DOI: 10.1002/anie.200905829

Molecular Beacons

# A Highly Sensitive, Excimer-Controlled Molecular Beacon**

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Molecular beacons (MBs) are widely used hairpin probes for the specific detection of DNA and RNA targets.[1–8] First proposed by Tyagi and Kramer,[1] the concept of a MB is based on the interaction between a fluorescent and a quenching molecule. In the absence of the target, the MB adopts a hairpin structure resulting in close proximity of the two terminally attached chromophores and, hence, fluorescence quenching, whereas hybridization of the target to the MB loop region leads to spatial separation of fluorophore and quencher and concomitant signal appearance. The choice of the stem is a crucial aspect in the design of MBs. Its stability must be finely tuned to ensure close proximity of the dyes in the native form (→low background signal) and, at the same time, allow efficient hybridization with the target (→high sensitivity). Additionally, it should not take part in unintended target hybridization, which might adversely affect selectivity, or interfere with formation of the hairpin structure, for example, by binding to the loop sequence which may also lead to an increase in background or a loss in signal intensity. Several types of stem-modified beacons have been presented to address these issues.[9–15] Incomplete fluorescence quenching in the hairpin form is a well-recognized drawback, and different directions have been described to overcome this problem, including the use of time-resolved fluorescence techniques,[16] wavelength-shifted[17–19] or super-quenched beacons,[20] the formation of triple-helical stems,[21,22] and stemless peptide nucleic acid beacons.[23] We propose here a molecular beacon in which signal control is accomplished by formation of a donor–acceptor (D–A) complex.[24–28] As illustrated in Figure 1, the stem contains pairs of non-nucleosidic pyrenes (Y) and perylenediimides (PDIs, E) that can interact by interstrand stacking.[29–31] In the native structure this leads to efficient signal suppression, whereas the hybridized form is characterized by an excimer signal[32–35] produced by the two adjacent pyrenes.[36] Additionally, the formation of a stable D–A complex helps to minimize the number of natural bases in the stem, thus reducing the chances of unwanted base-pairing interactions.

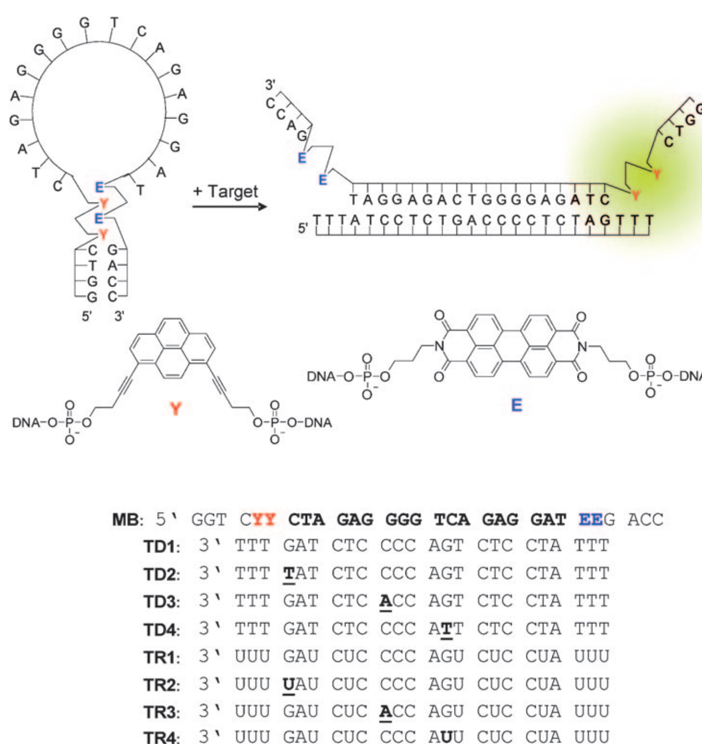


***Figure 1.*** Top: Illustration of an excimer-controlled molecular beacon; excimer formation between pyrene derivatives (Y) is prevented by D–A complex formation with PDI (E) in the native form. Upon hybridization with the target, PDI and pyrene units are separated whereby excimer formation is enabled. Bottom: **MB** and target DNA and RNA sequences; bold: target region, bold and underlined: mismatches.

The oligomers used in this study are shown in Figure 1. An 18-mer target sequence was chosen arbitrarily. In our molecular beacon **MB** the two pairs of pyrenes and PDIs are located immediately adjacent to either side of the loop region. 1,8-Dialkynylpyrene **Y** is used because it forms a strongly fluorescent excimer with both a large extinction coefficient and a high quantum yield.[37,38] Moreover, the extension of the pyrene aromatic core with two triple bonds renders this pyrene a particularly electron-rich component for a D–A complex. With four additional base pairs, the stem is of comparable length to that in conventional MBs (5–7 base pairs). D–A interstrand stacking interactions between the electron-rich pyrenes and the electron-poor PDIs support a highly stable secondary structure (see the Supporting Information). This interaction prevents excimer formation and fluorescence emission. Upon titration of our **MB** with the fully matched DNA target **TD1**, a strong excimer signal

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[**] This work was supported financially by the Swiss National Foundation (grant 200020-117617).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200905829.


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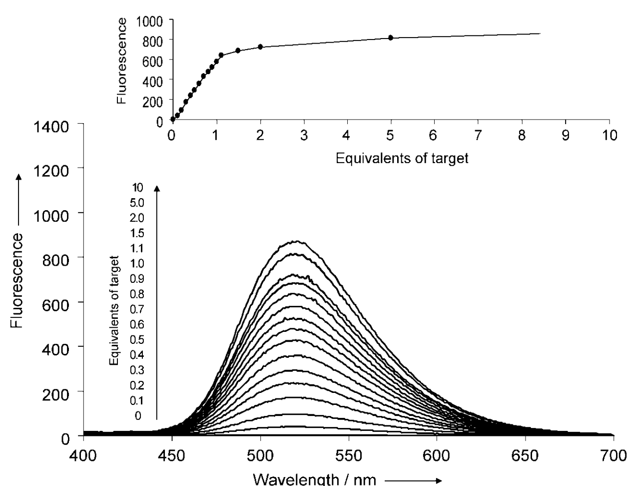


*Figure 2.* Fluorescence read-out obtained with **MB** ($10^{-6}$ M) on hybridization with the DNA target **TD1** (0 to 10 equiv, in steps as indicated). Conditions: Excitation at 370 nm, slits 10/5 nm; 10 mM phosphate buffer, pH 7.0, 100 mM NaCl, 37 °C. Inset: Concentration-dependent signal intensities at 520 nm. Signal-to-background (S/B)[39] at 520 nm: 434; quenching efficiency (Q): 99.8 %.

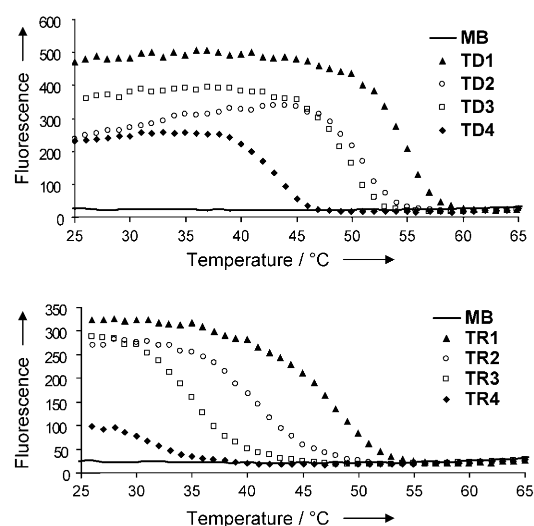


*Figure 3.* **MB** excimer signal intensities obtained in the presence of 1 equiv of matched and mismatched DNA (top; **TD1**–**TD4**) and RNA (bottom; **TR1**–**TR4**) targets. Conditions: **MB** ($1\times10^{-7}$ M), target ($1\times10^{-7}$ M); excitation at 370 nm, emission at 520 nm, slits 5/5 nm; 10 mM phosphate buffer, pH 7.0, 100 mM NaCl); only heating ramps are shown.

around 520 nm is generated, which increases linearly up to 1 equivalent ($R^2 = 0.997$) of target before leveling off (Figure 2).

The excimer-controlled **MB** efficiently differentiates between matched and mismatched DNA and RNA targets (Table 1). Mismatches located near the middle of the target

*Table 1:* Melting temperatures of **MB** hybridized to DNA and RNA targets obtained from fluorescence measurements (see Figure 3).

| DNA hybrid[a] | $T_m$ [°C][b] | RNA hybrid[a] | $T_m$ [°C][b] |
|---|---|---|---|
| **MB·TD1** | 55 | **MB·TR1** | 47 |
| **MB·TD2** | 51 | **MB·TR2** | 41 |
| **MB·TD3** | 50 | **MB·TR3** | 35 |
| **MB·TD4** | 44 | **MB·TR4** | 28 |

[a] Conditions are given in Figure 3. [b] Estimated error ±1 °C.

sequence or at the pyrene-bearing end were tested (Figure 1). Temperature-variable fluorescence curves (Figure 3) show a loss of signal intensity with all mismatched targets at substantially lower temperatures than with the matched target. The quantum yield of excimer fluorescence ($\phi$) of **MB** ($1\times10^{-6}$ M concentration) in the presence of 1 equivalent of DNA target **TD1** was 0.12 (see the Supporting Information). Together with the high absorptivity of the two bis-alkynylpyrenes ($\varepsilon \approx 70000$ L mol$^{-1}$ cm$^{-1}$)[37] this translates to a brightness of approximately 8400 L mol$^{-1}$ cm$^{-1}$. Spectral overlap between excimer emission and PDI absorbance is excellent and may, therefore, lead to a partial reduction in signal intensity by means of a FRET mechanism. This potential signal loss, however, is compensated by the high bis-pyrenyl absorptivity.

Fluorescence quenching in conventional MBs is described in the literature as dynamic (FRET mechanism) or static (ground-state complex) quenching.[40,41] Both static and dynamic quenching are often incomplete, resulting in a strong background signal, which is a major drawback for the detection of target molecules at low concentrations.[2,6] In the present type of beacon, the excimer signal from the YY dimer that appears in the open form is entirely cancelled in the hairpin structure because of the formation of a D–A complex in the stem with two PDIs.[42–44] Based on the spectroscopic data formation an EYEY complex seems most likely. UV/Vis and circular dichroism (CD) spectroscopy indicate the stacking of PDI and pyrene units. PDIs are involved in stacking interactions over the whole temperature range (20–90 °C, see the Supporting Information) as indicated by the vibronic band pattern.[45] The vibronic band pattern of the pyrenes significantly differs from those of previously reported pyrene dimeric stacks.[37] Furthermore, direct PDI–PDI (E–E) interactions are unlikely because of very weak exciton-coupled CD in the closed form (see the Supporting Information). In the presence of the target (open form) the two PDI units are in direct proximity, and this is accompanied by an increase in the CD couplet. Taken together, these data suggest that an EYEY complex is dominant in the closed form of the beacon.

Fluorescence in the present beacon is suppressed by the physical separation of the two pyrenes. This chromophoric system results in very robust signal characteristics: high signal intensity, largely red-shifted emission, and low background fluorescence. These features allow the detection of the DNA target **TD1** at low nanomolar concentrations (Figure 4). In the presence of $10^{-7}$ M **MB**, a resolved signal is still obtained at $1\times10^{-9}$ M. Thus, the target is detectable at a concentration corresponding to 1 % of the beacon concentration. The calculated detection limit corresponds to a value of 0.3 nM (see the Supporting Information).[15] To the best of our knowledge such sensitivity has not been reported for molecular beacons in a simple hybridization assay.

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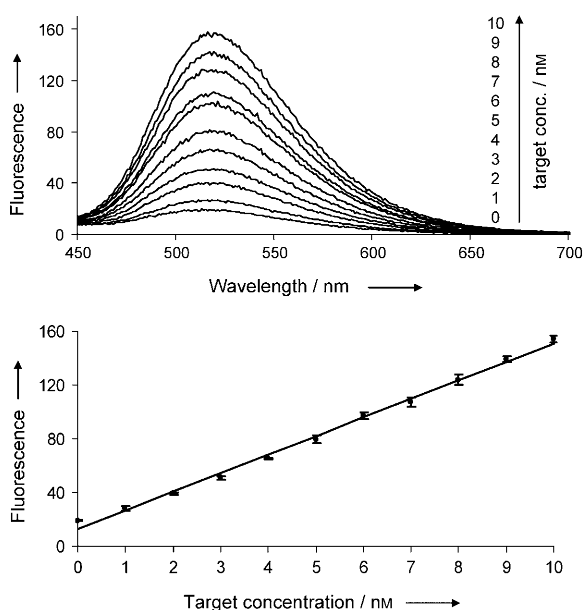


*Figure 4.* Top: Fluorescence intensities obtained with **MB** ($1\times10^{-7}$ M) in the presence of the DNA target **TD1** ($1\times10^{-8}$ to $1\times10^{-9}$ M). Bottom: Concentration dependence of the fluorescence signal ($R^2=0.996$, independent triplicate experiments); fluorescence measurements were taken after 10 min. Conditions: Excitation at 370 nm, slits 10/5 nm; 10 mM phosphate buffer, pH 7.0, 100 mM NaCl, 37 °C.

In conclusion, we have presented an excimer-controlled molecular beacon in which the interaction between two pairs of non-nucleosidic chromophores (pyrene and perylenediimide) located in the stem is used for signal control. Excimer fluorescence is effectively inhibited by formation of a donor–acceptor complex between pyrene and PDI units that prevent the formation of an excited pyrene dimer. The high efficiency of excimer inhibition allows target detection in the presence of a large excess of beacon. In combination with the bright excimer fluorescence of the alkynylpyrene used, this enables the detection of target sequences at low nanomolar concentrations. The excellent sensitivity renders this type of beacon attractive for cellular imaging as well as for screening applications without prior amplification.

Received: October 16, 2009
Revised: November 19, 2009
Published online: ■■ ■■, 2009

**Keywords:** DNA · donor–acceptor systems · fluorescence · molecular beacons · RNA

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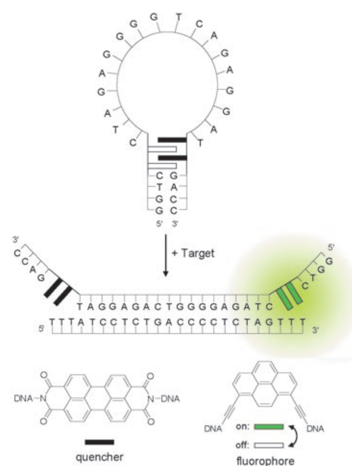


**Non-nucleosidic chromophores** in the stem of a molecular beacon inhibit excimer fluorescence through the formation of a donor–acceptor complex (see picture). The excellent reduction of the background fluorescence allows the detection of DNA and RNA targets in the presence of a significant excess of the probe.


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