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ARTICLE TYPE

A G-quadruplex based platform for label-free monitoring of DNA reaction kinetics

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The research on the kinetic characteristics and mechanisms of DNA reactions is crucial for bioengineering and biosensing. G-quadruplex, which can form peroxidase-mimicking DNAzyme with hemin, was first used to establish a versatile platform for kinetic investigation on DNA reaction. G-quadruplex sequence EAD2 was designed into the corresponding nucleic acid reaction as product. The kinetic curves can be obtained rapidly and simply via the quantification of created DNAzyme. In this paper, isothermal linear strand displacement amplification reaction with different DNA lengths and isothermal exponential amplification reaction were successfully elucidated via the G-quadruplex based monitoring platform. As a safe and accessible alternative to the traditional methods, this robust, label-free, time-saving and high-throughput platform shows great potential to explore more novel DNA reactions or circuits in an ingenious manner.

1. Introduction

Nucleic acid (NA), the carrier of genetic information, can act as biomarker that plays crucial roles in the diagnosis of genetic disorders, pathogenic infections and the inspection of biological warfare agents. Many effective and sensitive amplification methods, such as helicase-dependent amplification,[1] strand displacement amplification[2] and rolling circle amplification[3], have been developed to meet the needs of accurately and flexibly designed NA assays. Apart from these, NAs are also versatile and programmable building materials to construct artificial circuits for logic operation,[4] cascading,[5] signal gain,[6] self-sustained replication[7] and molecular programming[8]. The investigation on the kinetics of these NA reactions can provide us the illuminating insight into the essential mechanisms and functions, which can give practical guidance to the design of biosensors and circuits towards various targets and demands.

Monitoring the products or intermediate oligonucleotides of NA reaction is an important part of kinetic research. Different kinds of methods have been adopted by researchers, like radio-isotopic assay, real-time fluorescence measurement, fluorescence imaging of gels and mass spectrometry (MS). The most common used method is traditional radio-isotopic assay. Radioactive tracers allow trace amounts of substances to be detected. Simultaneously, the labeled and unlabeled probes show similar chemical properties that reduce the discrimination.[9] The radiation can be detected by autoradiography analysis, phosphor imaging or liquid scintillation counting. However, these costly tracers with short life-time have hazards to health and environment. Fluorescence imaging of gels is also popular and convenient for researchers to quantify the NA products,[10] but the intercalated fluorescent dyes used (eg. ethidium bromide) are harmful to the experimenters. Besides, the ability of sequence-specific identification is still not satisfactory, i.e., it cannot distinguish and quantify the specific oligonucleotides with the same or similar molecular weight via electrophoresis and dye only. In addition, the time-consuming gel electrophoresis operation reduces the efficiency of rapid quantification. To improve the performance of separation and identification, liquid chromatography (LC)-MS is used to analyze the complex components in DNA reaction.[11] The DNA products under investigation can be separated by LC and accurately detected by MS. This method is limited by low throughput and the poor ionization efficiency of long single-strand DNA and double-strand DNA (dsDNA) in electrospray-ionization condition, which directly affects the MS signals to quantify and even identify. Real-time fluorescence monitoring is often adopted in the exploration of kinetics in artificial circuits (eg. toehold-mediated NA strategy).[6,12] The well-designed labeling of fluorophores and quenchers makes the assays expensive. For real-time fluorescence monitoring based on intercalated dyes, the increase of fluorescence intensity that is correlated to the amount of dsDNA region cannot point out the kinetics of the individual product or step clearly, especially in some sophisticated multi-steps NA strategies. Therefore, the development of a simple, safe, efficient and label-free monitoring platform to meet more challenges is an attractive field.

G-quadruplex, consisted of repetitive G-rich sequence motifs, is a kind of higher-order DNA structure.[13] It can interact with hemin to form G-quadruplex/hemin DNAzyme that mimics horseradish peroxidase (HRP) activity. In recent researches, G-quadruplex sequence was widely used for signal transduction in biosensors towards small molecules[14] and bio-macromolecules[15]. The signal readout, which is proportional to the amount of G-quadruplex, can be measured by colorimetry,[16,17] chemiluminescence,[18]

fluorescence[19] or electrochemical assays[20]. Acting as a smart reporter, G-quadruplex shows great potential to construct a robust monitoring platform in order to replenish the current quantification assays adopted in DNA reaction kinetics.

Herein, taking advantages of simple, label-free, rapid and high throughput detection for G-quadruplex, a novel G-quadruplex based platform for the monitoring of DNA reaction kinetics was established. G-quadruplex sequence EAD2 was elaborately designed into the corresponding DNA reaction that being generated or released as the product. Using colorimetry to quantify EAD2 produced during DNA reaction, we investigated the kinetic curves of isothermal strand displacement amplification (SDA) reaction with different lengths of DNA products and monitored the exponential growth of isothermal exponential amplification reaction (EXPAR) successfully. It's the first time that G-quadruplex sequences are used as a versatile and programmable tool for the kinetics of DNA biochemical reactions.

2. Experimental Section

2.1 Reagents and Apparatus

All HPLC-purified DNA oligonucleotides listed in Table S1 of ESI were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The vent (exo-) DNA polymerase and Nt.BstNBI were purchased from New England Biolabs (Beverly, MA). Also, 10 × Nt.BstNBI buffer (500 mM Tris-HCl, 1000 mM NaCl, 100 mM $MgCl_2$, 1 mg/mL BSA, pH 7.9) and 10×ThermoPol buffer (200 mM Tris-HCl,100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1% Triton X-100, pH 8.8) were provided by New England Biolabs. The deoxynucleotide mixture (dNTPs) was purchased from Takara Biotechnology Co. Ltd (Dalian, China). Hydrogen peroxide (H_2O_2) was obtained from Beijing Chemical Reagent Company (Beijing, China). TMB 2HCl (TMB: 4,4'-diamino-3,3',5,5'-tetramethylbiphenyl) was bought from Ameresco (USA). Hemin was bought from Sigma-Aldrich (St.Louis, MO, USA). It was dissolved in dimethyl sulfoxide as 5 mM stock solution and stored at -20℃. All other reagents were at least of analytical reagent grade. Ultrapure water from a Milli-Q water purification system (Bedford, MA, U.S.A) was used throughout.

TC-512 PCR (TECHNE, UK) was used for all the isothermal amplification reaction. Absorption signals were recorded by Thermo Scientific Multiskan FC Microplate Photometer (USA).

2.2 The Detection of EAD2

The 10 μL different concentrations of EAD2 (in 1 ×ThermoPol buffer and 0.5 ×Nt.BstNBI buffer), 4 μL of 50 μM hemin and 14 μL 1 ×ThermoPol buffer were mixed. A 5 μL final solution was dropped into 96-well plate. Then 100 μL TMB-H_2O_2 solution (0.12 mg/mL TMB 2HCl, 18 mM H_2O_2, 0.1 M NaH_2PO_4-Na_2HPO_4, pH 6.0) was added to start the chromogenic reaction. G-quadruplex/hemin DNAzyme can catalyze H_2O_2-mediated oxidation of TMB. After 3 minutes incubation at room temperature (avoiding direct light exposure), the reaction was terminated by 50 μL 2 M H_2SO_4. Then the absorbance detection, collected as (A_{450}-A_{620}), was carried out by a Microplate Photometer via dual wavelengths mode.

2.3 SDA Strategy

SDA strategy was performed at a volume of 10 μL containing target X (200 nM), X′-Y′ template (200 nM), dNTPs (375 μM), Nt.BstNBI (0.4 U/μL), vent (exo-) DNA polymerase (0.05 U/μL), 1 ×ThermoPol buffer and 0.5 × Nt.BstNBI buffer. The reaction was incubated at 55℃ for different time and stored at 4℃ to terminate the reaction. The reaction product was used for further EAD2 quantification.

2.4 EXPAR Strategy

Part A and Part B solutions were prepared separately. Part A consisted of Y′-Y′ template, EAD2 sequence (denoted as Y), dNTP and Nt.BstNBI buffer. Part B consisted of Nt.BstNBI, vent (exo-) DNA polymerase and ThermoPol buffer. Part A and Part B were mixed immediately containing Y′-Y′ template (200 nM), Y (0, 1 pM, 10 pM or 100 pM), Nt.BstNBI (0.4 U/μL), vent (exo-) DNA polymerase (0.05 U/μL), dNTPs (375 μM), 1 ×ThermoPol buffer and 0.5 ×Nt.BstNBI buffer. The 10 μL mixture was incubated at 57℃ for fixed time and quenched at 4℃ for further measurement.

3. Results and Discussion

3.1 The quantification of G-quadruplex sequences

The G-quadruplex sequence EAD2,[13] with excellent HRP-mimicking DNAzyme activity in the presence of hemin, was selected to perform the monitoring. As EAD2 is served as the label-free tag to indicate the kinetic process in DNA reactions, a robust assay to quantify the amount of created or released EAD2 sequence is required. In our experiment, the TMB-H_2O_2 colorimetric system was adopted in microplate for rapid and high-throughput optical signal readout. With the optimization of chromogenic time to 3 min (Figure S1), the calibration curve of EAD2 was obtained (Figure 1). The lowest detectable concentration is 10 nM (100 fmol in 10 μL) and the linear equation is $\triangle A = 5.30\times10^5 C_{EAD2} + 0.113$ (R^2=0.992) ranging from 100 nM to 4 μM. The linear equation was used for the quantitative calculation of EAD2 product in the following DNA reaction and circuit system.

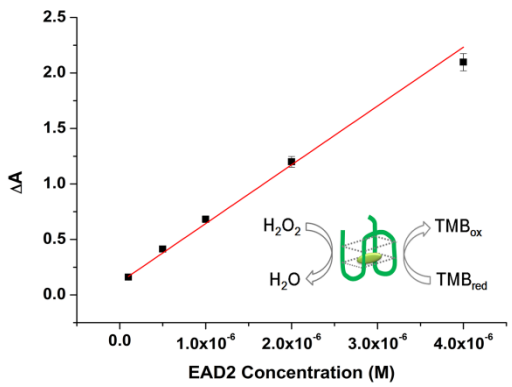
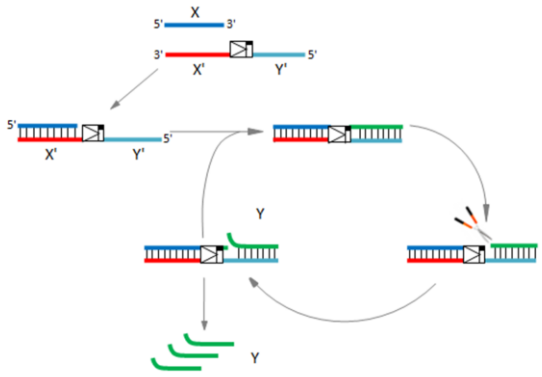


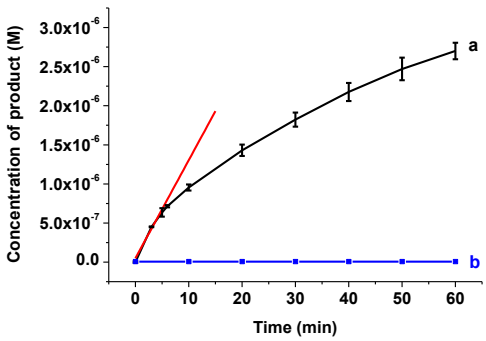
Figure 1 The calibration curve of △A (A_{450}-A_{620}) vs. EAD2 concentrations. △A means the signal response of individual concentration that the background value has been extracted. Error bars: Standard Deviation (SD), *n*=3.

3.2 The kinetic investigation of SDA strategy

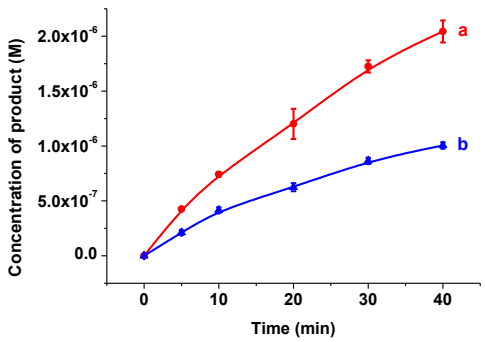
SDA is a basic and popular enzyme-based DNA reaction (Figure 2A). It's generally carried out by the hybridization of target DNA X and template DNA X'-Y'. The primer/template X/X'-Y' can be extended by polymerase to form a complete dsDNA. Then nicking endonucleases enzyme cleaves a phosphodiester on the one strand of the dsDNA structure. Following that, the polymerase can extend the 3'-end at the nicking recognition site and displace the downstream strand, which leads to the continuous generation of oligonucleotide Y via linear amplification. To test the feasibility of G-quadruplex based platform to monitor the kinetic process of SDA, the Y' region is designed to be the complementary of EAD2 sequence. The generated Y, EAD2, was quantified by colorimetry over the incubation time. We observed that the concentration of EAD2 increased rapidly during the first 10 min and then the reaction rate gradually slowed down (Figure 2B, curve a). About 2.7 μM EAD2 was produced in 60 min incubation, which meant that about 13.5 folds transduction efficiency was obtained from target X to reporter Y. At the same time, the SDA kinetic proceeding can be expressed as the mass action equation: $d[Y]/dt = r \times [X/X' - Y']$, r is the rate of oligonucleotide (Y) produced by each primer/template X/X'-Y'. According to the kinetic curve a in Figure 2B, the apparent rate constant was calculated to be $r_{initial}$ = 0.64 min^{-1} at beginning and r_{final} = 0.12 min^{-1} at the end time point of incubation. The rate decline was also observed in relevant reference,[21] in which said that the phenomenon might be due to the inactivation of one or more essential components in the SDA reaction. There was no detectable amount of EAD2 generated in the control of SDA reaction along the one hour incubation time (Figure 2B, curve b), which was consistent with the reference by real-time fluorescence monitoring.[22]



(A)



(B)



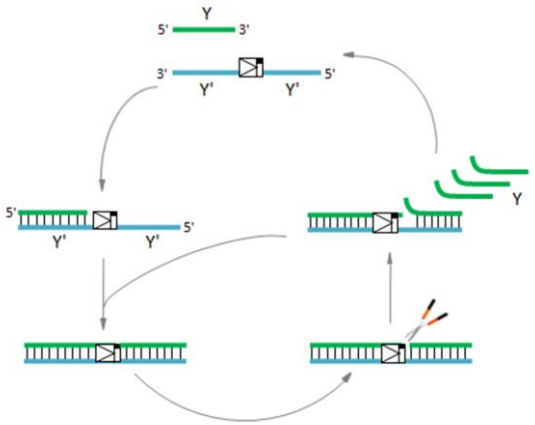
(C)

Figure 2 (A) Principle of SDA. The created EAD2 sequence (Y) is marked in green. (B) The kinetic curve of SDA via template X'-Y' in the presence (a) or absence (b) of trigger X. (C) The comparison of the SDA kinetic curves performed by templates X'-Y'-A5 and X'-Y'-A10. Two corresponding products EAD2-T5 (a) and EAD2-T10 (b) are detected, respectively. The trigger X and template were present at 200 nM both in 10 μL reaction solution. Error bars: SD, n=3.

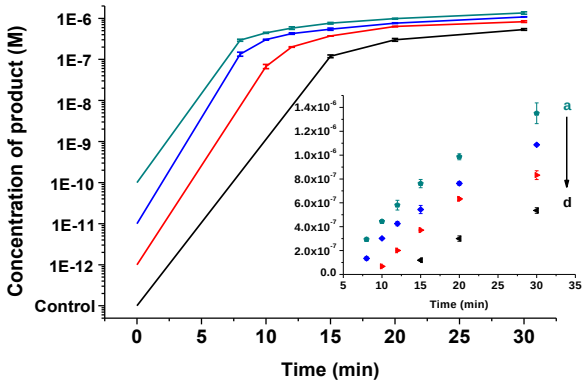
To investigate the SDA efficiencies towards different lengths of product oligonucleotides, we added five and ten adenines (A) to the 5'-end of template X'-Y' to form two new templates named X'-Y'-A5 and X'-Y'-A10 respectively. That means the products are EAD2-T5 and EAD2-T10 (T, thymine) performed by these two templates, which are five or ten bases longer than the previous EAD2 sequences. We had tested that the added T5 and T10 hardly affected the activity of EAD2/hemin DNAzyme (Figure S2). The two templates were triggered under the same experimental conditions and the results were showed in Figure 2C. It's obvious that as the length of created oligonucleotide increased, the corresponding yield of them decreased. The amount of EAD2-T10 created in 40 min was only a half of that obtained by EAD2-T5. The initial rates of X/X'-Y'-T5 and X/X'-Y'-T10 were calculated to be 0.43 min^{-1} and 0.21 min^{-1}. If the extension rate and nicking rate kept constant during the whole SDA strategy involved here, longer Y would need more time to obtain the same amount of products comparing with that of a shorter one. The initial rates and kinetic curves in our experiment coincide with the above hypothesis. It indicates that our G-quadruplex-based monitoring platform is feasible and robust for the research of kinetic information in SDA strategy. Furthermore, the platform can be applied to study the effects of enzyme concentrations, generation rate influenced by different base components and the affinity discrimination of DNA polymerase in SDA reactions.

3.3 The kinetic investigation of EXPAR strategy

The G-quadruplex based monitoring platform was further applied to complex and rapid exponential amplification reactions. EXPAR is an isothermal molecular chain reaction that synthesizes short oligonucleotides with excellent magnification (10^6).[21] The biochemical circuit with positive feedback design is contributed to the rapid kinetic performance and amplification efficiency. To obtain the kinetic curve of EXPAR by G-quadurplex based colorimetry, we used a template Y'-Y'



(A)



(B)

Figure 3 (A) The principle of EXPAR. The created Y is EAD2 sequence. (B) The concentrations of EAD2 generated along the amplification time in the presence of different concentrations of Y: (a) 100 pM; (b) 10 pM; (c) 1 pM and (d) 0.The starting point in (B) is not measurable by colorimetry and is the initial concentration added into the reaction. Error bars: SD, n=3.

containing two complementary regions of EAD2 sequences separated by nicking recognition site (Figure 3A). While the system is triggered by EAD2, the product oligonucleotide EAD2 can be created in linear manner and touch off another unoccupied template subsequently, leading to exponential amplification circuit.

The semilogarithmic plot in Figure 3B figured out the kinetic information: as the strategy would perform exponential amplification phase before the template (200 nM) was used up, we can see the concentration of product EAD2 was amplified to several magnitudes less than 10 minutes; then a linear amplification phase was carried out accompanying with declining trend of rate constant, which was similar to that observed in SDA kinetic curves. The whole kinetic curve of EXPAR observed by our developed method was consistent with that by LC-MS.[21] During 10 min incubation at 57℃, 68 nM, 302 nM and 444 nM EAD2 are detected while triggered by 1 pM, 10 pM and 100 pM Y respectively. However, there was no detectable amount of EAD2 oligonucleotide existed in the control sample until 15 min, at which 119 nM non-specifically generated EAD2 was found. The non-specific amplification of EXPAR is a normal phenomenon after a certain period of incubation time, which was also observed by gel electrophoresis and real-time fluorescence PCR monitoring.[22-24] The final amount of amplified EAD2 was limited by the concentration of template Y'-Y'. About 10^{-6} M created EAD2 product was produced in 30 min by our monitoring platform, which is almost compatible with the amplification efficiency mentioned in reference.[11, 21] The data in the late stage of exponential phase (less than 200 nM EAD2 was detected) can be used to estimate the apparent parameter of exponential performance roughly since the amount of product created in the system can be expressed by an exponential equation: $\sigma = \sigma_0 e^{\beta t}$, where σ means the concentration of product, (σ_0 is the concentration of triggering EAD2). Product EAD2 that was not released or displaced into solution was neglected here. The average value of β in the three triggering events (1 pM, 10 pM and 100 pM) was 1.10 min^{-1} (RSD=8.7%). β is the most important apparent rate parameter for the evaluation of exponential phase in EXPAR. The values are varied in different experiments due to various factors like enzyme concentrations, incubation temperature, the length and GC% of specific oligonucleotides and so on. It's clear that the G-quadruplex based monitoring platform can simply and rapidly illustrate intuitionistic kinetic curves in complex DNA amplification reactions, measure useful rate parameters and give us accurate understanding of the specific DNA reaction kinetics.

4. Conclusions

We first developed a G-quadruplex based monitoring platform for the investigation of DNA reaction kinetics. G-quadruplex sequence EAD2 was elaborately designed into the DNA reaction under exploration and generated as the product oligonucleotides in individual system. Taking advantages of the rapid, easy-handling colorimetry for G-quadruplex/hemin DNAzyme, we can quantify the concentration of G-quadruplex created in the specific DNA reactions and draw product vs. time curves for kinetic investigation. We monitored SDA and EXPAR by our platform and got some crucial kinetic parameters and qualitative cognitions successfully. Our platform showed outstanding characteristics like safety, simplicity, robustness, low cost, and high throughput. Despite the G-quadruplex based platform cannot fulfill continuous on-line observation of DNA reaction, it can be utilized for the monitoring of some fatal intermediated steps happened in complex non-enzyme toe-hold mediated DNA reactions as a powerful complement to the universal real-time fluorescence detection. The platform has great potential to study more novel DNA reactions or circuits conveniently and rapidly that contributes to the development and regulation of nucleic acid based biotechnology, biomedicine and biosensing.

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Notes and references

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Graphical Abstract

A G-quadruplex based platform was first established for the simple, label-free, rapid and high-throughput monitoring of DNA reaction kinetics.