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Amplified Nucleic Acid Sensing Using Programmed Self-Cleaving DNAzyme

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As the number of diseases caused by genetic defects continues to grow, there have been increasing demands for rapid nucleic acid detection. The recent progress in this area is remarkable[1−3] in terms of simplicity (*mix-and-read* assay), selectivity (SNP detection), and wide applicability, as illustrated for example by the target-assisted chemical ligation method of Kool et al.[2,3] Sensitivity is another factor of vital importance. The present work is concerned about signal amplification, which may be achieved when a sensing reaction occurs catalytically.[2c,4] We designed TASC probes as a new type of enzyme- and reagent-free nucleic acid sensors, which undergo target-assisted self-cleavage (TASC). We report here that the TASC probes are indeed capable of selective and multiple-turnover sensing under isothermal conditions.

The TASC probe (Figure 1) contains three essential structural motifs, a target-binding sequence, a DNAzyme sequence,[5] and a cleavage site, colored in red, blue, and green, respectively. The basic design follows that of Wang et al., for the target-induced formation of a three-way junction to activate the DNAzyme.[6b−d] In the present system, however, the three motifs are linked together in a single chain in such a way (Figure 1) as to promote isothermal amplification of the sensing reaction through three steps, i.e., activation, cleavage, and turnover (Scheme 1). Target−probe hybridization in step 1 stabilizes the internal hairpin structure of the probe to yield an "active" DNAzyme, which in step 2 cleaves the self-strand at the facing ribonucleotide moiety shown with an arrow in the presence of Mg^{2+};[6,7] the two shortened fragments of the probe come off to leave the probe-free target (step 3), to which a new TASC probe is bound (step 1) to drive a catalytic or amplification cycle.

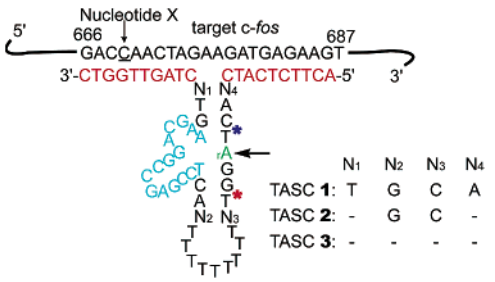
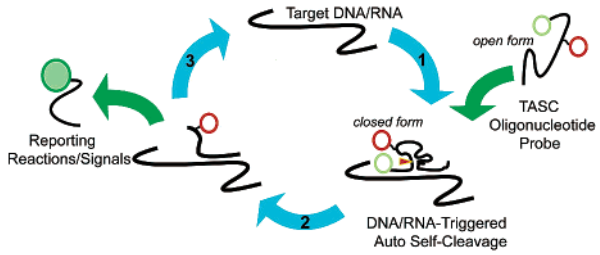


Figure 1. Target c-*fos* 666−687 sequence and TASC probes **1**, **2**, and **3** composed of target-binding (red) and DNAzyme motifs (blue) linked together via intervening nucleotide chains, where ribonucleotide rA marked with an arrow is the site of self-cleavage.

We chose the c-*fos* 666−687 nucleotide sequence as the target (Figure 1). TASC probes **1**−**3**[8] with fluorescein-labeling at the 5′-end were prepared (details in Supporting Information). Incubation of TASC probe **1** (59-mer) with the target c-*fos* sequence oligonucleotide at 37 °C in Tris-HCl buffer (10 mM, pH 7.2) containing Mg^{2+} (25 mM) gave two fragmented oligonucleotides (nonfluorescent 44-mer and fluorescent 15-mer),[8] indicating that cleavage had taken place at the 3′-side of the ribonucleotide rA moiety

Scheme 1. Turnover Sensing of Target DNA/RNA with TASC Probe



(Figure 1). Gel electrophoresis shows a distinct self-cleavage product of the probe even after 5 min of incubation with the target oligonucleotide (Figure 2). In its absence, self-cleavage was just detected only after 120 min. On the other hand, the truncated 39-mer control oligonucleotide lacking the target-binding site was completely intact even after 12 h (data not shown). There is thus little doubt that, upon hybridization, the target acts as an allosteric effector to trigger a conformational change (Scheme 1) of the otherwise inactive 39-mer domain of the probe into a cleavage-responsible active DNAzyme structure (steps 1 and 2 in Scheme 1), although the probe retains a small but unnegligible activity in the absence of the target.

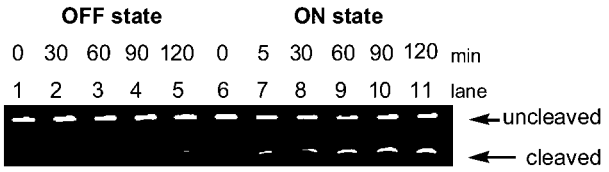


Figure 2. Self-cleaving reactions of 5′-fluorescein-labeled TASC probe **1** as monitored by gel electrophoresis with fluorescence detection after incubation for 0−120 min at 37 °C in the presence (ON) or absence (OFF) of target oligonucleotide.

The self-cleavage rate constants of TASC probe **1** in the presence (5-fold molar excess) and absence of the target oligonucleotide are $k_{obs}^{+} = 14 \times 10^{-3}$ min^{-1} and $k_{obs}^{-} = 0.9 \times 10^{-3}$ min^{-1}, respectively, giving rise to an allosteric-activation or target-induced rate-acceleration factor of $k_{obs}^{+}/k_{obs}^{-} \approx 16$. The rate constants, both k_{obs}^{+} and more sensitively k_{obs}^{-}, decrease upon successive removal of a complementary base-pair (N_1 and N_4 and N_2 and N_3) from the stem part, i.e., on going from probe **1** through **2** to **3** (Table 1 as well as Figure S1 for the PAGE monitoring of the reactions). As a consequence, probe **3** exhibits the highest rate enhancement (k_{obs}^{+}/k_{obs}^{-}), while the highest sensing rate (k_{obs}^{+}) is still marked by probe **1**. From the viewpoint of rapid sensing, we chose probe **1** for further characterization.

We then moved on analysis of multiple-turnover kinetics. The known enzyme- and reagent-free homogeneous DNA sensing probes including widely used molecular beacons[1a] produce one signal from one target at best, since the sensing reaction occurring

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Table 1. Self-Cleaving Rate Constants of 5′-Fluorescein-Labeled TASC Probe **1** in the Presence (k_{obs}^+) and Absence (k_{obs}^-) of Target Oligonucleotide and Allosteric Activation Factor (k_{obs}^+/k_{obs}^-)[a]

	k_{obs}^- ($\times 10^{-3}$ min^{-1})[b]	k_{obs}^+ ($\times 10^{-3}$ min^{-1})[b]	k_{obs}^+/k_{obs}^-
TASC **1**	0.9	14.0	15.6
TASC **2**	0.2	8.2	41.0
TASC **3**	0.05	4.1	82.0

[a] [probe] = 1 μM and [target] = 5 μM at 37 °C. [b] First-order rate constants were obtained as the slopes of ln P_f vs t plots where P_f is fraction of uncleaved probe and t = 0−30 min.

on the target is stoichiometric; in other words, the target as a possible catalyst undergoes stoichiometric deactivation due to "product inhibition". In the present case, however, the sensing reaction is not associative but dissociative, where the fragmented products must have a better chance to leave the target (catalyst) and regenerate the active site (steps 2 and 3 in Scheme 1) than the original whole probe (reactant) as a single molecule. This is in fact the case. Under conditions of 10-fold probe-excess with probe **1** (1 μM, 100 pmol) and 0.1 equiv of target (100 nM, 10 pmol), the reaction proceeds catalytically beyond the 1:1 or 100% level and gives a ∼400% yield of cleavage product after 1.5 h (Figure 3). The catalytic reaction is sequence-selective, too. When a two-base mismatched target[9] is used, the time-course is hardly distinguishable from that of a target-free reaction.

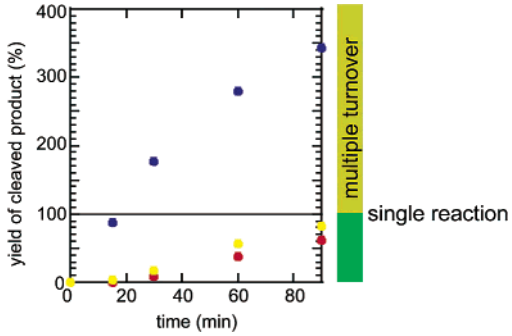

Figure 3. Time-courses of self-cleaving reactions of 5′-fluorescein-labeled TASC probe (1 μM) in the absence (red ●) or presence (100 nM) of fully matched target oligonucleotide (blue ●) or two-base mismatched target (yellow ●) at 37 °C.

We also devised a fluorescence-reporting TASC probe **1** (details of preparation in Supporting Information), having a FRET pair of fluorescein fluorophore and a dabsyl quencher attached at the T moieties (marked with blue and red asterisks, respectively) in close proximity across the site of cleavage (Figure 1). In the absence of target, the probe (FRET TASC probe **1**) is weakly fluorescent due to incomplete FRET. The fluorescence intensity is increased by ∼200% upon incubation of the probe with an equimolar amount of the target, as compared with a 20% increase without target under otherwise identical incubation conditions; the increases in intensity are shown in Figure 4a. The *mix-and-read* fluorescence assay using the FRET TASC probe **1** can be applied to C/A SNP discrimination at position 669 (nucleotide X in Figure 1). Fully matched C-allele gives rise to strong fluorescence, while one-base mismatched A-allele and a two-base mismatched target display just detectable fluorescence of a background (target-free) intensity (Figure 4b).

In summary, we report here a first-generation TASC probe, which, for its dissociative nature, acts as a multiple-turnover *substrate* for the target as a *catalyst*, thus amplifying the sequence information of the latter under non-PCR, i.e., isothermal and enzyme/reagent-free, conditions. The background fluorescence and

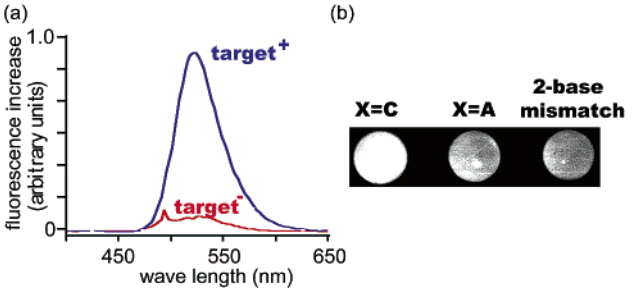

Figure 4. Fluorescence-sensing of target oligonucleotide with FRET TASC probe **1**. (a) Relative fluorescence intensities in the presence (target$^+$) and absence (target$^-$) of an equimolar amount of fully matched target. (b) Fluorescence images for incubation mixtures (after 3 h at 37 °C) with fully matched (X = C), one-base mismatched (X = A), or two-base mismatched[9] target oligonucleotide.

the spontaneous self-cleavage reactivity of the current probe should be minimized in the second generation. A particular application would be sensing of small-quantity or transient DNA/RNA targets in the cell. Any sequence of any given DNA/RNA can be a target of TASC probes, where, however, the choice of particular sequence may be important to enhance the accuracy of sensing in reference to k_{obs}^+/k_{obs}^-. This is because the hybridization domain of the probe, which is complementary to the target sequence, likely controls the conformation of the probe and hence the spontaneous self-cleavage rate constant (k_{obs}^-). Further work is now under way along these lines.

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Supporting Information Available: Details of synthesis and methods and Figure S1 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Probes **1**−**3** and their fragmentation products were characterized by gel electrophoresis. The 15-mer fluorescent product resulting from cleavage of probe **1** gave the correct molecular weight; MALDI-TOF [(M − H)$^-$] calcd 5103.4, found 5102.5.

(9) Mismatched sites are at position 666 (cytosine) and 669 (adenine).

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