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Detection of bacterial 16S rRNA using a molecular beacon-based X sensor

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ABSTRACT

We demonstrate how a long structurally constrained RNA can be analyzed in homogeneous solution at ambient temperatures with high specificity using a sophisticated biosensor. The sensor consists of a molecular beacon probe as a signal reporter and two DNA adaptor strands, which have fragments complementary to the reporter and to the analyzed RNA. One adaptor strand uses its long RNA-binding arm to unwind the RNA secondary structure. Second adaptor strand with a short RNA-binding arm hybridizes only to a completely complementary site, thus providing high recognition specificity. Overall the three-component sensor and the target RNA form a four-stranded DNA crossover (X) structure. Using this sensor, *Escherichia coli*16 S rRNA was detected in real time with the detection limit of ~0.17 nM. The high specificity of the analysis was proven by differentiating *Bacillus subtilis* from *E. coli* 16 S rRNA sequences. The sensor responds to the presence of the analyte within seconds.

1. Introduction

Hybridization-based techniques have been extensively employed for the analysis of specific DNA/RNA sequences (Guo et al., 2012; Silverman and Kool, 2007). The approach uses a short oligonucleotide probe to bind a specific DNA or RNA analyte by Watson–Crick base pairs, followed by the detection of the probe–analyte complex. In some cases; however, RNA and single-stranded DNA analytes form stable secondary structures, making the analysis of such nucleic acids complicated, since a region of interest becomes inaccessible for hybridization with an oligonucleotide probe. There is a common belief that targeting sequences within double stranded fragments of an RNA should be avoided (Kreil et al., 2006; Okten et al., in press; Santangelo et al., 2006; Vasilyeva et al., 2011). At the same time, species-specific variations are often found in the double-stranded RNA fragments (Fuchs et al., 1998; Wilks and Keevil, 2006; Yilmaz et al., 2006). This creates a serious limitation in the analysis of species-specific nucleotide variations in RNA. Furthermore, to find RNA fragments accessible for hybridization, a computational RNA analysis is required, which predicts RNA folding with certain degree of inaccuracy. The problem of specific RNA recognition becomes especially challenging in the case of RNA analysis in live cells (Rhee et al., 2008; Tyagi, 2009), where elevated temperatures and high excesses of hybridization probe cannot be used to achieve probe–RNA annealing. Hybridization probes made of peptide nucleic acids (PNA) or locked nucleic acids (LNA) can overcome this energetic barrier due to their exceptionally high affinity to DNA and RNA (Briones and Moreno, 2012; Hatamoto et al., 2010; Shiraishi et al., 2011; Tolstrup et al., 2003). Unfortunately, PNA and LNA remain to be expensive commercial products. Here, we demonstrate how a correctly designed 'smart' hybridization biosensor made of DNA oligonucleotides eliminates the aforementioned limitation by forming a sequence-specific fluorescent complex with an arbitrary chosen folded 16S ribosomal RNA (rRNA) fragment at ambient temperatures.

16S rRNA is a component of the small subunit of prokaryotic ribosomes. Analysis of 16S rRNA sequences is used in classification of bacteria species as well as in molecular diagnostics of infectious diseases (Balasingham et al., 2009; Hiyari and Bennett, 2011; Moschioni et al., 2010; Starke et al., 2007). In this study we have chosen *Escherichia coli* 16S rRNA as a model RNA analyte. It was reported earlier that 16S rRNAs, as well as their DNA amplicons, can fail to hybridize to the complementary probes, which represents a significant complication in the analysis of bacterial species (Chandler et al., 2003; Lane et al., 2004). Here we demonstrate how 16S rRNAs can be analyzed by a new type of multicomponent hybridization biosensor that uses a molecular beacon (MB) probe (Tyagi and Kramer, 1996; Li et al., 2008) as a real-time fluorescent reporter. MB probe is a fluorophore- and a quencher-labeled stem–loop folded DNA oligonucleotide, which is capable of increasing its fluorescence upon hybridization to the complementary nucleic acid sequence (Fig. 1a). The probe has found multiple applications in the analysis of DNA and RNA due to its ability to produce fluorescent signal in real-time when hybridized to the complementary sequences. The complications of MB probe in the analysis of folded RNAs is even more severe than that of linear oligonucleotide probes, since MB–analyte duplex formation requires unwinding of two secondary structure-folded nucleic

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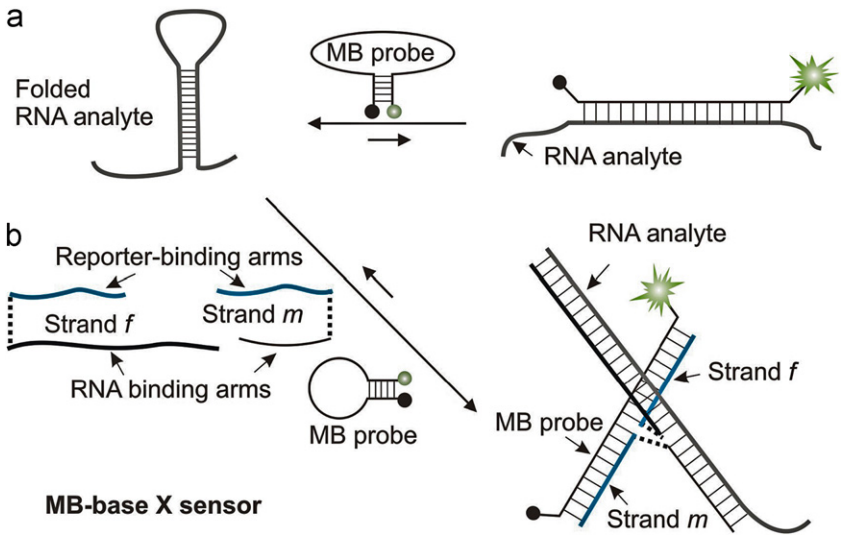

Fig. 1. Molecular beacon (MB)-based approaches for RNA analysis: (a) MB probe hybridizes to the folded RNA fragment: the equilibrium is shifted toward the two initial folded structures, and (b) MB-based X-sensor forms a thermodynamically stable fluorescent complex with the analyzed RNA. Strand **f** with long analyte-binding arm unwinds the secondary structure of RNA. Strand **m** with short RNA-binding arm hybridizes only to the fully complementary RNA fragment. MB probe fluorescently reports the complex formation. The equilibrium is shifted toward the fluorescent complex only if strand **m** is fully complementary to the RNA.

acids instead of one in case of linear probes. This requirement reduces both the thermodynamic and kinetic favorability of the event (Grimes et al., 2010; Nguyen et al., 2011; Tsourkas et al., 2003).

MB probe-based multicomponent sensors have been introduced and applied by us earlier for sequence-specific genotyping of short DNA fragments (Kolpashchikov, 2006; Gerasimova et al., 2010; Kolpashchikov et al., 2011). In this report we use one of the designs for the analysis of 16S rRNA obtained from bacterial source or by in vitro transcription or using short synthetic DNA strands as mimics of cDNA analytes. The multi-component sensor is named 'X sensor' since it forms a single DNA crossover structure (also known as DNA four way junction) upon hybridization to the target. The X sensor consists of the two DNA adaptor strands (strand **f** and strand **m** in Fig. 1b) and an MB probe as a reporter. Each adaptor strand has a fragment complementary to the MB probe (reporter-binding arm) and a fragment complementary to the target RNA sequence (RNA-binding arm). In the presence of a specific RNA analyte strand **f** hybridizes to the long RNA fragment and unwinds its secondary structure. In contrast to strand **f**, strand **m** possesses a short RNA-binding arm, which forms stable complex only with a fully complementary sequence. Only when hybridized to the adjacent positions of the analyte, strands **m** and **f** can cooperatively bind and open MB probe.

2. Materials and methods

2.1. Reagents and instruments

DNAse/RNAse-free water was purchased from Fisher Scientific, Inc. (Pittsburgh, PA) and used for all buffers and for the stock solutions of oligonucleotides. All oligonucleotides (for sequences see Table S1 in Supporting Information) were custom-made by Integrated DNA Technologies, Inc. (Coralville, IA). The enzymes were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Plasmid pEC16SM was kindly provided by Dr. Dedkova (ASU). The melting temperatures and the extinction coefficients of the oligonucleotides were predicted using Oligo Analyzer 3.1 software (IDT). Concentrations of oligonucleotides and rRNA transcript were calculated by measuring the absorbance of corresponding solutions at 260 nm using a Perkin–Elmer Lambda 35 UV/Vis spectrometer (San Jose, CA). Fluorescence spectra of the samples were recorded on a Perkin–Elmer (San Jose, CA) LS-55 Luminescence Spectrometer with a Hamamatsu xenon lamp (excitation at 485 nm; emission scan 500–550 nm).

2.2. Preparation of *E. coli* 16S rRNA transcript

E. coli 16S rRNA transcript was obtained by in vitro transcription using plasmid pEC16SM containing the 16S rRNA gene from *E. coli* strain A19. The full sequence of the transcript is provided in Supporting Information. The plasmid was linearized by PstI to serve as a template DNA (Nitta et al., 1998). The reaction mixture (100 μL) containing linearized pEC16SM (1 μg), Tris–HCl (40 mM), pH 7.9, $MgCl_2$ (20 mM), DTT (10 mM), spermidine (2 mM), NTP (4 mM each), bovine serum albumin (25 μg/mL), and T7 RNA polymerase (500 U) was incubated for 2 h at 37 °C. The transcript was collected by the conventional phenol treatment and ethanol precipitation. Concentrations of rRNA transcript in the transcription reaction mixture were estimated by the intensity of the band in 1% agarose gel using the gel-purified transcript of known concentration as a control (Fig. S1).

2.3. Detection of the 16S rRNA transcript using X sensor

For the detection of *E. coli* 16S rRNA, transcription reaction mixture containing ~1 μg/μL 16S rRNA transcript was used without the transcript isolation. A solution of MB1 (20 nM) and oligonucleotide adaptor strands **m16** (100 nM) and **f** (1000 nM) in a buffer containing Tris–HCl (50 mM), pH 7.4, and $MgCl_2$ (20 mM) was incubated either alone or in the presence of the transcript (5–80 ng). As a control, the MB probe was incubated in the same buffer in the absence of the adaptor strands and transcript. After 15 min incubation at room temperature (25 °C), the fluorescent spectra of the solutions (60 μL) were recorded, and the fluorescence intensity of the samples at 517 nm was measured.

2.4. Detection of oligonucleotide mimics for 16S rRNAs

To test the specificity of the X sensor, two oligonucleotides that mimic the targeted fragment of 16S rRNAs from *E. coli* or *Bacillus subtilis* were used as analytes. A solution of MB1 (20 nM) and oligonucleotide adaptor strands **m16, m12** or **m8** (100 nM) and **f** (1000 nM) in a buffer containing Tris–HCl (50 mM), pH 7.4, and $MgCl_2$ (20 mM) was incubated either alone or in the presence of each of the analytes (1–40 nM). As a control, MB1 was incubated in the same buffer in the absence of the adaptor strands and analytes. After 15 min incubation at room temperature (25 °C), the fluorescent spectra of the solutions (120 μL) were recorded, and the fluorescence intensities of the samples at 517 nm were measured. The data of three independent measurements is presented with an error margin of one standard deviation.

3. Results and discussion

We aimed to demonstrate that any randomly chosen sequence can be analyzed by the X sensor approach. Therefore, the fragment of *E. coli* 16S rRNA (1298–1341 nt, see Fig. 2a) targeted by the X sensor was chosen arbitrarily among the stem–loop folded fragments of the 1542-nt RNA for the analysis. This 43-nucleotide (nt) fragment folds in a stable stem–loop structure under the experimental conditions (T_m=84.5 °C, according to IDT Oligo Analyzer software). The design of the X sensor is shown in Fig. 2b. The sensor utilizes MB1 as a signal reporter. In the presence of the nucleic acid target, two DNA adaptor strands hybridize to the analyte and the MB1 probe, thus forming a fluorescent quadripartite complex (Fig. 2b). This fluorescent associate is stabilized by the formation of a DNA crossover structure. Such structures are known to exist as a mixture of two right-handed antiparallel crosses (Lilley, 2000). In case of MB-containing X-structure, the fluorescence of the complex would depend on how distant the fluorophore and the quencher groups of the MB probe are from each other: the conformer with the MB probe in the extended conformation would demonstrate the highest fluorescence. In general, the ratio between the two conformers depends on the nucleotide composition at the point of strands exchange (Lilley, 2000), which would be predetermined by the analyte sequence. In the design of the X sensor, introduction of triethylene glycol (TEG) linkers between the analyte- and reporter-binding arms of the adaptor strands forced the quadripartite associate to acquire the preferable conformation, since it made the strands to bend at the point of the TEG insertions (Kolpashchikov, 2006; Gerasimova et al., 2010).

In our design, the X sensor contained the adaptor strands, which had 9-nt A/T-rich reporter-binding arms. Such relatively short fragments ensured low background of the sensor due to the inability to form complex with the MB1 probe in the absence of the analyte. Adaptor strand **f** had 28-nt analyte-binding arms. Long analyte-binding arm allowed the probe to unwind the stable secondary structure of the targeted 43-nt fragment of *E. coli* 16S rRNA. The length of the analyte-binding arm of strand **m** employed to detect *E. coli* 16S rRNA was chosen to be 16-nt long (probe **m16/f**, Fig. 2b).

First, the sensor **m16/f** was used to detect *E. coli* 16S rRNA as a part of in vitro transcription reaction mixture. The analyte-dependent increase in fluorescence is shown in Fig. 3. The fluorescence depended on the amount of RNA linearly in the range of 5–40 ng RNA (Fig. 3, Inset). The probe was able to detect as low as 5 ng of 16S rRNA (corresponds to 10 fmol or 0.17 nM RNA in the sample, Fig. 3). This value is about one order of magnitude better than that observed for DNA analytes. This improvement can be attributed to the formation of more stable RNA–DNA hybrid in the case of 16S rRNA analysis. The background of the assay was low, in accordance with theoretical predictions. Indeed, the fluorescence intensity of the probe in the absence of the analyte was only about 20% higher than the intensity of the MB1 probe alone (Fig. 3).

To test the specificity of the X sensor, we used DNA oligonucleotides that mimicked the targeted fragment of 16S rRNA from *E. coli* and *B. subtilis* as analytes (Fig. 2c). These oligonucleotides

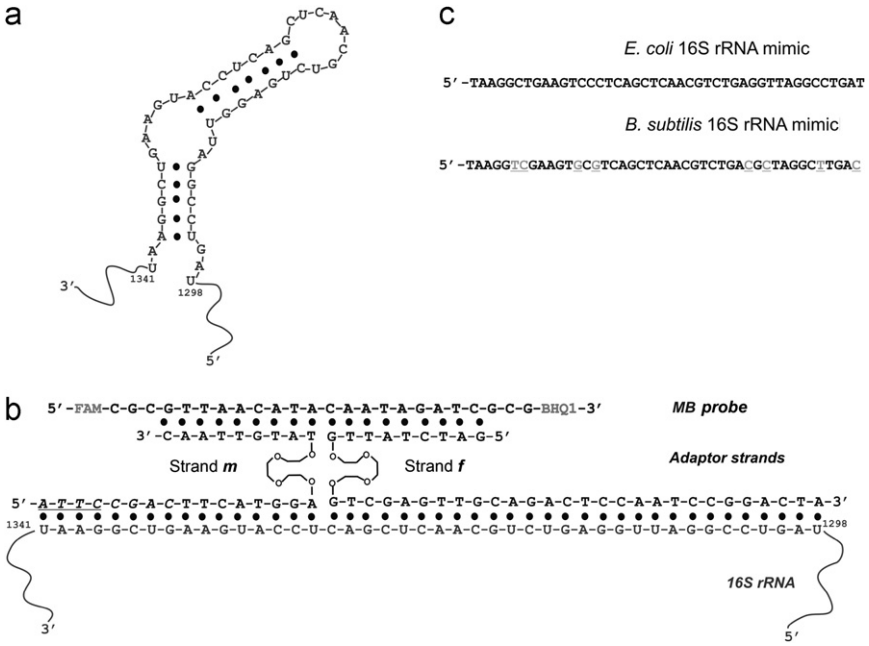

Fig. 2. X sensor for detection of a *E. coli* 16S rRNA fragment (1298–1341 nt): (a) predicted secondary structure of the rRNA fragment, (b) design of the X sensor **m16/f**. The nucleotides truncated in the design of the probe **m12/f** are underlined. In the design of the probe **m8/f** the analyte-binding arm of the adaptor strand **m** did not contain nucleotides shown in italic, and (c) the structure of two oligodeoxyribonucleotides that mimic fragments of 16S rRNA from *E. coli* (specific analyte) and *B. subtilis* (non-specific analyte). The nucleotides that are different in two analytes are shown in gray in the structure of *B. subtilis* 16S rRNA mimic.

are referred to as specific and non-specific analytes, respectively. The two analytes differed by eight nucleotides. The fragment complementary to the RNA-binding arm of strand **m** contained four nucleotides that were different in two analytes. The probe **m16/f** demonstrated rather poor specificity: the non-specific analyte triggered fluorescence only about three times lower than the specific one (Fig. 4a, first two bars; Fig. S2a). The specificity factor was calculated using a formula $SF = 1-(F_{ns}-F_0)/(F_s-F_0)$, where F_0, F_s, F_{ns} are fluorescence intensities of the probe in the absence or in the presence of specific or non-specific analyte, respectively. The *SF* of 0.78 was calculated for **m16/f**. Conclusively, the fluorescent associate formed between **m16/f** and the non-specific analyte was rather stable despite eight base pair mismatches.

It is obvious that the shorter the analyte-binding arms of the adaptor strands, the lower the affinity of the sensor to the non-perfectly complementary analyte. At the same time, the sensor should still be able to unwind the analyte's secondary structure. Therefore, in order to make the X sensor specific enough to differentiate between 16S rRNA from the two bacterial species, we varied the length of the analyte-binding arm of strand **m** leaving strand **f** the same. The new probes **m12/f** and **m8/f** contained 12- or 8-nt analyte-binding arms of strand **m**, respectively (Fig. 2b). Shortening of the analyte-binding arm of strand **m** down to eight nucleotides (probe **m8/f**) resulted in decreased signal both for the specific and non-specific analytes (Figs. 4a and S2c). In fact, the signal triggered by the non-specific analyte was not higher than the background, which improved probe selectivity (*SF*=0.97), but considerably lowered its sensitivity. Indeed, the signal-to-background ratio (S/B) for **m8/f** in the presence of the specific analyte was less than two, while the same parameter for **m16/f** reached 10 (Fig. 4a). When the analyte-binding arm of strand **m** was 12-nt long (probe **m12/f**), the high S/B of the probe in the presence of the specific analyte was preserved (Fig. S2b). Slightly higher values of S/B for **m12/f** in comparison with **m16/f** can be attributed to the higher background in the later design, resulted from partial complementarity of strands **m16** and **f**. Non-specific analyte triggered the fluorescence of **m12/f** close to the background. The specificity of the probe was near-ideal: the *SF* of 0.99 was achieved. Therefore, 12-nt analyte-binding arm was determined to be long enough to provide stable complex between the probe and the specific analyte, and to be short enough to differentiate between mimics of *E. coli* and *B. subtilis* 16 S rRNA fragments. The repeatability for probe **m12/f** was 11% relative standard deviation.

We further studied the performance of **m12/f** X sensor, using various concentrations of the specific or non-specific oligonucleotide analytes (Figs. S3 and S4). A concentration-dependent increase in fluorescence was observed in the experiments with the fully matched *E. coli* analyte, with the linear fluorescence increase within the concentration range of 1–10 nM (Fig. 4b, black circles). At the same time, no increase in fluorescence was triggered by the non-specific analyte (Fig. 4b, white circles). The fluorescence of the probe in the presence of the non-specific analyte remained close to the background even at high analyte concentrations (Fig. 4a, white bar in the middle).

The hybridization kinetics of **m12/f** sensor with the specific analyte is shown in Fig. 5. The fluorescence increase was detected in seconds after addition of 40 nM specific analyte (Fig. 5, inset) with linear increase within first 5 min. The fluorescence emission approached the plateau after 15–20 min reflecting the completion of the hybridization process. Therefore, we assumed that 15 min incubation of the probe with the analyzed nucleic acid was sufficient for achieving high signal.

Overall, the optimized X sensor detected *E. coli* 16S rRNA under mild hybridization conditions with high specificity. It contains three components, each of which plays a well-defined role in the RNA detection and, therefore, can be fine-tuned without the need to change other components. Among the advantages of the X sensor is the adjustable length of the analyte-binding arms of the adaptor strands. The analyte-binding arms of one or both adaptor strands can be made long enough to unwind the RNA's secondary structure. If it is important to differentiate between analytes containing nucleotide substitutions, one of the analyte-binding arms can be shortened to allow complex formation only with the fully complementary analyte. In addition, the multicomponent X sensor demonstrates the favorable hybridization

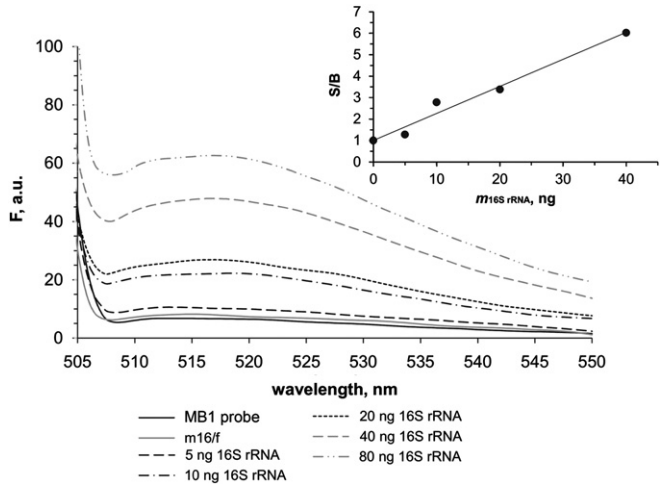

Fig. 3. Detection of *E. coli* 16S rRNA transcript using tricomponent sensor **m16/f.** Inset: the ratio (S/B) of the fluorescence intensities of the probe in the presence of different amount of the analyte to the background (no analyte).

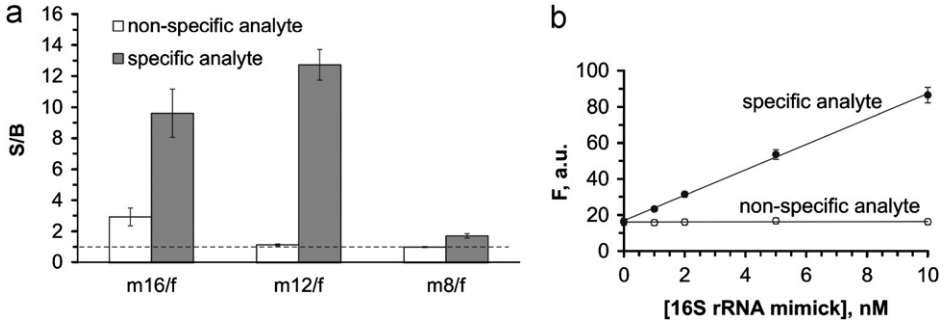

Fig. 4. Performance of the X sensor against *E. coli* 16S rRNA using oligodeoxynucleotides that mimic 16S rRNA from *E. coli* (specific analyte) or *B. subtilis* (non-specific analyte): (a) specificity of the tricomponent probes **m16/f**, **m12/f**, and **m8/f**. The concentration of the analytes is 40 nM. The black dashed line represents the background, and (b) the linear detection range for the probe **m12/f**. The data is averaged from three independent experiments, with standard deviations as error bars.

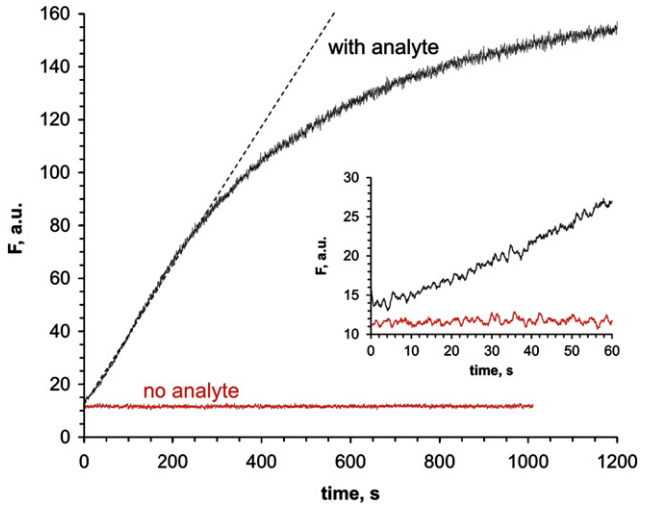

Fig. 5. Time-dependence for the fluorescence increase of the tricomponent probe **m12/f** in the absence (“no analyte“ curve) or presence of 40 nM specific analyte (“with analyte“ curve). Inset: enlargement of the curves for the first 1 min.

kinetics: the fluorescent signal was produced in seconds after mixing the sensor with the analyte.

4. Conclusions

It is commonly believed that using probes that demonstrate high hybridization affinities, such as PNA or LNA, is required for analysis of folded RNA structures. Here we have achieved detection of *E. coli* 16S rRNA under mild conditions using all DNA-based sensor, but innovative design principles. The proposed X sensor is able to (i) unwind the stable secondary structure of the analyzed nucleic acid at room temperature, (ii) fluorescently report the presence of the analyte in homogeneous solutions, and (iii) specifically discriminate the fully matched sequence from the mismatched ones. The approach is readily adaptable for the analysis of any given RNA sequence by simple replacement of RNA-binding arms of the adaptor strands. The optimized MB probe can be used as a universal reporter for any targeted RNA.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.08.058.

References

Balasingham, S.V., Davidsen, T., Szpinda, I., Frye, S.A., Tønjum, T., 2009. Molecular Diagnosis and Therapy 13, 137–151.
Briones, C., Moreno, M., 2012. Analytical and Bioanalytical Chemistry 402, 3071–3089.
Chandler, D.P., Newton, G.J., Small, J.A., Daly, D.S., 2003. Applied and Environmental Microbiology 69, 2950–2958.
Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W., Amann, R., 1998. Applied and Environmental Microbiology 64, 4973–4982.
Gerasimova, Y.V., Hayson, A., Ballantyne, J., Kolpashchikov, D.M., 2010. Chembiochem 11, 1762–1768.
Grimes, J., Gerasimova, Y.V., Kolpashchikov, D.M., 2010. Angewandte Chemie International Edition in English 49, 8950–8953.
Guo, J., Ju, J., Turro, N.J., 2012. Analytical and Bioanalytical Chemistry 402, 3115–3125.
Hatamoto, M., Ohashi, A., Imachi, H., 2010. Applied Microbiology and Biotechnology 86, 397–402.
Hiyari, S., Bennett, K.M., 2011. Journal of Dental Hygiene 85, 256–263.
Kolpashchikov, D.M., 2006. Journal of the American Chemical Society 128, 10625–10628.
Kolpashchikov, D.M., Gerasimova, Y.V., Khan, M.S., 2011. Chembiochem 12, 2564–2567.
Kreil, D.P., Russell, R.R., Russell, S., 2006. Methods in Enzymology 410, 73–98.
Lane, S., Evermann, J., Loge, F., Call, D.R., 2004. Biosensors and Bioelectronics 20, 728–735.
Li, Y., Zhou, X., Ye, D., 2008. Biochemical and Biophysical Research Communications 373, 457–461.
Lilley, D.M.J., 2000. Quarterly Review of Biophysics 33 (2), 109–159.
Moschioni, M., Pansegrau, W., Barocchi, M.A., 2010. Microbial Biotechnology 3, 370–388.
Nitta, I., Ueda, T., Watanabe, K., 1998. RNA 4, 257–267.
Nguyen, C., Grimes, J., Gerasimova, Y.V., Kolpashchikov, D.M., 2011. Chemisrty A European Journal 17, 13052–13058.
Okten, H.E.,Yilmaz,L.S.,Noguera,D.R. Systematic and Applied Microbiology http://dx.doi.org/10.1016/j.syapm.2011.11.001, in press.
Rhee, W.J., Santangelo, P.J., Jo, H., Bao, G., 2008. Nucleic Acid Research 36, e30.
Santangelo, P., Nitin, N., Bao, G., 2006. Annals of Biomedical Engineering 34, 39–50.
Shiraishi, T., Deborggraeve, S., Büscher, P., Nielsen, P.E., 2011. Artificial DNA PNA and XNA 2, 60–66.
Silverman, A.P., Kool, E.T., 2007. Advanced Clinical Chemistry 43, 79–115.
Starke, E.M., Smoot, J.C., Wu, J.H., Liu, W.T., Chandler, D., Stahl, D.A., 2007. Annals of the New York Academy of Sciences 1098, 345–361.
Tolstrup, N., Nielsen, P.S., Kolberg, J.G., Frankel, A.M., Vissing, H., Kauppinen, S., 2003. Nucleic Acids Research 31, 3758–3762.
Tsourkas, A., Behlke, M.A., Rose, S.D., Bao, G., 2003. Nucleic Acids Research 31, 1319–1330.
Tyagi, S., 2009. Nature Methods 6, 331–338.
Tyagi, S., Kramer, F.R., 1996. Nature Biotechnology 14, 303–308.
Vasilyeva, E., Lam, B., Fang, Z., Minden, M.D., Sargent, E.H., Kelley, S.O., 2011. Angewandte Chemie International Edition in English 50, 4137–4141.
Wilks, S.A., Keevil, C.W., 2006. Applied and Environmental Microbiology 72, 5453–5462.
Yilmaz, L.S., Okten, H.E., Noguera, D.R., 2006. Applied and Environmental Microbiology 72, 733–744.