Biosensors

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Fluorogenic DNAzyme Probes as Bacterial Indicators**

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The prevalence of food-borne pathogens, emergence of drug-resistant bacteria and viruses, and threat of bioterrorism are amongst the most pressing concerns of our time. The early detection of pathogens is a crucial step in preventing large-scale outbreaks, and is particularly important today as globalization of commerce and shorter journey times have significantly increased the rate and breadth of the spread of infectious agents.

Pathogen detection is traditionally performed by using microbiological techniques, which are highly accurate but can take days (even weeks) to obtain a result.[1] Both antibody- and PCR-based tests offer much-reduced detection times; however these tests still require multiple steps and specialized equipment.[2] There is a significant need for both simple methods that can achieve rapid detection of known pathogens and for new platforms that can be quickly put in place to create assays for a new pathogen in an unanticipated outbreak. These considerations have motivated us to develop a platform based on catalytic DNA molecules (DNAzymes), which are a special class of functional nucleic acids[3] that are artificial single-stranded DNA molecules that have a catalytic ability.[4] These molecules can be isolated from a random-sequence DNA pool by in vitro selection[5] and have been increasingly explored as molecular tools for various applications.[4,6] Herein we demonstrate that fluorogenic DNAzymes can be isolated from a DNA library to fluoresce in the crude extracellular mixture (CEM) that is produced by a specific bacterial pathogen, and that such probes can be used to develop a simple "mix-and-read" assay to detect this pathogen.

The ability to grow under nutritious conditions and exchange materials with its environment are distinct properties of a living cell. Thus, live microbes leave behind a mixture of small or macromolecular substances, some of which can be highly distinctive. However, the purification and identification of a suitable target from the CEM of a microbe for biosensor development can be a demanding task; it would become too laborious and costly to practice when multiple organisms are considered. We hypothesize, however, that it should be feasible to isolate fluorogenic DNAzymes in an in vitro selection experiment where the CEM from a given microbe is directly used as the complex target, thus bypassing all target separation and identification steps (Figure 1 a). The isolated DNA probes, which are encoded with a signal-generating capability, can then be used to develop a simple fluorescent assay for the detection of the target microbial organism.

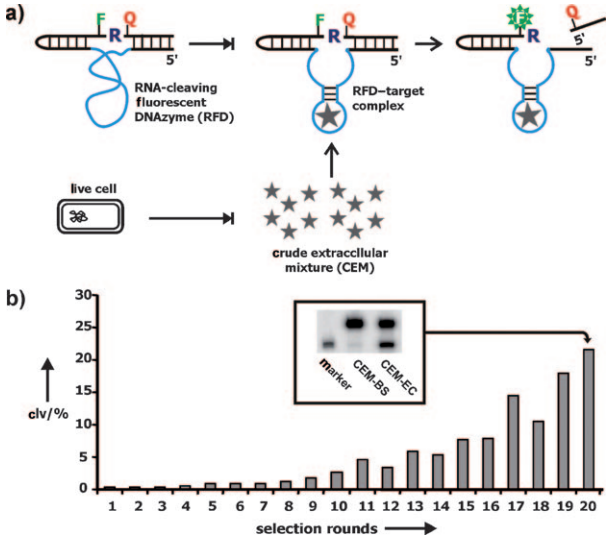

Figure 1. a) Conceptual design of fluorescent DNAzymes that fluoresce upon contact with the CEM produced by living bacterial cells. F = fluorescein–dT, Q = dabcyl–dT, R = adenosine ribonucleotide. b) In vitro selection progress. The selection progress was monitored through the percentage cleavage (clv%) of the DNA pool in each round of selection upon incubation with CEM-EC. The inset shows the image of a dPAGE gel used to analyze the activity of the 20th DNA pool in the presence of CEM-BS and CEM-EC. The top band is the full-length DNA pool and the bottom band is the cleaved product.

We chose to create such fluorogenic DNA probes based on an RNA-cleaving fluorescent DNAzyme (RFD) system that we previously developed.[7] These DNAzymes cleave a lone RNA linkage (R; Figure 1 a) embedded in a DNA chain and flanked by nucleotides labeled with a fluorophore (F) and a quencher (Q). These three moieties provide a convenient way to couple the activity of the DNAzyme to fluorescence signal generation.[7] To date, we have isolated and characterized several RFDs,[8] some of which have been explored for the design of fluorescent[9] or colorimetric[10] detection assays. In this study, we sought to derive RFDs that can respond directly to the CEM generated by the model microbe *Escherichia coli* (*E. coli*).

E. coli is a gram-negative bacterium commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are innocuous; some strains such as O157:H7,

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however, can cause deadly food poisoning in humans. In addition, *E. coli* is one of the most studied microbes and holds a special status in microbiology and biotechnology. For this study, we chose to work with *E. coli* K12, a nonpathogenic strain commonly used in research laboratories.

RFD isolation was achieved by using the DNA library (containing 70 random nucleotides) and the selection strategy (Figure S1 in the Supporting Information). Briefly, prior to the selection, two different CEM samples—CEM-EC (from *E. coli*) and CEM-BS (from *Bacillus subtilis* as a control)—were prepared by removing cells grown overnight in Luria–Bertani media (LB). The DNA library was incubated in the selection buffer (SB; 50 mM HEPES, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, and 0.01 % Tween 20) for 5 h, followed by 1 h incubation with CEM-BS (also in SB): this combined procedure served as the "negative selection" step to remove any self-cleaving and nonspecific DNAzymes. The uncleaved DNA molecules were purified by 10 % denaturing polyacrylamide gel electrophoresis (dPAGE) and then incubated with CEM-EC in SB for 30 min: this procedure was the positive selection step aimed at isolating DNAzymes specific to CEM-EC. The cleaved DNA sequences were purified by dPAGE, amplified by the polymerase chain reaction (PCR), and used for the next cycle of selective amplification (detailed experimental protocols are provided in the Supporting Information). After 20 iterations (Figure 1 b), a strong CEM-EC-dependant cleavage activity was established (> 30 % cleavage with CEM-EC, compared to < 1 % with CEM-BS; see the gel image in Figure 1 b).

The 20th DNA pool was cloned and sequenced. Three classes of DNAzymes were discovered; the sequence of the most dominant DNAzyme, named RFD-EC1, is provided in Figure 2 a. Note that RFD-EC1 is a cis-acting DNAzyme in which the substrate is covalently linked to the DNAzyme strand. The activity of synthetically produced RFD-EC1 was examined by fluorescence measurements (Figure 2 b) as well as by dPAGE analysis (Figure 2 c). A scrambled sequence, RFSS1 (Figure 2 a), was tested as a control. In the first experiment, CEM-EC and 2 × SB (double the concentration of SB) were mixed in a 1:1 ratio by volume in a quartz crystal cuvette; the fluorescence of this solution was maintained at a steady level (see the constant level of fluorescence in the initial 5 min of the fluorescence plot in Figure 2 b). However, the addition of RFD-EC1 (final concentration of 100 nM) caused a dramatic time-dependent increase in fluorescence (Figure 2 b; black curve). In contrast, no significant fluorescence increase was observed for RFSS1 (Figure 2 b, red curve; note that the initial fluorescence increase upon the addition of RFSS1 was attributed to the background fluorescence of the FRQ module because the fluorescence from F was not completely quenched by Q).

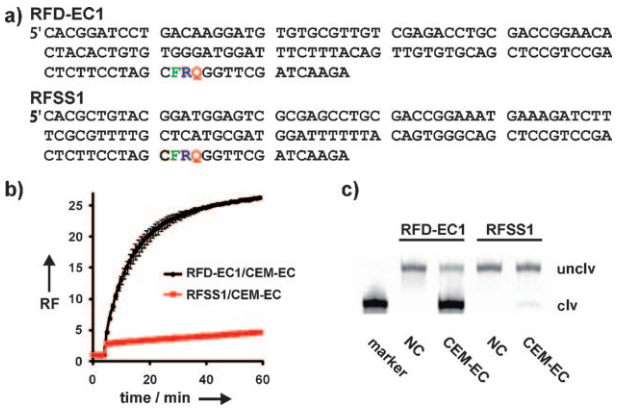

Figure 2. a) The sequences of RFD-EC1, the dominant DNAzyme obtained from in vitro selection, and RFSS1 (a control). b) Signaling profiles of 100 nM RFD-EC1 or RFSS1 in the presence of CEM-EC, which was incubated in SB alone for 5 min, followed by the addition of RFD-EC1 or RFSS1 and further incubation for 55 min. RF = relative fluorescence. c) 10 % dPAGE analysis of the cleavage reaction mixtures of RFD-EC1 and RFSS1. NC = negative control (reaction in SB), unclv = uncleaved.

To verify that the fluorescence increase of RFD-EC1 was indeed due to the cleavage of the RNA linkage, the cleavage mixture was analyzed by 10 % dPAGE. The cleavage of RFD-EC1 was expected to generate two DNA fragments; the 5′-cleavage fragment retains the fluorophore and can be detected by fluorescence imaging while the 3′-fragment does not fluoresce (see the marker lane in Figure 2 c, which shows a sample of RFD-EC1 after treatment with 0.25 N NaOH for 20 h at room temperature, a procedure that is known to cause the full cleavage of RNA[11]). The results shown in Figure 2 c are consistent with our hypothesis: RDF-EC1 cleaved itself in the presence of CEM-EC but not in SB alone (labeled as NC, negative control). In contrast, RFSS1/CEM-EC mixture produced only an extremely weak cleavage band.

We subsequently investigated the response of RFD-EC1 to CEM produced by other microbes. Nine other gram-negative bacteria and five gram-positive bacteria (including *B. subtilis*, the CEM of which was used during the negative selection step) were arbitrarily chosen for this experiment. Each bacterium was cultured in LB for a different period of time (because these bacteria have varying growth rates in LB) until the OD_{600} value (optical density at 600 nm) of each cell culture reached approximately 1. The CEM was then prepared and used to induce the cleavage of RFD-EC1 in a reaction for 1 h. None of the CEM from any of these randomly selected bacteria was able to activate RFD-EC1 (Figure 3 a). The lack of induction was also confirmed by fluorescence measurements (Figure S2). These experiments indicate that this DNAzyme is highly specific to the CEM of *E. coli*.

To investigate the nature of the targets (proteins or small molecules) in CEM-EC that activate RDF-EC1, we treated CEM-EC with two proteases, trypsin (TS) and proteinase K (PK). CEM-EC treated with either protease was no longer able to activate RFD-EC1 (Figure 3 b). The disappearance of the cleavage activity in the presence of protease-treated CEM-EC was also confirmed by fluorescence measurements (Figure S3). These observations strongly suggest that the responsive target is a protein.

To examine whether the observed cleavage activity of RFD-EC1 was simply caused by possible ribonucleases (RNases) that may exist in CEM-EC, we carried out two tests. In the first test, RiboLock (RI), which is an RNase inhibitor, was added to CEM-EC. As shown in Figure 3 c, the

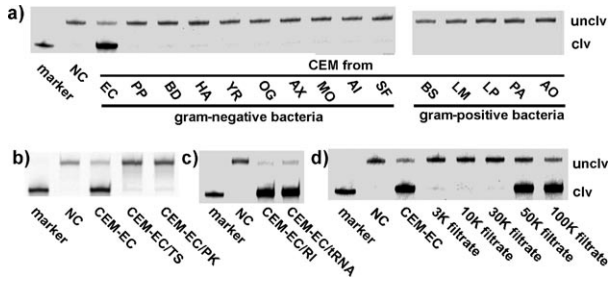

Figure 3. a) Responses of RFD-EC1 to the CEMs from various gram-negative and gram-positive bacteria. The gram-negative bacteria used were *Pseudomonas peli* (PP), *Brevundimonas diminuta* (BD), *Hafnia alvei* (HA), *Yersinia ruckeri* (YR), *Ochrobactrum grignonese* (OG), *Achromobacter xylosoxidans* (AX), *Moraxella osloensis* (MO), *Acinetobacter lwoffi* (AI), and *Serratia fonticola* (SF). The gram-positive bacteria used were *Bacillus subtilis* (BS), *Leuconostoc mesenteroides* (LM), *Lactobacillus planturum* (LP), *Pediococcus acidilactici* (PA), and *Actinomyces orientalis* (AO). b) Response of RFD-EC1 to CEM-EC pre-treated with trypsin (TS) and proteinase K (PK). c) Responses of RFD-EC1 to CEM-EC containing RI or a 100-fold excess of tRNA. d) Estimation of the molecular weight of the responsive protein target. The reaction time for all the cleavage reactions was 1 h.

addition of RI did not affect the cleavage activity. In the second test, the cleavage reaction was conducted in the presence of a 100-fold excess of tRNA. This treatment also did not cause any activity reduction. These results, together with the previous observation that RFSS1 (mutated RFD-EC1) failed to cleave upon contacting CEM-EC (Figure 2), strongly suggest that the cleavage of RFD-EC1 was not simply caused by an RNase.

We next probed the possible molecular weight of the target by using a molecular sizing column. CEM-EC was passed through centrifugal columns with a cut-off molecular weight of 3 K (3000 Daltons), 10 K, 30 K, 50 K, and 100 K. Although the filtrates from the 3 K, 10 K, and 30 K columns did not induce the cleavage of RFD-EC1, both the filtrates from the 50 K and 100 K columns were successful (Figure 3 d). The results indicate that the potential protein target has a molecular weight between 30000 and 50000 Daltons. The identification of the protein target (or targets) is beyond the scope of this report and will be pursued in a future study.

The ability to detect a single live cell is a hallmark of a bacterial detection method for many practical applications such as the detection of food-borne pathogens.[1] For this reason, bacterial detection methods in such practices have an essential cell-culturing step. Our method has an integrated cell-culturing step and is expected to offer the capability for single-live-cell detection. To verify this possibility, we prepared four *E. coli* stock solutions (in LB containing 15% glycerol) with 20, 2, 0.2, and 0.02 colony-forming units (CFUs; 1 CFU = a single live cell) per 100 μL, labeled as stocks A–D, respectively. Fifteen 100 μL aliquots were taken from each stock solution to inoculate 15 parallel solutions of LB. Theoretically, the individual tubes inoculated with stocks A, B, C, and D would contain an average of 20, 2, 0.2, and 0.02 CFUs, respectively. Thus we expected to observe bacterial growth in all the tubes inoculated with stocks A and B, three tubes with stock C ($0.2 \times 15 = 3$), and none with stock D ($0.02 \times 15 = 0.3$, i.e., < 1). All the inoculated solutions were incubated at 37 °C for 24 h; CEM-EC was then prepared from each sample and used to activate RFD-EC1 using dPAGE analysis. The results (Figure 4) are consistent with our

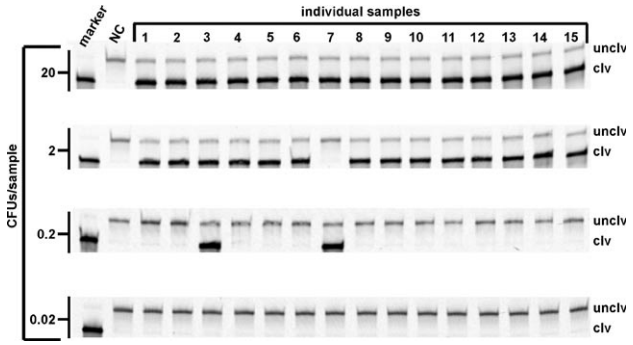

Figure 4. Single-live-cell detection. Each row contains the image of a dPAGE gel conducted to analyze 15 parallel cleavage reactions where RFD-EC1 was incubated with CEM-EC prepared from an overnight culture inoculated with 20 (top row), 2 (second row), 0.2 (third row), and 0.02 (bottom row) CFUs of *E. coli*. The reaction time for all the cleavage reactions was 1 h.

hypothesis: CEM-EC from all stock A inoculations, all but one stock B inoculations, two (instead of three) of the stock C inoculations and none of stock D inoculations produced a cleavage band (note that the OD_{600} value of each positive culture was between 1.0 and 1.2, whilst that of each negative sample was negligible). These results show that our DNAzyme-based method could indeed be used to achieve the detection of a single live cell.

We also investigated the time required to generate a detectable signal if 1 CFU of *E. coli* was used to initiate cell growth. From the data shown in Figure 5 a, 12 h of culturing was needed to achieve a robust signal, although a very weak signal was observed following 8 h of culturing.

Finally, we examined the number of seeding cells required to produce CEM-EC that was concentrated enough to induce a detectable signal of RFD-EC1 after 6 h of cell culturing (Figure 5 b). We found that only around 500 cells were required to produce sufficiently concentrated CEM to induce

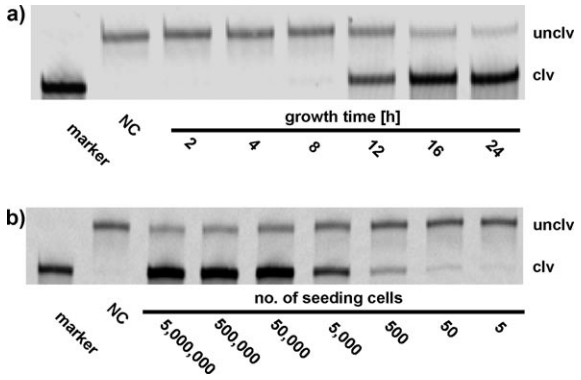

Figure 5. a) Growth time required for achieving single-cell detection. b) Detection limit of the assay when cells were cultured for 6 h. The reaction time for DNAzyme cleavage was 1 h.

the cleavage of RFD-EC1 that was significantly above the background level.

In summary, we have devised a novel approach for the detection of a specific bacterium by isolating fluorogenic DNAzymes from a random-sequence DNA library by using the unpurified complex extracellular mixture left behind by the target microbe. We have shown that these DNAzymes can be used to set up a simple "mix-and-read" bacterial detection assay. More importantly, we have demonstrated that our method has the capability to detect a single live cell. The most appealing feature of the method is that both probe isolation and subsequent assaying procedures bypass tedious and time-consuming target identification steps. Although our method was demonstrated using a nonpathogenic strain of *E. coli*, we believe that it can easily be implemented for pathogenic bacteria and viruses. Finally, although the demonstrated assay used fluorescence as the reporting mechanism, the same DNAzyme probe can also be used for the design of a colorimetric assay using the rolling circle amplification/organic dye strategy that we reported previously.[10]

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