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ARTICLE TYPE

Homogeneous chemiluminescent DNA assay based on allosteric activation of peroxidase-mimicking DNAzyme

Anastasia V. Gribas,[a] Shulin Zhao[b] and Ivan Yu. Sakharov*

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We developed a homogeneous chemiluminescent DNA assay based on allosteric activation of peroxidase-mimicking DNAzyme. This assay exhibits high detection sensitivity and high specificity for target DNA.

1. Introduction

The ability to sense and detect ultralow concentrations of specific DNA sequences using simple and inexpensive assays has become increasingly important, as scientists discovered the genetic basis of some diseases and begun to use this information to improve medical diagnosis.[1,2] Presently the detection of trace specific DNA sequences relies heavily upon various target amplification methods, such as polymerase chain reaction (PCR)[3] and rolling circle amplification.[4] However, these methods suffer from several drawbacks that include complicated procedures, high assay cost, high false positive rate arising from cross contaminations between samples, and lack of portability.

In recent years, novel and sensitive hybridization methods for the detection of DNA have been developed.[5-10] Some of them such as DNA chips with different kinds of detection are heterogeneous methods.[9] However, it is well known that hybridization of probes immobilized on solid surfaces proceeds slowly. By this, the heterogeneous methods usually are time-consuming. The detection of DNA in homogeneous solution is more rapid and simpler.[12,13] Here, we describe a novel homogeneous method for DNA detection based on an allosteric activation of peroxidase-mimicking DNAzyme (PMDNAzyme).

2. Experimental

2.1. Chemicals

Luminol, hemin, NaCl, KCl, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (USA). Graphene oxide (GO) was obtained from Nanjing XFNano Materials Tech Co., Ltd (Nanjing, China). Hydrogen peroxide (H_2O_2, 30%) was obtained from ChemMed (Russia). The concentration of H_2O_2 was estimated by measuring absorbance using ε_{240} = 43.6.[14] The designed DNA sequences were as follows: PMDNAzyme-containing probe with EAD2 (TCT-TCT-ATT-TCT-CCA-CAC-CAA-CTG-GGA-GGG-AGG-GAG-GGA), target DNA (TTG GTG TGG AGA AAT AGA AGA), single base mismatched target (TTG GTG TGG ACA AAT AGA AGA), double base mismatched target (TTG GTC TGG ACA AAT AGA AGA), non-complementary target (CTG GGA CTT TCC). All oligonucleotides were produced by Sintol (Russia).

2.2. Preparation of PMDNAzyme-containing probe and CL determination of target DNA

EAD2 aptamer associated with HIV oligonucleotide (1.3 nM - 2 μM) soluble in 25 mM Tris buffer (pH 8.0) were heated at 88°C for 15 min and then cooled to room temperature for 60 min. To form a G-quadruplex structure KCl, NaCl and Triton X100 were added to the oligonucleotide solutions up to concentrations of 20 mM, 200 mM and 0.05% respectively, and incubated for 40 min at room temperature. Then, hemin solution was added up to a concentration of 40 nM-1.0 μM and incubated for 60 min at room temperature. Next, target DNA was added in different concentration and incubated for 60 min at room temperature. Finally, the PMDNAzyme activity was measured.

CL determination of PMDNAzyme-containing probe was carried out in wells of black polystyrene plates for enzyme immunoassay (Costar, USA). For this, PMDNAzyme samples were mixed with aqueous solutions of luminol and H_2O_2. The final concentrations of luminol and H_2O_2 in the wells were 5 μM and 1.3 mM, respectively. CL intensity was measured 1 min after the initiation of luminol oxidation at room temperature on a luminometer (Spectra Max L, USA). The light intensity was expressed in relative luminescence units (RLU).

2.3. Circular dichromism experiments

CD experiments were performed on a JASCO J-815 spectropolarimeter at 25 °C in a 1 cm path length cuvette. The probe was produced by mixing of 2 μM oligonucleotide probe, preliminary heated to 88 °C and then cooled to room temperature for 60 min, with 1 μM hemin in 25 mM Tris buffer, pH 8.0 with 200 mM NaCl, 20 mM KCl and 0.5% Triton X100 and incubating for 40 min at room temperature. The complex of the probe with target DNA was prepared by adding TDNA up to 2.0 μM and incubating for 60 min. CD measurements from 230 to 290 nm were taken. The data pitch was 1 nm and the scan speed was 50 nm/min. The response was 2 sec. The band width was 1 nm. Each spectrum was corrected by subtracting the CD of the buffer.

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3. Results and discussion

Recently a homogeneous and sensitive method of DNA detection based on chemiluminescence resonance energy transfer (CRET) was reported.[15] In this assay a peroxidase-mimicking DNAzyme (PMDNAzyme), noncovalent complex of hemin and its G-quadruplex aptamer associated with a human immunodeficiency virus (HIV) oligonucleotide sequence catalyzed an oxidation of luminol with H_2O_2 with generation of chemiluminescence (CL). In the presence of graphene oxide (GO) and target DNA, which is able to hybridize with HIV oligonucleotide sequence of the PMDNAzyme, CL intensity was increased. The explanation of this phenomenon was based on a well-known fact that single-stranded oligonucleotide adsorbs on GO via π–π interactions and release from the support, if the oligonucleotide hybridizes with complementary DNA resulting in the formation of double-stranded DNA.[16-18] From the author's point of view[15], when the PMDNAzyme and GO were mixed in a buffer solution, the ssPMDNAzyme was adsorbed on GO and the CL generated upon PMDNAzyme-catalyzed oxidation of luminol is low because of its quenching with GO located into close proximity. But in the presence of the target DNA (TDNA), the PMDNAzyme reacted with the TDNA causing the desorption of the PMDNAzyme and the quenching of CL was not observed. Thus, the increase of CL emission in the presence of TDNA was explained by prevention of CRET.

Here, we developed for the first time a homogeneous method for DNA detection by using hemin aptamer in the probe with aptamer EAD2 (5'-CTG GGA GGG AGG GAG GGA-3'). Such method was presented, because the PMDNAzyme with EAD2 shows very high peroxidase-like activity.[19] As seen in Fig. 1, in the presence of 4.0 µg/mL GO, the increase of TDNA concentration in the reaction solution resulted in higher CL intensity generated by the PMDNAzyme-containing probe that is in a good agreement with the results reported by He's group.[15] However, we were surprised to find that the assay sensitivity was significantly increased at the decrease of GO concentration in the range 0.5-4.0 µg/mL (Fig. 1). Further decrease of GO concentration did not change a behavior of the calibration curve of TDNA. Interesting, the calibration curve for DNA determination obtained in the absence of GO was identical to that obtained with 0.5 µg/mL GO.

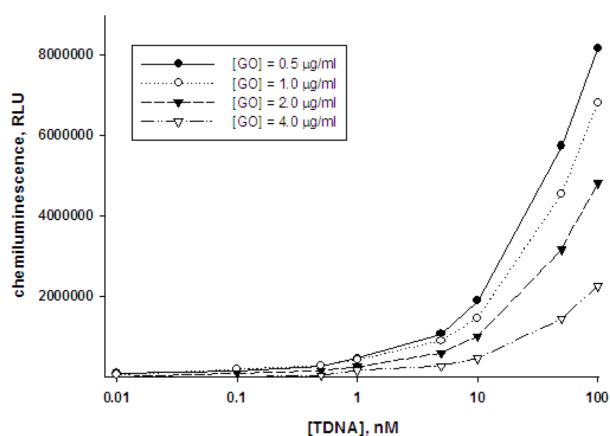

Fig. 1 Dependence of chemiluminescence intensity generated upon luminol oxidation catalyzed with DNAzyme on target DNA concentration in the presence of 0.5, 1.0, 2.0 and 4.0 µg/mL of graphene oxide. The DNAzyme was prepared at interaction of 0.1 µM of EAD2 associated with HIV oligonucleotide and 0.5 µM of hemin in 25 mM Tris buffer, pH 8.0 containing 20 mM KCl, 200 mM NaCl and 0.05% Triton X-100. The chemiluminescent activity of the PMDNAzyme was measured at adding 5 µM of luminol and 1.3 mM hydrogen peroxide at room temperature.

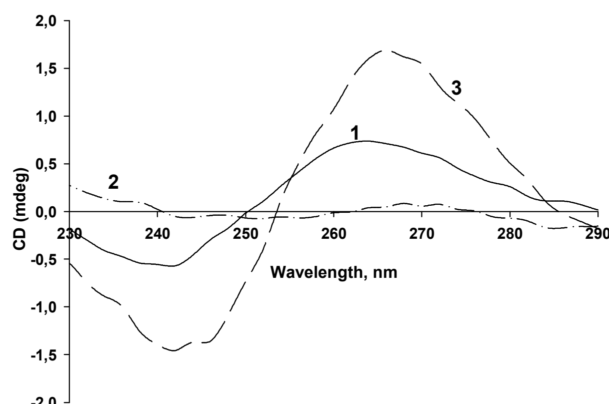

Fig. 2 CD spectra of the PMDNAzyme-containing probe (*1*), the target DNA (*2*) and the complex of the probe with the target DNA (*3*). The probe was produced by mixing of 2 µM EAD2 associated with HIV oligonucleotide sequence, preliminary heated to 88 °C and then cooled to room temperature for 60 min, with 1 µM hemin in 25 mM Tris buffer, pH 8.0 with 200 mM NaCl, 20 mM KCl and 0.5% Triton X-100 and incubating for 1 h at room temperature. The complex of the probe with target DNA was prepared by adding TDNA up to 2 µM and incubating for 1 h.

To study reasons of the observed phenomenon, the circular dichroism (CD) spectra of the probe with and without TDNA were recorded (Fig.2). As seen in Fig. 2 (*curve 1*), the spectrum of the probe (in the presence of K^+ and Na^+cations) has a positive signal at around 264 nm and a negative signal at 242 nm, which are characteristic of parallel G-quadruplexes.[20,21] The similar spectrum was recorded for the complex of the probe and TDNA (Fig. 2, *curve 3*). It means that the interaction of TDNA and the probe did not destroy parallel G-quadruplex structure.

At the same time, we found that the addition of TDNA to the probe solution significantly increased intensities of both peaks. The observed difference in the spectra of the probe with and without TDNA cannot be explained only with an effect of TDNA addition to the reaction solution, because under the experimental conditions the spectrum of TDNA itself has very low intensities (Fig. 2, *curve 2*). Therefore, these results demonstrated that the interaction of TDNA with the complementary sequence of PMDNAzyme-containing probe resulted in some changes of the spatial structure of the probe remaining its parallel G-quadruplex conformation.

The obtained results permit to assume the following mechanism of the allosteric activation of the PMDNAzyme-containing probe (Fig. 3). The probe containing PMDNAzyme in its structure has some peroxidase-like activity and its DNA fragment complementary to TDNA interact with G-quadruplex of the PMDNAzyme by unknown mechanism. In the presence of

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TDNA this interaction is destroyed, because DNA fragment forms stable duplex with TDNA. As a result of this, some re-organizations in 3-D structure of the probe are observed, which are accompanied with enhancement of catalytic activity of the PMDNAzyme. Presently the exact mechanism of the allosteric activation of PMDNAzyme is not clear and will be a subject of our further study.

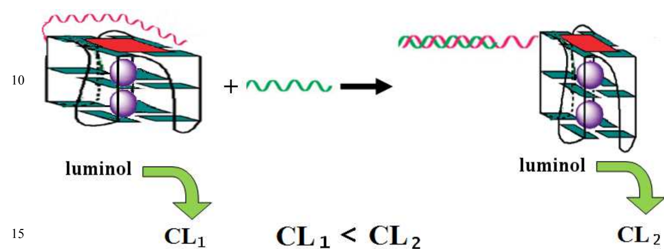

Fig. 3 Schematic illustration of the mechanism of allosteric activation of PMDNAzyme-containing probe with target DNA.

Taking into account of the above results, the development of the DNA assay was performed without GO use. To optimize the experimental conditions of the assay, the concentrations of hemin and the probe were tested. The experimental conditions for detection of the PMDNAzyme activity were optimized by us previously.[16]

As seen in Fig. 4, the sensitivity of the assay (a value of curve slope in linear range) was higher at hemin concentration of 250 nM. Since further increase of hemin concentration led to a sharp

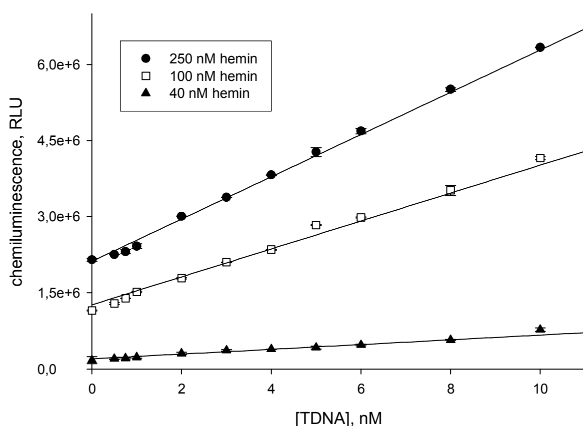

Fig. 4 Effect of hemin concentration in PMDNAzyme-containing probe on the sensitivity of the chemiluminescent determination of target DNA. EAD2 associated with HIV oligonucleotide (0.1 μM), preliminary heated to 88 °C and then cooled to room temperature for 60 min, with different concentrations of hemin (250, 100 and 40 nM) in 25 mM Tris buffer, pH 8.0 containing 20 mM KCl, 200 mM NaCl and 0.05% Triton X100 were incubated for 1 h at room temperature. The complex of the probe with TDNA was prepared by adding TDNA and incubating for 1 h at room temperature. The chemiluminescent activity of the PMDNAzyme-containing probe was measured at adding 5 μM of luminol and 1.3 mM hydrogen peroxide at room temperature.

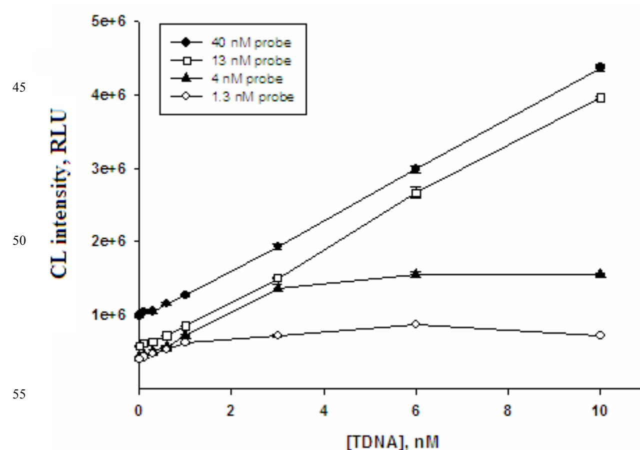

Fig. 5 Effect of concentration of PMDNAzyme-containing probe on the behavior of the calibration curve for target DNA determination. EAD2 aptamer associated with HIV oligonucleotide, preliminary heated to 88 °C and then cooled to room temperature for 60 min, was mixed with 0.25 μM hemin dissolved in 25 mM Tris buffer, pH 8.0 with 200 mM NaCl, 20 mM KCl and 0.5% Triton X-100 and incubated for 1 h at room temperature. TDNA was added in different concentrations (0-10 nM) and incubated for 60 min at room temperature. The chemiluminescent activity of the PMDNAzyme was measured at adding 5 μM of luminol and 1.3 mM hydrogen peroxide at room temperature.

increase in background CL signal, in this work we used hemin concentration of 250 nM.

Effect of concentrations of the probe on analytical parameters of the assay was also examined. As seen in Fig. 5, the assay sensitivity did not depend on the probe concentration. It should be noted that at low concentrations of the probe the working (linear) range was narrow. Increasing the probe concentration in the range 1.3 to 13 nM expanded the working range of the assay. Further increase of the probe concentration did not change the working range and sensitivity of the assay, but increased background signal.

The calibration curve for TDNA determination obtained under the optimized conditions (250 nM of hemin and 13 nM of the probe) allowed estimating the analytical parameters of the assay (Fig. 5). The detection limit (defined as 3 standard deviation (3σ) of background) value and the linear range (R^2=0.996) were shown to be 100 pM and 0.1–10 nM, respectively. These characteristics are identical to those published previously for CRET-based assay.[12] It should be also noted a high precision of the proposed assay, because the values of coefficient of variation (CV) measured within the working range varied from 0.3 to 2.0% (n=4).

The specificity of the proposed assay was also investigated. Comparison of cross-reactivity of TDNA, single base mismatched target (SMT), double base mismatched target (DMT) and non-complementary target (NT) is presented in Fig. 6. These data showed that a replacement of one base in TDNA sequence diminished the efficiency of its interaction with the probe by 13%. At the same time, DMT and NT slightly affected CL enhancement of the PMDNAzyme-containing probe having the

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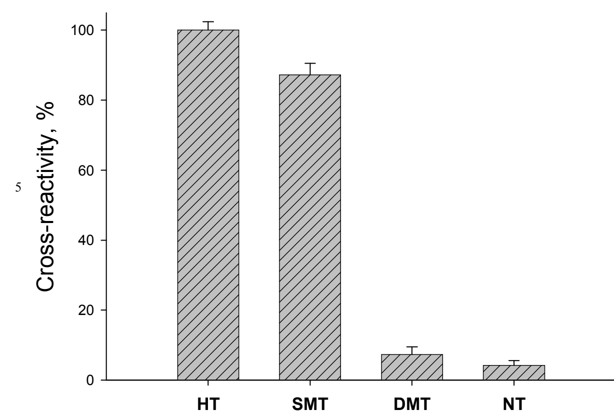

Fig. 6 Specificity of the proposed method for DNA determination (n=3). The experimental conditions: PMDNAzyme-containing probe was produced by mixing of 13 nM oligonucleotide probe, preliminary heated to 88 °C and then cooled to room temperature for 60 min, with 1 μM hemin in 25 mM Tris buffer, pH 8.0 with 200 mM NaCl, 20 mM KCl and 0.5% Triton X100 and incubating for 1 h at room temperature. The probe after its interaction with 3 nM target DNA, 3 nM single base mismatched target (SMT), 3 nM double base mismatched target (DMT) and 3 nM non-complementary target (NT) catalyzed the luminol oxidation. The chemiluminescent activity of the PMDNAzyme was measured at adding 5 μM of luminol and 1.3 mM hydrogen peroxide at room temperature.

cross-reactivity of 7.3 and 4.2%, respectively. This indicates a high specificity of the developed DNA assay.

In summary, we have developed a homogeneous chemiluminescent assay for DNA determination based on the allosteric activation of PMDNAzyme-containing probe resulting in enhancement of chemiluminescence. This method is simple, rapid, isothermal, sensitive, cost-effective and has good specificity. It opens up very promising perspectives for its use in analytical practice.

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Notes and references

[a]*Department of Chemistry, Lomonosov Moscow State University, Moscow 119991, Russia. E-mail: sakharovivan@gmail.com; fax: +7 (495) 9395417*

[b]*Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources (Ministry of Education), Guangxi Normal University, Guilin 541004, China.*

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