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A novel magneto-DNA duplex probe for bacterial DNA detection based on exonuclease III-aided cycling amplification

Yan Zeng [a,b], Yi Wan [a], Dun Zhang [a,*], Peng Qi [a,b]

[a] Key Laboratory of Marine Environmental Corrosion and Bio-fouling, Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China

[b] University of the Chinese Academy of Science, 19 (Jia) Yuquan Road, Beijing 100039, China

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ABSTRACT

A novel magneto-DNA duplex probe for bacterial DNA detection based on exonuclease III (Exo-III) aided cycling amplification has been developed. This magneto-DNA duplex probe contains a partly hybrid fluorophore-modified capture probe and a fluorophore-modified signal probe with magnetic microparticle as carrier. In the presence of a perfectly matched target bacterial DNA, blunt 3′-terminus of the capture probe is formed, activating the Exo-III aided cycling amplification. Thus, Exo-III catalyzes the stepwise removal of mononucleotides from this terminus, releasing both fluorophore-modified signal probe, fluorescent dyes of the capture probe and target DNA. The released target DNA then starts a new cycle, while released fluorescent fragments are recovered with magnetic separation for fluorescence signal collection. This system exhibited sensitive detection of bacterial DNA, with a detection limit of 14 pM because of the unique cleavage function of Exo-III, high fluorescence intensity, and separating function of magneto-DNA duplex probes. Besides this sensitivity, this strategy exhibited excellent selectivity with mismatched bacterial DNA targets and other bacterial species targets and good applicability in real seawater samples, hence, this strategy could be potentially used for qualitative and quantitative analysis of bacteria.

1. Introduction

The analysis of bacterial DNA, which is a commonly used genetic marker, is important in the molecular diagnosis of infectious diseases, rapid classification, and phenotyping of bacterial species [1–3]. Most diagnostic approaches related to 16S ribosomal RNA (16S rRNA) segment are based on polymerase chain reaction (PCR) [4,5]. However, some drawbacks, such as critical experiment conditions and complex operation [6], made it become a significant bottleneck in molecular diagnosis. There is therefore a need of detecting method of bacterial DNA segment in a more rapid, convenient and easily operated manner, which could also be used for bacteria and cell analysis.

A nuclease-assisted biosensor capable of amplified detection of single-stranded DNA is of great demand in nucleic acids, molecule detection, and gene diagnosis [7,8]. Various signal amplification strategies based on nuclease, including FokI enzyme [9], polymerase, and nicking endonuclease [10,11] have been developed. Compared with other nucleases, exonuclease III (Exo-III) does not require a specific recognition site, and is effective on the blunt or recessed 3′-terminus substrates of double-stranded DNA [12,13]. Moreover, Exo-III can provide a diverse platform for amplified DNA detection [14].

Most Exo-III aided signal amplification methods reported were carrier-mobilized strategies, including the use of nanoparticles [15], quantum dots [16], single-walled carbon nanotube [17] and graphene oxide [18]. However, most of them, such as quantum dots, or gold nanoparticles, suffer severe background interference from absorption, scattering, especially in complex biological media, and their detection capability would be greatly diminished. So, some advanced carriers need to be developed. Magnetic microparticles (MMPs) [19,20], which can be easily separated and are biocompatible and stable, were rarely reported in this field. In comparison with the above material, some advantages can be shown. First, most biological samples exhibit virtually no magnetic background, so the above background problem would be circumvented. Second, MMPs show fast binding kinetics with single-stranded DNA with larger surface area and high flexibility in homogeneous solution with easy separation. He and his coworkers recently designed an approach using Exo-III coded MMP probes that can simultaneously detect two kinds of DNA [21]. However, the fluorescence signal decreased correspondingly as the two different DNA strands co-assembled at MMPs. Lu and her coworkers fabricated a large diameter microsphere with high density of DNA probes to

* Corresponding author. Tel./fax: +86 532 82898960.
E-mail address: zhangdun@qdio.ac.cn (D. Zhang).

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increase the sensitivity of the analytical technique [14]. However, the sensitivity of analytical technique with a fixed diameter microsphere remains a challenging issue [22], as the coverage of single DNA strands on MMPs possibly reach saturation.

Based above, we presents a novel perspective for bacterial DNA detection based on fixed-diameter MMPs. Coupled with Exo-III aided cycling amplification, the magneto-DNA duplex probe was introduced to enhance sensitivity. The reaction mechanism of this strategy is based on the following: first, the construction of magneto-DNA duplex probes composed of two fluorophore-modified DNA probes easily captures target DNA and enhances final fluorescence signal. Second, fluorescent dyes and fluorophore-modified signal probes are recovered because of easy separation of MMPs. Third, Exo-III provides a unique cleavage function for amplifying reaction. To the best of our knowledge, our strategy for the first time reported novel perspective of magneto-DNA duplex probe to improve detection sensitivity based on fixed-diameter MMPs. Further more, this strategy offers the merits of high sensitivity, excellent selectivity, and easy operation.

2. Material and methods

2.1. Chemicals and instruments

All of the oligonucleotides were custom synthesized and high-performance liquid chromatography (HPLC) purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The library of oligonucleotide sequences used in this study is shown in Table S1. The stock solution of DNA were dispersed in TE buffer (10 mM Tris–HCl, 1 mM Ethylene diamine tetraacetic Acid/EDTA, pH 8.0), and diluted to different concentrations with the same TE buffer when needed. Streptavidin-modified MMPs (1.0 μm, 10 mg/mL) were purchased from HuiEr Nanotechnology Co., Ltd. (Henan, China). Exo-III was ordered from Sangon Biotechnology Co., Ltd. (Shanghai, China), and Tris (hydroxymethyl) aminomethane hydrochloride (Tris) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Seawater was collected from Huiquan Bay of Qingdao (zone approximately 120° 15′ to 120° 25′ N, 36° 00′ to 36° 06′ E, 10 m to 20 m deep), and then filtered with cellulose acetate ultra-filtration membrane (4 μm). Cellulose acetate ultra filtration membrane was purchased from Bioscience Biotechnology Co., Ltd. (Shanghai, China). All chemicals and solutions used throughout this study were dissolved or diluted with ultrapure water.

An 8-well magnetic rack was ordered from Sangon Biotechnology Co., Ltd. (Shanghai, China), and a KYC-1102C air constant temperature table was purchased from Jiangnan instrument factory (Ningbo, China). The fluorescence spectra data were recorded from F-4500 Fluorescence spectrophotometer (Hitachi High-technologies Corporation, Tokyo, Japan). A laser scanning confocal microscope image analysis system (MRC-1024, Carl Zeiss AG, Germany) and a DYCZ-24DN mini double vertical electrophoresis apparatus (Liuyi instrument factory, Beijing, China) were also used. The ultrapure water was produced by Storage & Distribution system (Millipore Corporation, USA).

2.2. Determining capture probe coverage on MMPs

A 100 μL fluorescence and biotinylated capture probe (500 nM) was mixed with varying amount of MMPs (0, 1, 3, 5, 7, 10, 15, and 20 μL). The supernatant was obtained with magnetic separation after 30 min of incubation at room temperature. Fluorescence signal was recovered with Hitachi F-4500 fluorescence spectrophotometer (excitation wavelength = 494 nm).

2.3. Magneto-DNA duplex probe preparation

Streptavidin-modified MMPs (10 μL) was washed five times with 1 mL of washing buffer (10 mM Tris–HCl, 1 mM EDTA, 2 M NaCl, pH 7.5). The ultimate magnetic absorptions were resuspended in 1 mL binding buffer (10 mM Tris–HCl, 1 mM EDTA, 2 M NaCl, pH 7.5). The streptavidin-modified MMPs suspension were mixed with 100 μL–500 nM biotin-modified capture probes (S1) and 100 μL of 500 nM signal probes (S2). The mixture was incubated at 25 °C for 120 min with gentle shaking. The weekly bound capture probes were removed after repeated magnetic separation and cleaning. The rest of the absorption was resuspended in 1 mL reaction buffer (20 mM Tris–HCl, 5 mM $MgCl_2$, pH 8.0) and stored at 4 °C for further use.

2.4. Fluorescence microscopy imaging of the magneto-DNA duplex probe

Streptavidin-modified MMPs and fluorescence probe-conjugated MMPs (10 μL) were added onto a glass slide, and a cover slip was glued onto the glass slide using nail polish. The sample was then imaged using a confocal laser scanning fluorescence microscope.

2.5. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed by using an unmodified capture probe, unmodified signal probe, and target DNA with the same sequence, as described in Table S1. The polyacrylamide gel was prepared with 30% (w/v) acrylamide solution, 10% (w/v) ammonium persulfate, 50 × Tris–acetate–EDTA (10 mM Tris, 5 M acetic acid and 0.625 M EDTA, pH 8.5, TAE), tetramethylethylenediamine with 1 × TAE buffer. Then, 10 μL of sample was mixed with 2 μL of loading buffer. The mixtures were run through PAGE at 170 V for 5 min and then 110 V for 60 min. The gel was stained with 0.5 μg/mL of anthodium bromide for 20 min, and a picture was taken using a digital camera under UV illumination.

2.6. Procedure for bacterial DNA detection

The detection of bacterial DNA based on Exo-III cycling amplification reaction was performed by mixing 10 μL magneto-DNA duplex probe, 10 μL of different concentrations of the target DNA, 4 μL of 10 × reaction buffer and 75 units of Exo-III to a final volume of 40 μL, followed by incubation at 37 °C for 90 min. The MMPs were then removed by magnetic separation, and the fluorescence signals of the supernatant were measured with synchronous scanning fluorescence spectrometry. Fluorescence was measured with 494 nm as the excitation wavelength, and the fluorescence emission spectra range was recorded from 500 nm to 650 nm. Excitation and emission slits were both set as 5 nm. Each experiment was repeated at least thrice to test reproducibility.

3. Results and discussion

3.1. Design and validation of the assay

We presented a novel magneto-DNA duplex probe for bacterial DNA detection. The magneto-DNA duplex probe was constructed as follows: fluorophore-modified capture probe (S1) was assembled on the streptavidin-modified MMPs, with optimized amount ratio of MMPs and S1 during the experiment. With the addition of fluorophore-modified signal probe (S2), S1 would hybridized with S2, leaving a 24-nt single-stranded DNA segment overhanging at its 3′-terminus (B part of Scheme 1). It should be noted that S2 only hybridizes partly with S1, leaving 5 nt single-stranded DNA segments overhanging at its 3′-terminus to resist the cleavage by Exo-III (Supplementary section S1.1.) because the resistance degree of Exo-III on single-stranded DNA at the 3′-protruding terminus is dependent on the length of the extension, with extensions 4 nt or longer being