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Lateral flow assay-based bacterial detection using engineered cell wall binding domains of a phage endolysin

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ABSTRACT

The development of a cost-effective and efficient bacterial detection assay is essential for diagnostic fields, particularly in resource-poor settings. Although antibodies have been widely used for bacterial capture, the production of soluble antibodies is still expensive and time-consuming. Here, we developed a nitrocellulose-based lateral flow assay using cell wall binding domains (CBDs) from phage as a recognition element and colloidal gold nanoparticles as a colorimetric signal for the detection of a model pathogenic bacterium, *Bacillus cereus (B. cereus)*. To improve conjugation efficiency and detection sensitivity, cysteine-glutathione-S-transferase-tagged CBDs and maltose-binding protein-tagged CBDs were produced in *Escherichia coli* (*E. coli*) and incorporated in our assays. The sensitivity of the strip to detect *B. cereus* was 1×10^4 CFU/mL and the overall assay time was 20 min. The assay showed superior results compared to the antibody-based approach, and did not show any significant cross-reactivity. This proof of concept study indicates that the lateral flow assay using engineered CBDs hold considerable promise as simple, rapid, and cost-effective biosensors for whole cell detection.

1. Introduction

Lateral flow assays (LFAs) are widely used as a rapid detection method for various monitoring and diagnostic purposes. LFAs are particularly useful for on-site use in resource-poor settings because they are easy to use and portable, their results can be interpreted without external equipment, and are usually obtained within tens of minutes (Posthuma-Trumpie et al., 2009). Most LFAs employ several types of antibodies (monoclonal, polyclonal, HRP-conjugated, AP-conjugated, etc.) to capture the analytes and produce detection signals. However, the production of these antibodies requires immunized animals and mammalian cell expression systems, which greatly increases the cost of the diagnostic assays. Despite the ongoing efforts to produce recombinant antibodies in microbial organisms, they are still associated with several problems, including inclusion body formation, inefficient secretion, and lack of post-translational modifications (Frenzel et al., 2013; Robinson et al., 2015; Spadiut et al., 2014). Moreover, there is an increasing concern about the specificity, sensitivity, and stability of these antibodies (Baker, 2015). The use of high-affinity aptamers could be another method to replace expensive antibodies in diagnostics (Chen and Yang, 2015), but the immobilization of small aptamers onto nitrocellulose or other analytical membranes often requires ultraviolet light, which could induce structural changes to the aptamers through thymine dimerization (Bruno, 2014; Smiley et al., 2013). For these reasons, the development of alternative receptor molecules in LFAs to reduce assay cost and provide reliable data has been a topic of great commercial and academic interest.

Bacteriophages produce highly evolved lytic enzymes called endolysins, which help release phage progeny. These endolysins usually have a modular structure consisting of one or several catalytic domains that break down the bacterial cell wall, in addition to a cell wall binding domain (CBD) that recognizes a highly specific ligand in the cell wall and targets the endolysin to its substrate (Schmelcher et al., 2012). Since CBDs have strong affinity and high specificity toward target bacteria and can be easily produced by *E. coli* expression system (Table S1), several groups have tested their potential as a biosensing tool. Walcher et al. combined CBD-based magnetic separation with real-time polymerase chain reaction (PCR) for the detection of *Listeria* cells

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in raw milk (Walcher et al., 2010). Tolba et al. immobilized CBDs from *Listeria* phage endolysins on a gold electrode, allowing bacterial detection by electrochemical impedance spectroscopy (EIS) (Tolba et al., 2012). Kong et al. reported a surface plasmon resonance (SPR)-based detection method using *Bacillus cereus* phage CBDs (Kong et al., 2015). In a recent study, Yu et al. used immunomagnetic separation and streptavidin–horseradish peroxidase (strep–HRP) labeled CBDs to detect *Staphylococcus aureus* cells (Yu et al., 2016). Although these CBD-based detection methods presented promising results with a detection limit ranged from 10^2 to 10^5 CFU/mL, sophisticated equipment (PCR, EIS, SPR, etc.) is often required to detect their signals and expensive enzymes/substrates (strep–HRP, 3,3′,5,5′-tetramethylbenzidine) are needed for the assays, hindering the production of low-cost devices.

Here we describe an engineered CBD-based lateral flow assay for the detection of *Bacillus cereus* (*B. cereus*). We chose *B. cereus* as a model organism because *B. cereus* is widely distributed in nature and is an important food pathogen that causes emetic and diarrheal syndromes (Bottone, 2010). The use of engineered CBDs allowed for simple immobilization onto gold nanoparticles and improved detection sensitivity. To the best of our knowledge, this is the first report to describe the use of an engineered CBD in a lateral flow assay format. In addition, this approach does not require primary antibodies or expensive enzyme-conjugated secondary antibodies, enabling the assay to be developed at low cost. This work can thus provide the basis for the development of rapid, cost-effective, and eco-friendly point-of-care diagnostic devices for bacterial detection.

2. Materials and methods

2.1. Materials

Nitrocellulose (NC) strips (15 μm pore size) were purchased from Advanced Microdevices (CNPC-SS12; Ambala Cantt, India) and absorbent pads were purchased from Ahlstrom (Grade 222; Helsinki, Finland). 20 nm gold nanoparticles (AuNPs) suspended in 0.1 mM phosphate-buffered saline (PBS) and AuNPs suspended in citrate buffer were purchased from Sigma-Aldrich (753610, 741965; St. Louis, MO, USA). Methoxypoly(ethylene glycol) functionalized with a thiol group (mPEG-SH, MW: 5000) was purchased from SunBio (Anyang, Korea). To prepare sample solutions containing target bacteria at known concentrations, each bacterial colony was inoculated into tryptic soy broth (TSB), which was purchased from BD Difco (211825; Sparks, MD, USA), and grown overnight. Next, 50 μL of the bacterial culture was diluted with fresh TSB solution (1/100 dilution) and grown until it reached the desired concentration.

2.2. Construction of plasmids

The gene fragment encoding the putative CBD was amplified from LysB4 (Son et al., 2012), digested with *BamHI* and *HindIII* (Takara Clontech, Kyoto, Japan), and ligated into pET28a (Novagen, Madison, WI, USA). For enhanced green fluorescent protein (EGFP)-fused CBD proteins, the *BamHI/HindIII*-digested CBD gene was subcloned into pET28a:EGFP (Kong et al., 2015). For mCherry tagging, the sequence encoding mCherry was subcloned from pmCherry-N1 (Clontech) into pET28a using the *NdeI* and *BamHI* sites. For Cys-glutathione-S-transferase (Cys-GST) tagging, the sequence encoding GST was amplified by PCR using primers that introduced an additional N-terminal single cysteine residue and C-terminal GSGSGS-linker residues. The amplified DNA product was double-digested using *NdeI* and *BamHI* and ligated into the pET28a vector. For maltose binding protein (MBP) tagging, the sequence encoding MBP was amplified using pMBP Parallel 1 as a template (Sheffield et al., 1999), digested with *NdeI*/*BamHI* (Takara Clontech), and subcloned into 10His-pET28a (Kong and Ryu, 2016). The native stop codons of EGFP, mCherry, GST, and MBP were omitted for translational fusions. The *BamHI/HindIII*-digested CBD gene was subcloned into the corresponding recombinant plasmids. DNA sequences were verified for all constructs. All constructs had an N-terminal His tag for protein purification. All primers and recombinant plasmids used in this study are listed in Table S2.

2.3. Engineered CBD production

The recombinant CBDs were expressed in *E. coli* BL21 (DE3) cells (Invitrogen, Carlsbad, CA, USA) grown at 37 °C in LB broth supplemented with kanamycin (50 μg/mL). When the optical density of the medium reached approximately 0.8, protein expression was induced using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), after which the cultures were incubated overnight at 18 °C. The cells were disrupted by sonication and the CBDs were purified by nickel affinity chromatography using Ni-NTA Superflow resin (Qiagen, Hilden, Germany) as described previously (Kong et al., 2015). For Cys-GST tagged CBD proteins, all purification buffers included 6.25 mM β-mercaptoethanol to maintain a reducing environment. The purified proteins were subjected to gel filtration chromatography through a Sephadex G-25 column (PD MidiTrap G-25; GE Healthcare, Buckinghamshire, UK) with PBS buffer. The final concentrations of the recombinant CBDs were determined by the Bradford assay.

2.4. Characterization of the CBD proteins

The binding properties of the EGFP-CBD fusion protein were examined as previously described (Loessner et al., 2002). Briefly, exponentially growing bacterial cells were transferred to Dulbecco's phosphate-buffered saline (DPBS, GenDepot, Barker, TX, USA) and incubated with 0.4 μM EGFP-CBD fusion protein for 5 min at room temperature. The cells were then washed twice with DPBS buffer and observed by epifluorescence microscopy (DE/Axio Imager A1 microscope; Carl Zeiss, Oberkochen, Germany) with a filter set for EGFP (excitation 470/40; emission 525/50). Since mCherry is more tolerant to pH changes than GFP (Doherty et al., 2010), mCherry-tagged CBD proteins were used to examine the effect of NaCl and pH on CBD binding. Quantitative fluorescence assays were conducted using a SpectraMax i3 multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation at 485 nm and emission at 535 nm. All quantitative assays were carried out in triplicate.

2.5. Conjugation of gold nanoparticles to CBDs

For CBD conjugation, 10 μL of Tris-Cl (pH 9.0) was added to 1 mL of citrate-stabilized gold nanoparticles (20 nm, Sigma). Then, 15 μg of Cys-GST-tagged CBD proteins was adsorbed at room temperature for 1 h on a rotator. To block and stabilize the CBD-conjugated AuNPs, 100 μL of 2 mM thiol-terminated PEG (5 kDa, dissolved in 10 mM Tris-Cl, pH 9.0) was added to 1 mL of conjugate solution and further incubated for 1 h. Unbound proteins and PEGs were removed by centrifugation (8000×*g*, 4 °C) for 30 min. The final conjugate was resuspended in 1 mL of storage buffer (PBS, pH 7.4, 0.02% bovine serum albumin (BSA)) and stored in a dark bottle at 4 °C.

2.6. Conjugation of gold nanoparticles to antibodies

To test the detection capability of the antibody, we used commercially available anti-*B. cereus* antibody (ab20556; Abcam, Cambridge, UK). For antibody conjugation, 12 μL of K_2CO_3 (0.2 M) was added to 600 μL of AuNP (20 nm, suspended in 0.1 mM PBS) solution to adjust the pH to 9.0. Then, 4 μg of the appropriate antibody was added to the solution, after which the mixture was incubated at room temperature for 20 min. To block nonspecific binding sites on the surfaces of the antibody-conjugated AuNPs, 100 μL of PBS containing 3% BSA was added, after which the mixture was incubated for 20 min. To remove

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