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# Light-Switching Excimer Beacon Assays For Ribonuclease H Kinetic Study

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RNase H is a ribonuclease that degrades the RNA strand in a RNA–DNA hybrid to produce 3′-hydroxyl- and 5′-phosphate-terminated products. It is a nonspecific endonuclease that catalyzes the cleavage of RNA through an endonucleolytic mechanism,[1] aided by an enzyme-bound divalent metal ion; however, DNA strands or unhybridized RNA strands are not degraded. The enzyme is involved in several important cellular processes including DNA replication, DNA repair, and transcription.[2] Members of the RNase H family can be found in nearly all organisms, from archaea and prokaryota to eukaryota. RNase H also has wide applications in molecular biology and biotechnology in terms of its unique cleaving property. Retroviral RNase H, a part of viral reverse transcriptase, is an important pharmaceutical target, as it is absolutely necessary for the proliferation of retroviruses, such as HIV. Inhibitors of this enzyme could therefore provide new drugs against diseases like AIDS. *E. coli* RNase H usually requires at least six base pairs of RNA–DNA hybrids as substrates to bind and cleave effectively in solution, while the hybrid length required in living cells might be somewhat greater.[3] The complete digestion of poly(rA):poly(dT) by *E. coli* RNase H yields oligoribonucleotides with varying chain lengths, ranging from monomers to hexamers.[4] In order to understand more about these functions and processes, and more importantly, to screen new drugs against retroviruses, it is necessary to develop a fast, real-time, sensitive, and isotope-label-free system to assay the cleavage activity of RNase H. A number of traditional methods have been used to assay the enzyme activities and evaluate the kinetic parameters, such as the acid-soluble release of RNA fragment,[5] gel electrophoresis,[4] and HPLC.[6] The acid-soluble and gel-electrophoresis techniques require radioisotope-labeled substrates, and the HPLC method needs micromolar concentrations of substrate. All of these methods are indirect, discontinuous, and time-consuming. Recently, an RNA–DNA duplex was incorporated into a fluorescent probe to study RNase H in real time; however, only very short substrate sequences could be analyzed. Moreover, the complexity in the RNA–DNA oligonucleotide synthesis can also result in a low yield and restrict its general utility in enzyme-activity studies.[7]

Here we describe a real-time fluorescence method in which the signal transduction is achieved by taking advantage of the light-switching excimer mechanism inherent to molecular beacons (MBs). A molecular beacon is a single-stranded DNA that can form an intramolecular hairpin structure with a fluorophore and quencher at either end.[8] DNA MB assays have been described for a few enzyme studies, such as single-stranded specific DNases, endonuclease *Bam*HI, and small nonenzyme DNA cleavage agents.[9–11] Enzyme activity was detected and characterized by taking advantage of the signal-transduction mechanism built into the MBs. The change in fluorescence signal reflects the conformational change of the MB, which is a result of enzyme cleavage.[10] Most beacon designs are based on fluorescence quenching[12–14] and fluorescence resonance energy transfer (FRET).[12] Though each technique has its own advantages, some limitations remain. For example, a quenching-based FRET molecular probe always has incomplete quenching, and this results in significant background noise.[15]

In this paper, we describe the molecular engineering of a light-switching excimer beacon probe for RNase H activity monitoring. Some spatially sensitive fluorescent dyes, such as pyrene[16–19] and BODIPY FL,[20,21] can form an excimer when an excited-state molecule is brought into close proximity with another ground-state molecule. The excimer results in a shift of the emission to a longer wavelength than that of the monomer. The formation of excimer between two pyrene molecules that are connected by a flexible covalent chain, such as DNA, is very useful for probing spatial arrangements. In a similar manner to FRET, the spatially dependent property of excimer formation can be used as signal transduction in the development of effective molecular probes. This unique technique is especially useful for the design of MBs, which can undergo conformational changes upon target binding. By attaching pyrene molecules[16] to both ends of a DNA strand, an excimer switching MB probe has been developed. In the absence of target molecules, the DNA beacon stays in its closed form of a loop–stem hairpin structure that brings the two pyrene moieties close together and allows the formation of an excimer that emits at ~485 nm. When binding to the complementary DNA or RNA, the MB opens up. Both pyrene molecules are spatially separated, and only the monomer emission peaks (at 378 and 397 nm) are observed. The change in emission wavelength serves as a unique, real-time method of tracing a series of conformational changes in the DNA-probe-based assays.[16] This emission-wavelength switching solves the problem of background signal that occurs with FRET molecular probes. Thus, it can help improve the precision of the kinetic parameter quantitation, which also takes the background signal into account

We have designed a highly sensitivity 31-mer light-switching pyrene beacon assay. The sequences are given in Table 1 and the mechanism for monitoring the activity is shown schematically in Scheme 1. A dual-pyrene-labeled DNA beacon is free in solution without target binding. The pyrene moieties are brought together by the beacon's hairpin structure, allowing the formation of an excimer. The excimer emission at 485 nm

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**Table 1.** Probes and oligonucleotides used in the ribonuclease H kinetic study.

| Name | Sequence |
|---|---|
| MB226 | 5′-pyr-CCTAGC TCT AAA TCA CTA TGG TCG CGC TAG G-pyr-3′ |
| MB226 shared stem cDNA-A | 5′-CCTAGC GCG ACC ATA GTG ATT TAG A-3′ |
| MB226 shared stem cDNA-G | 5′-GCG ACC ATA GTG ATT TAG AGC TAG G-3′ |
| MB226 shared stem RNA | 5′-GCG ACC AUA GUG AUU UAG AGC UAG G-3′ |
| MBS1 | 5′-pyr-CGC ACC TCT GGT CTG AAG GTT TAT TGG TGC G-pyr-3′ |
| MBS1 shared stem cDNA-A | 5′-CGC ACC AAT AAA CCT TCA G AC CAG A-3′ |
| MBS1 shared stem cDNA-G | 5′-AAT AAA CCT TCA GAC CAG AGG TGC G-3′ |
| MBS1 shared stem RNA | 5′-AAU AAA CCU UCA GAC CAG AGG UGC G-3′ |

Underlining indicates the stem sequences in the molecular beacon structures.

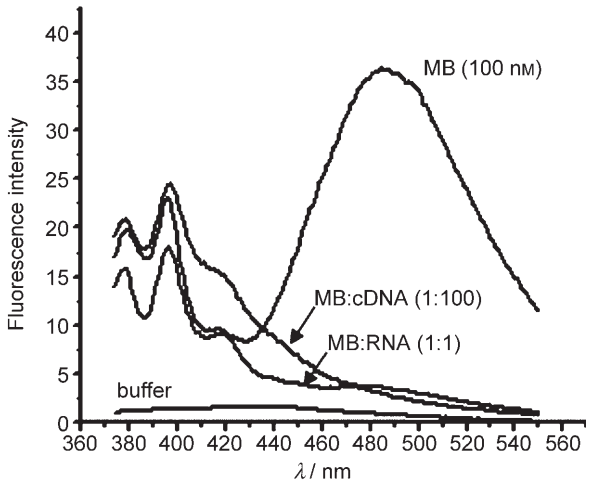
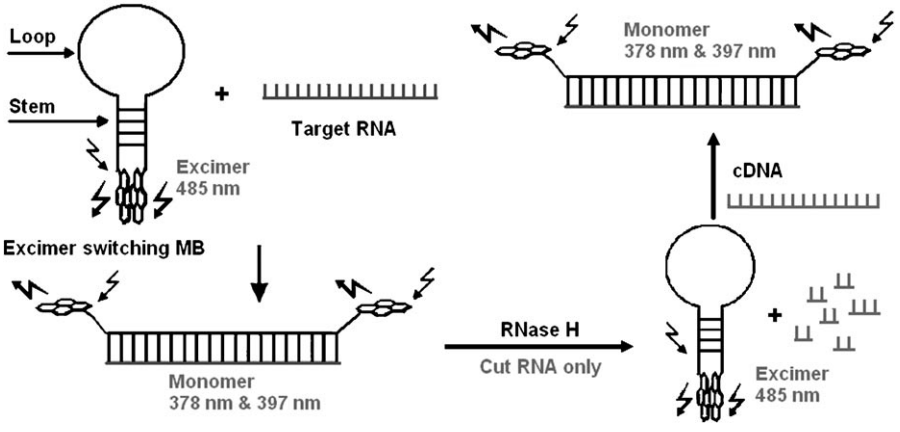



**Figure 1.** Steady-state fluorescence spectra from the MB226 pyrene beacon assay for studying RNase H activity. Excitation at 340 nm was used for all spectra. The 100 nM pyrene MB gives an ~37-fold signal-to-background fluorescence enhancement at 485 nm emission before target binding. After hybridization to the target RNA by annealing at a 1:1 ratio, the excimer emission at 485 nm decreased eightfold to a similar excimer background as that in the emission spectrum of the beacon–cDNA duplex at 1:100 ratio. A corresponding increase in monomer signals (378 and 397 nm) is also shown.

has a fluorescence enhancement of about 37-fold, as shown in Figure 1. Binding of the DNA beacon to RNA opens up the hairpin structure and thus spatially separates the pyrene moieties. The fluorescence emission experiences a blue shift to 378 and 397 nm (monomer emission). The RNA–DNA hybrid, which serves as the substrate for the RNase H cleavage, has a low fluorescence background at 485 nm (Figure 1). After the addition of the enzyme, only the RNA strand will be cleaved from the duplex;[1] this sets free the DNA beacon. The restoration of the hairpin structure brings the pyrene moieties back together and gives a dramatic fluorescence enhancement at the excimer emission at 485 nm. The real-time fluorescence monitoring is shown in Figure 2. This critical step is the one we used to study the RNase H kinetic parameters. Compared to the design of normal fluorophore-quencher-labeled MBs, whose fluorescence signal will be quenched at this step; the pyrene beacon assay results in a fluorescence increase and thus has a much higher sensitivity. It brings a lot more convenience to the calculation of kinetics with the signal enhancement design. Scheme 1 reveals another important advantage of the light-switching excimer signaling approach over traditional gel-electrophoresis experiments: real-time detection without separation. Because only the cleaved duplex gives excimer emission, the uncleaved targets do not have to be separated from the solution for detection. In addition, since it is a real-time detection, the fluorescence assay gives us a much clearer picture of the enzyme cleavage activity and also minimizes the inconvenience of being discontinuous in experiment manipulation. In order to confirm that the signal enhancement comes from the cleavage of the RNA strand by the RNase H, a complementary DNA is added to the solution after the enzyme cleavage. The free beacon hybridizes with the complementary DNA and opens up its hairpin structure again as shown by the dramatic decrease in excimer emission (Figure 2).




**Scheme 1.** Schematic representation of the fluorescence mechanism of using a light-switching excimer beacon to study RNase H activity.

The oligonucleotide targets for normal MBs have the complementary sequence to the loop sequence of the beacons; however, sometimes beacons can exhibit a substantial amount of intermolecular interactions as a result of sticky-end pairing of the beacon stems in the presence of target nucleic acids.[22] Two complementary sticky ends from two beacon hybrids can pair to form a short double helix, leading to association of

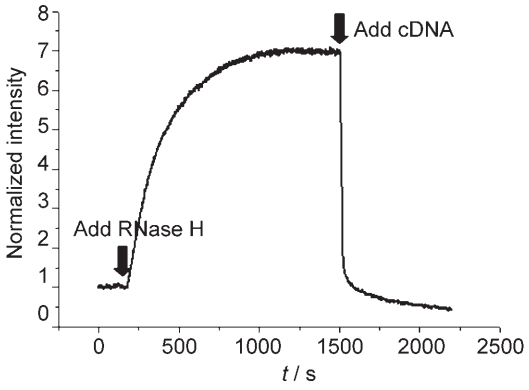



**Figure 2.** Time-base fluorescence monitoring of RNase H cleavage activity in MB226 assay. While monitoring the excimer signal at 485 nm in the system, the addition of RNase H induces the fluorescence enhancement due to the cleavage of the RNA strands from the pyrene beacons. After reaching plateau, shared stem cDNA is added to hybridize with the free beacon, which results in a dramatic decrease in excimer emission. This reaction is shown to confirm the cleavage mechanism of the pyrene beacon assays. Reaction conditions: [RNA–DNA hybrid] = 100 nM, [RNase H] = 6 nM (50 units mL$^{-1}$), [cDNA] = 10 μM. $\lambda_{ex}$ = 340 nm; $\lambda_{em}$ = 485 nm (excimer emission).

the two hybrids at one end. With sticky-end pairing, two separated pyrene molecules are drawn back together again, causing high background in excimer emission and false negative results in the enzyme activity study, as shown in Figure 3. This

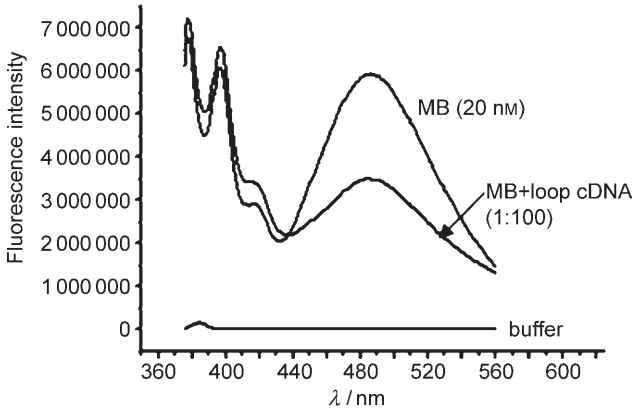



**Figure 3.** Steady-state fluorescence spectra of pyrene beacon (20 nM) with loop-cDNA (1:100) for optimization of RNA sequence in MB226 assay. The duplex of pyrene beacon and loop-cDNA gives a high background signal after hybridization due to the sticky-end pairing problem.

problem is more severe when the probe concentrations in solution are high. In order to avoid this sticky-end pairing problem, shared-stem oligonucleotide targets are used in the design of the pyrene beacon assays. We designed two different sequences of shared-stem cDNA (Table 1) to test the fluorescence signal for the optimization of the target RNA sequence and found that the sequence with a G base on the 5′-end gave a lower background at the excimer emission than the one with A. According to the literature,[23] this is because G on the 5′-end of the DNA strand can partially quench the fluorescence of the fluorophore through energy transfer. We used this sequence for the design of the RNA stand in our kinetic studies. Moreover, using shared-stem RNA to solve the sticky-end-pairing problem also has the potential to increase the assay concentration from 20 to 100 nM in order to obtain a higher fluorescence signal.

The excimer beacon assay was then used to obtain the kinetic parameters for *E. coli* RNase H by monitoring the cleavage of the RNA strand from the RNA–DNA hybrid. Figure 4

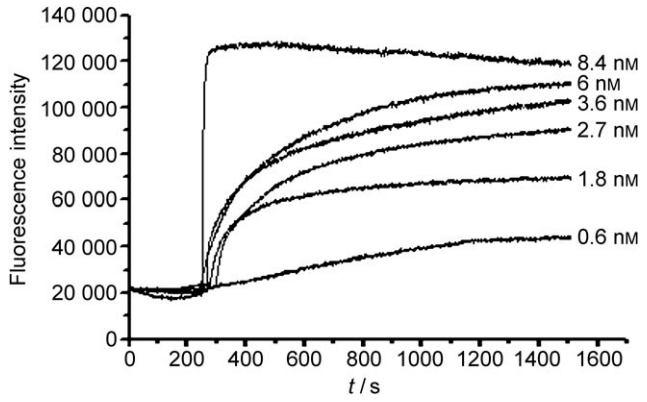



**Figure 4.** Time curves of cleavage of RNA strand from MB226 assay by *E. coli* RNase H at different enzyme concentration at 37 °C. Reaction conditions were: [RNA–DNA hybrid] = 100 nM; [RNase H] = 0.6 nM (5 units mL$^{-1}$), 1.8 nM (15 units mL$^{-1}$), 2.7 nM (22.5 units mL$^{-1}$), 3.6 nM (30 units mL$^{-1}$), 6 nM (50 units mL$^{-1}$), 8.4 nM (70 units mL$^{-1}$); 1 unit of *E. coli* RNase H = $1.2\times10^{-13}$ mol.

shows that the cleavage of RNA is enzyme concentration dependent, thus indicating that the excimer beacon assay can be used for RNase H detection over a range of enzyme concentration. The initial rate of hydrolysis can be used for RNase H kinetic studies. The kinetic parameters of RNase H for the 25 bp RNA–DNA hybrid are $K_m$ = 0.019 μM and $k_{cat}$ = 0.25 s$^{-1}$. Figure 5

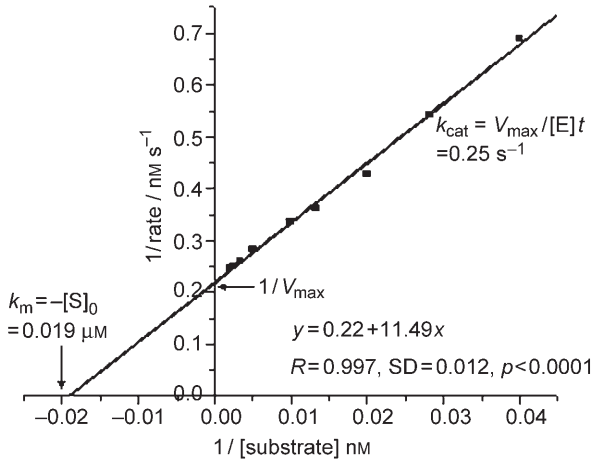



**Figure 5.** Lineweaver–Burk plot of the reciprocals of initial rate versus substrate concentration for the determination of kinetic parameters $K_m$, $k_{cat}$, and $V_{max}$ of RNase H in the MB226 assay; ◆: B, ——: linear fit of data B. From the plot, we got $K_m$ = 0.019 μM, $V_{max}$ = 1.65 nM s$^{-1}$ and $k_{cat}$ = 0.25 s$^{-1}$. Reaction conditions: [RNA–DNA hybrid] = 25, 50, 75, 100, 200, 300, 400, and 500 nM. [RNase H] = 6 nM (50 units mL$^{-1}$). $\lambda_{ex}$ = 340 nm, $\lambda_{em}$ = 485 nm.

shows how these parameters are obtained. The values are similar to those of radioisotope-labeled duplexes with similar length of base pairs (14-mer: $K_m = 0.08\ \mu M$ and $k_{cat} = 0.1\ s^{-1}$,[7] 22-mer: $K_m = 0.07\ \mu M$ and $k_{cat} = 1.1\ s^{-1}$,[4] 24-mer: $K_m = 0.02\ \mu M$;[23] Table 2) obtained by using gel electrophoresis. The variation in $K_m$ and $k_{cat}$ is the result of varied assay conditions, substrates, and methods. Previous reported methods such as gel electrophoresis and HPLC analysis resulted in a wide range for the RNase H kinetic parameters when using RNA–DNA hybrids.

**Table 2.** Kinetic parameters of pyrene beacon assays for *E. coli* RNase H.

| Substrate | $K_m$ [μM] | $k_{cat}$ [$s^{-1}$] |
|---|---|---|
| MB226 (25-mer) | 0.019 | 0.25 |
| MBS1 (25-mer) | 0.031 | 0.33 |
| 24-mer[23] | 0.02 | – |
| 22-mer[4] | 0.07 | 0.1 |
| 14-mer[7] | 0.08 | 1.12 |

Statistical analysis of kinetic parameters of MB226 and MBS1 are from the Lineweaver–Burk plot of initial rate versus substrate concentration, see Figure 5.

In addition, early observation confirms that the cleavage sites of RNase H are not sequence dependent.[4] However, the enzyme does exhibit a sequence-dependence effect on the kinetic properties of the cleavage activity.[7] We synthesized a 25-mer pyrene beacon, MBS1 (Table 1), that has the same GC content as the MB226 beacon in the whole hybrid, but a much higher GC content in the stem. The beacon was also applied to studying the kinetics. While similar results were obtained owing to the same length of the hybrids, there were slight increases in Michaelis constant ($K_m = 0.031\ \mu M$) and turnover rate ($k_{cat} = 0.33\ s^{-1}$) for the MBS1 assay (Table 2) that suggested that the kinetic characteristics of the enzyme's cleavage activities show a slight sequence preference. This is most likely due to the only difference between these two assays—the GC content of the beacon's stem sequence. A larger Michaelis constant means a higher ratio between the velocities of enzyme–substrate complex degradation and formation. A higher GC content in the stem makes the product after cleavage more stable in the beacon's hairpin structure, and this leads to a higher rate of complex degradation. The results indicate that *E. coli* RNase H does have slight sequence preference for the substrate with the higher stem GC content in the pyrene beacon assay.

In summary, with its unique properties, the dual-pyrene-label beacon is finding interesting applications in enzyme-activity studies. Integrated with a novel signal-transduction mechanism, the binding and cleaving elements can be used as sensitive probes for enzymatic monitoring and kinetic analysis. We have demonstrated that the light-switching excimer approach is an excellent signal transduction for MB development with a specific detection purpose. The switching of the excimer signal indicates that the conformational change of the pyrene beacon can be used to monitor enzymatic activity. This signaling approach has merits for application in kinetic analysis for several reasons. First, real-time detection from monitoring the signal transduction corresponding to conformational change gives a real-time portrayal of what is happening in the reaction. It avoids any indirect and time-consuming problems arising from stopping the reaction and taking out samples every few minutes, such as in gel electrophoresis methods. Moreover, by being free from any need to stop reactions in the middle with inhibitors, it can give a more precise response that can only come from the cleavage activity of the enzyme. Second, shorter detection times are needed. Real-time monitoring avoid any subsequent detection and analysis steps,[24] so it shortens the detection time for each sample and minimizes environmental effects, thereby affording more precise detection. The real-time light-switching assay we have developed here shows great advantages in these aspects for its simple, rapid method, easy construction, and high sensitivity. These properties will enable the construction of specially designed light-switching pyrene beacons for different types of enzyme activity studies in extracellular or even intracellular environments.

## Experimental Section

**Materials**: The sequences of DNA and RNA oligonucleotides prepared are listed in Table 1. DNA synthesis reagents were purchased from Glen Research (Sterling, VA). Two difference sequences of DNA MBs (excimer probes both with six base pairs in the stem, sequences shown in Table 1) were labeled at both ends with pyrene. The RNA sequences were synthesized and purified by Sigma-Proligo (The Woodlands, TX, USA.). *E. coli* RNase H with an enzyme activity of 2000 units $mL^{-1}$ (240 nM) was purchased from Sigma–Aldrich; 1 unit of RNase H hydrolyzes 1.0 nmol RNA in $^3$H-labeled poly(dA):poly(dT) to acid-soluble material in 20 min at 37 °C. The enzyme specific activity is 419972.4 units per mg of *E. coli* RNase H.[7] The RNase H inhibitor, EDTA, was purchased from Fisher Scientific. The calibration dye SYBR-Green was purchased from Invitrogen.

**Synthesis and purification**: A solid-phase synthesis was used to couple pyrene to MB sequences at both the 3′ and 5′-ends. The synthesis started with a 3′-amino-modifier C7 controlled-pore glass (CPG) column on the 1 μmol scale. After the synthesis of the DNA beacon, a 5′-amine was added to the sequence by using 5′-amino-modifier C6 linker phosphoramidite. The column was then flushed slowly with dimethylformamide (DMF; 15 mL), piperidine (20%) in DMF (15 mL), trichloroacetic acid (3%) in dichloromethane (15 mL), and then more DMF (15 mL). The CPG contained within the column was released into DMF solution (1 mL) containing pyrene butyric acid (57.7 mg, 200 μmol), dicyclocarbodiimide (41.3 mg, 200 μmol), and dimethylaminopyridine (24.4 μg, 200 μmol). After being stirred for 3 h, the solution was centrifuged, and the supernatant was discarded. The pellet was washed with DMF, methanol, and water (3× each), before being incubated in a solution of methylamine (50%) in ammonia at 65 °C for ~10 min. The resulting clear and colorless supernatant was collected. Under UV radiation, an intense green fluorescence was observed from the collected solution. The beacon solution was desalted on a Sephadex G-25 column (NAP-5, Amersham Pharmacia) and dried in a SpeedVac. The dried product was purified by HPLC on a C18 column with a linear elution gradient with TEAA (triethylammonium acetate) in

acetonitrile changing from 25 to 75% over 25 min at a flow rate of 1 mL min$^{-1}$. The second chromatography peak, which absorbed at 260 and 350 nm and emitted at 485 nm with 350 nm excitation, was collected as the product. The collected product then was vacuum dried, desalted with a G-25 column, and stored at −20 °C for future use.[25]

**Instruments**: An ABI3400 DNA/RNA synthesizer (Applied Biosystems) was used for DNA synthesis. Probe purification was performed on a ProStar HPLC (Varian) with a C18 column (Econosil, 5U, 250×4.6 mm) from Alltech Associates. UV/Vis measurements for probe quantitation were performed with a Cary Bio-300UV spectrometer (Varian). Steady-state fluorescence measurements were performed on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon, Edison, NJ, USA). For emission spectra, 340 nm was used for excitation.

**Pyrene beacon assays for RNase H**: Assays were carried out in USB RNase H buffer (100 μL; Tris-HCl (20 mM, pH 7.5), KCl (20 mM), $MgCl_2$ (10 mM), EDTA (0.1 mM), and DTT (0.1 mM)),[7] containing RNase H (6 nM, 50 units mL$^{-1}$) and DNA beacon–RNA hybrid (100 nM) at a 1:1 ratio. An increase in fluorescence emission at 485 nm, upon excitation at 340 nm, indicated the hydrolysis progress of the hybrids. The maximum fluorescence emission was determined by incubating the hybrids with excess RNase H. To determine the Michaelis–Menten kinetic parameters, the beacon assay concentration was varied from 25 to 500 nM, over the range of the previously reported $K_m$ values for RNase H.[18,21] A dye calibration curve using SYBR® GreenER was applied to determine accurately the concentration of the hybrid. SYBR® GreenER is a double-strand oligonucleotide-binding dye that can quantitatively differentiate duplex from single-strand oligonucleotides after hybridization. In all kinetic experiments, initial-rate measurements were obtained in the first 30 s, with an enzyme concentration of 6 nM (50 units mL$^{-1}$). All experiments were performed at 37 °C and repeated 2–3 times. Average values were used for calculations. Data in Tables 1 and 2 were obtained by performing curve fitting to the Michaelis–Menten equation by using OriginPro 7.0 (Microcal Software Inc.).

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**Keywords:** DNA · light-switching excimer beacon · pyrene · ribonucleases · RNA

[1] *Ribonuclease H* (Eds.: R. J. Crouch, J. J. Toulme), Libbey, Paris, **1998**.
[2] H. I. Miller, A. D. Riggs, G. N. Gill, *J. Biol. Chem.* **1973**, *248*, 2621–2624.
[3] C. A. Stein, *J. Clin. Invest.* **2001**, *108*, 641–644.
[4] E. Kanaya, S. Kanaya, *Eur. J. Biochem.* **1995**, *231*, 557–562.
[5] M. L. Dirksen, R. J. Crouch, *J. Biol. Chem.* **1981**, *256*, 69–73.
[6] H. H. Hogrefe, R. I. Hogrefe, R. Y. Walder, J. A. Walder, *J. Biol. Chem.* **1990**, *265*, 5561–5566.
[7] J. Rizzo, L. K. Gifford, X. Zhang, A. M. Gewirtz, P. Lu, *Mol. Cell. Probes* **2002**, *16*, 277–283.
[8] S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303–308.
[9] R. J. Strouse, F. Z. Hakki, S. Wang, A. DeFusco, *BioPharm (Duluth, MN)* **2000**, *13*, 40–47.
[10] J. J. Li, R. Geyer, W. H. Tan, *Nucleic Acids Res.* **2000**, *28*, e52.
[11] J. B. Biggins, J. R. Prudent, D. J. Marshall, M. Ruppen, J. S. Thorson, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13537–13542.
[12] J. W. J. Li, X. H. Fang, W. H. Tan, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 31–40.
[13] X. H. Fang, A. Sen, M. Vicens, W. H. Tan, *ChemBioChem* **2003**, *4*, 829–834.
[14] R. Nutiu, Y. F. Li, *Chem. Eur. J.* **2004**, *10*, 1868–1876.
[15] S. T. Crooke, K. M. Lemonidis, L. Neilson, R. Griffey, E. Lesnik, B. P. Monia, *Biochem. J.* **1995**, *312*, 599–608.
[16] K. Fujimoto, H. Shimizu, M. Inouye, *J. Org. Chem.* **2004**, *69*, 3271–3275.
[17] J. B. Birks, *Photophysics of Aromatic Molecules*, Wiley–Interscience, London, **1970**.
[18] F. M. Winnik, *Chem. Rev.* **1993**, *93*, 587–614.
[19] J. R. Lakowicz, *Principles of Fluorescent Spectroscopy*, Kluwer Academic/Plenum, New York, **1999**.
[20] M. Dahim, N. K. Mizuno, X.-M. Li, W. E. Momsen, M. M. Momsen, H. L. Brockman, *Biophys. J.* **2002**, *83*, 1511–1524.
[21] R. E. Pagano, O. C. Martin, H. C. Kang, R. P. Haugland, *J. Cell Biol.* **1991**, *113*, 1267–1279.
[22] L. Tan, Y. Li, T. J. Drake, L. Moroz, K. Wang, J. Li, A. Munteanu, C. J. Yang, K. Martinez, W. H. Tan, *Analyst (Cambridge, UK)* **2005**, *130*, 1002–1005.
[23] M. Haruki, E. Noguchi, S. Kanaya, R. J. Crouch, *J. Biol. Chem.* **1997**, *272*, 22015–22022.
[24] E. Kanaya, Y. Uchiyama, E. Ohtsuka, Y. Ueno, M. Ikehara, S. Kanaya, *FEBS Lett.* **1994**, *354*, 227–231.
[25] C. J. Yang, S. Jockusch, M. Vicens, N. J. Turro, W. H. Tan, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 17278–17283.

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