

## Design of an ultimate quencher free molecular beacon containing pyrrolocytidine-guanine base pair

Yoshio Saito, Yuta Shinohara, Subhendu Sekhar Bag, Yoshiki Takeuchi, Katsuhiko Matsumoto, and Isao Saito

Department of Materials Chemistry and Engineering and Newcat Institute, School of Engineering, Nihon University, Koriyama, Fukushima 963-8642, Japan.

### ABSTRACT

A novel quencher free molecular beacon was designed in which fluorophore-labelled pyrrolocytidine was placed away from the stem terminal. This new type of MB was used for the detection of a target DNA with an excellent efficiency.

### INTRODUCTION

One of the most versatile probes for DNA and RNA detection is the fluorescent molecular beacon (MB) which is widely used both in homogeneous and in microarray technology.<sup>1</sup> Since its first introduction by Tyagi *et al.*, in 1996, an unprecedented flurry of research activity is centered on it along with its modifications needed for its better selectivity and for getting an excellent signal to noise ratio in DNA detection defining its tremendous sensitivity. As a result of tremendous research effort from various laboratories, several conceptually new MB probes<sup>2</sup> were developed including our more recently developed of quencher free molecular beacon<sup>3</sup>.

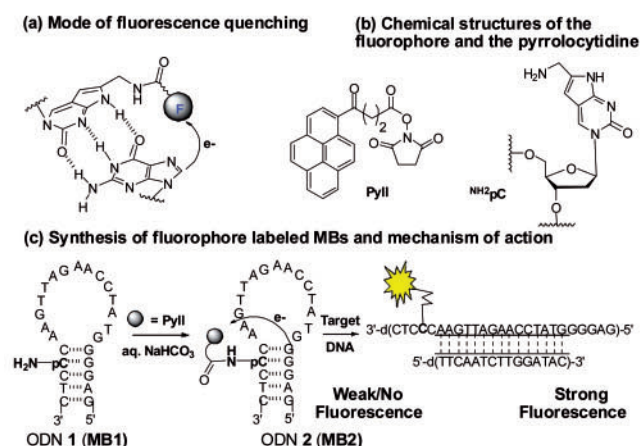
All the recent advances in the design of quencher free molecular beacon rely on the introduction of a fluorophore at the stem terminal.<sup>3</sup> Therefore, we thought that if we can design a MB with the fluorophore covalently attached to a base placed away from the stem terminal then we can yield a newer quencher free MB. In that case the quenching would occur only from its opposite guanosine base and we can use it at any stem sequences and at any position of the stem with the aim to detect the target complementary loop sequence with high efficiency. Therefore, our long term efforts in designing fluorescently labeled oligonucleotide probes<sup>4</sup> for high throughput genetic analyses, lead us to devise quencher-free MBs in which the labeled nucleotide was placed away from the stem terminal.

### RESULTS AND DISCUSSION

**Table 1:** Sequence of DNAs used in this study

ODNs	Sequences
1 MB1	5'-d(GAGGGGTATCCAAGATTGAAC <sup>NH<sub>2</sub></sup> pCCTC)-3'
2 MB2	5'-d(GAGGGGTATCCAAGATTGAAC <sup>PyII</sup> pCCTC)-3'
3	5'-d(TTCAATCTTGGATAC)-3'
4 MB3	5'-d(TGAGGAGTATCCAAGATTGAATC <sup>NH<sub>2</sub></sup> pCTCA)-3'
5 MB4	5'-d(TGAGGAGTATCCAAGATTGAATC <sup>PyII</sup> pCTCA)-3'

To achieve our goal and because of the recent renewed interest in pyrrolocytidine<sup>5</sup> as fluorescent nucleoside analogues to pair with guanosine *via* Watson–Crick base-pairing similar to cytosine, we were motivated to synthesise alkylamino substituted pyrrolocytidine containing MBs. The fluorophore was incorporated into the new alkylamino substituted pyrrolocytidine *via* post synthetic techniques and were used to investigate the sensitivity with respect to fluorescence response to the detection of target DNA. The molecular beacons presented here were designed in which the labeled pyrrolocytidine was placed three base pairs away from the 3'-end. The quenching of the fluorophore in the hairpin state is the result of close proximity to the opposite guanosine in the other stem as shown in Fig. 1a. However in the presence of the complementary fully matched target loop sequence the stem will open up with



**Fig. 1:** Schematic illustration of (a) concept of the mechanism of fluorescence quenching, (b) chemical structures of the fluorophore and the alkylamino substituted pC, and (c) the synthesis of MB and its mechanism of action

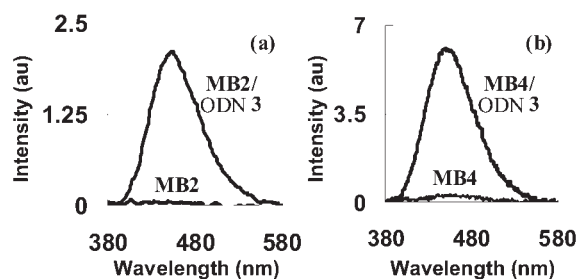
the separation of the fluorophore from the G-base yielding a large fluorescence signal from the fluorophore (Fig. 1c).

To realize our conceptual basis in practice, we first synthesized trifluoroacetylalkylamido substituted pyrrolocytidine (Fig. 1b) according to standard synthetic protocol<sup>6</sup> and was incorporated into DNA by automated DNA synthesizer. Finally, the fluorophore (Fig. 1b) was post-synthetically incorporated into **MB1** to get the desired molecular beacons, **MB2** with **PyII** as a labeled fluorophore, (Fig. 1c). As target loop strand, we used ODN **3** as a fully matched sequence.

**Table2:** Thermal melting properties and the fluorescence quantum yield of the ODNs

ODNs	$T_m$ (°C)	$\Phi_f$
<b>MB2</b>	45.7	0.001
<b>MB2/ODN 3</b>	47.4	0.037
<b>MB4</b>	47.5	0.008
<b>MB4/ODN 3</b>	50.4	0.130

To evaluate the efficiency of the MBs, we first studied the response for sensing the target DNA by comparing fluorescent-signal generated from the fluorophores. Thus, in the resulting fluorescence spectra of ODN **MB2**, we observed a sharp difference in the fluorescence of the hairpin MBs and their duplexes with complementary loop strands (ODN **MB2/ODN 3**). The **MB2** is excellent showing almost no background signal and thus allowing us to detect the target DNA with an extremely high efficiency (Fig. 3a). This is due to the pyrene carbonyl moiety which is much sensitive to quenching by electron transfer from opposite guanine. Therefore, efficient quenching in the hairpin state lead to the fluorescence off state.



**Fig. 2:** Fluorescence spectra of hairpin **MB2** (a) and **MB4** (b), and of the duplexes formed by hybridization with ODN **3** (2.5  $\mu$ M, pH 7.0, r.t.). Excitation wavelength was 354 nm.

To check whether our MB containing **PyII**-pC-G pair could be use irrespective of both the stem sequence and the position in the stem, we have synthesised **MB3** in which the **NH<sub>2</sub>**-pC-G pair placed three bases away from the 3'-stem end having no terminal G-C base pair.<sup>3a</sup> Thus, **MB4** was synthesised from **MB3** via post synthesis. The fluorescence

response in presence of target DNA is quite similar to that of other stem sequences *i.e.* for the case of **MB2** (Fig. 2b). Thus, it is clear that our fluorophore-labeled pC-G pair is capable of detecting the target DNA with an excellent efficiency irrespective of the sequence as well as of the position in the stem.

## CONCLUSION

In conclusion, we have introduced a conceptually new quencher free MBs in which the fluorophore and the intrinsic quencher, the guanosine, are placed away from the stem terminal and thus it could be used in any stem sequence for the detection of target loop strand with high efficiency. The newly designed MBs shown here, unlike the traditional ones, produce efficient on/off signal that can be used as a more sensitive probe. These MBs are extremely important with almost zero background signal and is capable of sensing the specific target DNA sequence with an excellent selectivity and ultimate sensitivity. Looking after the fluorescence brightness, development of newer probe is underway in our laboratory.

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\*Corresponding Author. E-mail: [saito@chem.ce.nihon-u.ac.jp](mailto:saito@chem.ce.nihon-u.ac.jp)