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Title Page

Title: G-quadruplex DNA biosensor for sensitive visible detection of genetically modified food

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Abstract

In this paper, a novel label-free G-quadruplex DNzyme sensor has been proposed for colorimetric identification of GMO using CaMV 35S promoter sequence as the target. The binary probes can fold into G-quadruplex structure in the presence of DNA-T (Target DNA) and then combine with hemin to form a DNzyme resembling horseradish peroxidase. The detection system consists of two G-rich probes with 2:2 split mode by using the absorbance and color of ABTS²⁻ as signal reporter. Upon the addition of a target sequence, two probes both hybridize with target and then their G-rich sequences combine to form a G-quadruplex DNzyme, and the DNzyme can catalyze the reaction of ABTS²⁻ with H₂O₂. Then the linear range is from 0.05 to 0.5 μM while detection limit is 5 nM. These results demonstrate that the proposed G-quadruplex DNzyme method could be used as a simple, sensitive and cost-effective approach for assays of GMO.

Keyword: GMO; G-quadruplex; colorimetric

1. Introduction

In order to increase the output and improve quality of agricultural products, genetically modified organisms (GMO) have been developed greatly in recent years. GMO means an organism whose genetic characteristics has been altered by introduction of a novel DNA sequence bringing a new character, e.g., insect or herbicide tolerance. However, it also arouses dispute about the effect of GMO on our health and environment. The safety of GMO still hasn't got the unified understanding and many countries had established their own labeling system. The consumer

concerns about GM food has affected food regulation policies worldwide and prompted the development or changes in GM food labeling legislation in many countries.¹ For example, the European Union (EU) requires a compulsory labeling for food products which contain more than 0.9% transgene of food and feed.² Hence, simply and quickly qualitative and quantitative analysis of GMO is in great need.

The common methods for detecting GMO mainly contain Enzyme-Linked Immunosorbent Assay (ELISA),^{3,4} polymerase chain reaction (PCR)^{5,6}, microarray⁷ and electrochemistry^{8,9}. And the PCR-based and chromatography-based methods are always the preferred choice^{10,11}. However, they require expensive instrument and specialized technicians, which makes them impractical for non-wealthy areas and for field testing. As a result, the development of an easy, rapid and reliable method for determination of GMO has become an issue of great interest.

G-rich nucleic acid sequences are prone to fold into tetragonal arrays called G-tetrads stacking to shape a G-quadruplex helix.¹² When binding with hemin, the G-quadruplex becomes a DNAzyme possessing peroxidase-like activities, which can catalyze the H_2O_2 mediated oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS²⁻) into a colored radical anion ABTS⁻ and then produce a detectable color change.¹³⁻¹⁵ The DNAzyme has been utilized as a catalytic beacon in many areas, such as analysis of telomerase activity¹⁴ or DNA¹⁶, amplified detection of small molecule^{17,18} or protein¹⁶, and so on. Moreover, dividing the G-quadruplex into two parts would induce an extraordinary selectivity for both protein and nucleic acid recognition.¹⁹ For example, Xiao *et al*²⁰ have reformed

G-quadruplex structure as possessing G-rich sequence and extended single-stranded DNA for sensing target DNA, and utilized luminol/H₂O₂ reaction system as signal output. Based on the similar principle, Kolpashchikov²¹ has also utilized the binary probe to visually analyze single nucleotide polymorphisms (SNPs). Deng *et al*¹³ have designed an 3:1 asymmetrically split mode for visual detecting nucleic acid.

In this paper, we reported a convenient visible G-quadruplex sensor for detecting GMO by using soybean as an example. Also, many influencing factors, such as split mode of probes, hemin concentrations, incubation time of hemin with G-quadruplex, and time of probes with target DNA were investigated in detail. Under the optimum conditions, soybean having over 0.9% transgene can be obviously identified through absorption and color signal.

2. Materials and Methods

2.1 Instrumentation and Reagents

All DNA sequences were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China). DNA stock solutions were 100 μ M in a Tris-HCl buffer (pH 7.6) and stored at 4 °C until used. Both hemin and ABTS were purchased from Aladdin Reagents (Shanghai, China). Hemin stock solution (6.75 mM) was prepared in Dimethyl sulfoxide (DMSO) (Sangon Biotechnology Co., Ltd., Shanghai, China) and stored at -20 °C. Hydrogen peroxide (H₂O₂) was purchased from Fuchen Chemical Reagents (Tianjin, China). Hemin, ABTS²⁻ and H₂O₂ working solutions were freshly prepared in a total buffer (pH 7.6) containing 25 mM Tris-HCl, 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100, and 1% DMSO before used. Lambda 750

UV-Vis spectrophotometer (PerkinElmer, Shelton, USA) was used to collect the absorbance signal.

2.2 Assembly of the DNAzyme

1.5 μ L Probe 1 (or Probe-A and -B) for 100 μ M and different concentrations of DNA-T for 100 μ M were mixed equably and then incubated at room temperature for 40 min in Tris-HCl buffer (pH 7.6). Afterwards, 2.2 μ L hemin for 67.5 μ M was added and then the mixture was incubated for 80 min. Subsequently, total buffer (pH 7.6) was added until the probe concentration was 0.5 μ M and hemin was 0.5 μ M. At last, ABTS and H₂O₂ were added for final concentration to be 2 mM respectively. The color change was observed and absorbance spectrum was obtained by Lambda 750 UV-Vis spectrophotometer.

3. Results and Discussion

3.1 Principle of the proposed sensor

The principle of the proposed sensor was shown in Scheme 1. With CaMV 35S promoter as target, lectin, an endogenous gene for soybean, was chosen as reference gene. At the same time, we designed three types of probe with different split mode: complete mode, 2:2 split mode, 3:1 split mode to detect target DNA fragment. Herein, while the probes hybridize with target DNA, the overhanging GGG repeats get closer and combine to shape a G-quadruplex structure. Once hemin is added, a DNAzyme with peroxidase-like activities is formed and then catalyze H₂O₂ mediated ABTS²⁻ to obtain visible oxidation product ABTS⁻, which also can be detected with UV-Vis spectrophotometer.

The preferred position for Scheme 1

3.2 Probe design

By utilizing the software Primer Premier 5.0 (Premier Co.,Canada), a specific nucleotide fragment DNA-T was screened from CaMV 35S promoter sequence and was used as target. A specific sequence DNA-R was also screened from lectin gene by the same software as a reference gene. Three probes with different modes: complete mode, 2:2 split mode, and 3:1 split mode (see Table 1) were designed and used to detect target (DNA-T) gene. It was found that probes with different modes had different sensitivities. As shown in Figure 1, for probe with complete mode, its catalytic activity of target system was little higher than that of blank system and their colors are similar. For probe with 2:2 split mode, target system has much higher catalytic activity than that of blank system, and its color is also significantly more blue than the blank system observed with naked eyes. As far as 3:1 split mode, although the contrast of catalytic activity between target system and blank system is similar to that of 2:2 mode, its blank system has stronger color than those of other probes. It means that 3:1 split has larger background value and higher blank response, so it's not suitable for colorimetric detection of target DNA. Thereby, probe with 2:2 split mode was chosen in the following experiments.

The preferred position for Table 1

The preferred position for Figure 1

Subsequently, probe with 2:2 split mode was used to detect target DNA-T. From Figure 2, we could find that both blank and DNA-R systems had light color and weak catalytic activities. Only in the presence of DNA-T, the system acquired high catalytic

activity and changed to obvious cyan color. It may be because that both probe 2-A and probe 2-B could hybridize with DNA-T, which results in their overhanging GGG-repeats getting closer and being easier to form a G-quadruplex structure. After that, the G-quadruplex binds hemin to produce a DNAzyme which could catalyze H_2O_2 mediated ABTS^{2-} to generate visible cyan ABTS^- as signal output. The proposed method is simple, convenient and without any expensive instrument, therefore it is worth to be developed as a kit. Moreover, the method can be used to identify GMO by detecting CaMV 35S promoter sequence or other transgenes.

The preferred position for Figure 2

3.3 Optimization of affecting factors

As far as we know, hemin could stabilize the second structure of a DNAzyme and the influence of hemin concentration will be discussed later. As shown in Figure S1 (see Supporting Information), the absorbance value has reached its maximum when the concentration of hemin is 0.5 μM . As a result, 0.5 μM hemin was used in the following experiments.

The optimal incubation time of hemin with G-quadruplex has been surveyed too. Figure S2 (see Supporting Information) indicated that the system had high absorbance when the incubation time was 80 min or more. Hence, 80 min was chosen as the optimal incubation time for hemin and G-quadruplex.

The incubation time between probes and target was investigated, and the result was shown in Figure S3 (see Supporting Information). When the incubation time was prolonged to 40 min, the absorbance increased dramatically, namely the DNAzyme

activity enhances greatly. Nevertheless, the absorbance value hardly varies as incubation time prolonging. Consequently, 40 min was used as the best incubation time.

As we know, many countries have GM food labeling legislation. EU dictates indication of all food containing GMO or made from GMO in the EU Regulation 1831/2003 and the threshold value is 0.9% in food and feed.²² The relative percentile quantification demands the absolute quantification of transgene in the samples.²³ Therefore we explore the relationship of transgene concentration with UV-Vis absorbance and colorimetric intensity.

As shown in Figure 3(B), DNA-T concentration has linear relationship with the absorption value. And the linear equation is $y = 0.2630 + 0.5769x$ ($R = 0.9977$, $n=6$). Therein, y represents the maximal UV-Vis absorption value, and x represents the DNA-T concentration. The linear range is from 0.05 to 0.5 μM with a detection limit of 5 nm.

As seen in Figure 3A (b-g), the color becomes deeper and deeper regularly while the DNA-T concentration varies from 0.05 to 0.5 μM , respectively.. Consequently, we can judge the transgene content based on the color. Furthermore, the color of **h** (0.6 μM) is darker than that of **g** (0.5 μM) in the same Figure 3A. Thus, we can judge whether a transgene concentration is over the threshold value or not according to the color.

The preferred position for Figure 3

3.4 Interference

In the actual samples, the endogenous gene of soybean and other genetically modified composition are widespread. In this work, the endogenous gene-lectins (lectin, DNA-R) and the NOS terminator had been investigated to certify the interference to the target DNA. The results were shown in Figure 4. When there is 100 times reference gene (DNA-R) or 80 times the NOS terminator sequence coexist with the DNA-T, the absorbance signal system is almost no change. Therefore, we can believe that the coexist DNA sequences do not interfere the determination of target sequences of DNA CaMV 35S promoter.

The preferred position for Figure 4

3.5 Detection of GMO

As mentioned above, many countries have regulated labeling criteria, with threshold for unintentional mixing of GMO defined as 0.9% in the EU,²² 3% in Korea and 5% in Japan.²⁴ Also china has required labeling for the existence of GMO since 2001 and much effect has been expanded. Since the upper limit for admixtures with GM plants that have been judged as safe and approved in the EU is 0.9%, DNA-T system of 0.9% has been compared with pure DNA-R system as follows.

As shown in Figure 5, the absorbance of the system containing 0.9% transgene is always larger than that of pure DNA-R system, and its color is darker than that of pure DNA-R system. Therefore, we can conclude that GMO containing over 0.9% transgene can be detected by both UV-Vis spectrophotometer and naked eye.

The preferred position for Figure 5

4. Conclusion

In this study, we introduced a simple, label-free and visible method without expensive instrument to detect the genetically modified soybean. We also demonstrated G-rich probes with 2:2 split mode could hybridize with target CaMV 35S promoter fragment, and then combined with hemin to form a G-quadruplex DNAzyme. The DNAzyme could catalyze H_2O_2 mediated oxidation of ABTS^{2-} into a colored radical anion $\text{ABTS}^{\cdot-}$ and then produce a detectable color change. In addition, the method also can be used to quantify transgene composition in the range from 0.05 to 0.5 μM with a detection limit of 5 nM. It was expected that the proposed G-quadruplex DNA biosensor assay would be widely applied in the genetically modified organism detection as well as food safety control.

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Figure Captions

Figure 1 Comparison of probe detective ability. The absorbance changes within 600 s of the oxygenation product $\text{ABTS}^{\cdot-}$ in buffer containing 25 mM Tris-HCl, 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100, and 1% DMSO at 419 nm. The reaction systems contain hemin (0.5 μM); ABTS^{2-} (2 mM), H_2O_2 (2 mM) and (a) probe 1 (0.5 μM), without DNA-T (b) probe 1 (0.5 μM), with DNA-T (0.3 μM); (c) probe 2-A (0.5 μM), -B (0.5 μM), without DNA-T; (d) probe 2-A (0.5 μM), -B (0.5 μM) with DNA-T (0.3 μM) (e) probe 3-A (0.5 μM), -B (0.5 μM), without DNA-T; (f) probe 3-A (0.5 μM), -B (0.5 μM) with DNA-T (0.3 μM). The incubation time of probes/DNA-T (or only probes without DNA-T) is 1 h, and that of hemin/G-quadruplex is 1 h too.

Figure 2 Identification of target DNA-T. The absorbance changes within 300 s of the oxygenation product $\text{ABTS}^{\cdot-}$ in a buffer containing 25 mM Tris-HCl, 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100, and 1% DMSO at 419 nm. The reaction systems contain that probe 2-A, -B (0.5 μM); hemin (0.5 μM); ABTS^{2-} (2 mM), H_2O_2 (2 mM) and (a) without DNA-T or -R (b) DNA-R (0.3 μM); (c) DNA-T (0.3 μM). The incubation time of probes/DNA-T (or -R) is 1 h, and that of hemin/G-quadruplex is 1 h too.

Figure 3 A. Pictures of the systems with different concentration DNA-T; **B.** Relationship between DNA-T concentration and the catalytic activity of the DNzyme. The UV-Vis absorption after 600 s of the oxygenation products $\text{ABTS}^{\cdot-}$ in a buffer containing 25 mM Tris-HCl, 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100, and 1% DMSO; the reaction system also consists of probe 2-A, -B (0.5 μM),

hemin (0.5 μM), ABTS (2 mM) and H_2O_2 (2 mM). The incubation time of probes and DNA-T is 40 min, and that of hemin and G-quadruplex is 80 min. The concentration of DNA-T is (a) 0, (b) 0.05, (c) 0.1, (d) 0.2, (e) 0.3, (f) 0.4, (g) 0.5 μM respectively. Inset: the linear relationship of concentrations of DNA-T with the absorbance values.

Figure 4 The effect of other DNAs. Absorbance wavelength, 419 nm; buffer, 25 mM Tris-HCl containing 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100 and 1% DMSO; probe 2-A, -B, 0.5 μM ; hemin, 0.5 μM ; ABTS^{2-} , 2 mM; H_2O_2 , 2 mM; incubation time of probes and DNA-T, 40 min; incubation time of hemin/G-quadruplex, 80 min. (a) DNA-T (0.3 μM) (b) DNA-T (0.3 μM) with DNA-R (30.0 μM) (c) DNA-T (0.3 μM) with NOS terminator DNA (24.0 μM).

Figure 5 Identification of 0.9% target DNA-T. The absorbance changes within 600s of the oxygenation product $\text{ABTS}^{\cdot-}$ in buffer containing 25 mM Tris-HCl, 150 mM NaCl, 20 mM KCl, 0.03% Triton X-100, and 1% DMSO at 419 nm. The reaction systems contain probe -A, -B (0.5 μM); hemin (0.5 μM), ABTS (2 mM), H_2O_2 (2 mM) and (a) DNA-R (33.3 μM) (b) 0.9% DNA-T with DNA-T 0.3 μM and DNA-R 33 μM . The incubation time of probes/DNA-T (or -R) is 40 min, and that of hemin/G-quadruplex is 80 min.

Scheme 1 Principle scheme of the proposed sensor

Table 1 Sequences of Oligomers used in this experiment

oligomer	Sequence (from 5' to 3')	Description
DNA-T	ATTGTGCGTCATCCCTTACGT CAGTGGAG	From nucleotides of CaMV 35S promoter genome (the GenBank access number: M74305.1)
DNA-R	GAGGATGGATTAAACCACT CAGCACCG	From nucleotides 649-676 of lectin genome (the GenBank access number: K00821)
Probe 1	CTCCACTGACGTAAGGGATG ACGCACAATGTGGGTAGGGC GGGTTGG	The first 29nt from 3'-terminal match the 5'-terminal of DNA-T
Probe 2-A	CTCCACTGACGTAATGGGTA GGG	The first 14nt from 5'-terminal match the 3'-terminal of DNA-T
Probe 2-B	GGGTTGGGCGGATGACGCAC AAT	The first 14nt from 3'-terminal match the 5'-terminal of DNA-T
Probe 3-A	CTCCACTGACGTAAGTGGGT AGGGCGGG	The first 15nt from 5'-terminal match the 3'-terminal of DNA-T
Probe 3-B	TGGGTGATGACGCACAAT	The first 13nt from 3'-terminal match the 5'-terminal of DNA-T
PS2.M	GTGGGTAGGGCGGGTTGG	Hemin-binding aptamer reported by Travascio [Travascio, et al., 1998]

Figure 1

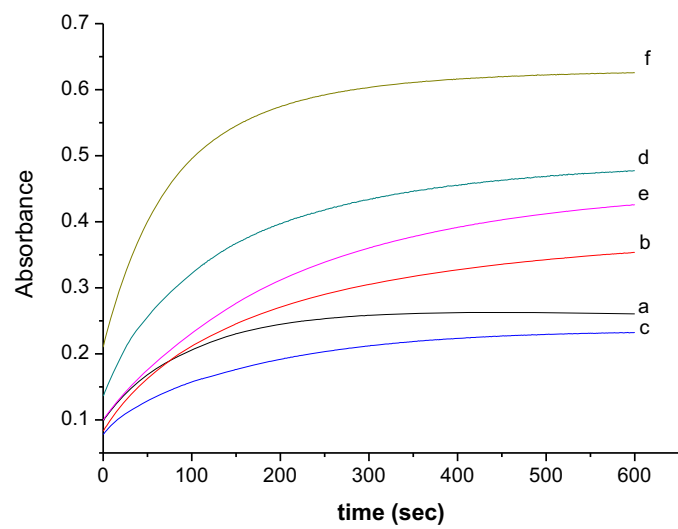


Figure 2

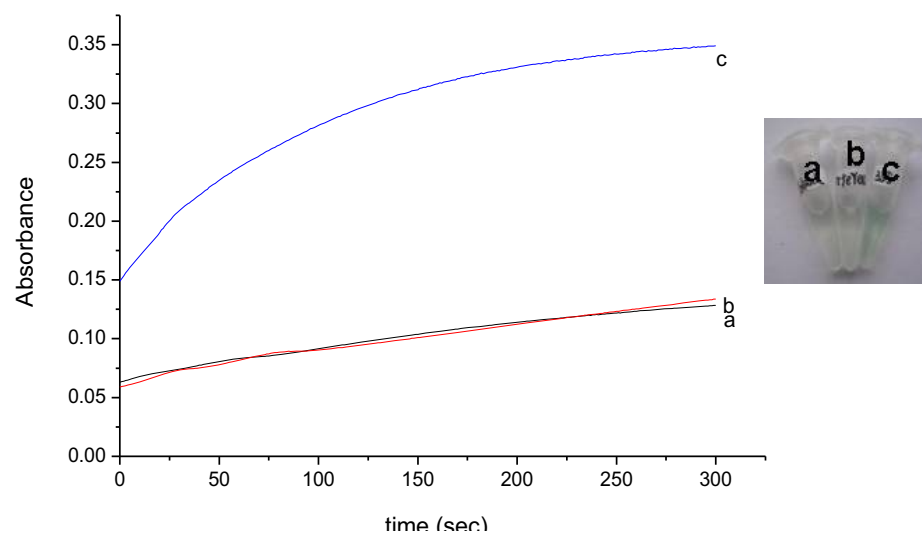
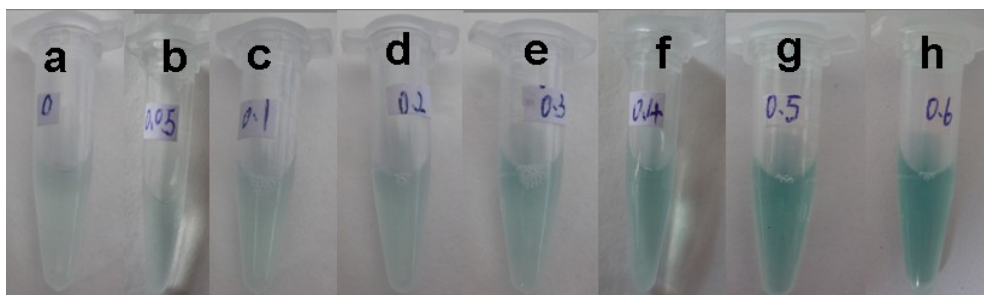


Figure 3

A



B

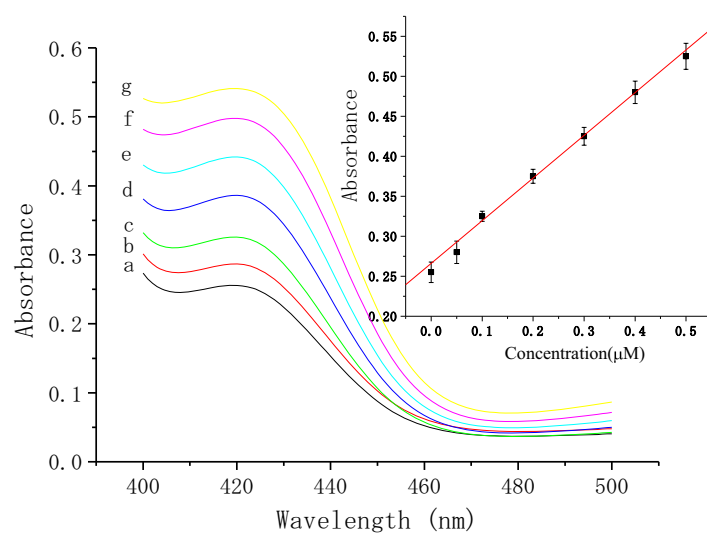


Figure 4

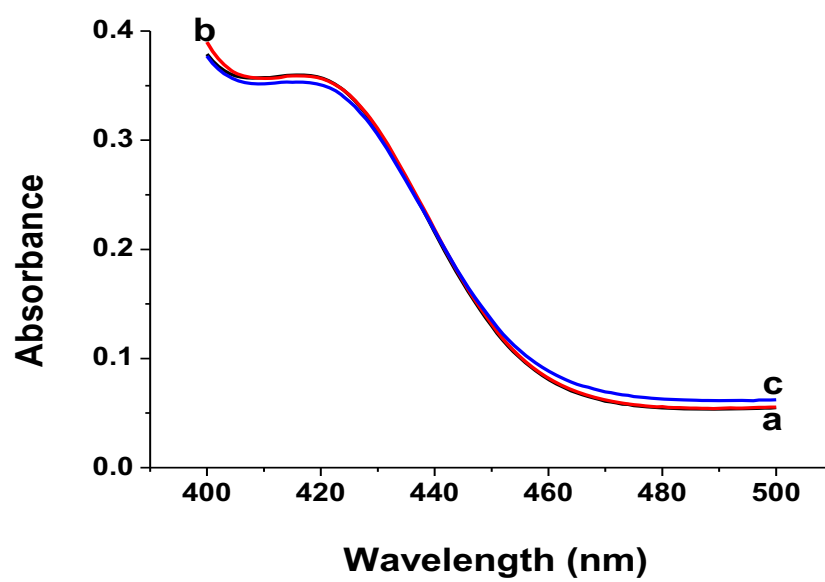
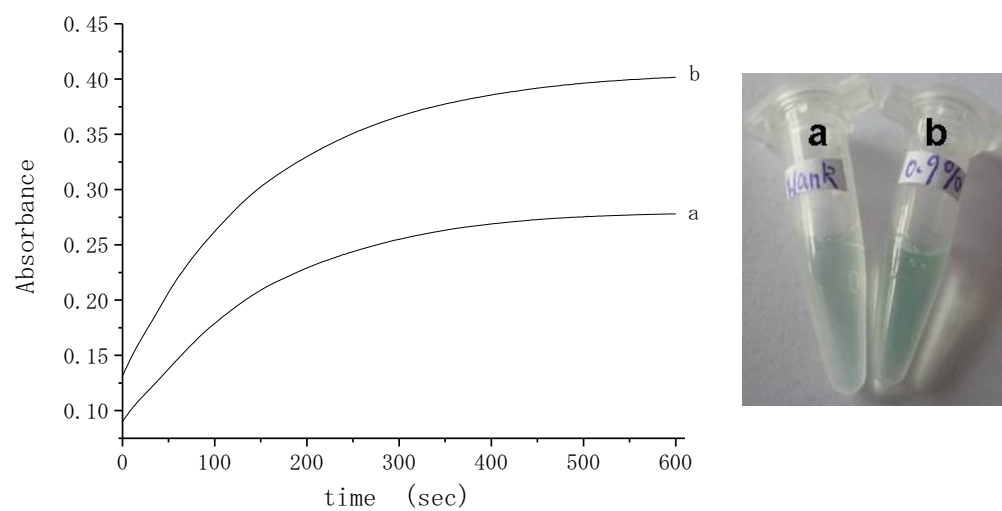
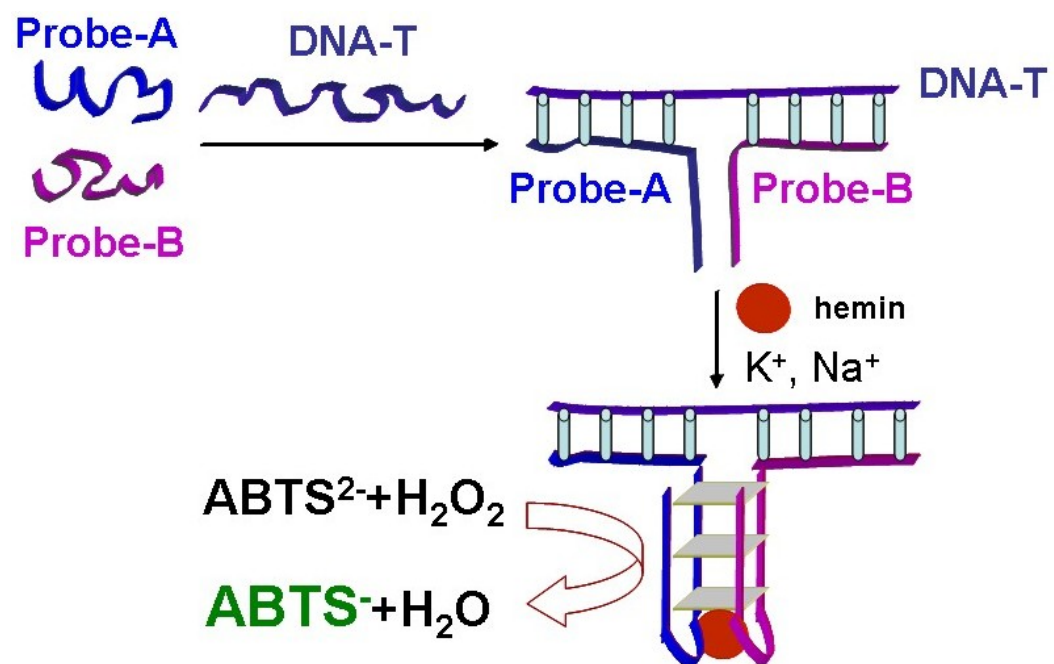


Figure 5



Scheme 1



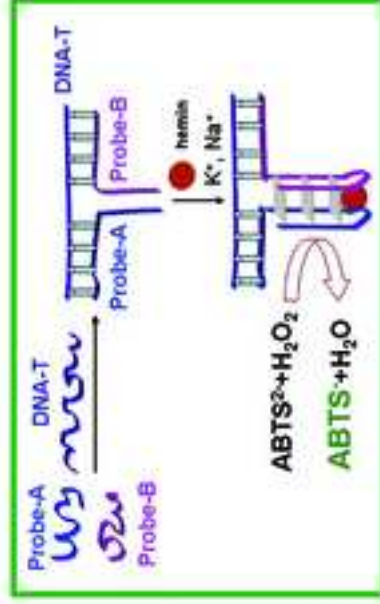
Research Highlights

- ◆ Simple , visible , and label-free G-quadruplex DNAzyme biosensor is proposed for GMO.
- ◆ G-rich probes hybridize with target fragment DNA-T and form a G-quadruplex DNAzyme.
- ◆ The DNAzyme could catalyze H_2O_2 mediated oxidation of ABTS^{2-} into a $\text{ABTS}^\cdot$.
- ◆ The quantitative range is from 0.05 to 0.5 μM with a detection limit of 5 nM.

Genetically Modified(GM) food



G-quadruplex DNA biosensor



simple, sensitive and visible detection of GM food.

