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Short communication

Aptamer cocktails: Enhancement of sensing signals compared to single use of aptamers for detection of bacteria

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ABSTRACT

Microbial cells have many binding moieties on their surface for binding to their specific bioreceptors. The whole-cell SELEX process enables the isolation of various aptamers that can bind to different components on the cell surface such as proteins, polysaccharides, or flagella with high affinity and specificity. Here, we examine the binding capacity of an aptamer mixture (aptamer cocktail) composed of various combinations of 3 different DNA aptamers isolated from *Escherichia coli* and compare it with one of the single aptamers using fluorescence-tagged aptamers. The aptamer mixtures showed higher fluorescence signal than did any single aptamer used, which suggests that use of aptamer mixtures can enhance the sensitivity of detection of microbial cells. To further evaluate this effect, the signal enhancement and improvement of sensitivity provided by combinatorial use of aptamers were examined in an electrochemical detection system. With regard to current decreases, the aptamer cocktail immobilized on gold electrodes performed better than a single aptamer immobilized on gold electrodes did. Consequently, the detection limit achieved using the aptamers individually was approximately 18 times that when the 3 aptamers were used in combination. These results support the use of aptamer cocktails for detection of complex targets such as *E. coli* with enhanced sensitivity.

1. Introduction

Conspicuous and rapid targeting of specific pathogens or their subpopulations in complex environments are regarded as the greatest challenge in the field of microbial diagnosis. Ligands having high affinity and specificity make diagnosis precise and efficient (Eaton et al., 1995). Antibodies and their variants are widely used as ligands, and their capabilities have been demonstrated in many applications in biotechnology, diagnostics, and disease treatment; however, preparation and modification of these antibodies are challenging and laborious tasks (Mayer et al., 2010; Zichi et al., 2008). Nucleic acid-based aptamers have been verified to be useful for generating high-affinity ligands for an array of targets ranging from small molecules to cells by SELEX (systematic evolution of ligands by exponential amplification) (Ellington and Szostak, 1990, 1992; Sefah et al., 2010; Tuerk and Gold, 1990). In contrast to antibodies, nucleic acid-based aptamers are relatively rigid because the backbone of nucleic acids has less torsional conformations than that of proteins, where the side chains have various degrees of torsional freedom; therefore, nucleic acid-based aptamers are tolerant to harsh environmental conditions (Eaton et al., 1995). Aptamers can be isolated *in vitro* and do not require immunization, tissue culture, or purification from serum for mass production. After determination of the aptamer sequence, large quantities of the specific oligomer can be synthesized by chemical or enzymatic processes at low cost and high efficiency (Graham and Zarbl, 2012).

Cell-SELEX is a recent application of SELEX, in which representative cellular ligands (aptamers) are prepared against the surface of cells by repeated binding and amplification of ssDNA or RNA to living cells (Sefah et al., 2010). Cell-SELEX offers flexibility, because it can be applied to various cell types, from bacterial cells to cancer cells, to discover unknown biomarkers exposed on the surface of the cells (Kim et al., 2013; Sefah et al., 2010). In particular, bacterial cell-SELEX can be operated in non-adherent cell cultures to identify specific aptamers, and many components of the cellular surface such as polysaccharides, surface proteins, or flagella can be targets. The aptamers isolated against the bacterial cellular surface can be manipulated to construct bacterial diagnostic sensors that can be operated in a non-invasive and label-free manner without requiring cell lysis

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(Labib et al., 2012; Ohk et al., 2010). In our previous study (Kim et al., 2013), using bacterial cell-SELEX, we isolated several aptamers with high affinity and specificity owing to the various possible targets on the surface of cellular membrane; however, exact targets of these aptamers on *Escherichia coli* cells remain to be identified and verified. Considering the complexity of the cellular surface, we suspect that all aptamers do not share the same targets.

Herein, we investigate the advantage of the combination of aptamers (aptamer cocktail) targeting the same bacterium to enhance the sensing signal, thereby enhancing the sensitivity of detection, over using a single aptamer as the sensing ligand in a biosensor. We performed a binding assay for single aptamers and aptamer cocktails to compare signal generation when the aptamers bound to the bacterium *E. coli*. Further, aptamer cocktails were immobilized on a screen-printed gold electrode for electrochemical detection of *E. coli* and their sensitivity and limit of detection were compared that of a single immobilized aptamer. These results indicate that aptamer cocktails, not only single aptamers, can be used to identify useful ligands for the construction of biosensors with enhanced sensitivity to detect complex targets that have many possible binding moieties for the ligands.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli (KCTC 2571) was used as the target strain and grown in nutrient broth medium. *Klebsiella pneumoniae* (KCTC 2208), *Citrobacter freundii* (KCTC 2006), *Enterobacter aerogenes* (KCTC 2190), and *Staphylococcus epidermidis* (KCTC 1917) were used for specificity testing and were grown in nutrient broth. All strains were cultured at 37 °C, except *C. freundii* and *E. aerogenes*, which were cultured at 30 °C. All bacterial strains were obtained from the Korean Collection for Type Culture (KCTC).

2.2. ssDNA aptamers

Three different ssDNA aptamers specific to *E. coli*, which were isolated in a previous study, were used in this study (Kim et al., 2013). The sequence of each aptamer was as follows: E1, 5′-GCA ATG GTA CGG TAC TTC CAC TTA GGT CGA GGT TAG TTT GTC TTG CTG GCG CAT CCA CTG AGC GCA AAA GTG CAC GCT ACT TTG CTA A-3′; E2, 5′-GCA ATG GTA CG G TAC TTC CCC ATG AGT GTT GTG AAA TGT TGG GAC ACT AGG TGG CAT AGA GCC GCA AAA GTG CAC GCT ACT TTG CTA A-3′; E10, 5′-GCA ATG GTA CGG TAC TTC CGT TGC A CT GTG CGG CCG AGC TGC CCC CTG GTT TGT GAA TAC CCT GGG CAA AAG TGC ACG CTA CTT TGC TAA-3′. All aptamers were custom-synthesized by Genotech Inc. (Daejeon, Korea), and all aptamers were dissolved into a concentration of 10 μM in distilled water for further use.

2.3. Fluorescence analysis for aptamer binding

E. coli (KCTC 2571) were cultured in nutrient broth to the middle growth phase (10^8 CFU/ml) and centrifuged at 13,000 g for 10 min to remove the media. Subsequently, cells were washed 3 times in PBS (pH 7.4). FAM-labeled ssDNA aptamers were prepared as single aptamers (E1, E2, and E10) or aptamer cocktails (E1+E2, E1+E10, E2+E10, and E1+E2+E10) with various concentrations (0, 10, 25, 50, 100, 250, and 500 nM) in PBS. The molar ratio of each aptamer in the cocktails was the same. Then, 100 μl of the cell suspension (10^7 cells) was incubated with 100 μl of aptamer solution for 45 min at 25 °C with mild shaking. Cells were washed 2 times to remove unbound aptamers from cells by centrifugation (13,000 rpm for 10 min) and were resuspended in PBS. Finally, the fluorescence intensity of each sample was measured using a fluorospectrophotometer (LS50B, PerkinElmer Co., USA).

2.4. Fluorescence imaging of E. coli with QD-labeled DNA aptamers

To observe the binding of different aptamers on a single *E. coli* cell surface, the aptamers were labeled with 3 different quantum dots (QDs). Two QDs (NSQDs-AC530 and NSQDs-AC580, Nanosquare Inc.) were conjugated using the E1 and E2 aptamers, respectively. Another QD (Qdot®655 ITK™ carboxyl, Invitrogen) was conjugated with the E10 aptamer. Carboxyl-functionalized QDs (5 μl, 8 μM) were incubated with 5 μl of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) (each 10 mM) for 2 h at 25 °C. The amine-modified aptamer (85 μl, 2 μM as the final concentration) in PBS buffer was added to the QD solution and incubated for 2 h at 25 °C with mild shaking. Finally, non-reacted COOH groups on QDs were blocked with ethanolamine (10 mM) for 2 h. Then, 3 QD/aptamer conjugate pairs were incubated with *E. coli* (10^7 cells) for 45 min at 25 °C. The cells were washed 2 times by centrifugation and resuspended in 40 μl of PBS buffer. The cell suspension was dropped onto a glass slide, and a cover slide was used to make a thin smear of the bacteria. Fluorescent images of the bacteria were observed under a fluorescence microscope (Olympus BX50).

2.5. Electrochemical detection of E. coli using aptamers immobilized on gold electrode chip

Electrochemical analysis was performed at room temperature using an electrochemical analyzer CHI640D (CH Instrument, USA). The screen-printed electrodes composed of working (gold), reference (silver), and counter electrodes (gold). All electrodes were integrated in a single chip, and the working electrode had a diameter of 1.6 mm (DropSens, S.L., Spain). To fabricate the chip, each *E. coli*-specific DNA aptamer was first labeled with an amine group at 3′-end, and then immobilized as a single aptamer (E1, E2, or E10) or aptamer cocktails (E1+E2, E1+E10, E2+E10, or E1+E2+E10). The electrodes were washed with 10 mM H_2SO_4 for 10 min before immobilization. To immobilize the aptamers on the electrode, the working electrode was treated with 10 mM 3,3′-dithiodipropionic acid for 2 h to form a self-assembled monolayer (SAM) and then washed thoroughly with distilled water. The CVs of every electrode at this stage were measured for quality control and further aptamer modification after SAM modification (Fig. S1). Activation of carboxylic groups was performed on the electrode after incubation with 40 mM EDC and 40 mM sulfo-NHS for 1 h. Finally, 1 μM amine-modified DNA aptamer was incubated on an electrode for 1 h and washed thoroughly with distilled water. Then, ethanolamine (20 mM, pH 8.5) was applied to block the non-reacted COOH group for 1 h.

Various concentrations of *E. coli* suspension (0–10^8 CFU/ml) in PBS were dropped onto the electrode and incubated for 1 h. After washing with distilled water, 70 μl of 5 mM $K_3Fe(CN)_6$ solution containing 100 mM KCl was dropped on electrode chip until all 3 electrodes are immersed. Then, cyclic voltametry (CV) was performed under electric potentials ranging from –0.2 to 0.4 V with a scan rate of 20 mV/s and a sample interval of 2 mV. To verify that the current change was caused by only specific interactions between *E. coli* and the aptamer, other bacteria species (*K. pneumoniae*, *C. freundii*, *E. aerogenes*, and *S. epidermidis*) were tested under identical conditions. Electrochemical data analysis was conducted, and the percent decrease in the current before and after the sample treatment ($\Delta I=(I_0-I_1)/I_0\times 100$) was