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Sensitive and Selective Amplified Fluorescence DNA Detection Based on Exonuclease III-Aided Target Recycling

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Molecular beacons (MBs) have rapidly become a mainstay for the detection of oligonucleotides because they rapidly and specifically report the presence of a given nucleic acid sequence in homogeneous solution.[1−7] Typically, MBs are composed of a single-stranded oligonucleotide with self-complementary 5′ and 3′ ends that, in the absence of target, forms a stem−loop structure that brings an attached fluorophore/quencher pair into close proximity, reducing fluorescence emission. Hybridization to a complementary target disrupts the stem−loop structure, segregating the fluorophore from the quencher and thereby producing a large increase in fluorescence.[1−3]

A limitation of traditional MBs is that each target strand hybridizes with (and thus activates) only a single copy of the probe (Figure 1, top). This 1:1 hybridization ratio limits the gain of the approach and thus its sensitivity. In order to overcome this, the polymerase chain reaction (PCR) has historically been employed amplify the oligonucleotide targets, improving the signaling to the extent that the single-molecule detection limit is often realized.[8−10] PCR, however, is a relatively complex multistep process prone to false positives arising from artifactual amplification (e.g., of primer dimers, etc.).[8−10] In response, several groups have recently employed nicking endonucleases to amplify the signals obtained using various DNA/RNA detection schemes.[11,12] Specifically, these assays rely on the target-dependent cleavage (and thus activation) of the molecular beacon by a DNA nicking enzyme. Unfortunately, however, nicking endonucleases are sequence-specific and thus are limited in the number of target sequences against which they can be deployed.[11,12] Here, in contrast, we demonstrate a more versatile nuclease-amplified DNA detection scheme. In this scheme, exonuclease III, which is sequence-independent, is used to "recycle" target molecules, thus leading to improved sensitivity relative to traditional molecular beacons without any significant restriction in the choice of target sequences (Figure 1, bottom).

Exonuclease III catalyzes the stepwise removal of mononucleotides from 3′-hydroxyl termini of duplex DNAs.[13−16] However, its activity on single-stranded DNA is limited, and thus, the enzyme's preferred substrates are blunt or recessed 3′ termini.[15,17] Critically, exonuclease III does not require a specific recognition site, so cleavage occurs irrespective of the sequence present at the blunt terminus. This contrasts sharply with the nicking endonucleases utilized in earlier MB amplification schemes.[11,12]

Using the cleavage function of exonuclease III on double-stranded DNA, we have designed an amplified DNA detection scheme employing a stem−loop DNA molecular beacon as the signaling probe. The molecular beacon we have employed, which is modified with a CAL Fluor Red 610 (FR610) fluorophore at its 5′ terminus and a Black Hole Quencher (BHQ) at an internal position, self-hybridizes to form a stem−loop structure that contains exonuclease III-resistant 3′ protruding termini (Figure 1, bottom). This closed structure holds the fluorophore in close proximity to the quencher, which results in very weak fluorescence. After challenge with a perfectly matched target, the stem−loop structure is opened and forms a double-stranded structure with a blunt 3′

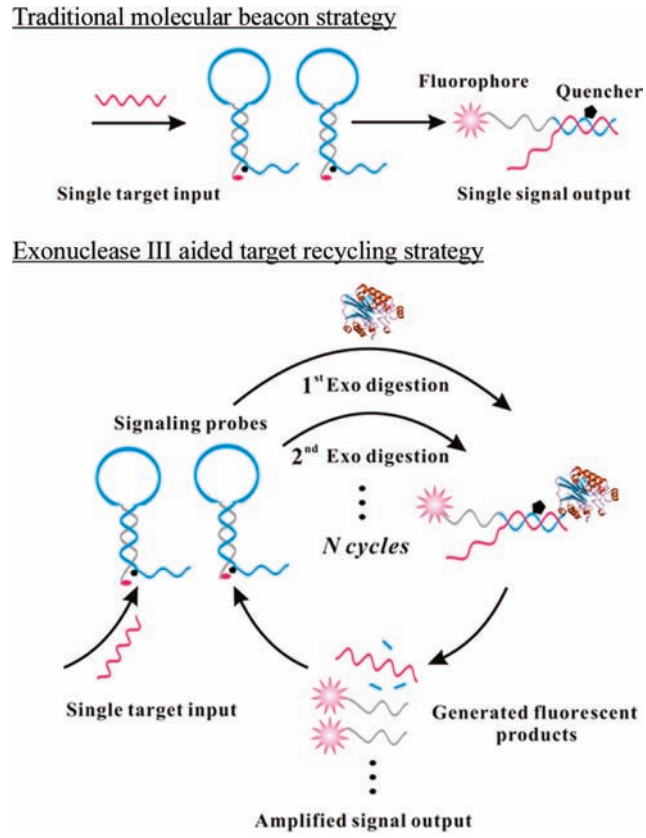

Figure 1. (top) A limitation of traditional molecular beacons (MBs) is that each target strand hybridizes with (and thus activates) only a single copy of the probe. This 1:1 hybridization ratio limits the gain of the approach and thus its sensitivity. (bottom) Using the blunt-end cleavage activity of exonuclease III, we have designed an amplified DNA assay in which, after challenge with target, the stem−loop structure is opened and forms a blunt 3′ terminus. Exonuclease III then catalyzes the stepwise removal of mononucleotides from this terminus, liberating the fluorophore and ultimately releasing the target. The released target can then hybridize with a second probe, whence the cycle starts anew, leading to significant amplification of the signal. The amplification provided by exonuclease III leads to a 10-fold increase in the final fluorescence intensity observed at 37 °C upon addition of 1 μM target.

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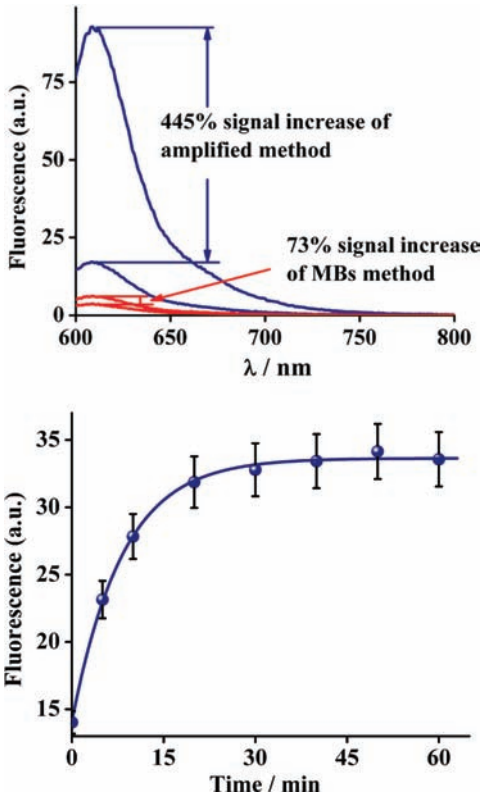

Figure 2. (top) The net signal gain produced by this exonuclease-amplified approach is enhanced significantly: whereas we observed a 445 ± 36% signal increase for 100 nM DNA target using the exonuclease III amplification, the corresponding signal gain in the absence of exonuclease amplification was only 73 ± 6%. (bottom) When performed at 37 °C, the assay is rapid and exhibits a nearly saturated signal within 30 min of the addition of 10 nM target DNA. Shown here and in the following figures are mean values and standard deviations obtained from at least three independent experiments.

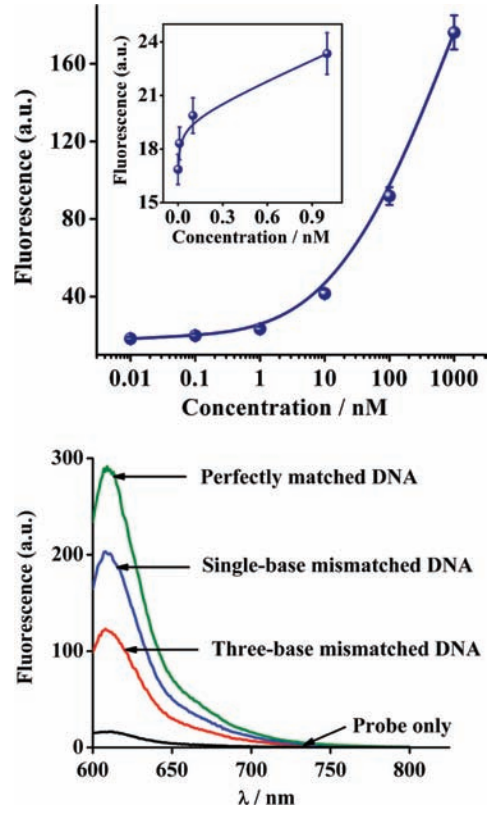

Figure 3. (top) We achieved a 10 pM detection limit of target with a 30 min incubation at 37 °C, corresponding to the detection of 0.5 fmol in a 50 μL sample volume. Above this concentration, we observed a monotonic increase in emission intensity with increasing target concentration. Shown are mean values and standard deviations obtained from at least three independent experiments. (bottom) The exonuclease-amplified assay readily differentiates between perfectly matched and mismatched DNA targets. The data shown were collected at 1 μM target concentrations.

terminus. Exonuclease III catalyzes the stepwise removal of mononucleotides from this terminus, liberating the fluorophore (which, separated from the quencher, now fluoresces) before ultimately releasing the target. The released target then hybridizes with another MB probe, whence the cycle starts anew. Thus, a single copy of the target generates many fluorescent MB fragments (Figure 1, bottom).

When the assay is conducted at 37 °C, the amplification provided by exonuclease III leads to a 4-fold increase in the final fluorescence upon addition of the relevant target (Figure 2, top). Unfortunately, however, under these conditions the exonuclease also degrades some of the closed molecular beacon, increasing the background fluorescence in concert with the observed increase in signal. Nevertheless, the net signal gain produced by this approach is significantly enhanced: whereas we observed a 445 ± 36% signal increase when exonuclease III amplification was employed, the corresponding signal gain in the absence of exonuclease amplification was only 73 ± 6% (Figure 2, top; both at the target concentration of 100 nM). Indeed, the gain achieved upon exonuclease amplification is great enough to produce a naked-eye color change from blue to purple in our samples, an effect that is not observed in the absence of exonuclease (see Figure S1 in the Supporting Information). Under these conditions, the exonuclease-amplified assay is also rapid, responding to its complementary target DNA with a time constant of ∼40 min (Figure 2, bottom).

The exonuclease-amplified MB assay is sensitive and specific. For example, we readily achieved a 10 pM detection limit within 30 min, corresponding to the detection of 0.5 fmol of target in a 50 μL sample volume (Figure 3, top, inset). Above this limit, we observed a monotonic increase in emission intensity with increasing target concentration until a 10-fold gain was achieved at target concentrations of 1 μM (Figure 3, top). In contrast, we obtained a 7.8 nM detection limit (see Figure S2) using the modified MB probe without exonuclease amplification, the sensitivity of which was almost 3 orders of magnitude poorer than that of the exonuclease-amplified method. The exonuclease-amplified assay is also specific. In order to evaluate this, we challenged our assay using single-base- and three-base-mismatched targets and found that it readily discriminates between single nucleotide polymorphisms (Figure 3, bottom).

The residual exonuclease activity against the unbound molecular beacon, which increases our background and reduces our signal gain at 37 °C, is almost entirely abolished at 4 °C. This in turn leads to significantly enhanced signal gain and significantly improved detection limits. Under these conditions, the gain of our assay is so great that we can readily detect target over the range from 20 aM to 200 fM (Figure 4), corresponding to as few as ∼600 molecules in a 50 μL sample. The gain obtained under these conditions is so great that naked-eye observations alone are sufficient to reveal a color change from blue to purple with increasing target concentration over much of this same range (Figure 4).

While the sensitivity of the exonuclease-amplified assay at lower temperatures is significantly improved, it nevertheless represents a

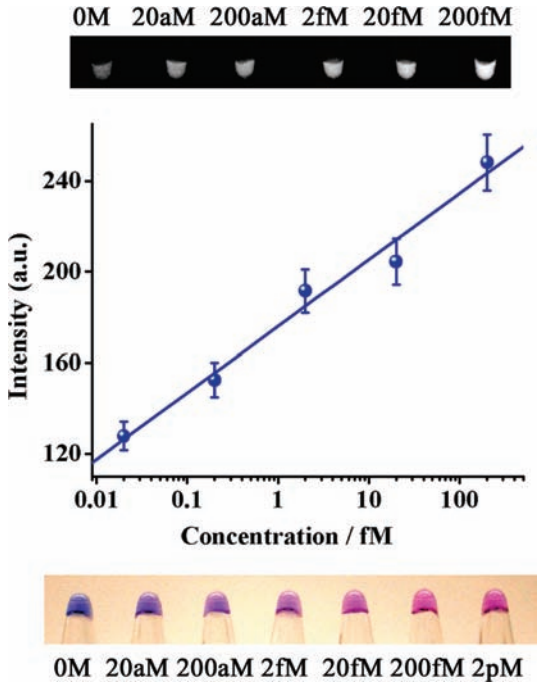

Figure 4. (top and middle) The reduced background achieved at 4 °C allowed us to detect target over the range from 20 aM to 200 fM via changes in fluorescence emission. (bottom) Even naked-eye observations reveal a color change from blue to purple with increasing target concentration. In comparison with other colorimetric detection schemes, such as the gold nanoparticle-based colorimetric method of Rothberg,[26] the sensitivity of our method is improved by 2 to 3 orders of magnitude.

trade-off: while a detection limit of 10 pM was obtained in only 30 min at 37 °C, achieving a 20 aM detection limit required 24 h at 4 °C. However, though the latter may be slow, it has the advantage of being significantly simpler than other reported amplification-based methods and competes well with them in terms of sensitivity. For example, there is no need for multiple self-assembly steps, as are required in gold nanoparticle-based amplification assays.[12,18−22] Neither does the approach require the complicated denaturation−annealing cycles involved in Taqman-based PCR.[8−10] This detection sensitivity exceeds those of many amplification assays, such as nanoparticle-based amplification (femtomolar detection limit)[18−22] or HRP-based enzyme amplification (femtomolar detection limit),[18,23−25] and is close to the detection limit in PCR-based detection schemes.[8−10] Moreover, as our assay can be interrogated with the naked eye, it is amenable to deployment in the developing world, where fluorometric detection may be problematic. Finally, in comparison with other colorimetric detection schemes, such as the gold nanoparticle-based colorimetric method of Rothberg,[26] the sensitivity of our approach is improved by 2 to 3 orders of magnitude.

In conclusion, we have demonstrated a simple, rapid, and extremely sensitive method for the detection of DNA molecules. The approach achieves picomolar detection simply by mixing the modified molecular beacon, exonuclease III, and complementary target DNA and incubating for 2 h at 37 °C. If incubated instead at 4 °C for 24 h, the method achieves a detection limit as low as 20 aM (600 target molecules in a 50 μL sample). This approach thus greatly improves upon the sensitivity of traditional, unamplified molecular beacons.

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Supporting Information Available: Detailed description of the experimental procedures and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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