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Label-free and dual-amplified detection of protein via small molecule-ligand linked DNA and a cooperative DNA machine

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ABSTRACT

Sensitive detection of protein is essential for both molecular diagnostics and biomedical research. Here, taking folate receptor as the model analyte, we developed a label-free and dual-amplified strategy via small molecular-ligand linked DNA and a cooperative DNA machine which could perform primary amplification and mediate secondary amplification simultaneously. Firstly, the specific binding of folate receptor to the small-molecule folate which linked to a trigger DNA could protect the trigger DNA from exonuclease I digestion, translating folate receptor detection into trigger DNA detection. Subsequently, trigger DNA initiated the DNA machine through hybridizing with the hairpin of the DNA machine, resulting in hairpin conformational change and stem open. The open stem further hybridized with a primer which initiated circular strand-displacement polymerization reaction; meanwhile the rolling circle amplification templates which were initially blocked in the DNA machine were liberated to mediate rolling circle amplification. In such a working model, the DNA machine achieved cooperatively controlling circular strand-displacement polymerization reaction and rolling circle amplification, realizing dual-amplification. Finally, the rolling circle amplification process synthesized a long repeated G-quadruplex sequence, which strongly interacted with N-methyl mesoporphyrin IX, bringing label-free fluorescence signal. This strategy could detect folate receptor as low as 0.23 pM. A recovery over 90% was obtained when folate receptor was detected in spiked human serum, demonstrating the feasibility of this detection strategy in biological samples.

1. Introduction

Sensitive and selective detection of protein is necessary in molecular diagnostics as well as biomedical research. To achieve the specific recognition of proteins, antibodies and aptamers are typically used as affinity ligands (Nam et al., 2003; Mani et al., 2009; Wang et al., 2011; Zhou et al., 2009). Compared with antibodies, aptamers have the advantages of reusability, readily produced by chemical synthesis and easily incorporated in oligonucleotide-based signal amplification processes (Lee et al., 2010; Liu et al., 2011; Zuk et al., 1979). Despite those advantages, aptamers for some proteins (α-fetoprotein and prostate-specific membrane antigen) are RNA, which are unstable since they can be easily degraded by ribonucleases (Lee and Lee, 2012; Lupold et al., 2002). And aptamers for some proteins have not been screened out yet (He et al., 2013).

Small molecule-ligand linked DNA can selectively capture proteins (Harris et al., 2008, 2009). And the DNA part which can be randomly coded provides enormous possibilities for signal transforming and amplification. Due to those two characteristics, DNA terminally tethered with small molecule-ligand serves as attractive complement to aptamer. In 2009, Wu and the co-workers found that the DNA tethered small molecular at 3′-end could be protected from exonuclease I (Exo I) digesting after binding to target protein, which is called terminal protection (Wu et al., 2009). This principle provides a basis for constructing selective and sensitive protein detection methods via small molecular-ligand linked DNA.

Based on terminal protection, a series of protein assays have been designed by combining with several transducing techniques, including fluorescence (He et al., 2013; Wei et al., 2012), electrochemical (Wu et al., 2009; Cao et al., 2012; Wang et al., 2013), chemiluminiscence (Zhao et al., 2014), and colorimetric transducer (Yang and Gao, 2014). Among them, the fluorescence methods have attracted wide attentions due to the advantages of

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safety, simplicity and little proclivity to sample damage. To further improve the detection sensitivity, a number of signal amplification approaches including one-stage amplification strategies (exonuclease III assisted DNA recycling reaction, nicking enzyme assisted DNA recycling reaction, DNAzyme assisted amplification) (Zhou et al., 2013; Zhen et al., 2012; Zhao et al., 2013) as well as two-stage amplification strategies (Ou et al., 2013) have been employed in fluorescence methods. In comparison with the one-stage amplification strategies, the two-stage amplification strategies significantly improve amplification efficiency. However, the two-stage amplification strategies still require labels to obtain detectable signals. The labeling process may decrease the performance of the signal probe since the incomplete quench between fluorophore and quencher. Additionally, the complexity and costliness also restrict their universal applications. Hence, there is still an urgent demand for developing a label-free and sensitive protein detection strategy.

"DNA machine" is a DNA assembly that performs a mechanical function, where the mechanical function is triggered by a fuel substrate and the mechanical operation yields a product that provides a readout signal (Zhang et al., 2011; Qi et al., 2013). DNA machine can perform different functions, acting as switches Tian and Mao (2004), walkers (Weizmann et al., 2006), or amplified sensors (Weizmann et al., 2008; Li et al., 2014; Wen et al., 2012). Amplified DNA machines can carry out the function of circular amplification autonomously and effectively only under simple operations, which offer them the possibility to be utilized for mediating other amplification process (Zhuang et al., 2014). Here, we developed a label-free and dual-amplified strategy based on small molecular-ligand linked DNA and a cooperative DNA machine which could perform primary amplification and mediate secondary amplification simultaneously. Folate receptor (FR) is a tumor biomarker and therapeutic target in tumor treatment. It has been proven that elevated serum FR levels are clinically correlated with metastatic cancers, active granulomatous disease, and liver disease (Eichner et al., 1978). The elevated FR levels may also serve as useful clinical biomarker for specific disease states Elnakat and Ratnam (2004). FR level of metastatic disease can increase to 650 pg/mL (22 pM). Given that normal serum is virtually free of FR (Eichner et al., 1978), the detection limit should reach 22 pM at least to realize metastatic disease detection and much lower to realize early diagnosis of cancer. In this method, FR is used as the model target. The specific binding of FR to folate (FA) which linked to trigger DNA could protect trigger DNA from degradation by Exo I, translating FR detection into trigger DNA detection. Trigger DNA further hybridize with the hairpin of the DNA machine to activate the DNA machine, which would perform circular strand-displacement polymerization reaction (CSDPR), along with the liberating of rolling circle amplification (RCA) templates to trigger multiple RCA. RCA process synthesized long repeated G-quadruplex sequences. N-methyl mesoporphyrin IX (NMM), which has a pronounced structural selectivity for G-quadruplex, is added to obtain label-free fluorescence signal (Hu et al. 2011). It is demonstrated that the cooperative DNA machine is reliable and effective signal amplifier and the detection limit for FR is achieved as low as 0.23 pM.

2. Experimental section

2.1. Reagents and apparatus

Trigger DNA used in this work was synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China). The other DNA oligonucleotides were obtained from Sangon Inc. (Shanghai, China) and the sequences of the oligonucleotides are listed in Table S1. FR was obtained from Sino Biological Inc. (Beijing, China). Human tumor necrosis factor-α (TNF-α) and recombinant human interferon-γ (IFN-γ) were purchased from Haematologic Technologies, Inc. (Beverly, USA) Abcam. Human thrombin was purchased from Ding Guo Biotech Co., Ltd. (Beijing, China). Klenow fragment exopolymerase (KF polymerase) was obtained from New England Biolabs LTD (Beijing, China). Exo I, T4 DNA Ligase, phi29 DNA polymerase and the deoxynucleotide triphosphates (dNTPs) were purchased from Sangon Inc. (Shanghai, China). NMM was purchased from Frontier Scientific Inc. (Utah, USA). The NMM stock solution was prepared in dimethyl sulfoxideand stored in the dark at −20 °C. All other chemicals were of analytical grade and used as received. The ultrapure water obtained from a Millipore Milli-Q water purification system (> 18.25 MΩ) was used to prepare all of the solutions.

Fluorescence emission spectroscopy was performed on a Hitachi F-7000 fluorescence spectrometer (Hitachi. Ltd., Japan) at room temperature. The mixtures in square quartz cuvettes were excited at 399 nm and the emission spectra were collected from 550 to 710 nm. The fluorescence intensity at 618 nm was used to evaluate the performance of the proposed strategy. The slits of excitation and emission were both set at 10 nm and the photomultiplier tube voltage was 700 V.

2.2. Preparation of DNA machine

300 nM hairpin oligonucleotide and 300 nM RCA template oligonucleotide were mixed in 90 μL buffer (10 mM Tris–HCl, 100 mM $MgCl_2$, 50 mM NaCl, 1.0 mM dithiothreitol, pH 7.9). The solution was incubated at 95 °C for 5 min and then allowed to slowly cool to room temperature over 30 min to enable the hairpin to fold into the correct structure. The solution was divided into 10 μL aliquots and stored at −20 °C.

2.3. Terminal protection assay for FR recognition and signal translation

A 1.5 μL aliquot of FR sample at a certain concentration and 1.5 μL 0.8 μM trigger DNA were mixed in NEB buffer2 (10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1.0 mM dithiothreitol, pH 7.9), and water was added to make 10 μL volume. The mixture was incubated for 1 h at 37 °C in dark. 0.025–0.125 μL 40 U/μL Exo I solution was mixed and incubated for 40 min at 37 °C, then the solution was heated to 85 °C for 15 min to terminate the reaction.

2.4. Operation of the cooperative DNA machine: CSDPR and RCA mediation

After the digestion reaction, 10 μL solution containing 6.7 μL hairpin/RCA template complex solution, 2 μL 1.0 μM primer1, 1 μL 10 × NEB buffer2, 1 μL dNTPs, 0.10–0.35 μL 5 U/μL KF polymerase was added. Then the CSDPR in a volume of 20 μL was carried out at 37 °C for 40 min. The solution was kept at 85 °C for 20 min to inactive the polymerase.

2.5. RCA

Afterward, the ligation reaction was performed by adding 10 μL reaction mixture containing 0.2 μL 5 U/μL T4 DNA ligase, 3 μL 10 × T4 DNA ligase buffer (400 mM Tris–HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8), 2 μL 1.0 μM primer2, 4.8 μL water to the circularization mixture. Then the mixture was incubated at 37 °C for 1 h, followed by heating to 75 °C for 10 min. Then, 0.15–0.35 μL 10 U/μL phi29 DNA polymerase, 8 μL 10 mM dNTPs, and 4 μL 10 × reaction buffer (330 mM Tris-acetate, 100 mM Mg-acetate,