Talanta 179 (2018) 331–336

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Target binding protection mediated rolling circle amplification for sensitive detection of transcription factors

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ARTICLE INFO

Keywords:
Transcription factors
Target binding protection
Nt.BbvCI cleavage
Rolling circle amplification

ABSTRACT

Transcription factors (TFs) play central roles in the regulation of gene expression by binding with specific DNA sequences. As a potential diagnostic marker, sensitive detection of TFs is essential for pharmacological research development and clinical disease diagnosis. Here, a new fluorescent method based on target binding protection mediated rolling circle amplification (RCA) was developed for TFs detection. A hairpin probe with recognition site for target binding, cleavage site for Nt.BbvCI digestion and two hanging DNA strands with part of G-quadruplex complementary sequences for signal output was designed. Moreover, the hairpin probe could serve as template of RCA after being ligated. Firstly, TFs bound with hairpin probes and protected signal complementary sequences against cleavage by Nt.BbvCI due to space hindrance effect, while the excess hairpin probes were effectively digested to avoid false positive signal. Then, TFs and Nt.BbvCI were dissociated from hairpin probes by heating, complete hairpin probes being preserved. Next, protected hairpin probes were specifically connected to dumbbell templates under the action of T4 DNA ligase. Subsequently, dumbbell templates hybridized with primer to initiate the RCA reaction, obtaining numerous G-quadruplex sequences. Finally, N-methyl-mesoporphyrin IX (NMM) bound with G-quadruplex to generate enhanced fluorescence signal. The proposed assay system achieved excellent specificity and sensitivity toward TATA-binding protein (TBP) with a detection limit as low as 88 pM, and with a linear range from 100 pM to 40 nM. The strategy proposed here was looking forward to offer a powerful tool for TFs related bioanalysis and disease diagnostics.

1. Introduction

Transcription factors (TFs) are DNA-binding proteins that can regulate gene expression and play vital roles in modulating a variety of cellular processes, such as cell development, differentiation and growth [1–4]. The dysregulation of TFs is involved in numerous pathological processes and the changes of their expression level sensitively reflect related disease states [5–8]. Therefore, TFs have been taken as a potential target in clinical diagnosis. Sensitive and specific detection of TFs has an important significance for clinical diagnosis and basic medical researches [9].

Many conventional techniques were established for detecting TFs, such as DNase footprinting [10], electrophoretic mobility shift assay (EMSA) [11], western blots [12] and enzyme-linked immunosorbent assay (ELISA) [13]. Additionally, diverse new strategies including electrochemical [14,15], colorimetric [16,17] and fluorescent methods were proposed to reduce cost, simplify operation process and improve the sensitivity. Among them, fluorescence-based detection methods attracted attention due to the advantages of simplicity, safety and sensitivity [18]. Up to now, two types of fluorescence-based method for detecting TFs had been explored. The first was the conformational transition based on target-DNA binding [19,20]. Representatively, the TFs recognition site was designed in two short DNA duplexes that were labeled with two different fluorophores. Two short DNA duplexes could generate combination to arise fluorescence resonance energy transfer (FRET) with the target protein binding [19]. A labeled double stem-loop structure molecular beacon was also designed that could convert into single stem-loop structure to produce fluorescence recovery, on account of occurring conformational transition with the specific binding of TFs [20]. However, conformational change was an equilibrium process that was easy to cause false positive signal, thus having higher requirement for design of probes. The second was nuclease cleavage inhibition based on target binding protection principle [21–23], which could avoid the participation of conformational transition. TFs specifically bound to the recognition probe, which hindered nuclease cleavage and protected the recognition probes from being digested. Exonuclease III (Exo III) was usually used to digest excess probes for TFs detection [21,22]. However, Exo III was a non-sequence-

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https://doi.org/10.1016/j.talanta.2017.11.028
Received 31 July 2017; Received in revised form 27 October 2017; Accepted 16 November 2017
Available online 21 November 2017
0039-9140/

specific enzyme that gradually digested the duplex DNA from 3′ to 5′ termini, thus causing non-specificity cleavage of DNA probe. In addition, Exo III could displace some TFs to bind with DNA and led to undesired digestion of probes, resulting in false negative signal [24]. Both of them could affect the detection accuracy. Compared with Exo III, ApoI was a sequence-specific cleavage enzyme and could produce specific digestion to DNA probes for TFs detection [23]. However, it was required that the recognition site of TFs must contain the ApoI cleavage site, which limited the versatility of this assay.

In order to solve those problems, we developed a fluorescence method based on target binding protection mediated rolling circle amplification (RCA) for sensitive, accurate and versatile detection of TFs. We designed a hairpin probe with two hanging DNA strands, target recognition site and Nt.BbvCI cleavage site. Nt.BbvCI was a sequence-specific enzyme and could cleave redundant probes, avoiding non-specificity cleavage and false negative signal, which could improve the accuracy of the detection. And we designed that recognition sequence of Nt.BbvCI was tightly close rather than inserted to the target recognition site at the stem of the hairpin, which was helpful to achieve versatility detection of TFs. Firstly, the binding of TFs with recognition site produced a space hindrance to occupy the upstream of cleavage site of Nt.BbvCI, which could protect hairpin probe against cleavage by Nt.BbvCI. Then, after deactivating TFs and Nt.BbvCI by heating, complete hairpin probes were preserved. Subsequently, with the addition of T4 DNA ligase, the ends of two hanging DNA single strands were specifically ligated to form a dumbbell template which contained the complete G-quadruplex complementary sequence. Next, the dumbbell template hybridized with primer to trigger RCA process in which the primer was elongated to obtain multiple G-quadruplex sequences under the action of phi29 DNA polymerase. Finally, N-methyl-mesoporphyrin IX (NMM) bound with G-quadruplex to produce fluorescence signal [25]. In the proposed method, due to target binding protection effect by space hindrance and rolling circle amplification (RCA) process, the sensitive and specific detection toward TATA-binding protein (TBP) was achieved with 88 pM detection limit and a linear range from 100 pM to 40 nM. In the meantime, the specific cleavage of Nt.BbvCI for hairpin probes could effectively avoid false positive signal and low fluorescent background was achieved. The method proposed here was looking forward to offer a powerful tool for TFs related bioanalysis and disease diagnostics.

2. Experimental section

2.1. Materials and reagents

All synthetic DNA oligonucleotides and the deoxynucleotide triphosphates (dNTPs) were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). The DNA oligonucleotides sequences used in the study were shown in Table S1. TBP was obtained from Abnova (Taiwan, China). Human thrombin, human immunoglobulin G (IgG) and bovine serum albumin (BSA) were provided by Sigma-Aldrich Co. (St Louis, MO, USA). NF-κB was supplied by Cayman Chemical (Ann Arbor, MI, USA). Nt.BbvCI nicking endonuclease was purchased from New England Biolabs Ltd. (Beijing, China). Phi29 DNA polymerase and T4 DNA ligase were obtained from Thermo Fisher Scientific Ltd. (Shanghai, China). N-methyl mesoporphyrin IX (NMM) was provided by J&K Scientific Ltd. (Beijing, China). All other reagents didn't need to be further purified or modified and belonged to analytical grade reagents. We prepared all solutions by using ultrapure water (> 18.25 MΩ cm) produced from a Millipore Milli-Q water purification system.

2.2. Protection effect of transcription factor-DNA binding

The binding reaction was carried out in 10 μL of mixture containing target TBP at a certain concentration, 60 nM hairpin probe, 1 × reaction buffer (10 mM Tris-HAc, pH 7.5, 100 mM KAc, 2 mM Mg (Ac) $_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT) at 37 °C for 4 h. For insuring the formation of hairpin structure, the recognition probe needed to be heated for 5 min at 95 °C and then gradually cooled down to room temperature. Subsequently, 6 U Nt.BbvCI, 10 × CutSmart (20 mM Tris-HAc, 50 mM KAc, 10 mM Mg (Ac) $_2$, 100 μg/mL BSA, pH 7.9) were added and incubated at 37 °C for 1.5 h to digest the extra hairpin probes. The following process was to inactivate Nt.BbvCI and target TBP by heating the solution for 20 min at 80 °C.

2.3. Ligation process and RCA reaction

For the ligation process, 240 nM primer, 1.0 U T4 DNA ligase and 10 × T4 ligase buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8) were added to above reaction mixture and reacted at 37 °C for 40 min to obtain dumbbell template probe. Next, T4 DNA ligase was deactivated by heating for 5 min at 75 °C. After that, 1 mM dNTPs, 3.5 U phi29 DNA polymerase, 10 × phi29 reaction buffer (330 mM Tris-Ac, pH 7.9, 100 mM Mg (Ac) $_2$, 660 mM KAc, 1% Tween 20, 10 mM DTT) were put into the mixture to perform the RCA process. The reaction occurred at 37 °C for 3 h and was terminated by heating at 65 °C for 10 min.

2.4. Fluorescent spectra measurement

The products obtained from RCA process were mixed to final volume of 40 μL with 40 μM KCl and 2.5 μM NMM and incubated at 37 °C for 30 min. Then we used Hitachi F-7000 fluorescence spectrometer (Hitachi. Ltd., Japan) to measure fluorescence spectra. We set 399 nm as the excitation wavelength and collected the emission spectra from 560 to 680 nm. The slit widths and the voltage were set at 10.0 nm and 700 V, respectively. The fluorescence emission maximum was at 618 nm.

2.5. Gel electrophoresis analysis

The gel electrophoresis was used for further validation of experiment process. The target and DNA binding protection and Nt.BbvCI digestion process were analyzed by 15% non-denaturating polyacrylamide gel electrophoresis (PAGE). The products of RCA reaction were analyzed by 0.7% (w/v) agarose gel electrophoresis. The PAGE and agarose gel electrophoresis were carried at 30 mA for 1.5 h and 200 V for 1 h in 1 × Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM Boric Acid, 2.0 mM EDTA, pH 8.3), respectively. The ethidium bromide was chosen as a stain and the condition was 5 min at room temperature. Finally, the gels were destained in ultrapure water and photographed using UV imaging system (Bio-RAD Laboratories Inc., USA).

3. Results and discussion

3.1. The principle of target binding protection mediated RCA for sensitive detection of TFs

The proposed method of TFs detection is illustrated in Scheme 1. We designed a probe that was a hairpin structure with stem, loop and two hanging DNA single strands. The stem of hairpin probe included TBP recognition site (in red) and Nt.BbvCI cleavage site (in green), thus achieving the binding of target and Nt.BbvCI cleavage. Two hanging DNA single strands contained respectively a part of G-quadruplex complementary sequences, thus being regarded as signal carrier. Moreover, the hairpin probe could be connected to form a dumbbell probe as the template of RCA. First, the binding of TFs with recognition site produced a space hindrance to occupy the upstream of cleavage site of Nt.BbvCI, which could protect hairpin probes against cleavage by Nt.BbvCI. TFs and Nt.BbvCI were deactivated after heating and complete hairpin probes were preserved. Next, under the action of primer and T4 DNA ligase, the ends of two hanging DNA single strands were