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Detection of RNA

Folding of 16S rRNA in a Signal-Producing Structure for the Detection of Bacteria**

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Conventional methods for the detection of pathogenic bacteria rely on either time-consuming culture growth or qPCR. These assays are sensitive to contaminations and require qualified personal or sophisticated equipment. Detection of nucleic acids directly from unamplified samples is of great importance for point-of-care (POC) diagnostic formats.[1] Such techniques would enable simple and straightforward tests that do not require expensive equipment or user expertise. Importantly, POC assays should allow fast and efficient recovery of nucleic acids from the sample and detect low concentrations of bacteria. Herein, we propose a straightforward and simple assay that analyzes bacterial RNA both in samples of total RNA and in whole bacterial cells with high selectivity and low detection limit.

The idea of the assay was inspired by the DNA-origami technology.[2] In DNA origami, the phage M13mp18 DNA is folded into desired shapes with the help of hundreds of short oligonucleotide staple strands (Figure 1 A). Importantly, the yield of the desired shape can reach up to 90 %.[2,3] Misfolded structures are unfavorable owing to the energetic sink that drives the hybridization towards the formation of only the most stable complete associate in the presence of excess amount of the staple strands.[2,3] If the produced origami shape could be easily detected by conventional methods, the approach would become adaptable for the detection of long nucleic acids, such as natural RNA molecules. In this study, we propose folding native bacterial RNA with the help of a series of functional "staple" strands into a specific nanostructure that produces fluorescent signal.

We have chosen bacterial 16S ribosomal RNA (rRNA) as a target for the proof-of-concept study. It is approximately 1500 nucleotides in length and is a component of small subunit of prokaryotic ribosome. 16S rRNA is highly conserved between different species of bacteria and archaea.[4] Along with highly conserved regions, it also contains hypervariable sequences that can provide species-specific signatures used for identification of prokaryotic organisms.[5] Moreover, multiple copies of rRNA are present in a single bacterial cell, which makes it an attractive target for the detection of low-abundance bacteria without PCR amplification.

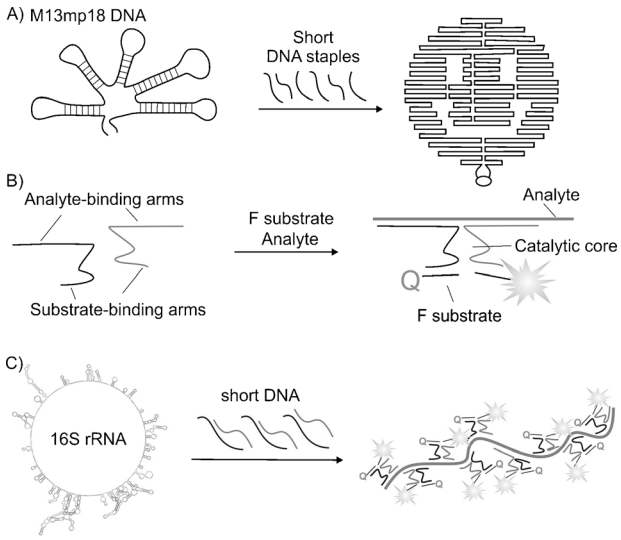

Figure 1. The design of "deoxyribozyme-on-a-string structure" for nucleic acid analysis. A) Concept of DNA origami. B) A single binary (split) deoxyribozyme probe re-forms a catalytic core in the presence of a specific analyte. C) Detection of 16S rRNA using a "deoxyribozymes-on-a-string" complex.

To develop a highly sensitive assay we took advantage of signal amplification property of deoxyribozymes (Figure 1 B). In split or binary deoxyribozyme sensors,[6] two DNA strands containing fragments complementary to a target analyte (analyte-binding arms) and fragments complementary to a fluorophore- and quencher-labeled fluorogenic reporter substrate (F substrate) reform a catalytic core in the presence of a specific analyte. Active enzyme binds and cleaves the fluorogenic substrate, thus producing high fluorescence that can be easily detected by a conventional fluorometer.

In the present work, we combined the binary deoxyribozyme approach with the staple-assisted folding of a long nucleic acid inspired by the DNA origami to design a highly sensitive sensor for RNA detection. In the proposed assay, an RNA folded in a stable secondary structure is incubated with a multitude of functional staple strands (deoxyribozyme precursors) and a fluorogenic reporter substrate. Hybridization of the staples to the RNA unwinds its secondary structure to form "deoxyribozymes-on-a-string" complex (Figure 1 C). This associate contains multiple catalytically active deoxyribozymes, which cleave the reporter substrate, thus producing fluorescent signal. The entire length of RNA molecule is used for the signal production, and each active

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deoxyribozyme cleaves several copies of the fluorogenic substrate owing to multiple catalytic turnovers. The combination of these two factors offers high level of signal amplification.

We used binary 10–23 deoxyribozyme developed by Mokany et al.[6b] to design 32 binary deoxyribozyme sensors that cover almost the entire sequence of *E.coli* 16S rRNA (Supporting Information, Table S1). The design of the sensors was uniform; the sequences of the analyte-binding arms were the only change made for each binary 10–23 probe (Supporting Information, Figure S1).

Testing the five individual 10–23 probes with 16S rRNA transcript revealed the detection limits of 1.3–0.4 ng (16–5 pM) of 16S rRNA (Supporting Information, Figure S2), which correlated with the limit of detection (LOD) of 5 pM for 10–23 split deoxyribozyme probe reported earlier.[6b] To enable the 32 deoxyribozyme probes operate together in the "deoxyribozyme-on-a-string" complex, the adjacent catalytic cores were designed to face the opposite sides of the double-stranded RNA-DNA helix by separating them by about 4.5 helical turns. The performance of the sensor was evaluated using a purified transcript of 16S rRNA. The 64 DNA staple strands formed an associate with the RNA transcript, which had an electrophoretic mobility corresponding to the predicted "deoxyribozyme-on-a-string structure" (Supporting Information, Figure S3). The sensor was able to detect as low as about 50 pg of 16S rRNA transcript in a 150 μL sample, or about 0.6 pM RNA analyte (Supporting Information, Figure S4). This LOD was significantly lower than that of the individual binary 10–23 probes. This indicates that the series of binary deoxyribozyme probes bound to the RNA "string" retained their catalytic functions and were able to cleave the fluorogenic substrate simultaneously according to the hypothesis shown in Figure 1 C.

The sequence of 16S rRNA is highly conserved among bacteria species. For example, *B. subtilis* and *E. coli* have 79 % of 16S rRNA sequence identical (Supporting Information, Figure S5). For the reliable detection of a targeted bacterium, the sensor should be selective enough to differentiate between bacterial species. To test the selectivity of the "deoxyribozyme-on-a-string" approach, we designed the sensor targeting *B. subtilis* 16S rRNA (see the Supporting Information, Table S1 for sequences).

We tested the performance of both *E. coli*- and *B. subtilis*-specific sensors in the presence of total RNA from either *E. coli* or *B. subtilis*. It was found that the signal above the background can be achieved in the presence of about 0.3 ng of total bacterial RNA (Supporting Information, Figure S6). For both sensors, an about two-fold increase in fluorescence was observed in the presence of the target RNA, while the non-target RNA triggered fluorescence almost as low as the background (Figure 2), thus confirming the high selectivity of the assay. This result is not surprising, because for the catalytic core to be re-formed, a pair of deoxyribozyme strands needs to be bound to the target simultaneously. Therefore, for good selectivity it is important to avoid perfect complementarity of both strands of each non-specific sensor to the target RNA. In our design, there was no completely complementary pair of strands (Supporting Information, Figure S5). Comparative

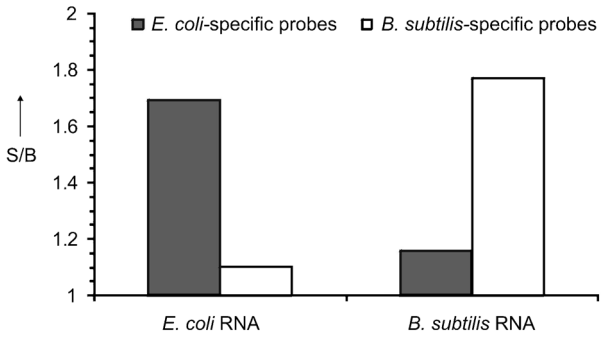

Figure 2. Selectivity of the "deoxyribozymes-on-a-string" sensor. *E. coli*- or *B. subtilis*-specific probes (gray or white bars, respectively) were incubated in the absence or presence of 10 ng total RNA from *E. coli* or from *B. subtilis* for 1 h. The data is an average value from two independent measurements.

analysis of the two bacterial 16S rRNA sequences revealed that only three deoxyribozyme strands were fully complementary to both specific and non-specific 16S rRNA molecules (Supporting Information, Table S1 and Figure S5). Even though some strands have analyte-binding arms perfectly complementary to both bacterial RNAs, if a considerable number of the strands are out of the complex, the equilibrium might be totally shifted towards the free folded rRNA.

RNA isolation is a simple but time-consuming procedure. Therefore, we next tested the possibility to detect 16S rRNA in a single assay starting form the whole bacterial cells. Different amounts of *E. coli* cells were boiled in the presence of *E. coli*-specific multicomponent deoxyribozyme sensor, followed by incubation of the complex in the presence of the fluorogenic reporter. Fluorescence of the samples increased with increasing the cells amount (Figure 3 A; Supporting Information, Figure S7). According to our estimation, 1×10^5 *E. coli* cells were sufficient to produce the signal above the background after 1–3 h assay. Prolonged incubation allowed reducing the detection limit to 3×10^4 cells, which are present in about 20 nL of a bacterial culture at the end of the exponential growth phase. By lowering the sample volume to 2–10 μL, which can be easily measured by the NanoDrop 3300 fluorospectrometer, for example, it is possible to decrease the limit of detection to about 4×10^2–2×10^3 cells after overnight incubation. Similarly to isolated bacterial RNA, the whole cell-based assay was highly selective. Indeed, non-targeted cells trigger fluorescence only slightly above the background (Figure 3 B).

In conclusion, we have applied recent findings of DNA nanotechnology[2] and the advantages in the design of deoxyribozyme sensors[6] to develop a PCR-free assay that can detect specific RNA molecules in a sample of total bacterial RNA or starting from the whole cells. The limit of 16S rRNA detection was found to be 0.6 pM in case of isolated RNA transcript, or about 3×10^4 *E.coli* cells, which potentially can be further lowered to 400 cells. These limits of detection are within the range of the amount of some pathogenic bacteria in clinical samples. For example, a typical sputum sample of a *Mycobacterium tuberculosis*-infected individual may contain 10^5–10^6 cells mL^{-1}.[7] Using RNAse inhibitors

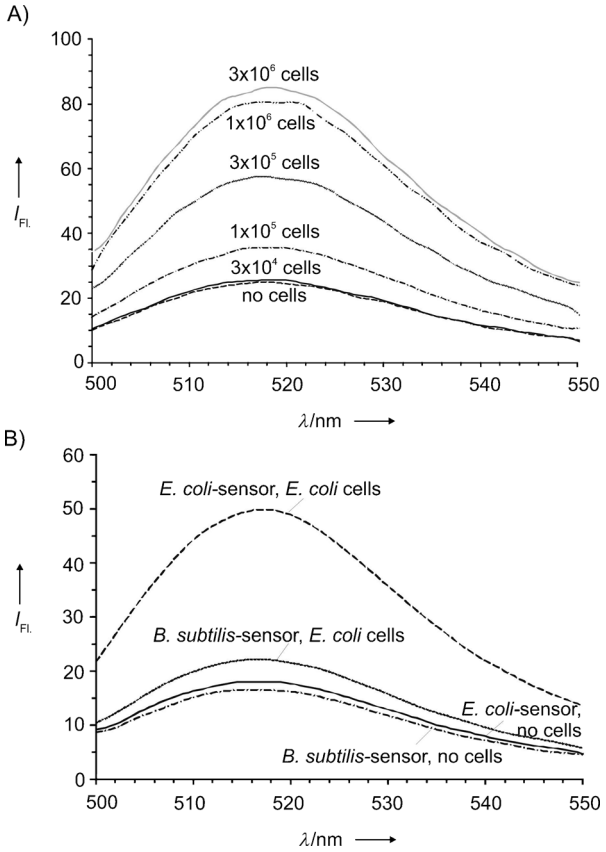

Figure 3. Detection of whole bacterial cells. A) Dependence of fluorescence for the *E. coli*-specific sensor on the amount of *E. coli* cells. B) Sensor selectivity. Fluorescent spectra for *E. coli*- and *B. subtilis*-specific probes in the absence or presence of *E. coli* cells. All of the spectra were recorded after 3 h incubation at 54 °C.

that prevent RNA degradation in the whole-cell assay may further improve the detection limit. The assay is selective, as little or no fluorescence increase was observed in the presence of RNA from a non-targeted bacterium. The design of the sensor was straightforward and universal. Despite using multiple sensors, the reagent cost is expected to be low, as the deoxyribozyme sensors are inexpensive synthetic oligonucleotides, while the fluorogenic signal reporter is universal for all probes and therefore can be synthesized in bulk amount and used efficiently. The approach is potentially applicable to the detection of any folded RNA molecules including mRNA and thus can be adopted for the detection of drug-resistant bacterial strains expressing a gene responsible for the resistance. Further development of this approach may deliver a straightforward, fast, and inexpensive test for POC detection of bacterial infections.

Experimental Section

For the analysis of *E. coli* 16S rRNA, *E. coli*-specific strands A1-A32 and B1-B32 (10 nM each) were annealed with isolated 16S rRNA (0.16–1.3 ng mL^{-1}) by incubating the mixtures in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01 % Triton X-100, 25 mM $MgCl_2$ (reaction buffer) at 95 °C for 5 min. The mixtures were cooled down to room temperature for 20 min. The complexes formed between 16S rRNA and the strands were isolated using centrifugal filters with MWCO 30 kDa (Millipore), washed two times with the reaction buffer, and mixed with the reporter oligonucleotide (200 nM) in the reaction buffer. The final reaction mixtures were incubated at 54 °C for 1–3 h or overnight. Fluorescence spectra of the samples were recorded on a PerkinElmer (San Jose, CA) LS-55 luminescence spectrometer with a Hamamatsu xenon lamp (excitation at 485 nm; emission 517 nm). More details of the experimental procedure are given in the Supporting Information.

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