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Sensitive quantitative detection of bacterial DNA based on lysozyme signal probe and Exo III-aided cycling amplification reaction

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ABSTRACT

A sensitive bacterial DNA detection method based on lysozyme signal probe and exonuclease III (Exo-III) aided cycling amplification is developed. First, the capture DNA conjugated with lysozyme is assembled on magnetic beads (MBs). In the presence of a perfectly matched target bacterial DNA, blunt 3′-terminus of the capture probe is formed, thereby activating Exo-III aided cycling amplification. Thus, Exo-III catalyzes the stepwise removal of mononucleotides from this terminus, releasing lysozyme and target DNA. The released target DNA then finds another capture probe to start a new cycle, while the released lysozyme is recovered by magnetic separation for fluorescence signal collection. Because of the separating function of MBs, unique cleavage function of Exo-III, and catalytic signal amplification of lysozyme, this system achieves sensitive bacterial DNA detection, with a detection limit of 3.5 fM. Besides this sensitivity, this strategy exhibits excellent selectivity with mismatched bacterial DNA targets and other bacterial species targets, as well as good applicability in real human serum samples. As a result, this strategy shows considerable potential for qualitative and quantitative analysis of bacteria.

1. Introduction

Bacterial DNA, a commonly used genetic marker, is crucial in the molecular diagnosis of phenotyping, and rapid classification of bacterial species [1,2], as any self-replicating organism can be discriminated from another on the basis of nucleic acid sequences that are unique to that particular organism [3]. Most diagnostic approaches for 16S ribosomal RNA (16S rRNA) segment detection are based on real time polymerase chain reaction (RT-PCR)[4,5], which is still restricted by certain disadvantages, including complex operation and critical experiment conditions [6]. Therefore, a simple, sensitive detection method for bacterial DNA segments that could also be used for bacteria and cell analysis is urgently needed [7,8].

DNA-protein conjugates are artificial combinations of biomolecular building blocks of two large families of biopolymers, namely, nucleic acids and proteins [9,10]. These DNA-protein conjugates possess the combined properties of nucleic acids and protein. On one hand, with the extremely high specificity of Watson-Crick base pairing, physicochemical stability, and mechanical rigidity, the DNA molecule is proven to be extraordinarily useful in flexible molecular nanoconstruction [11,12]. On the other hand, proteins combine high chemical specificity via various enzymatic reactions and sensitive detection with inherent catalytic signal amplification after billions of years of evolution [13,14]. Thus, with the above mentioned properties, DNA-protein conjugates possess a broad range of bio-analytical applications, especially for nucleic acid detection. In 2011, Xiang et al. introduced DNA-invertase conjugates to quantify a variety of analytical targets [15]. Since then, a functional DNA sensor linked with a commercially available personal glucose meter (PGM) has been further demonstrated by using the conversion of invertase. With this sandwich assay, a detection limit of only 40 pM DNA target was obtained [16]. Wang et al. reported a simple assay for quantitating the target DNA based on a personal uric acid meter and xanthine oxidase-modified DNA, and the detection limit was 10 pM for p53 DNA [17]. Song et al. also reported a multistage volumetric bar chart chip for visualized quantification of DNA with catalase-DNA aided catalytic reaction and three stages of platinum-catalyzed propulsion, however, the detection limit was only 20 pM for DNA targets [18]. Based on the

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above, new stages are needed to be added to further improve the sensitivity of DNA detection assay based on DNA-protein conjugates.

Recently, several DNA amplification techniques, including rolling circle amplification [19,20], polymerase chain reaction [21] and DNA strand-displacement polymerization [22,23], have been reported for DNA detection. All these strategies are based on nucleases, such as FokI enzyme [24], polymerase [25], and nicking endonuclease [26]. Compared with other nucleases, exonuclease III (Exo-III) does not require a specific recognition site, and is effective for the blunt or recessed 3′-terminus of double-stranded DNA [27]. Moreover, Exo-III can provide a diverse platform for amplified DNA detection [28].

In this paper, a novel detection method for bacterial DNA detection based on lysozyme signal probe and Exo-III aided cycling amplification was proposed. Lysozyme is an alkaline enzyme that has been thoroughly characterized [29]. With the catalytically active site of Asp52 and Glu35, lysozyme can hydrolyze the substrate 4-methylumbelliferyl β-D-*N*, *N*′, *N*′′-triacetylchitotrioside and produce a fluorescent product 4-methylumbelliferone, which can be detected with fluorescence spectroscopy [30]. Coupled with lysozyme as catalytic signal probe, Exo-III aided cycling amplification was introduced to enhance sensitivity. The reaction mechanics of this strategy is based on the following. First, the lysozyme-modified capture probe immobilized on MBs easily captures target DNA and enhances the final fluorescence signal. Second, lysozyme are recovered because of easy separation of MBs. Third, the target DNA triggers several rounds of amplification cycle because of unique cleavage function and amplifying reaction of Exo-III. To the best of our knowledge, our strategy for the first time reports a novel perspective of lysozyme-based detecting method for bacterial DNA. Furthermore, this strategy offers the merits of high sensitivity, excellent selectivity, and easy operation.

2. Material and methods

2.1. Chemicals and instruments

Lysozyme, 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (sulfo-SMCC), 4-methylumbelliferyl β-D-*N*, *N*′, *N*′′-triacetylchitotrioside, dithiothreitol (DTT) were ordered from Sigma-Aldrich (St. Louis, MO). All oligonucleotides were custom-synthesized and purified by high-performance liquid chromatography (HPLC) 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 was 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 MBs (1.0 μm, 10 mg/mL) were purchased from Life Technologies (Norway). Exo-III was ordered from Sangon Biotechnology Co., Ltd. (Shanghai, China), and Tris (hydroxymethyl) aminomethane hydrochloride was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Human serum was obtained from Hai Ci Hospital (Qingdao, 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). Fluorescence spectra data were recorded by F-4500 fluorescence spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Ultrapure water was produced by Storage & Distribution System (Millipore Corporation, USA).

2.2. Lysozyme-DNA conjugation

For lysozyme-DNA conjugation, 200 μL of 10 mg/mL lysozyme in buffer A (0.1 M NaCl, 0.1 M sodium phosphate buffer) was mixed with 0.25 mg of sulfo-SMCC. After being vortexed for 5 min, the solution was placed on a shaker for 1 h at room temperature. Then, the mixture was centrifuged to remove the insoluble sulfo-SMCC, and the supernatant was purified for 8 times by Amicon-3K using buffer A. For DNA activation, Thiol-modified oligonucleotide probes (0.1 mM, 200 μL) were treated with 125 mM dithiothreitol in PBS with 10 mM sodium hydroxide for 2 h and then purified by Amicon-3K using buffer A for eight times. The above solution was then mixed with sulfo-SMCC activated lysozyme, and the mixed solution was kept at room temperature for 48 h. Then, the solution was purified by Amicon-3K using buffer A for eight times to remove unconjugated DNAs and lysozyme.

2.3. Capture probe immobilization on MBs

Streptavidin-modified MBs (10 μL) were washed for 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, and 2 M NaCl, pH 7.5). The streptavidin-modified MB suspension was mixed with 100 μL of 500 nM biotin-modified capture probes. The resulting mixture was incubated at 25 °C for 120 min with gentle shaking. The weakly 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. Procedure for bacterial DNA detection

Detection of bacterial DNA based on lysozyme signal probe and Exo-III cycling amplification reaction was performed by mixing 10 μL MB immobilized capture probe, 10 μL of different concentrations of the target DNA, 4.0 μL of 10 × reaction buffer and 3 μL of Exo-III to a final volume of 40 μL, followed by incubation at 37 °C for 60 min. MBs were then removed by magnetic separation, and the above mentioned resulting supernatant was mixed with 100 μL of lysozyme fluorescence substrate (1.5 mg/mL). The mixed solution was kept at 50 °C for 1.5 h with reaction buffer (20 mM PBS pH 5.0). At the end of the incubation period, the reaction mixture was terminated by the addition of 200 μL bicarbonate buffer (pH 10.4). For fluorescence collection, the resulting fluorescent product was measured with 360 nm as excitation wavelength, and the fluorescence emission spectra range was recorded from 360 nm to 650 nm. Excitation and emission slits were both set as 1 nm. Each experiment was repeated for a minimum of three times to test reproducibility.

3. Results and discussion

3.1. Principle of the sensitive detection of bacterial DNA

The design principle for sensitive detection of bacterial DNA is schematically described in Scheme 1. First, lysozyme was conjugated with the capture probe (in red color, S1 for short), and the conjugation procedure is shown in the experiment section. Afterwards, the capture probe conjugated with lysozyme was fixed on streptavidin-MBs. With the introduction of the target DNA (in blue color), the DNA would hybridize with the capture probe. Notably, the capture DNA is designed to completely hybridize with the target bacterial DNA, leaving 5 nt single-stranded DNA segments overhanging at target DNA 3′-terminus to resist the cleavage by Exo-III. This resistance degree of Exo-III on single-stranded DNA at the 3′-protruding terminus is dependent on the length of the extension,