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A target-triggered exponential amplification-based DNAzyme biosensor for ultrasensitive detection of folate receptors†

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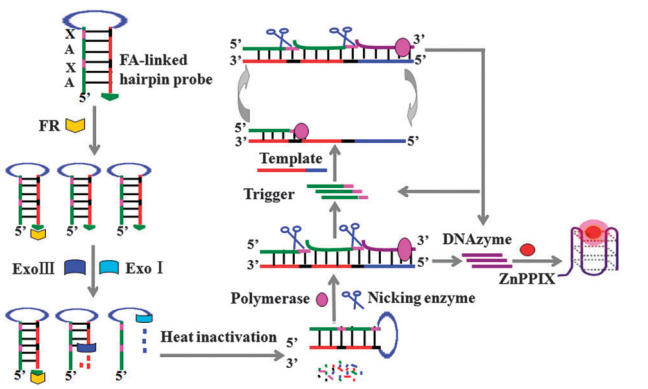
We develop a new method for ultrasensitive detection of folate receptors (FRs) using a target-triggered isothermally exponential amplification reaction (EXPAR)-based DNAzyme biosensor. This method exhibits excellent specificity and high sensitivity with a detection limit as low as 0.23 fM and a large dynamic range of 6 orders of magnitude from 1 fM to 1 nM. It might be further applied for the detection of various small molecule-binding proteins by simply changing the linked small molecule moiety of the hairpin probes.

Folate receptors (FRs), glycosylphosphatidylinositol (GPI)-anchored cell surface glycoproteins with a strong binding affinity for folate ($K_d \sim 10^{-10}$ M), play an essential role in cell growth and development.[1,2] FRs are over-expressed in the vast majority of cancers, but are absent in the healthy tissues except for choroid plexus, placenta, lungs and kidneys,[3,4] making them highly selective tumor markers. Due to the high affinity of FRs for folic acid (FA), the FA/FR interaction has been widely employed in bioimaging and therapeutic applications.[5–7] Moreover, the expression level of FRs can be used to evaluate the evolution of malignancy and specific disease states.[8] Therefore, the accurate quantification of FRs is of great importance in both clinical diagnosis and drug development.

The conventional methods used for the detection of small molecules (*e.g.* FA) and their protein receptors (*e.g.* FR) include fluorescence resonant energy transfer (FRET),[9] affinity chromatography,[10] kinetic capillary electrophoresis,[11] fluorescence anisotropy[12] and surface plasmon resonance (SPR).[13,14] However, these methods usually suffer from poor sensitivity, susceptibility to nonspecific interactions and the involvement of surface-based molecular immobilization. Recently, the small-molecule-linked DNA has been employed to monitor the interaction between small organic molecules and their protein receptors.[15–17] The small-molecule-linked DNA can be randomly coded with the capability of selectively capturing the target proteins, facilitating the signal transduction and amplification.[15–17] Moreover, binding of small-molecule-linked DNA with the target proteins can protect DNA from digestion by exonuclease I.[17–19] Nevertheless, in the reported FR assay the small-molecule-linked DNA usually works with the electrochemical method with the involvement of complicated electrode modifications and poor sensitivity.[17,18] The development of a simple and sensitive method for FR assay still remains a great challenge.

Here, we develop a new fluorescence method for ultrasensitive detection of FRs using the FA-linked hairpin probe in combination with the isothermally exponential amplification reaction (EXPAR) (Scheme 1). The EXPAR can proceed at a constant temperature with high amplification efficiency and rapid amplification kinetics (10^6–10^9-fold amplification within minutes),[20] and has been successfully applied for sensitive detection of DNA, RNA and proteins.[20–23] In conventional EXPAR assay, SYBR Green I is usually used as the label, but its preferential binding to the GC-rich sequences limits its further applications in multiplex PCR assay.[24] In addition, SYBR Green I might not only adversely inhibit the PCR reaction in a concentration-dependent manner, but also promote the nonspecific amplification.[25–27] To overcome



Scheme 1 Schematic illustration of a target-triggered exponential amplification-based DNAzyme biosensor for FR assay.

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the limitations of SYBR Green I, we turn to a novel fluorophore, Zinc(II)-Protoporphyrin IX (ZnPPIX).[28] ZnPPIX can bind to the G-quadruplex to form a supramolecular ZnPPIX/G-quadruplex structure.[28] The ZnPPIX/G-quadruplex structures have the advantages such as excellent photostability, nontoxicity, biocompatibility and a low background signal, and have been applied for the detection of DNA,[28] adenosine-5-triphosphate (ATP),[28] telomerase activity[28] and metal ions.[29]

As shown in Scheme 1, the FA-linked hairpin probe has two complementary sequences at two ends which can be self-assembled into a hairpin structure. Region X in the stem of the hairpin probe is the recognition sequence of DNA endonuclease. Region A in the stem can initiate signal transduction and DNA amplification when the recognition site (region X) is nicked by the nicking enzyme upon the formation of DNA duplexes. The loop region has a sequence complementary to the functional DNAzyme which can react with ZnPPIX to induce an enhanced fluorescence signal. The 3′ terminal of the hairpin probe is covalently conjugated with a FA molecule which can specifically bind with an FR. In the presence of an FR, the binding of the FR with FA can prevent the hairpin probes from being digested by Exo III and Exo I, and the hairpin probes retain the stem-loop structure. With the addition of polymerase and endonuclease, the recognition sites (region X) in the stem are nicked by the nicking enzyme, generating two new replication sites for the polymerase. Region A can subsequently function as a primer to initiate the strand displacement amplification (SDA), producing a large number of region A and functional DNAzymes. With the addition of new templates, the linear SDA reaction can be turned into an exponential amplification with the generated region A as the trigger for the EXPAR. The new template contains two repeat complementary sequences of region A, two repeat complementary sequences of region X and a loop region (see ESI,† Table S1). The generated region A can bind with the new templates to initiate the cycles of polymerization, nicking and displacement, leading to an exponential amplification and consequently the generation of a large number of DNAzymes and DNA triggers (region A).

The process of the FR-induced EXPAR can be monitored in real time and the amplification products can be confirmed by non-denaturating polyacrylamide gel electrophoresis (PAGE) (see ESI,† Fig. S1). The resultant DNAzyme, a kind of G-rich oligonucleotide, can bind with ZnPPIX fluorophores to form the supramolecular ZnPPIX/G-quadruplex structure.[28] The ZnPPIX/G-quadruplex structure displays fluorescence emission at 590 nm upon excitation at 420 nm,[28] which can be used as a fluorescent label for FR assay. It should be noted that no extra primer is needed to initiate the amplification reaction in this assay. While in the absence of FRs, the FA-linked hairpin probes are completely digested by Exo III and Exo I, resulting in neither the initiation of EXPAR nor the formation of ZnPPIX/G-quadruplex structures. Consequently, no significant fluorescence signal is observed in the absence of FRs.

To investigate the sensitivity of the proposed method, we measured FRs at various concentrations under optimal conditions (see ESI,† Fig. S2). As shown in Fig. 1A, the fluorescence intensity

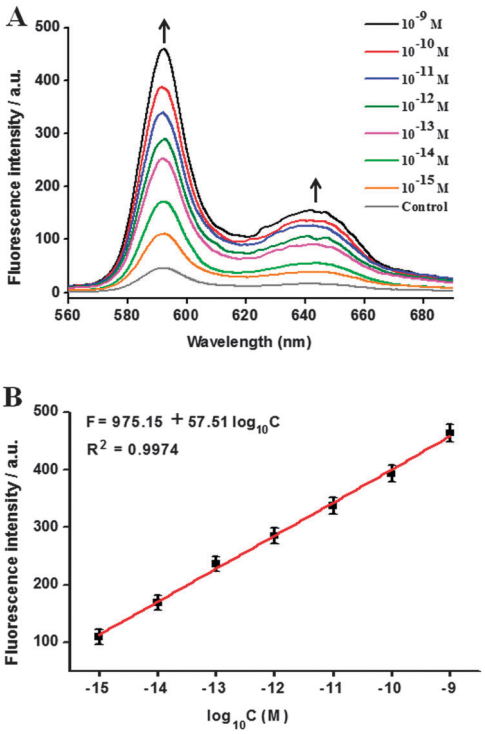

Fig. 1 (A) Fluorescence spectra in response to different concentrations of FRs. (B) Linear relationship between the fluorescence intensity at 590 nm and the logarithm of FR concentration. Error bars show the standard deviations of three experiments.

increases with the increasing concentration of FRs. In the logarithmic scales, the fluorescence intensity at 590 nm exhibits a linear correlation with the concentration of FRs over a range of 6 orders of magnitude from 1 fM to 1 nM (Fig. 1B). The regression equation is $F = 975.15 + 57.51 \log_{10} C$ with a correlation coefficient of 0.9974, where F and C are the fluorescence intensity at 590 nm and the concentration of FRs (molar), respectively. The detection limit was calculated to be 0.23 fM by evaluating the average signal of blank plus three times standard deviation. Notably, the sensitivity of the proposed method has improved by as much as 4 orders of magnitude as compared with that of the carbon nanotube-based electrochemical method (3 pM),[17] and 6 orders of magnitude as compared with that of the G-quadruplex-quinaldine red-based fluorescence method (0.3 nM).[19] The improved sensitivity might be attributed to the high amplification efficiency of EXPAR (10^6–10^9-fold amplification)[20] and the enhanced signal of ZnPPIX/G-quadruplex structure-induced fluorescence.[28]

To investigate the selectivity of the proposed method, we measured some irrelevant proteins, such as thrombin, BSA and IgG, which cannot specifically bind to FA. In the presence of these nonspecific proteins, the hairpin probes will be completely digested by Exo III and Exo I due to lack of protection, leading to neither the initiation of EXPAR nor the formation of ZnPPIX/G-quadruplex structures. As expected, a significant fluorescence enhancement is observed only in the presence of FRs, while no distinct fluorescence signal is observed in the presence of

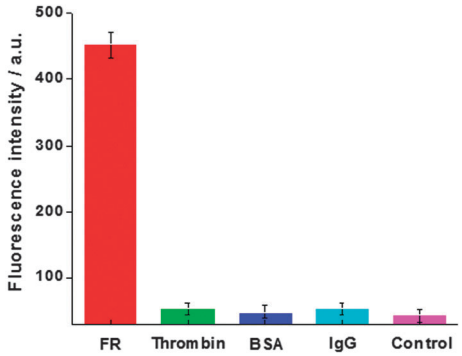

Fig. 2 Specificity of the proposed method for FR assay. The samples tested are 1 nM FR, 1 μM thrombin, 1 μM BSA, 1 μM IgG, and a negative control without any proteins. Error bars show the standard deviations of three experiments.

Table 1 Detection of FRs in the human serum samples

Sample	Added FR concentration	Measured FR concentration	Recovery ratio (%)	Relative error (%)
1	1.00 nM	0.96 nM	96.0	3.80
2	10.00 pM	9.65 pM	96.5	3.50
3	100.00 fM	96.67 fM	96.7	3.33
4	1.00 fM	0.95 fM	95.0	4.72

nonspecific proteins including thrombin, BSA and IgG even at an extremely high concentration of 1 μM (Fig. 2). These results clearly demonstrate the high specificity of the proposed method for FR assay.

Because FRs are absent in normal human serum, their abnormal level in blood can be used to indicate the occurrence of diseases.[18] To evaluate the feasibility of the proposed method for real sample analysis, we spiked different-concentrations of FRs in the normal human serum and then measured the recovery ratio. As shown in Table 1, the measured recovery ratios are more than 95% with a relative error of less than 5% (n = 3). These results demonstrate that the proposed method can sensitively detect FRs in the human serum samples.

We further employed the proposed method to measure FRs in total membrane proteins extracted from HeLa cells (human cervical carcinoma cell line) and A549 cells (human lung adenocarcinoma cell line). As shown in Fig. 3A, a distinct fluorescence signal is detected in HeLa cells, but no significant fluorescence signal is observed in either A549 cells or the control group with the extraction buffer only, consistent with the over-expression of FRs in HeLa cells and the negligible expression of FRs in A549 cells.[30,31] Moreover, the fluorescence intensity increases with the increasing amount of total membrane proteins extracted from HeLa cells, with a linear correlation in the range from 5 μg to 200 μg (Fig. 3B). The regression equation is $F = 198 + 0.56\,C$ with a correlation coefficient of 0.9618, where F and C are the fluorescence intensity at 590 nm and the amount of total membrane proteins (μg), respectively. These results clearly demonstrate that the proposed method can be used to measure FRs in different kinds of cells with high specificity.

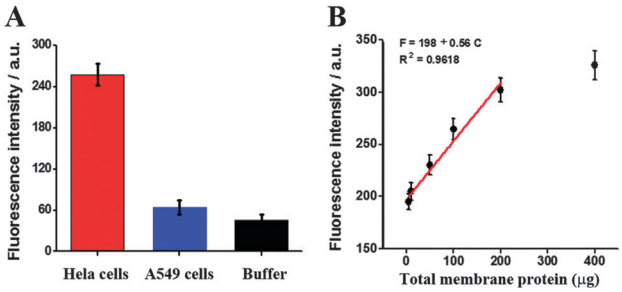

Fig. 3 (A) Measurement of fluorescence intensity in 50 μg of total membrane proteins extracted from HeLa cells (red column), A549 cells (blue column) and the control group with the extraction buffer only (black column). (B) Linear relationship between fluorescence intensity and the amount of total membrane proteins extracted from HeLa cells. Error bars show the standard deviations of three experiments.

In summary, we have developed a new method for ultrasensitive detection of FRs using a target-triggered EXPAR-based DNAzyme biosensor in combination with the ZnPPIX/G-quadruplex labels. In this research, we employed a FA-linked hairpin probe to convert the protein signal to a DNA signal. The hairpin probe with a recognition sequence of DNA endonuclease in the stem can be nicked by nicking enzymes to initiate the EXPAR without the requirement of any extra primers, generating abundant functional DNAzymes. With the digestion of excess hairpin probes by the exonucleases and the use of ZnPPIX as the fluorescent label, a high signal-to-noise ratio can be obtained. Due to the strong affinity of FRs for FA, the high amplification efficiency of the EXPAR and the enhanced fluorescence signal of ZnPPIX/G-quadruplex structures, the proposed method can sensitively measure FRs with a large dynamic range of 6 orders of magnitude from 1 fM to 1 nM and a detection limit as low as 0.23 fM, which is superior to most currently used approaches for FR assay.[17,19] In addition, the proposed method exhibits excellent selectivity and functions well in real sample analysis. Importantly, this method might be further extended to detect various small molecule-binding proteins by simply changing the linked small molecule moiety of the hairpin probes.

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