G-quadruplex-based DNAzyme for facile colorimetric detection of thrombin

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Received (in Cambridge, UK) 2nd April 2008, Accepted 23rd April 2008
First published as an Advance Article on the web 9th June 2008
DOI: 10.1039/b805565c

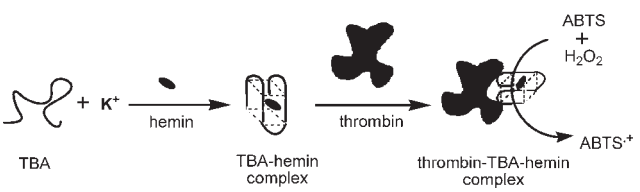
Thrombin-binding aptamer is found to bind hemin to form a catalytic complex whose activity is significantly promoted by the addition of thrombin, which enables the colorimetric detection of thrombin with high specificity and sensitivity in a facile way.

DNAzymes are a kind of artificial enzyme that have exhibited surprising potential as new biocatalysts.[1,2] They have been widely applied to numerous biochemical reactions such as DNA or RNA cleavage,[3–6] porphyrin metalation,[7] and DNA self-modification.[8–10] A DNA enzyme formed from hemin and a G-quadruplex aptamer (*i.e.* G-quadruplex-based DNAzyme) mimics horseradish peroxidase and catalyzes the H_2O_2-mediated oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or luminol,[11–15] accompanied by a color change or chemiluminescence emission. In comparison with protein peroxidases, the G-quadruplex-based DNAzyme can be facilely tethered to DNA sequences or other targets,[14–17] serving as a novel kind of catalytic label or beacon. With this advantage, they have been employed to develop many colorimetric and/or chemiluminescence biosensors for the sensitive and specific detection of proteins, DNA and other biomolecules,[14–19] indicating their great potential in biological applications.

A 15-mer DNA aptamer d(GGTTGGTGTGGTTGG), also known as thrombin-binding aptamer (TBA),[20] is a widely used sensing element for constructing various aptasensors.[15,21–25] This aptamer is thought to adopt a random coil structure in the absence of K^+ and thrombin. Upon addition of K^+ or thrombin, TBA folds into a stable G-quadruplex. This conformational change enables TBA to sense thrombin and K^+ after it is labelled with appropriate indicators.[22,25] Considering this structural characteristic of TBA, here we explore the interaction between this G-quadruplex aptamer and hemin. Interestingly, TBA is found to bind hemin in the folded state to form a complex with peroxidase-like activity. More importantly, we find that the addition of thrombin significantly promotes the enzyme activity of the TBA–hemin complex, which provides a facile colorimetric approach to sensing thrombin with high sensitivity.

Scheme 1 depicts the designed colorimetric method for thrombin detection, with TBA as the sensing element. In the presence of K^+, the folded TBA can bind hemin loosely to form a complex with very low peroxidase-like activity. After incubation with thrombin, the TBA–hemin complex is bound to form a supramolecular complex (thrombin–TBA–hemin), which is accompanied by an increase in the catalytic activity. This will be reflected by a color change when monitored in the ABTS–H_2O_2 system, thus enabling the specific detection of thrombin by using UV-Vis absorption spectroscopy.

Besides the 15-mer TBA, another 29-mer DNA aptamer d(AGTCCGTGGTAGGGCAGGTTGGGGTGACT)[26] has been proved to bind thrombin strongly as well. Here, the two TBAs are under investigation to select the better sensing element for thrombin detection. Before use, these oligonucleotides were dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) and quantified using UV-Vis absorption spectroscopy with the following extinction coefficients ($\varepsilon_{260\ nm}$, $M^{-1}\ cm^{-1}$): A = 15 400, G = 11 500, C = 7400, T = 8700. The DNA solutions were heated at 88 °C for 10 min and gradually cooled to room temperature. After pretreatment, an equal volume of 2× HEPES buffer (50 mM HEPES, pH 7.4, 40 mM KCl, 400 mM NaCl, 0.1% (w/v) Triton X-100, 2% (v/v) DMSO) was added to the above DNA solutions, allowing the DNA to properly fold for 40 min to form the G-quadruplex structures. Then, to these DNA solutions was added an equal volume of the same concentration of hemin dissolved in HEPES buffer, keeping the mixtures at room temperature for 2 h to form the TBA–hemin complexes. Finally, equal volumes of different concentrations of thrombin dissolved in the binding buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) were added to the solutions of TBA–hemin, and the resulting solutions were incubated at room temperature for 3 h to form the thrombin–TBA–hemin supramolecular complex. The formed complexes were then analyzed in the ABTS–H_2O_2 system by UV-Vis absorption spectroscopy. The absorption spectra of the reaction product ($ABTS^{\bullet +}$) in the wavelength range from 500 to 390 nm were



Scheme 1 Colorimetric approach to sensing thrombin based on the DNAzyme formation between hemin, TBA and the target protein. TBA serves as the sensing element for specific detection of the thrombin protein, and also provides a platform for binding hemin to form a G-quadruplex-based DNAzyme.

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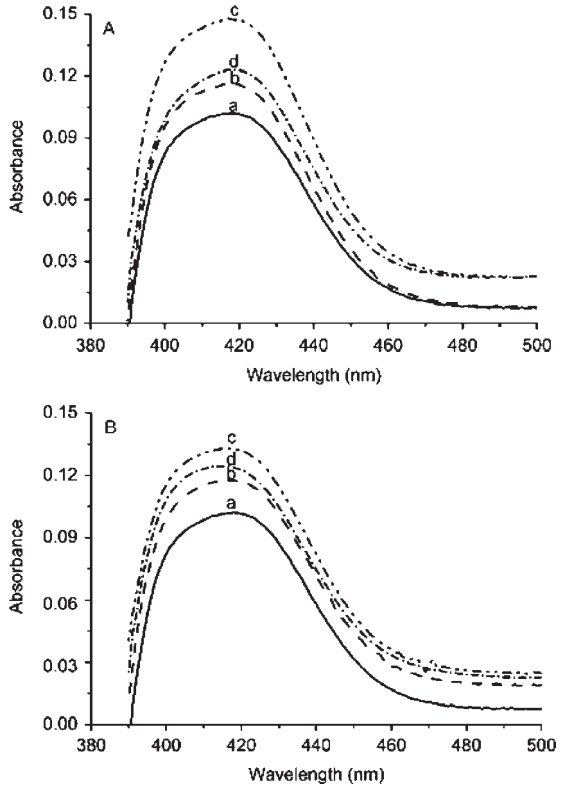

Fig. 1 Colorimetric analyses of several catalysts using UV-Vis absorption spectroscopy: (a) 0.5 μM hemin, (b) 0.5 μM hemin plus 0.5 μM TBA, (c) conditions in (b) plus 0.5 μM thrombin, (d) conditions in (b) plus 0.5 μM BSA. (A) 15-mer TBA is employed as the sensing element; (B) 29-mer TBA serves as the sensing element. Experimental conditions: [ABTS] = 5.9 mM, [H_2O_2] = 0.6 mM, in 25 mM HEPES buffer (pH = 8.0) containing 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO.

collected every 1 min by a Cary 500 Scan UV-Vis-NIR Spectrophotometer (Varian, USA).

Fig. 1 depicts a comparison between the use of the 15-mer or 29-mer TBA for thrombin detection. The two TBAs are both found to enhance the catalytic activity of hemin (curve b), indicating the binding of the TBAs to hemin. After the addition of thrombin (curve c), there is a large improvement in the enzyme activity of the TBA–hemin complex when the 15-mer TBA is used (Fig. 1A), whereas a smaller improvement is observed in the case of the 29-mer TBA (Fig. 1B). The control experiments reveal that the addition of a foreign protein such as BSA has little influence on the catalytic activity (curve d), indicating that the TBA–hemin complexes specifically respond to thrombin. Taking into account the above factors, the 15-mer TBA is considered as the better sensing element, and so it is adopted below.

To evaluate the affinities for binding of hemin to TBA and the thrombin–TBA complex, the binding assays were performed under the experimental conditions shown in Fig. 1, based on the peroxidase-like activities of the formed TBA–hemin and thrombin–TBA–hemin complexes. The results indicate that hemin binds to TBA with a supramicromolar affinity ($K_d = 37 \pm 2$ μM), whereas the interaction between hemin and the thrombin–TBA complex is relatively strong ($K_d \approx 23$ μM).

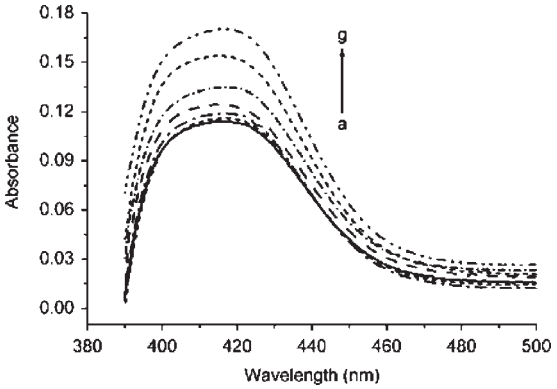

Fig. 2 UV-Vis absorption spectra (after 4 min) analyzing different concentrations of thrombin: (a) 0, (b) 0.01, (c) 0.02, (d) 0.05, (e) 0.1, (f) 0.2, (g) 2 μM. The experimental conditions are identical to those in Fig. 1.

That is, the addition of thrombin promotes the binding of TBA to hemin and thus a more catalytic complex is formed, giving rise to an increase in the enzyme activity. It should be noted that the G-quadruplex structure is generally stabilized by alkali cations such as K^+ and Na^+,[27] and so the ionic conditions have a potential influence on the folding of TBA and hemin binding. We find that TBA does not bind hemin under salt-deficient conditions even in the presence of thrombin, whereas the presence of more than 5 mM K^+ sufficiently favors the formation of TBA–hemin and thrombin–TBA–hemin complexes (data not shown), indicating K^+ is absolutely required for hemin binding. According to a previous report,[11] the aptamer–hemin complexes possess the best DNAzyme function in HEPES buffer (25 mM HEPES, pH = 8.0, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO). Therefore, this buffer condition is used throughout.

Because the thrombin–TBA–hemin complex is always in dissociation equilibrium, it is conceivable that the addition of more thrombin will shift this equilibrium towards the complex. Fig. 2 shows the changes in the UV-Vis absorption spectra upon adding different concentrations of thrombin into a 0.5 μM mixture of hemin and TBA when monitored in the ABTS–H_2O_2 reaction. As the concentration of thrombin increases up to 2 μM, a gradual increase in the spectrum intensity is observed. These results indicate that more thrombin–TBA–hemin complex is formed in the mixture when more thrombin is added, as expected.

Fig. 3 outlines the relationship between the absorbance at 421 nm (the maximal absorption of $ABTS^{\bullet+}$) and thrombin concentration in the range from 0 to 2 μM. From Fig. 3, it is found that the addition of 0.02 μM thrombin gives rise to an obvious increase in the enzyme activity, indicating a detection limit of 20 nM for analyzing thrombin. A good linear relationship ($R = 0.9997$) between the absorbance and thrombin concentration is observed in the range of 0.02–0.2 μM (Fig. 3, inset). These results reveal this facile colorimetric method for thrombin detection is sensitive and effective.

In conclusion, we have introduced a facile method for the sensitive colorimetric detection of thrombin, with the 15-mer TBA as the sensing element. This G-quadruplex aptamer is found to bind hemin in the folded state to form a complex with

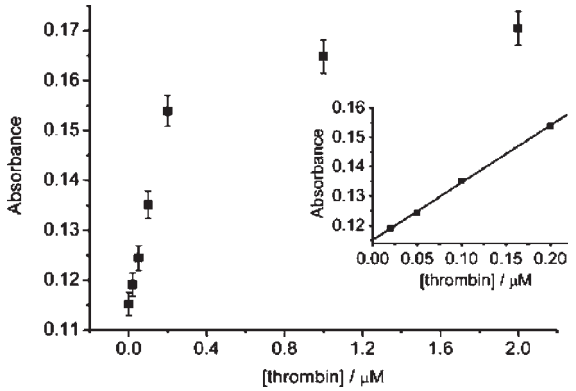

Fig. 3 Dependence of the absorbance at 421 nm on thrombin concentration. The inset shows a linear relationship between the absorbance and thrombin concentration in the range from 0.02 to 0.2 μM.

weak peroxidase-like activity. After incubation with thrombin, the TBA–hemin complex is bound to form a supramolecular DNAzyme, giving rise to an increase in the catalytic activity. This is reflected by an absorbance change when monitored in the ABTS–H_2O_2 system using UV-Vis absorption spectroscopy, thus providing a facile approach to sensing thrombin with a detection limit of 20 nM. By changing the sensing element, this DNAzyme-based method will be applicable to the colorimetric detection of other proteins (*e.g.* nucleolin[28] and HIV-1 integrase[29]) whose G-quadruplex aptamers have been selected *in vitro*.

This work is supported by the National Natural Science Foundation of China with the Grants 20675078 and 20735003, 973 project 2007CB714500, and the Chinese Academy of Sciences KJCX2.YW.H11.

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