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Catalytic activity of a dual-hemin labelled oligonucleotide: conformational dependence and fluorescent DNA sensing†

Quanbo Wang,[a] Nan Xu,[a] Zhen Gui,[b] Jianping Lei,[a] Huangxian Ju*[a] and Feng Yan*[b]

The conformation-dependent peroxidase activity of a dual-hemin labelled oligonucleotide was identified and conveniently utilized to design a sensitive homogenous fluorescent method for DNA sensing.

The sensitive, selective and fast detection of sequence-specific DNA fragments has attracted considerable interest in various fields such as cancer diagnosis and food safety.[1] Great effort has been devoted to this area by designing and optimizing DNA probes labelled with different signal molecules. Fluorescein or rhodamine derivatives labelled probes such as a molecular beacon have been widely used in fluorescent DNA sensing.[2] Electrochemical indicators such as ferrocene and methylene blue have also been used to label DNA probes for the development of electrochemical DNA biosensors.[3] Typically, both the fluorescent and electrochemical DNA biosensors transduce the target DNA hybridization event into the structural change of the probe, and then output detectable signal directly through the labelled molecule in a ''one target to one signal molecule'' manner.

In order to improve the sensitivity, different signal amplification strategies have been designed to transduce a one-target recognition event to multiple signal molecules. One of the most feasible transduction pathways is to incorporate multiple signal probes into the detection system using a target DNA cycle strategy such as an endonuclease cycle,[4] an exonuclease cycle[5] or strand-displacement amplification.[6] Another convenient and promising avenue is to introduce an enzymatic cycle for producing the multiple signal molecules, in which the enzyme or the catalyst can be labelled to a DNA probe[1b,7] or formed upon the target DNA recognition.[8] This work used small hemin molecules as the biomimetic peroxidase to label the two ends of an oligonucleotide and design a target DNA regulated enzymatic cycle for producing a sensitive fluorescent signal.

Hemin is an important catalytic cofactor in many natural enzymes. The regulation of its catalytic activity has become an effective way to utilize its biomimetic catalysis for obtaining tunable detection signals and developing highly efficient analytical protocols. For example, based on the formation of a G-quadruplex/hemin DNAzyme with peroxidase activity by incorporating hemin into a guanine quadruplex, many DNA, RNA or protein sensing strategies have been proposed.[8] The peroxidase activity of hemin can also be regulated by its adsorption on some carbon nanomaterials such as graphene oxide (GO), which is achieved by assembling the hemin labelled single-stranded DNA (ssDNA) on GO.[7a] However, the quenching properties of GO[2c] on the fluorescence of catalytically generated dityramine and the adsorption of some formed probe/target duplex on GO surface greatly decreases the DNA detection sensitivity.[9] Therefore, it is highly desired that other ways to effectively regulate the catalytic activity of hemin be sought.

Due to the weak solubility, hemin can usually self-aggregate into catalytically inactive dimer in aqueous solution.[10] Grafting hemin to DNA can significantly improve its solubility, and thus dissociate the hemin aggregate to monomer to recover its peroxidase activity.[11] This work proposed a ''DNA switch'' to regulate the aggregation of hemin and dissociation of the hemin dimer by labelling two hemin molecules to two ends of an oligonucleotide. The dual-hemin labelled DNA probe was synthesized *via* an amide reaction (Scheme 1A) and characterized using mass spectroscopy (Fig. S1–S3, ESI†). This probe could spontaneously form an intramolecular dimer of hemin, which formed a circular structure and led to a low peroxidase activity of hemin (Scheme 1B). Upon the hybridization of the probe with complementary target DNA, the intramolecular hemin dimer dissociated into highly active hemin monomers. Thus the hybridization recognition could be used to introduce an enzymatic cycle for producing multiple signal molecules. Using the oxidation reaction of non-fluorescent tyramine using hydrogen peroxide

[a] State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P.R. China. E-mail: hxju@nju.edu.cn; Fax: +86 25 83593593; Tel: +86 25 83593593

[b] Department of Clinical Laboratory, Nanjing Medical University Cancer Hospital & Jiangsu Cancer Hospital, 42 Baiziting Road, Nanjing 210009, P.R. China. E-mail: yanfeng2007@sohu.com

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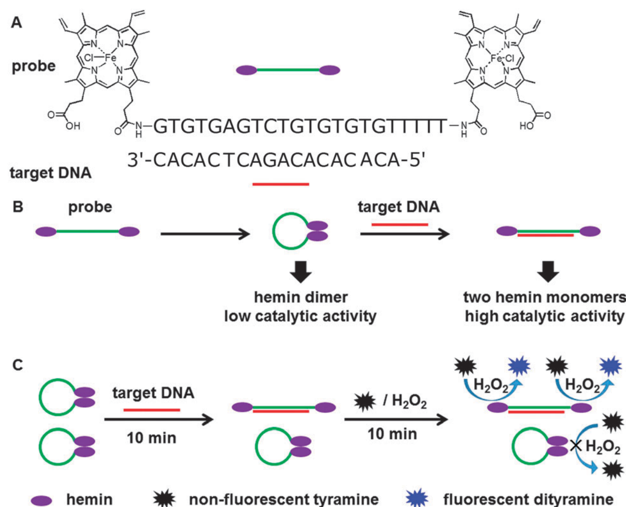

Scheme 1 Schematic illustration of (A) chemical structure of dual-hemin labelled probe, (B) target DNA regulated catalytic activity of probe, and (C) homogenous fluorescence strategy for DNA sensing.

(H_2O_2) as a model, the enzymatic catalysis produced fluorescent dityramine (Scheme S1, ESI†). The reaction possessed quick dynamics and could be completed within 10 min, leading to a relatively fast fluorescence method for target DNA detection (Scheme 1C). This method was highly sensitive and convenient, showing a promising application in practice.

To investigate the structural change of the probe upon its recognition to target DNA, the UV-visible absorption spectra of the probe and the formed probe/target duplex were first measured. The absorption spectrum of probe/target duplex, which was formed by mixing a 1 μM probe and 1 μM target DNA and allowing the mixture to incubate for 10 min, showed a characteristic peak of the hemin monomer at 402 nm (Fig. 1A, curve a), while the absorption spectrum of the probe showed a blue-shifted, broadened and hypochromic peak at 378 nm (Fig. 1A, curve b). This could be attributed to the formation of a face-to-face dimer of hemin,[10] which benefited from the flexible structure of the ssDNA probe. Similarly, the absorption spectrum of a control stem-loop structured reference probe (rP1) bearing a conclusive intramolecular hemin dimer also showed a broadened hemin peak at 378 nm (Fig. S4, ESI†), which further confirmed the formation of the circular structure and hemin dimer in the probe.

Another reference probe (rP2) was designed to examine the dissociation of the face-to-face dimer by replacing one hemin molecule labelled at the 5′ end of the probe with a fluorescent reporter X-rhodamine (ROX) (Scheme S2, ESI†). In the absence of target DNA, the fluorescence of ROX on rP2 was greatly quenched by hemin according to a photoinduced electron transfer mechanism[12] (Fig. 1B, curve a), which indicated the formation of a hemin-ROX heterodimer.[13] The quenching efficiency of photoinduced electron transfer was very sensitive to the distance between hemin and ROX. After rP2 hybridized with target DNA, the fluorescence of ROX was apparently recovered (Fig. 1B, curve b), which demonstrated the separation of ROX and hemin and significantly increased the distance between hemin and ROX due to the formation of a rigid probe/target duplex structure. Thus the spontaneously formed intramolecular dimer could be dissociated by hybridizing the probe with target DNA.

The fluorescence of dityramine was used to trace the peroxidase activity change of the probe upon its recognition to target DNA. The fluorescent spectra were recorded after mixing 0.7 mM tyramine and 2.0 mM H_2O_2 with DNA or the mixture of target and probe to initiate the catalytic reaction for 10 min. In the absence of the probe, the fluorescent spectra of the mixtures of 0.7 mM tyramine and 2.0 mM H_2O_2 without/with 10 nM target DNA showed a negligible fluorescent peak at 410 nm (Fig. 2A, black and red), which could be attributed to the slow oxidation of tyramine by H_2O_2 to produce a small quantity of dityramine. In the presence of a 10 nM probe, the fluorescent peak at 410 nm slightly increased due to the weak catalytic activity of the hemin dimer (Fig. 2A, blue), which produced more dityramine. However, after 0.7 mM tyramine and 2.0 mM H_2O_2 were mixed into the mixture of the 10 nM probe and 10 nM target DNA for 10 min, the fluorescent peak of dityramine increased sharply (Fig. 2A, pink), indicating the high peroxidase activity of the probe due to the dissociation of the hemin dimer after the hybridization of the probe with target DNA. The conformation-dependent peroxidase activity could be used for the fluorescent detection of target DNA.

As mentioned above, the hemin dimer showed weak catalytic activity, thus the fluorescent spectrum showed a slightly increased fluorescent signal over time (Fig. 2B). Upon increasing the amount of target DNA from 2.5 to 10 nM in the 10 nM probe, the

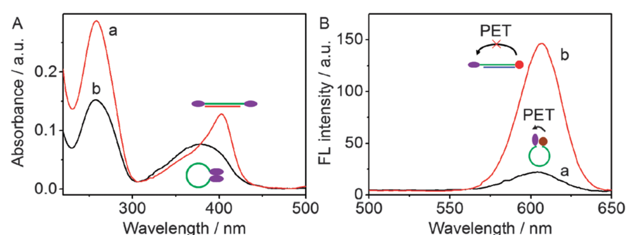

Fig. 1 (A) UV-visible spectra of 1 μM probe/target duplex (a) and probe (b). (B) Fluorescence spectra of 10 nM rP2 (a) and rP2/target duplex (b).

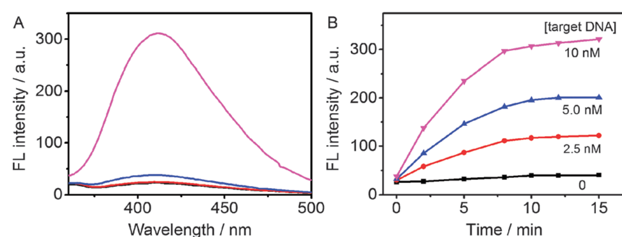

Fig. 2 (A) Fluorescent spectra of the mixtures of 0.7 mM tyramine, 2.0 mM H_2O_2 without (black) or with (red) 10 nM target DNA for 10 min, and the mixtures of 0.7 mM tyramine, 2.0 mM H_2O_2 after reaction with 10 nM probe (blue) and 10 nM probe/target duplex (pink) for 10 min. (B) Fluorescence kinetic curves of tyramine oxidation catalyzed by the hybridization product of 10 nM probe with 0, 2.5, 5.0 and 10 nM target DNA in 50 mM Tris-HCl (pH 7.4, containing 100 mM NaCl).

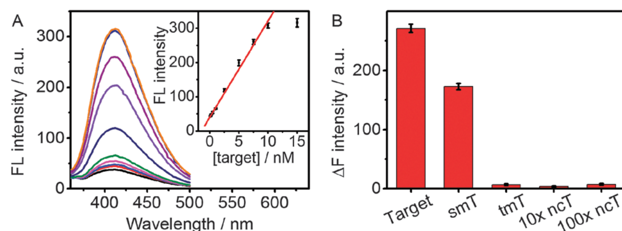

Fig. 3 (A) Fluorescent spectra of dityramine formed in presence of 10 nM probe at 0, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, 10 and 15 nM target (from bottom to top). Inset: calibration curve. (B) Fluorescence intensity increment (ΔF) in presence of 10 nM probe and 10 nM complementary target, single-base (smT), three-base mismatched target (tmT), or 100 and 1000 nM non-complementary target (ncT).

fluorescent signal of the mixture of 0.7 mM tyramine and 2.0 mM H_2O_2 with the hybridization product at the same reaction time increased obviously (Fig. 2B). For 10 nM target DNA, the time-dependent curve reached a plateau at 10 min, indicating a relatively fast enzymatic reaction kinetics. Thus this work selected 10 min as the optimal reaction time.

The ratio of fluorescent signals in the presence of the 10 nM probe/target duplex or probe was used to optimize the detection conditions (Fig. S5, ESI†). At 2.0 mM H_2O_2, the ratio increased with the increasing tyramine concentration and reached the maximum value at 0.7 mM. Higher concentration of tyramine led to a high fluorescence background and decreased ratio. Thus 0.7 mM tyramine was used as the optimal condition, under which the maximum ratio occurred at the H_2O_2 concentration of 2.0 mM. The ion strength and pH of reaction buffer greatly influenced both the hybridization and enzymatic reaction rate. High NaCl concentrations decreased the fluorescent signal due to the inhibited peroxidase activity of hemin.[7a] The optimal NaCl concentration and pH were 100 mM and 7.4, respectively (Fig. S6, ESI†).

The peroxidase activity change of hemin upon the recognition of the designed probe to target DNA to dissociate the hemin dimer offered a method for homogeneous fluorescent detection of target DNA. Theoretically, one target DNA could recover the peroxidase activity of two hemin molecules labelled on the same probe for catalyzing the oxidation reaction of tyramine to form fluorescent dityramine. Thus "one target" could lead to the formation of "multiple signal molecules", which provided a novel signal amplification strategy. The FL intensity was proportional to target DNA concentration ranging from 0.1 to 10 nM (R^2 = 0.993) (Fig. 3A). The detection limit was estimated at 3σ to be 19 pM, which was 10 times lower than that using GO to regulate the peroxidase activity of the ssDNA-hemin probe,[7a] and also lower than some fluorescent (1 nM),[8d] chemluminescent (1 nM)[7e] and colorimetric (0.2 μM)[8e] DNA detection methods based on G-quadruplex/hemin DNAzyme. Compared with DNAzyme, the preparation of dual-hemin labelled probe needs a labelling step. However, it is a ready-to-use probe, while hemin-DNAzyme must be labelled to a recognition unit for specific detection (ESI†). The specificity of this protocol was demonstrated to be acceptable for discriminating the single-(smT) and three-base mismatched target (tmT) from the complementary target (Fig. 3B). In addition, 10 or 100-fold concentration of non-complementary target (ncT) showed a much lower FL intensity increment than target DNA, indicating the excellent selectivity for DNA detection.

This work designed a dual-hemin labelled oligonucleotide probe as a novel "DNA switch" with regulative peroxidase activity, which was achieved by the hybridization of the probe with target DNA. Using the fluorescent oxidation product of tyramine as the tracing molecule, the intramolecular hemin dimer with low peroxidase activity ensured a low fluorescence background. The formation of probe/target duplex dissociated the hemin dimer and thus significantly increased the catalytic activity of hemin to produce fluorescent dityramine. Based on the conformation-dependent peroxidase activity, the strategy of "one target" to "multiple signal molecules" and a sensitive, specific and homogeneous fluorescent DNA sensing method was proposed. This method can be easily extended to detect different target DNA by simply changing the base sequence of the probe. This work paved a promising approach for developing highly sensitive detection protocols by regulating the catalytic activity of a labelled small molecule catalyst on an oligonucleotide probe.

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