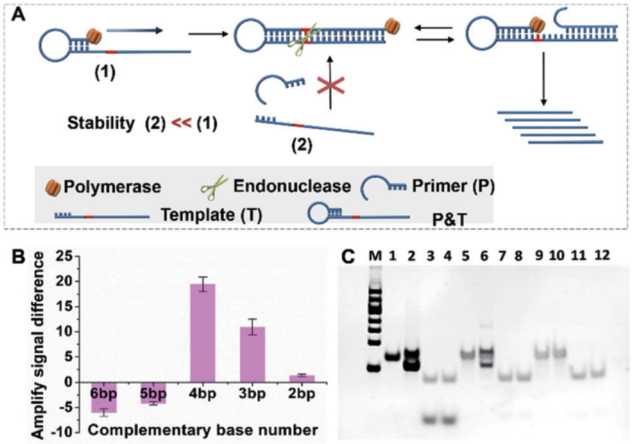

Fig. 2 The selection of suitable complementary base number between primer and template to regulate the nucleic acid polymerization by proximity induction. (A) Schematic diagram of optimization process. (B) The effect of complementary base number on nucleic acid amplification reaction. (C) PAGE analysis of nucleic acid amplification results of primers and templates with different complementary bases. Lane M, DNA ladder; lane 1, 1 μM 4bp without enzyme; lane 2, 1 μM 4bp with enzyme; lane 3, 1 μM 4bp-a and 4bp-b without enzyme; lane 4, 1 μM 4bp-a and 4bp-b with enzyme; lane 5, 1 μM 3bp without enzyme; lane 6, 1 μM 3bp with enzyme; lane 7, 1 μM 3bp-a and 3bp-b without enzyme; lane 8, 1 μM 3bp-a and 3bp-b without enzyme; lane 9, 1 μM 2bp without enzyme; lane 10, 1 μM 2bp without enzyme; lane 11, 1 μM 2bp-a and 2bp-b with enzyme; lane 12, 1 μM 2bp-a and 2bp-b without enzyme.

blue sequence that can be complementary with the excessive templates (not coupled to the target) to act as a primer, because its complementary base is longer than the primer sequence and can hybridize stably without target binding. The other is the G-rich purple sequence, which can combine with ThT to produce specific fluorescence.

In many excellent proximity assays, researchers usually considered the DNA loops as a mimic of the probe-target complexes.[20,29] We also used the hairpin DNA to simulate the binding of the recognition units of primer and template on one target. In order to make the assembly in a metastable state, that is, only under the condition of proximity induction can the assembly take place, we optimized the appropriate complementary base number. As shown in Fig. 2A, we constructed a simple strand displacement amplification (SDA) to explore the effect of complementary base number on nucleic acid polymerization. When the base number is too large, the primer can assemble with the template stably without the participation of target recognition process. When the base number is too small, even if the primer combined with the template by binging on one target, it may not initiate SDA reaction. Only when the complementary base number is suitable, the reaction is in a critical state.

The fluorescence intensity of Super GelRed in the system can reflect the amount of nucleic acid. The amplify signal difference in Fig. 2B was the difference between the fluorescence increment of probe–target complex mimic and that of split primer and template. It can be seen from Fig. 2B that when complementary base number were 6, 5 and 2 bp, there was almost no difference, indicating that the formation of probe–target complex has no effect on nucleic acid polymerization. The fluorescence increment of 4 bp was the largest, which indicated that 4 bp could realize the metastable state. As a result, we achieved the purpose of regulating nucleic acid polymerization through proximity induction. The simulation of DNA folding also supports our experimental results (Table S2 and Fig. S1, ESI†).

We showed the results more intuitively by polyacrylamide gel electrophoresis. As shown in 1–4 swim lanes of Fig. 2C, when the complementary number of the separated templates and primers was 4 bp, no new bands were found, indicating that there was almost no reaction. However, the hairpin of the simulated probe–target complex had an obvious new band after the enzyme participated in the reaction, which indicated that the SDA occurred after the proximity induction. When the complementary base number was 3 bp, the same result could be seen, but the new band was much shallower than that of 4 bp. The experimental results showed that when the complementary base number was 3 bp, polymerization would occur, but this length might be not conducive to enzyme binding. When the complementary number was 2 bp, there was no new band in the lane under any circumstances. In general, the electrophoretic results was also consistent with the fluorescence results. It was further demonstrated that 4 bp was the most suitable complementary number, which could achieve our regulation purpose.

To verify that the sensor based on the proximity-induced EXPAR can detect proteins, we employed streptavidin as a representative target for ultrasensitive assay. The reasons for choosing streptavidin as representative are as follows: (1) it has a high binding affinity with biotin ($K_d = 10^{-14}$ M).[21] This strong

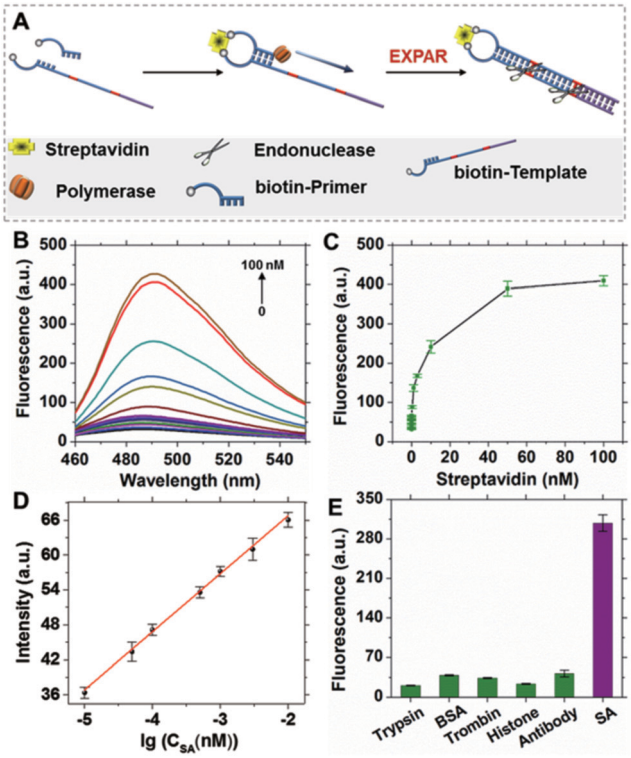

Fig. 3 (A) Exponential amplification reaction triggered by streptavidin. (B) Fluorescence spectra of streptavidin sensing system with different contents (0–100 nM). (C) Working curve of the relationship between 485 nm fluorescence intensity and streptavidin content (0–100 nM). (D) Linear fitting between fluorescence intensity at 485 nm and logarithm of the streptavidin content. (E) Selectivity of the exponential amplification reaction triggered by streptavidin.

interaction ensures the proximity-induced DNA assembly performance. (2) As streptavidin has a wide range of molecular biological applications, the assay of streptavidin can be a representative of protein detection. The electrophoretic experiment proved the feasibility of the reaction Fig. S2A (ESI†). As shown in Fig. 3A, when streptavidin is present in the system, biotin modified sequences will recognize streptavidin at the same time, and then stable DNA assembly will occur under its induction. On this basis, an EXPAR occurs, which produces a large number of G-quadruplex that specifically binds to ThT, producing fluorescence signal (the detailed amplification process shows in Fig. 1D). By the way, EXPAR is often accompanied by large background interference because of non-specific DNA amplification and fluorescence response of SYBR Green I. In our work, only the G-rich sequence could produce fluorescence, so the background was weak. It has also been proved by our previous work.[28]

We first optimized the reaction conditions (Fig. S3, ESI†). The content of SA in the system was determined under the optimum reaction conditions. As shown in Fig. 3B and C, with the increase of SA concentration, the fluorescence intensity of the sensing system gradually increased (0–100 nM). When the concentration of SA was in the range of 0–10 pm, the spectral curve was shown in the Fig. S6A (ESI†). As Fig. 3D shown, in the concentration ranges (10 fM–10 pM), we got a good linear fitting results, $Y = 9.9642\ X + 86.6820$, $R^2 = 0.9963$ (Y is the fluorescence intensity and X is the logarithm of SA concentration). Compared to previous approaches for SA activity detection (Table S3, ESI†), a lower detection limit (2.9 fM) was calculated, based on 3 times standard deviation over the blank response (3δ/slope).

We further studied detection capability for SA in the presence of other proteins such as BSA, trypsin, thrombin, histone and antibody to evaluate the specificity of the sensor. As shown in Fig. 3E, although other protein concentrations (100 μg mL^{-1}) were higher than SA (1 μg mL^{-1}), the fluorescence intensity of SA was stronger than others. The double recognitions between SA and biotin contributed greatly to the selectivity of the reaction.

With adenosine triphosphate (ATP) as the model to represent small molecules, we verified that the proximity-induced EXPAR has the ability to detect small molecules. The reasons for choosing ATP as the representative are as follows: (1) although the complete aptamer is split into two parts, the binding affinity between ATP and target still exists, and the specificity is improved. This ensures that DNA can successfully assemble through binding induction.[30] (2) ATP is one of the most common substances in biological detection, which can be a representative of biological small molecule detection.[31,32] The electrophoretic experiment proved the feasibility of the reaction Fig. S2B (ESI†).

In the presence of ATP in the system, as shown in Fig. 4A, the primer sequence and template sequence linked with ATP split aptamers could recognize ATP cooperatively, inducing stable DNA assembly. ATP aptamer combined with ThT would have strong fluorescence signal (as shown in Fig. S4, ESI†) which was selected as the baseline. Our fluorescence intensity was the increment of fluorescence obtained by subtracting the background signal measured before the reaction starts. We first optimized the reaction conditions (Fig. S5, ESI†). As shown in Fig. 4B and C, the fluorescence signal of the detection system gradually increased with the increase of ATP concentration (0–300 nM). The fluorescence spectral curve of low concentration is shown in Fig. S6B (ESI†). As shown in Fig. 4D, in the concentration range (100 fM–30 pM), we got a good linear fitting result, and the linear fitting equation was $Y = 10.1432\ X + 43.8203$, $R^2 = 0.9941$(Y is the fluorescence intensity and X is the logarithm of ATP concentration). Compared to previous approaches for ATP content detection (Table S4, ESI†), a lower detection limit (31.3 fM) was calculated (based on 3δ/slope). In order to evaluate the selectivity to ATP, we used UTP CTP, and GTP to conduct control experiments. The concentrations of UTP, CTP and GTP were 100 mM respectively, while the concentration of ATP was 1 mM. As shown in Fig. 4E, it could be seen that the reaction system containing ATP had a strong signal, while the signal changed caused by the other three analogues were weak, showing good selectivity.

In this work, we have conclusively demonstrated the potential of the proximity to regulate nucleic acid polymerization and designed a proximity-induced EXPAR to build biosensors for SA

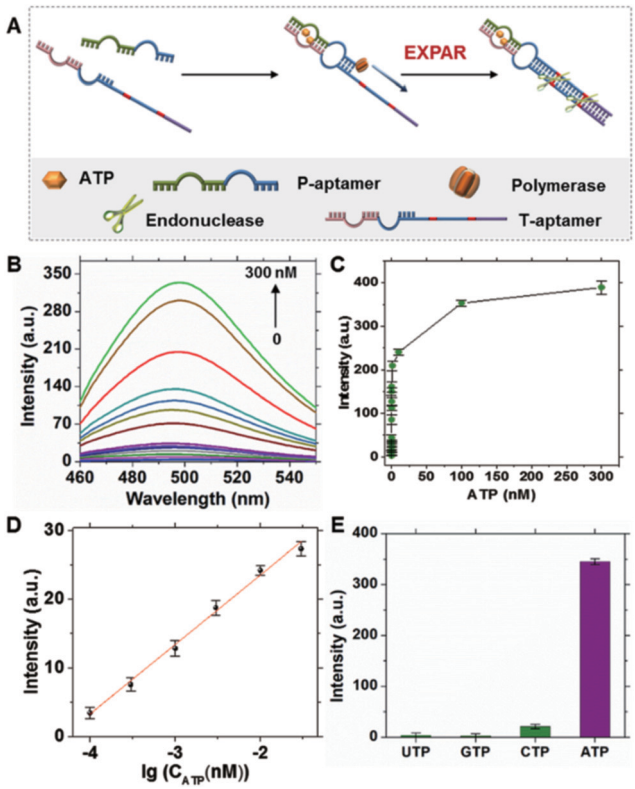

Fig. 4 (A) Exponential amplification reaction triggered by ATP. (B) Fluorescence spectra of ATP sensing system with different contents (0–300 nM). (C) Working curve of the relation-ship between 485 nm fluorescence intensity and ATP content (0–300 nM). (D) Linear fitting between fluorescence intensity at 485 nm and logarithm of the ATP content. (E) Selectivity of the exponential amplification reaction triggered by ATP.

and ATP detection. Obviously, only when the two probes combine with the target at the same time, can the EXPAR take place, which enriches the scope of application of the principle of proximity regulation reaction. On this basis, by changing the probe recognition units, we successfully applied this system to the detection of protein (SA) and small molecules (ATP), two substances having completely different properties, indicating that our reaction system has extensive universality. All experimental data pave the way for understanding the interaction between controlled nucleic acid assembly and nucleic acid polymerization. They also provide an avenue for the design of sensors/biosensors. Combined with the flexibility of DNA assembly and the simplicity of one-step isothermal nucleic acid amplification, the proximity-induced exponential amplification reaction should have great practical potential in a variety of future environments, including medical diagnosis, biological research and point-of-care testing.

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Conflicts of interest

There are no conflicts to declare.

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