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A simple mix-and-read bacteria detection system based on DNAzyme and molecular beacon

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Here we describe a simple mix-and-read method for detection of specific bacterial strains that uses DNAzyme and molecular beacon to generate signal. We have greatly improved upon previously described DNAzyme-based bacteria detection method by eliminating a tedious preparation step while maintaining detection sensitivity.

Bacteria are very closely related to human health. Pathogenic bacteria can cause severe disease and death, while species comprising the human microbiome are essential for health by helping provide, for example, essential vitamins.[1, 2] Classically, bacteria detection involves colony counting following culturing on appropriate plates.[3] This method is accurate and sensitive but usually requires days or weeks to culture cells into visible colonies. To reduce detection time, many non-culture-based bacteria detections have been proposed, such as enzyme-linked immunosorbent assays (ELISA), PCR-based amplification, and cell-imprinted microplates.[4-6] But these methods generally require multiple steps and specialized experimental equipment. Recently, a rapid and simple platform based on DNAzymes was developed for bacteria detection.[7]

DNAzymes, also called Deoxyribozymes or catalytic DNAs, are DNA oligonucleotides that can perform a specific catalytic reaction, including cleavage or ligation of nucleic acids.[8-10] The catalytic cleavable DNAzymes are artificial single-stranded DNA molecules that are isolated from a random DNA sequence pool by *in vitro* selection.[11] These DNAzymes can catalyze the cleavage of a substrate RNA or DNA with the help of corresponding cofactors. They have attracted increasing attention due to their high reaction sensitivity, fast reaction rate, high stability, low synthesis cost, and mild reaction conditions. To date, DNAzymes have been used in various applications, such as molecular devices, DNA computing circuits and biosensors.[12-14]

For use as biosensors, researchers take advantage of DNAyzme requirement for co-factors. While metal ions are the most common cofactor,[15-17] UO_2^{2+}, histidine or proteins are also used.[18-21] The first DNAzyme developed to detect bacteria uses a kind of protein found in crude extracellular mixture (CEM) or crude intracellular mixture (CIM) of *Escherichia coli* (*E. coli*) as the cofactor.[7,22] This *E. coli* DNAzyme system can rapidly respond to *E. coli*. It has been used to create nanosensors, test bacterial contamination in food, and detect single bacteria in unprocessed blood.[23-25] In addition to the DNAzyme *E. coli* indicator, Li et al. also obtained, through *in vitro* selection, a catalytic DNAzyme strand activated by a pathogenic strain of *Clostridium difficile* (*C. difficile*)[20] and an RNA-cleaving DNAzyme probe for highly specific recognition of breast tumors[21]. Although the signal generation step in most DNAzyme-based detection assays is simple, these assays all require complex, expensive and inefficient steps to prepare the DNAzyme strand. These shortcomings restrict further application of DNAzyme-based systems for bacteria detection.

In this paper, we report a much simpler and less expensive bacteria detection method using a modified DNAzyme.[7] We use *E. coli* as a model microbe, and demonstrate a new mix-and-read bacterial DNAzyme detection system that uses molecular beacon (MB) as the signal reporter.[26] The DNA strand in this new biosensor is derived from the *E. coli* DNAzyme (Figure S1) and lacks the 5'-Primer Binding Site (5'-PBS), which is required in amplification during *in vitro* selection but is not required for DNAzyme activity.[22] We name this new DNA strand as s-DNAzyme-Ecoli (short for DNAzyme strand for *E. coli*). This

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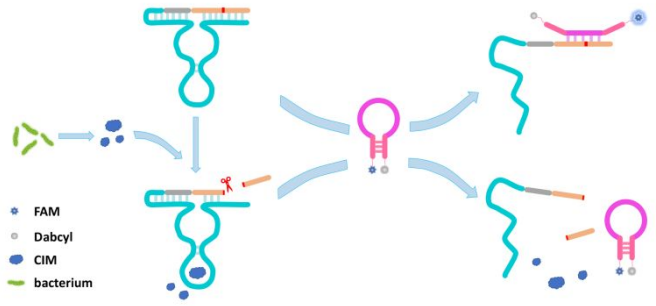
detection method simplifies the workflow, reduces hands-on time, and lowers experimental cost.

The workflow of the method is shown in Scheme 1. Intact single stranded DNA containing the DNAzyme catalytic domain and substrate domain is incubated at room temperature with *E. coli* CIM. After one hour, a molecular beacon is added to the reaction system. The loop of the MB is complimentary to the partial sequence of s-DNAzyme-Ecoli exactly covering the cleavage site. We label this MB as MB-rA. If the s-DNAzyme-Ecoli cannot respond to CIM, MB-rA will be opened and the fluorescence signal will be released. By contrast, if the s-DNAzyme-Ecoli is cleaved by CIM, each of the two cleavage DNA strand products is only partly complimentary to the loop region of MB-rA. The binding strength now is not enough to open the stem-loop structure of MB-rA and the fluorescence signal will remain unchanged.



Scheme 1. Schematic principle of the mix-and-read DNAzyme system for bacterial detection.

The original DNAzyme-based bacterial detection system contains at least three necessary experimental procedures: phosphorylation of the FQ-DNAzyme substrate strand, ligation with the DNAzyme catalytic strand, separation and purification of the intact DNAzyme strands (Scheme S1).[7, 24, 25] The purpose of the ligation is to promote the binding probability of CIM with DNAzyme strands and thus improve the cleavage efficiency.[22] The pre-treatment is necessary since the commercial DNAzyme strand containing catalytic domain, substrate domain, fluorophore and quencher factors are much more difficult and expensive to synthesize directly. Although the subsequent process of mixing and reacting with CIM of *E. coli* is simple, the preparation and synthesis of the intact DNAzyme sequence are much more complex. They need long experimental operation times, have a high cost, and uncertain synthesis efficiency. These drawbacks prevent bacterial detection based on DNAzyme system from wider development and practical applications. By contrast, in our experimental design, a shortened DNAzyme strand (s-DNAzyme-Ecoli) is used as target sequence for the interaction with CIM of bacteria. Using a molecular beacon strand to replace the fluorophore and quencher modification in the DNAzyme itself as a signal reporter maintains the fluorescence readout and, at the same time, decreases the experimental cost. The whole experiment mainly depends on the reaction of s-DNAzyme-Ecoli with CIM, avoiding the complex pre-treatment process. DNAzyme substrate strands for other protein targets serially established via *in vitro* selection by Li et al share the same DNA substrate sequence.[20, 21] This means that the MB-rA can be used as a universal signal reporter for applications such as *C. dif* detection and breast tumor recognition for the DNAzyme-based detection. Besides, compared with the molecular beacon design targeting cleaved oligo products,[27] the molecular beacon design targeting the cleavage site can eliminate the induced background by the DNAzyme sequence and can be flexibly designed for different DNAzyme systems. Thus, our s-DNAzyme design for bacteria detection is a simple mix-and-read system. It has a short reaction time, an easy experimental operation and low cost.

We first demonstrate the feasibility of this system. As shown in Figure 1A, MB-rA itself showed a weak fluorescence signal, which meant a stable formation of the stem-loop structure and the fluorophore could be efficiently quenched by the quencher. When this MB-rA mixed with s-DNAzyme-Ecoli, in the absence of CIM of *E. coli,* the stem-loop structure was opened and gave an increased fluorescence signal. However, if this s-DNAzyme-Ecoli was first treated with CIM of *E. coli*, the MB-rA was not opened and showed a decreased fluorescence signal again. To further demonstrate the signal difference resulting from the cleavage of s-DNAzyme-Ecoli, HPLC-MS was conducted for the reaction product. A short DNA oligo product was extracted from the reaction solution (Figure 1B), which had the same molecular weight with the theoretical cleavage product clp-s in MS (Table S1, Figure S2). According to this data, we speculated that the cleavage action was caused by the electron-rich elements of the protein in CIM or hydroxyl function group in the solution (Figure S3).

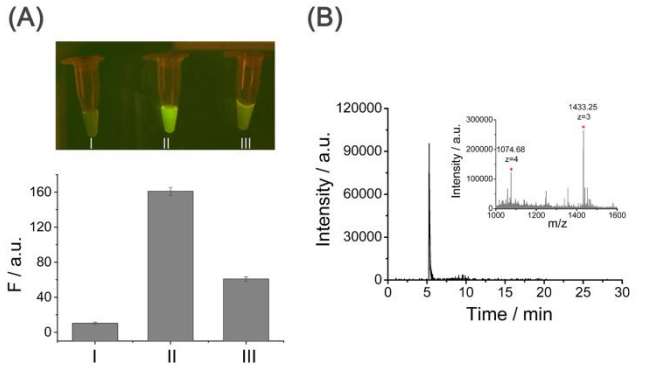


Figure 1. (A) The fluorescence value and images of different solution: (I) MB-rA solution (II) MB-rA solution mixing with s-DNAzyme-Ecoli (III) MB-rA solution mixing with CIM treated S-DNAzyme-Ecoli. (B) Chromatograms of the short cleavage DNA oligo product (insert picture is corresponding mass spectrum data).

For this s-DNAzyme system, we explored the influence of some critical experimental parameters. The result was shown in Figure 2. Each measurement was done by varying one

parameter while maintaining all other experimental factors constant. For different s-DNAzyme-Ecoli concentration (Figure 2A), the opened MB-rA derived from the interaction with s-DNAzyme-Ecoli increased with the increase of the s-DNAzyme-Ecoli concentration (grey data). The opened MB derived from the interaction with cleaved s-DNAzyme-Ecoli and the background signal of MB also increased (light purple data). The signal difference reached the highest point when its concentration was 1 μM. Figure 2B showed the effects of MB-rA concentration. Both the signals of s-DNAzyme-Ecoli system without and with CIM increased as MB-rA concentration increased because of its signal reporter function. When the concentration of MB was 0.2 μM, the highest F_0/F was obtained. For this assay, reaction temperature had a large effect (Figure 2C). A higher reaction temperature was unfavourable to the conformation of s-DNAzyme-Ecoli and had a negative impact on the binding of s-DNAzyme-Ecoli with CIM. Experimentally we found that 30 °C was an optimum temperature for detection. The magnesium ion concentration affected the conformation stability of s-DNAzyme-Ecoli, especially the stem-loop stability of MB. The more magnesium ions that existed in the reaction system, the more stable the stem-loop structure of MB was. Correspondingly, it would be more difficult to open the hairpin structure of MB-rA in detection system without CIM. Figure 2D showed the fluorescence signal difference of s-DNAzyme-Ecoli system decreased gradually when magnesium ion concentration was larger than 0.5 μM.

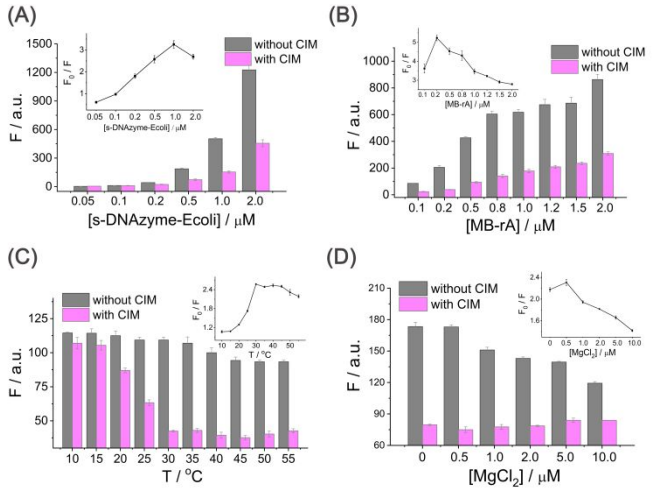


Figure 2. The influence of the experimental parameters: (A) s-DNAzyme-Ecoli concentration (B) MB-rA concentration (C) reaction temperature (D) $MgCl_2$ concentration. Insert graph means F_0/F (fluorescence value without CIM / fluorescence value with CIM) at the same condition.

Using the optimal experimental conditions, we assessed the specificity and sensitivity of the assay. We first explored the response of different common bacteria on the s-DNAzyme-Ecoli system. From Figure 3A, the DNAzyme system responded to K12 and BL21, but not to BS, KP, SH SL and SS bacteria. K12 and BL21 were two different strains of *E. coli*. Because the original DNAzyme sequence was selected with K12 strain model by *in vitro* selection, it gave the strongest signal change with the same culture and reaction condition. The signal decrease of BL21 indicated the unknown protein cleaving DNAzyme strand also existed but at a lower content in BL21 bacterium. *E. coli* and KP are Gram-negative bacteria (red star in Figure 3A). But here KP could not cause the decrease of the signal. BS, SH, SL and SS are Gram-positive bacteria (black star in Figure 3A). BS is a member of the genus Bacillus and found in gastrointestinal tract of ruminants and humans. SH, SL and SS all come from Staphylococcus family. These Gram-positive bacteria also could not reduce fluorescence signal. For each of these bacteria, successful detection would require specific selection of a DNAzyme.

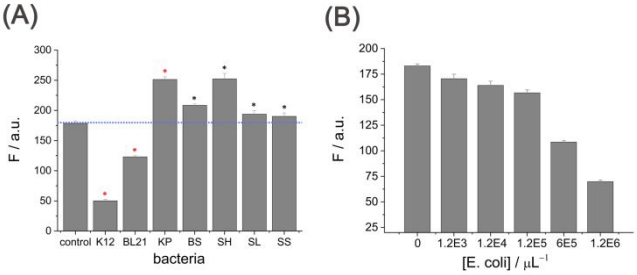


Figure 3. The fluorescence readout results of (A) different bacteria and (B) different E. coli concentration.

We also achieved the detection of E. coli bacterium number in 50 μL reaction solution (Figure 3B). The detection limit was 1.2E3/μL, which was the same order of magnitude as the original DNAzyme detection method for *E. coli*.[22] Thus, our method using MB to replace FQ modification did not sacrifice the sensitivity of the detection. Integrating with the cell-culturing step, we split 1 mL *E. coli* LB solution (mean 1.57 E. coli bacterium in total) into ten 100 μL aliquots to verify its capability for single live bacterium cell detection. We placed these ten 100 μL aliquots into 2 mL LB broth. After culturing overnight, we found that only one aliquot exhibited a reduced signal (Figure S4). Thus, this assay has the capability for single live bacterium detection, provided it is integrated with a cell culturing step.[7]

In conclusion, we have constructed a simple and fast mix-and-read bacteria detection based on the DNAzyme and the molecular beacon using E. coli as a model. DNAzymes are very promising as a bacteria detection tool because of their high stability, short reaction times and low cost. Conventional DNAzyme detection method for bacteria needs pre-treatment processing, which is complex, expensive and time-consuming. By modifying the DNAzyme sequence, introducing a molecular beacon and optimizing the experimental design, we developed a new bacteria detection based on s-DNAzyme-Ecoli. We have explored its feasibility and also have proven that it is insensitive to other non-target bacteria. The detection limit is the same as that of previous work. Our detection method simplifies the workflow, reduces hands-on time, and lowers the experimental

cost. It will facilitate further practical applications of nonculture-based bacterial detection based on the DNAzyme.

The authors declare no conflicts.

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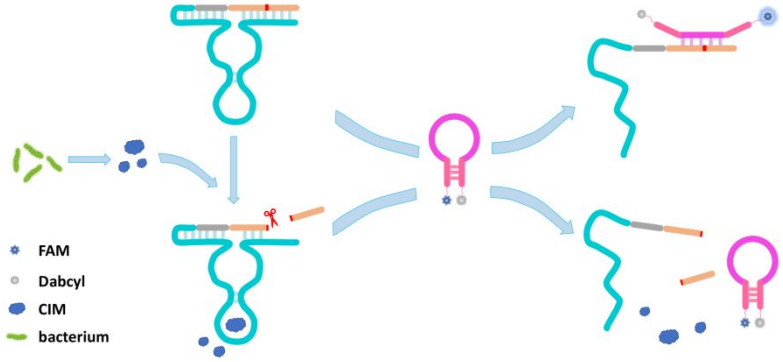
Graphical Abstract



A simple improved mix-and-read method for detection of bacteria is developed based on DNAzyme and molecular beacon.