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Enzyme-assisted binary probe for sensitive detection of RNA and DNA[†]

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Abstract

The new enzyme-assisted assay for DNA/RNA detection provides real-time fluorescent signal readout along with low limit of detection and high discrimination power toward a single-base substitution. Requiring only two new unmodified DNA oligonucleotides for the detection of each new analyte, the assay is an efficient tool for low-cost analysis of multiple analytes.

Analysis of low quantities of specific DNA/RNA sequences is of great interest for molecular diagnostics, drug development, linkage analysis and identity testing in forensic applications. To achieve high sensitivity, polymerase chain reaction (PCR)-based techniques are conventionally employed. However, PCR is susceptible to contaminations, which decreases the assay reliability. Moreover, it lacks portability and requires highly qualified personnel. To develop straightforward, inexpensive and easily reproducible methods for nucleic acid detection, a number of PCR-free strategies, which employ signal amplification approaches, have been suggested recently.¹

Molecular beacon (MB) probes are state-of-the-art tools for real-time nucleic acid analysis.² The MB probe is a stem-loop folded oligonucleotide with a fluorophore at the 5'-end and a quencher at its 3'-end. Hybridization to a complementary single-stranded DNA or RNA switches the MB probe into the elongated conformation, in which the fluorophore is remote from the quencher, resulting in high fluorescence. The intrinsic limitation for sensitivity of MB probes is the stoichiometric hybridization between the probes and the analytes. Recently, several enzyme-assisted assays for MB probes have been suggested.³ In these assays, an MB probe complementary to a specific analyte formed a duplex structure, which could be recognized by a DNA-nicking enzyme, for example (Scheme 1a). The enzyme catalyzed the cleavage of MB, thus separating the fluorophore from the quencher. This approach benefited from the increased turnover allowing a single analyte molecule to initiate cleavage of several MB probes. However, each new analyte required synthesis of an analyte-specific MB probe, which is an important disadvantage in application of these techniques in detecting multiple nucleic acids. Moreover, in most of these assays a sequence-specific enzyme was employed, which applied restrictions on an analyzed nucleic acid in terms of both type (only DNA molecules could be detected) and sequence. Here, we propose to use indirect binding of an MB-like reporter oligonucleotide with the analyzed nucleic acids. The approach avoids the aforementioned disadvantages and offers greater selectivity allowing

[†]Electronic supplementary information (ESI) available: Experimental detail, oligonucleotide sequences, electrophoretic analysis of the enzyme-assisted binary probe in polyacrylamide gel, calibration curve for the dependence of fluorescent signal enhancement from RO cleavage degree. See DOI: 10.1039/c0cc03248d

discrimination between two analytes that differ by a single nucleotide at ambient temperatures without the need for precise temperature control.

The approach uses two synthetic DNA strands α and β and an MB-like reporter oligonucleotide (RO) (Scheme 1b). Each strand contains a fragment complementary to RO (reporter-binding arm) and a fragment complementary to a nucleic acid analyte (analyte-binding arm). In the presence of the analyte, the probe forms a quadripartite complex, in which strands α and β serve as adaptors to connect the analyte and the reporter (Scheme 1b, top). The quadripartite complex contains a well-characterized⁴ DNA four-way junction motif, in which all nucleotides form base pairs at the point of strand exchange, and the strands undergo pairwise coaxial stacking. Reporter-binding arms of strands α and β are complementary to two RO sites, each of which contains a single ribonucleotide embedded into DNA sequence (shown by green inserts in Scheme 1b). The duplex fragments 5'-DNA-RNA-DNA-3'/3'-DNA-5' are recognized by the enzyme RNase HII, which catalyzes the hydrolysis of phosphodiester bond 5' to the ribonucleotide at the DNA-RNA junction.⁵ After nicking, the hybrid between RO and the reporter-binding arms of the probe becomes less stable, and the RO fragments dissociate from the quadripartite complex into solution. The released analyte-strand α -strand β tripartite complex can bind another molecule of the intact quenched reporter from solution. Therefore, one analyte molecule can trigger cleavage of many RO molecules, thus allowing amplification of the fluorescent signal.

In this proof-of-concept study we (i) validated the feasibility of RNase HII-assisted cleavage of the quadripartite complexes for both DNA and RNA analytes; (ii) proved that the signal amplification is achieved due to a catalytic turnover; (iii) demonstrated high selectivity of the RNase HII-assisted binary probe; and (iv) determined the limit of detection for the probe.

The sequences of oligonucleotides used in the study are listed in Table S1, ESI.† For the proof-of-concept experiments we designed RNase HII-assisted binary probes that recognize a fragment of hepatitis C virus mRNA (HCV-DNA and HCV-RNA) or a fragment of human genomic DNA containing a site of single nucleotide polymorphism (SNP) rs717302-G.

Incubation of RO with HCV-strand α , HCV-strand β and the cognated analyte HCV-DNA in the presence of RNase HII results in time-dependent fluorescent increase (Fig. 1). The detectable signal is achieved within first few minutes of the addition of 2 nM analyte. The RO cleavage was confirmed by denaturing polyacrylamide gel-electrophoresis (Fig. S1, ESI†). It was found that ~10-fold fluorescence increase was achieved after 3 hours of incubation. Such fluorescence increase corresponded to the cleavage of about 70% of RO (Fig. S2, ESI†). This means that 3.5 molecules of RO were cleaved per one analyte molecule. These data support the hypothesis of multiple turnover shown in Scheme 1b.

In order to determine the limit of detection of the assay, the probe was incubated with different concentrations of HCV-DNA analyte, and the fluorescence intensities of the samples were measured after 1 hour. It was found that the probe was able to generate fluorescence reliably above the background in the presence of 0.3 nM analyte (Fig. 2a). In comparison, the limit of detection for a conventional MB probe is about 8 nM.^{3b} Therefore, the improvement in sensitivity by more than an order of magnitude is possible using this approach. Longer incubation times and further optimization of the probe design and assay conditions can result in even lower detection limits.

Interestingly, RNase HII preferably cleaves a double stranded DNA duplex containing a single ribonucleotide over a RNA/DNA hybrid substrate.⁶ Therefore, single stranded RNA analyte or RNA in complex with DNA-based probe will not be hydrolyzed by the enzyme. This enables application of the assay for RNA analysis. Indeed, RNase HII-assisted binary

probe had similar limit of detection for DNA and RNA of the same sequence (Fig. 2, compare panels (a) and (b)). In the course of the assay no cleavage of the RNA analyte was detected by polyacrylamide gel electrophoresis (data not shown).

The selectivity of RNase HIII-assisted binary probe was tested using the probe designed to detect a fragment of human genomic DNA corresponding to the SNP rs717302 (allele G). In a typical hybridization assay, accurate allele discrimination is achieved at elevated temperatures (30–65 °C). Room temperature represents a challenge for SNPs analysis. Therefore, to demonstrate the efficiency of the suggested approach in single mismatch discrimination, we designed the probe for rs717302 that is able to operate at ambient temperature (22 °C).

It was shown earlier that hybridization of two short oligonucleotide fragments to the adjacent positions of the analyte provides excellent detection selectivity.⁷ Indeed, in our assay the allele-specific analyte-binding arm of rs717302-strand α was only 9 nucleotides long. The 9-nucleotide hybrids are stable at ambient temperatures only if perfectly complementary to the target. At the same time, the probe recognizes 25-nucleotide-long analyte fragment (strand β binds 16-nucleotide analyte fragment), which provides enough specificity for recognition of a particular fragment in a genome-large nucleic acid sequence. It was found that the assay reliably distinguishes between single-nucleotide substituted analytes. Indeed, the fully complementary rs717302-G generated fluorescent signal ~6 times above the background, while a mismatched rs717302-A triggered fluorescence increase that was statistically indistinguishable from the background (Fig. 3).

One of the main advantages of this technique over earlier reported enzyme-assisted MB assays³ is its high selectivity at ambient temperatures. For example, Zuo and colleagues^{3b} reported poor discrimination between single-base substituted analytes for an exonuclease III-aided MB probe (~1.5 difference in fluorescence triggered in the presence of matched or single-base mismatched DNA) even at 37 °C. In contrast, the selectivity of the approach suggested in this study is excellent: the single-base mismatched analyte does not trigger fluorescence increase that is statistically significantly above the background. High selectivity is achieved due to the short length of the analyte-binding arm of rs717302-strand α , which is extremely sensitive to even one nucleotide difference in the analyte sequence. Another significant advantage is the possibility to employ a universal RO for the analysis of each new analyte. Since the reporter oligonucleotide does not directly bind the analyte, only one double-labeled RO is required for the analysis of DNA or RNA of an arbitrary sequence, which decreases the cost of the assay in case of multiple analytes. Therefore, the structure of RO can be optimized to provide the best probe performance with respect to low background and high turn-on properties. It is important that strands α and β are unmodified and unpurified synthetic DNA oligonucleotides, which are easily affordable even by low-budget laboratories. Finally, indirect binding of the reporter oligonucleotide to the analyzed nucleic acid overcomes the sequence limitation for the analyte. In the present study, we demonstrate that both DNA and RNA analytes can be detected using the same approach. Moreover, two arbitrary chosen analyte sequences (a fragment of viral nucleic acid and an SNP site of the human genome) were analyzed using the same RO. The suggested enzyme-assisted binary probe represents a promising tool for highly selective, sensitive and inexpensive detection of specific RNA and DNA molecules for which the full potential is yet to be realized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

(a) Signal amplification in the case of enzyme-assisted MB probes.^{3a} (b) Signal amplification scheme for RNase HII-assisted binary probe. The ribonucleotides embedded in DNA sequence of the reporter are shown as green inserts.

Fig. 1.

Fluorescence-based detection of a DNA analyte corresponding to the fragment of hepatitis C virus mRNA (HCV DNA) using RNase HIII-assisted binary probe in the absence ($\circ$) or in the presence ($\bullet$) of the enzyme. RO (10 nM, 130 μL) was incubated with HCV-probe-strands α and β (100 nM) and DNA-HCV (2 nM) in the absence or presence of RNase HIII (10 U) at 30 $^{\circ}\text{C}$.

Fig. 2.

Fluorescence enhancement of the RNase HII-assisted binary probe in the presence of various concentrations of HCV-DNA (a) or HCV-RNA (b). Horizontal dashed line shows the fluorescence level corresponding to the limit of detection, which equals the average value plus three standard deviations of the background fluorescence. RO (10 nM, 130 μ L) was incubated with HCV-strands α and β (100 nM) and HCV-DNA or HCV-RNA (0.1–3 nM) in the presence of RNase HII (10 U) at 30 °C. Fluorescence intensities of the samples were measured after one hour of incubation. The dots represent average values of three independent experiments. The error bars represent one standard deviation. The analyte concentration range 0.1–1 nM is shown in the figure to demonstrate the probe's detection limit, while the actual dynamic range of the probe is 0.3–100 nM (data not shown).

Fig. 3. Selectivity of RNase HII-assisted binary probe. RO (10 nM, 130 μL) and rs717302-strands α and β (100 nM) were incubated with either fully matched rs717302-G or mismatched rs717302-A (10 nM) in the presence of RNase HII (10U) at 22 $^{\circ}\text{C}$. The average values of three independent experiments are shown. The errors are given as one standard deviation of the average.