

Translating Bacterial Detection by DNazymes into a Litmus Test**

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Abstract: Microbial pathogens pose serious threats to public health and safety, and results in millions of illnesses and deaths as well as huge economic losses annually. Laborious and expensive pathogen tests often represent a significant hindrance to implementing effective front-line preventative care, particularly in resource-limited regions. Thus, there is a significant need to develop low-cost and easy-to-use methods for pathogen detection. Herein, we present a simple and inexpensive litmus test for bacterial detection. The method takes advantage of a bacteria-specific RNA-cleaving DNzyme probe as the molecular recognition element and the ability of urease to hydrolyze urea and elevate the pH value of the test solution. By coupling urease to the DNzyme on magnetic beads, the detection of bacteria is translated into a pH increase, which can be readily detected using a litmus dye or pH paper. The simplicity, low cost, and broad adaptability make this litmus test attractive for field applications, particularly in the developing world.

Portable sensors are highly desirable for environmental monitoring, food safety control, and medical surveillance, particularly in resource-limited regions.^[1–3] Colorimetric sensors represent an attractive option as the change of color can be easily detected by the naked eye. The Litmus test for pH is a well-established and cheap colorimetric sensor that is still being widely used today. Existing litmus dyes and pH papers respond to pH changes by producing a color signal. By devising a method that links a molecular recognition event to the pH change of the sensing solution, we can take advantage of these inexpensive litmus dyes and pH papers to detect other targets.

Urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia.^[4–6] The hydrolytic reaction raises the pH value of the solution. Urease is highly efficient, speeding up the hydrolysis of urea by about 10^{14} times. Urease is also a stable enzyme and various forms of ureases are commercially available.^[7,8] Thus, we postulate that urease in combination with litmus dyes (or pH papers) are suited for the development of colorimetric biosensors. To do so, a strategy is required to couple a molecular recognition event to the activity of urease.

Functional nucleic acids, particularly DNA aptamers and aptazymes (aptamer-regulated DNazymes), have been shown to be excellent molecular recognition elements because they offer high affinity and specificity for their cognate targets, and they are stable and cost-effective.^[9–18] Many aptazymes have been engineered using RNA-cleaving DNazymes, where target binding triggers the cleavage of an RNA-containing substrate.^[18] Some of these aptazymes have been linked to signal-generation modules to produce fluorescent, colorimetric, and electrochemical readouts.^[19] As we will show herein, the RNA cleavage system also offers a convenient way to link the action of an aptazyme to the activity of urease through the use of magnetic beads. The ease of separation makes magnetic beads (MBs) an attractive option to immobilize biomacromolecules,^[20] and thus they have been widely used to set up bioassays.^[21]

The conceptual framework is illustrated in Figure 1. Four components are utilized: streptavidin-coated MBs, an aptazyme, urease conjugated to a DNA oligonucleotide (UrDNA), and a pH-sensitive dye (or pH paper). The aptazyme contains a biotin moiety at its 5' end for streptavidin binding and a sequence extension at its 3' end for hybrid-

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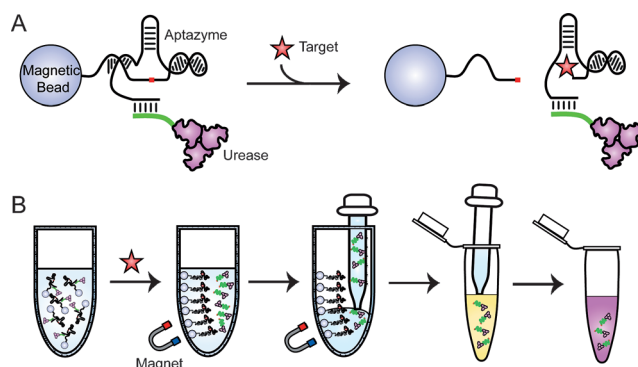


Figure 1. Conceptual schematic representation. A) Cleavage reaction. The binding of the cognate target to the aptazyme on the magnetic bead triggers its cleavage activity, thereby resulting in the release of urease. B) Colorimetric reporting assay. Upon target-induced cleavage and magnetic separation, the urease is used to hydrolyze urea in the presence of a litmus dye.

ization with UrDNA. Thus, simple mixing of the MBs, the aptazyme, and the UrDNA results in functional MBs that can release urease in response to the target of the aptazyme (Figure 1 A). Upon magnetic separation, the freed urease can be used to hydrolyze urea in the presence of a litmus dye for color generation (Figure 1 B).

The above design is compatible with any RNA-cleaving aptazyme; however for the current demonstration, we employed a DNAzyme, EC1, previously developed in our laboratory for the specific detection of *Escherichia coli* (*E. coli*), a model bacterial pathogen.^[22,23] Pathogenic bacteria pose a grave threat to public health and safety, and early detection of specific pathogens is an important step towards preventing a potential outbreak. However, laborious and expensive pathogen tests often represent a bottleneck in such efforts, particularly in resource-limited regions. A simple litmus test for pathogen detection offers a very attractive option.

A bifunctional linker, maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), was used to achieve the conjugation of a 5'-amino-modified DNA oligonucleotide (H₂N-DNA) to urease (Figure 2 A). H₂N-DNA was first allowed to react with MBS, which resulted in maleimidobenzoic DNA amide (MDA). This was followed by the coupling of urease to MDA through the addition of thiol to the double bond of the maleimide. By using a fluorescently labeled DNA, we showed that this method was able to achieve successful coupling of H₂N-DNA to urease (Figure 2 B).

The functionality of the MB-EC1-UrDNA was examined by treating the MB conjugates with the crude cellular extract

(CCE) prepared from *E. coli* (EC; intended bacteria) or *Bacillus subtilis* (BS; a negative control; we have previously shown that EC1 can not be activated by CCEs from a host of bacteria including *B. subtilis*).^[22,23] The cleavage activity was analyzed by denaturing polyacrylamide gel electrophoresis (dPAGE); for this reason, EC1 was internally labeled with ³²P so that the cleavage of EC1 would result in a DNA fragment that could be detected by dPAGE. We found MB-EC1-UrDNA can indeed be activated by CCE-EC, but not by CCE-BS (Figure 2 C).

We next carried out the litmus test for *E. coli* using phenol red as the litmus dye because in our preliminary test it produced a rather sharp, yellow-to-pink transition. The procedure consisted of two separate reactions: an *E. coli* induced probe cleavage reaction and a urease-mediated reporting reaction. The cleavage reaction was conducted at room temperature for 60 min in 1 × reaction buffer (1 × RB; 1 mM HEPES, pH 7.4, 150 mM NaCl, 15 mM MgCl₂, 0.01 % tween 20) containing CCE-EC or CCE-BS prepared from 10⁷ *E. coli* or *B. subtilis* cells, respectively (total reaction volume was 10 μL; see the Supporting Information for experimental details). This was followed by a 10-fold dilution with H₂O to facilitate the magnetic separation and minimize the impact of the buffering agent on the reporting reaction. After magnetic separation, 70 μL of the diluted cleavage solution were mixed with 100 μL of a urea-containing solution (2 M NaCl, 60 mM MgCl₂, 50 mM urea, 1 mM HCl) and 10 μL of 0.04 % phenol red. This resulted in a new reaction mixture with an initial pH value of approximately 5.5; at this pH value, phenol red exhibits a yellow color. As shown in Figure 3 the reaction mixture from CCE-EC changed its color within 15 min from yellow to brownish pink, which continued to intensify into bright pink within 60 min. In sharp contrast, the color of the reaction mixture originating either from RB alone or from CCE-BS remained unchanged.

We then examined the functionality of the litmus test in complex sample matrices represented by apple juice, milk, and lake water (see Figure S1 in the Supporting Information). This experiment shows that background materials present in

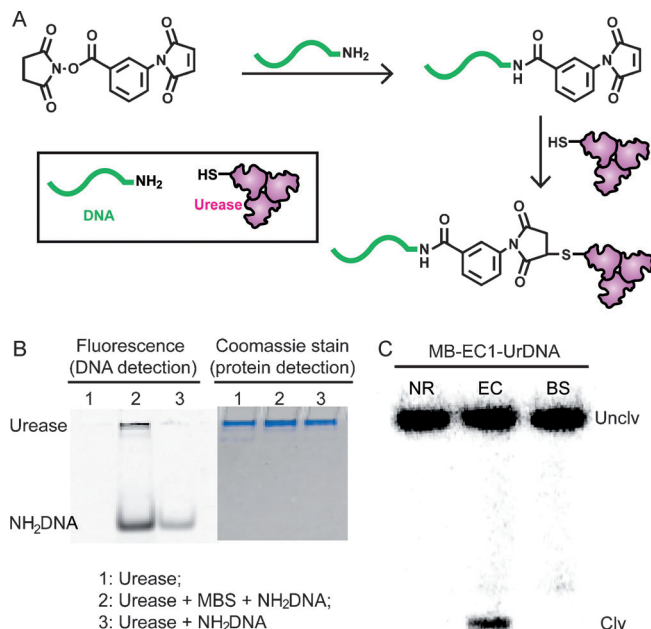


Figure 2. Synthesis and functional test of the sensor construct. A) Conjugation of 5'-amino-modified DNA to urease using MBS. B) Analysis of DNA-urease conjugation mixtures using non-denaturing PAGE. C) Functional test. NR: no reaction; EC and BS: MB conjugates treated with CCE-EC and CCE-BS, respectively; Civ: cleavage product; Unclv: uncleaved construct. Note: EC1 was radioactively labeled.

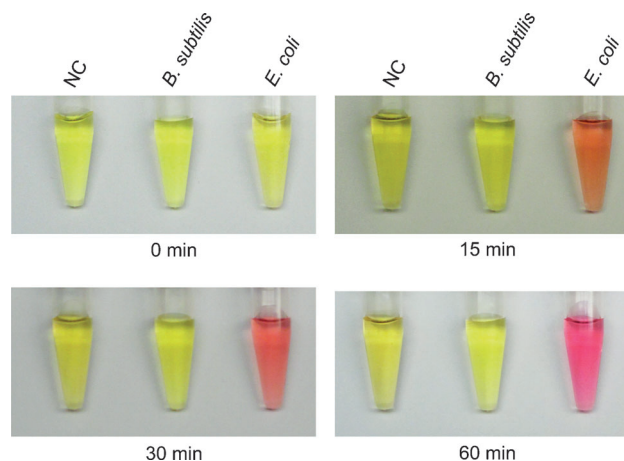


Figure 3. Litmus test with reaction buffer alone (left tube of each panel), CCE prepared from 10⁷ *B. subtilis* cells (middle), or *E. coli* cells (right). The photographs were taken at 0, 15, 30, and 60 min.

these complex samples do not significantly affect the outcome of the litmus test.

Several other dyes were then examined for the same assay, including bromothymol blue, neutral red, cresol red, *m*-cresol purple, and *o*-cresolphthalein complexone (see Figure S2 in the Supporting Information). It is apparent from the results that any of these dyes are compatible with our assay.

We next measured the time-dependent increase in the pH value of the reporting solution using a hand-held pH meter (Figure 4A). The pH value increased nearly 3 pH units for the *E. coli* sample, while the pH value of the control samples (either buffer only or *B. subtilis* samples) remained unchanged.

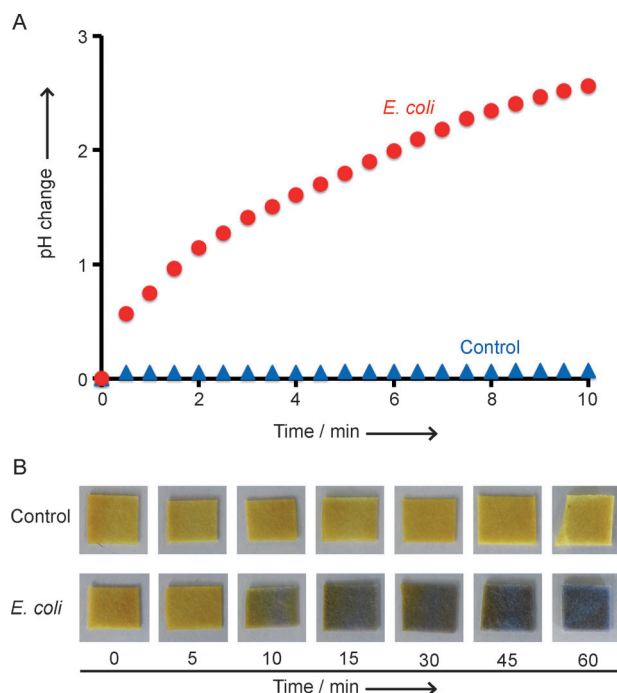


Figure 4. Monitoring pH changes caused by the presence of *E. coli* through electronic reading with a hand-held pH meter (A) and pH paper strips (B). 10^7 bacterial cells were used in the experiments.

We also monitored the changes in the pH value of these samples using commercially available pH paper strips (Figure 4B). Once again, while the control samples produced no detectable color change on the pH paper, a notable color change can be detected within 10 min with the *E. coli* sample. The results from all the experiments above show that the devised method can indeed be used to achieve target-specific detection using simple methods that include the color change of litmus dyes in solution, color change of a pH paper, and electronic readings using a hand-held pH meter.

We determined the sensitivity of our assay using phenol red. Eight CCE-EC samples were prepared from serially diluted *E. coli* samples, each of which contained the specific number of cells shown in Figure 5. A sharp color transition was observed for the sample containing 5×10^5 cells after color development for both 1 hour (top panel) or 2 hours (bottom panel). A subtle but detectable color transition, in

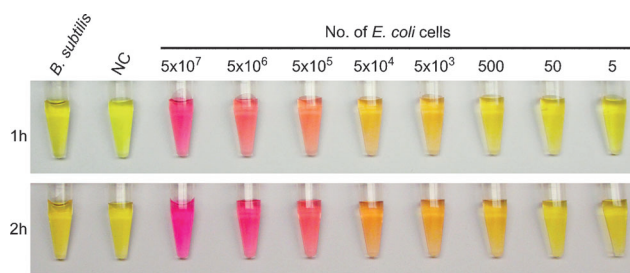


Figure 5. Litmus test with CCE-EC prepared from various numbers of *E. coli* cells. The photograph was taken after a signal-producing time of 1 h (top panel) or 2 h (bottom).

comparison to the two reference samples (5×10^7 *B. subtilis* cells and RB alone), was observed for the sample containing 5000 cells after incubation for 1 hour and 500 cells after incubation for 2 hours. For comparison, polymerase chain reaction (PCR) and sandwich enzyme-linked immunosorbent assay (ELISA) approaches, two popular pathogen detection methods, offer detection limits of approximately 10^4 – 10^5 *E. coli* cells.^[24,25] Thus, our litmus test offers comparable detection sensitivity.

Methods for the practical detection of food or water-borne pathogens such as *E. coli* are required to detect as low as 1–100 colony-forming units (CFUs). To achieve this level of detection sensitivity, an enrichment step through culturing is necessary. For this consideration, we also examined the ability of the litmus test to detect a single CFU of *E. coli* with a culturing step. The combined culturing/litmus test can easily detect a single CFU after 7 h of culturing (see Figure S3 in the Supporting Information), which is comparable to the popular PCR (ca. 10 h) and ELISA (ca. 16 h) methods.^[24,25]

In summary, we have developed a litmus test for *E. coli* that uses an RNA-cleaving DNazyme as the molecular recognition element and protein enzyme urease as the signal transducer. Our sensing system also takes advantage of magnetic separation, which is easy to implement, and pH-sensitive dyes or pH paper strips, which are cheap and widely available. To our knowledge, this is the first example where a molecular recognition event of an aptazyme (or any functional nucleic acid) is translated into a pH change. The litmus test exhibits a sensitivity similar to that of the fluorescence-based detection method we published earlier using the same DNA probe,^[22,23] however the colorimetric test is simple to perform and does not require specialized equipment, and therefore is better suited for field applications, particularly in developing countries.

Although an *E. coli*-sensing aptazyme was used in the current study, the sensor design can be easily extended to any RNA-cleaving aptazyme. Similarly, the design principle should be broadly compatible with any system in which a cleavable substrate (for the detection for an enzyme or factors that activate the enzyme) can be coupled to urease.

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