Design of a novel G-quenched molecular beacon: A simple and efficient strategy for DNA sequence analysis†

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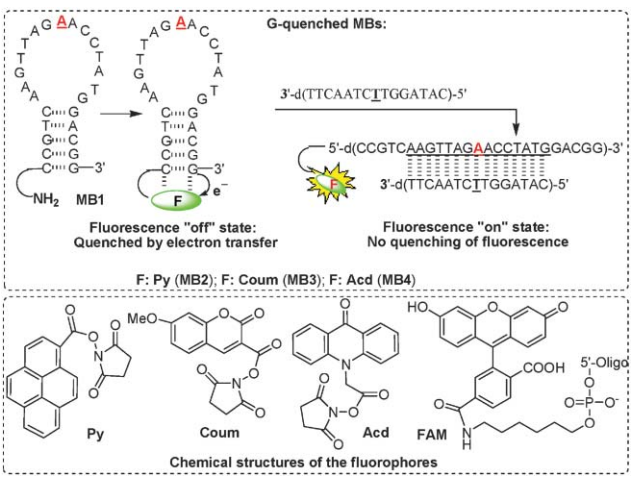
G-quenched MBs are devised from readily available starting materials and used for sequence specific DNA detection with high efficiency.

Sequence specific DNA analysis is of great importance in molecular biology and chemical genomics. DNA hybridization is a major tool for the diagnosis of genetic disease, linked to single nucleotide polymorphisms (SNPs).[1] Thus, rapid and efficient mutation detection has become the focus of many research efforts. One of the most commonly used methods for genetic analysis is "molecular beacon".[2] Molecular beacons (MBs) are hairpin-shaped oligonucleotide probes with an internally quenched fluorophore, in which florescence is restored when bound to its complementary target oligonucleotide sequence. While MBs have been used successfully in DNA assays, their utility for quantification is limited mainly because residual fluorescence arises from the hairpin state which greatly limits the detection sensitivity of MBs. In addition, traditional MBs with fluorophore/quencher type can rarely be used to detect the target quantitatively. To address these problems several research groups are engaged in the modification of MBs. As a result of various research efforts, "metallobeacons",[3a,b] "HyBeacon",[3c] LNA MB[3d] and superquencher MB[3e] were developed as effective genetic probes. A linear dual-labelled fluorescent MB probe[4a] and a MB probe with two fluorophores[4b] that did not rely on the mechanism of traditional MBs probes were also reported to detect specific nucleic acids in homogeneous solution .

It is well known that the charge transfer type interaction between guanine base (G), particularly a GG-doublet[5a] and an electron accepting fluorophore results in a strong quenching of the fluorophore.[5] Thus, if one can take the advantage of the phenomenon of G-base stacking and thereby quenching of the fluorescence, the design of a molecular beacon having no quencher at the 3′-end of the stem is possible. Thus, the quencher-less MB can be realized with an improved signal to noise ratio and a high target selectivity. Our long term efforts in designing fluorescently labeled oligonucleotide probes[6] for inexpensive, simple, less time consuming and high throughput genetic analyses, lead us to devise G-quenched MBs with no quencher at the 3′-end. The MBs designed here are advantageous as the hybridization of such MBs with target sequences is easier, faster and more efficient as compared with other genetic analysis systems. Secondly, other fluorophores can easily be inserted into the MBs. Finally, the synthesis is very simple, inexpensive and less time consuming as the probe does not contain any quencher.

To achieve our goal, three molecular beacons were designed. These are pyrene (**Py**),[6f] coumarin (**Coum**), and acridone (**Acd**)[6g] containing MBs at the 5′-end as a fluorophore. **MB1** was synthesized by automated DNA synthesizer. Finally, the fluorophores were post-synthetically incorporated into **MB1** to get the desired molecular beacons, **MB2, 3**, and **4**, in which the fluorophores are attached simply *via* a six-carbon alkylamino linker (ESI, Fig. 1†). The MBs were characterized by MALDI-TOF mass spectrometry and used to evaluate their selectivity in sensing target ODNs (Scheme 1). As target loop strands, we synthesised ODN **11**, a fully matched sequence, and ODNs **8–10**, one-base-mismatched sequences at the central position of the loop (Table 1).

Initially, we have investigated the thermal stability of the MBs. Thus, the thermal melting temperature behaviour showed an enhanced stability of the hairpin states of all the MBs compared to unmodified hairpin **MB1** (T_m = 54.6 °C). The melting temperature indicates that the **MB3** (T_m = 59.6 °C) and **MB4** (T_m = 58.7 °C) form more stable stem hairpins than **MB2** (T_m = 55.9 °C), suggesting that **Coum** and **Acd** rings are involved in a more stable stacking interaction such as intercalation between G–C base pairs as compared to **Py** (Table 2).



Scheme 1 Synthesis, structures and schematic representation of the action of G-quenched molecular beacons.

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Table 1 Sequences of ODNs used in this study

(ODNs)	Sequences	
1	**MB1**	5′-d(**NH_2**–$(CH_2)_6$–CCGTCAAGTTAG***A***ACCTATGGACGG)-3′
2	**MB2**	5′-d(**Py**CONH–$(CH_2)_6$–CCGTCAAGTTAG***A***ACCTATGGACGG)-3′
3	**MB3**	5′-d(**Coum**CONH–$(CH_2)_6$–CCGTCAAGTTAG***A***ACCTATGGACGG)-3′
4	**MB4**	5′-d(**Acd**CONH–$(CH_2)_6$–CCGTCAAGTTAG***A***ACCTATGGACGG)-3′
5	**MB5**	5′-d(**FAM**CONH–$(CH_2)_6$–CCGTCAAGTTAG***A***ACCTATGGACGG)-3′
6	**MB6**	5′-d(**NH_2**–$(CH_2)_6$–ATTATTAAAGTTAG***A***ACCTATGTAATAAT)-3′
7	**MB7**	5′-d(**Acd**CONH–$(CH_2)_6$–ATTATTAAAGTTAG***A***ACCTATGTAATAAT)-3′
8		5′-d(CATAGGT***A***CTAACTT)-3′
9		5′-d(CATAGGT***G***CTAACTT)-3′
10		5′-d(CATAGGT***C***CTAACTT)-3′
11		5′-d(CATAGGT***T***CTAACTT)-3′

The hybridization properties were also tested by means of fluorescence measurements. Thus, upon hybridization with the complementary fully matched sequence, **MB2** and **MB4** show 24.3 and 14.6 fold increase in fluorescence intensity, while **MB3** showed only a 4.6 fold increase in intensity as compared with the hairpin state. On the other hand, the single base mismatched duplexes [**MB2, MB3**, or **MB4**/ODN **8**, **9**, or **10**] showed a weaker fluorescence emission (Fig. 1). The hairpin **MB2** and **MB4** showed a highly quenched hairpin state compared to **MB3**, indicating that the quenching efficiency with neighbouring G is highly dependent upon the nature of the electron withdrawing property of the fluorophores.

Thus, it is clear that the novel **Py-**, **Coum-**, **Acd**-labeled MBs are capable of sensing a fully matched sequence with a good to excellent discrimination factor (D.F. 18.5, 4.2, and 14.2, respectively). This behaviour in fluorescence response suggests that the effect of the terminal base G in quenching the fluorescence *via* electron transfer in the hairpin state is much greater in **Py**-labeled **MB2** and **Acd**-labeled **MB4** than in case of coumarin labeled **MB3** (Table 2). Thus, it is clear that **Py**- and **Acd**-labeled G-quenched **MB2** and **MB4** are better candidates than the coumarin-labeled **MB3** both in selectivity and in discrimination factor. **MB4** is superior in terms of longer emission wavelength in selecting the target DNA sequence and hence can be used for chip based detection technology as well.

After establishing the fact that the MBs with **Py, Coum**, and **Acd** as fluorophore, are capable of sensing the specific DNA sequences with high efficiency, we turned our attention to another fluorophore, 5-fluorescein (**FAM**) to determine the effect of fluorophores on the G-quenching and to discover whether or not the interaction with the neighbouring G base at the 3′-end is necessary for realising efficient G-quenched MBs. While all the aforementioned fluorophores have well known intercalating properties with DNA bases, **FAM** is not an intercalator and is widely used as reporter dye. Thus, we have synthesized **MB5** containing **FAM** as a fluorophore attached *via* a six-carbon alkylamino linker at the 5′-end and evaluated its photophysical properties. Thermal melting temperature behaviour showed no enhanced stability of the hairpin ODN **MB5** (T_m = 54.7 °C) as compared to the unmodified hairpin **MB1**, indicating that **FAM** has no such stabilising interactions with the neighbouring DNA base pair.

The photophysical properties of **FAM**-labeled **MB5** also reflects a similar non-interacting nature of the fluorophore. Thus, upon hybridization with the complementary fully matched ODN **11**, **MB5** shows only a 1.1 fold increase in the fluorescence intensity with a very poor discrimination factor (D.F. 1.2) Thus, it is clear that FAM-labeled MB (**MB5**) is unable to discriminate a specific DNA sequence (Fig. 2a).

Table 2 Melting temperatures and photophysical properties of hairpin MBs and their duplexes

ODNs	T_m/°C	D.F.[a]	Φ_F[b]
MB1	54.6	—	—
MB1/ODN**11**	55.6	—	—
MB2	55.9	18.5	0.007
MB2/ODN**11**	56.1	—	0.12
MB3	59.6	4.2	0.008
MB3/ODN**11**	54.6	—	0.04
MB4	58.7	14.2	0.006
MB4/ODN**11**	60.1	—	0.13
MB5	54.7	1.2	0.40
MB5/ODN**11**	55.3	—	0.46
MB7	40.0	0.87	0.57
MB7/ODN**11**	50.1	—	0.50

[a] D.F. (Discrimination Factor): the ratio of the integrated area of the emission of the duplex and that of the hairpin state. [b] Measured according to Ref. 6(a).

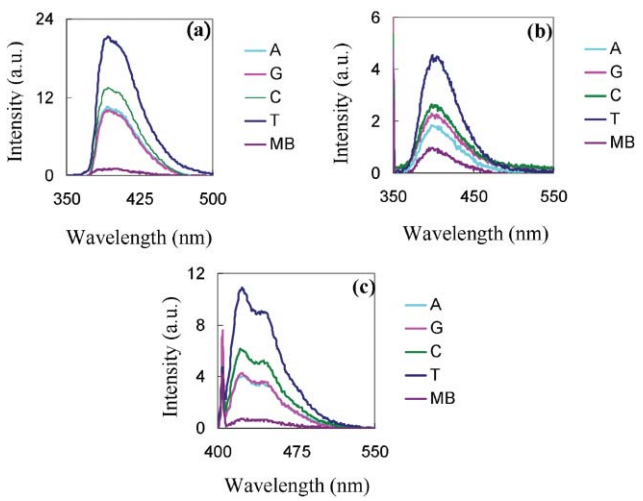

Fig. 1 Fluorescence spectra of hairpin **MB2** (a), **MB3** (b), and **MB4** (c) [(2.5 μM) and the duplexes formed by hybridization with ODN **8–11** (2.5 μM, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, RT)]. Excitation wavelengths were 346, 348 and 403 nm for **MB2**, **MB3**, and **MB4**, respectively. ''MB''denotes the hairpin states.

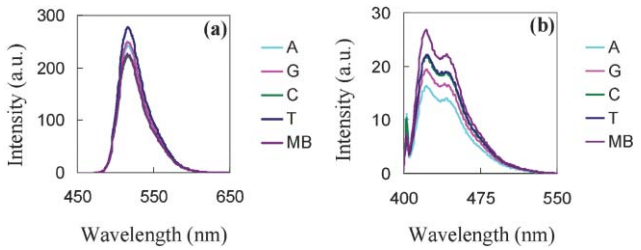

Fig. 2 Fluorescence spectra of hairpin **MB5** (a), **MB7** (b) [(2.5 μM) and the duplexes formed by hybridization with ODN **8–11** (2.5 μM, 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, rt)]. Excitation wavelengths were 496 and 403 nm for **MB5** and **MB7**, respectively. "MB" denotes the hairpin states.

To re-establish the fact that to achieve stable and efficient G-quenched MBs, the fluorophores have to be strongly involved in π-stacking interaction with the neighbouring 3′-end G–C base pair in the hairpin state and to show that the 3′-end G base is responsible for the fluorescence quenching, we have designed and synthesised **MB7** from **MB6** having a stem with seven A–T base pairs (Table 1).

We have studied the thermal stability and the photophysical properties of **MB7** upon binding with the complementary ODNs. The thermal melting temperature showed a stable stem hairpin structure **MB7** (T_m = 40.0 °C), with a lower T_m value as compared to the MBs containing G–C base pairs in the stem part (Table 2). As shown in Fig. 2b, **MB7** showed a relatively strong fluorescence emission even in the hairpin state and no selectivity for sensing the target sequence, reflecting that the 3′-end G base is indispensable for quenching the fluorescence the in hairpin state.

While there has been a report of a quencher-free **MB**[7,8] our designed **MB2**, **3**, and **4** are examples of G-quenched MBs which utilise simple mixing and steady-state fluorescence reading for efficient SNP detection with excellent discrimination factors. Taking acridone as the test fluorophore, we have designed, synthesized and studied the photophysical properties in the presence of target sequences of a number of MBs having the same stem length (total 5 base pairs) but varying number of GC contents at the 3′-termini (ESI, Fig. S10–S12†). Thus, we arrive at the conclusion that to achieve efficient SNP typing using our concept based designed MBs, only a single opposite terminal G base is indispensable. The designed MBs unlike the traditional ones, produce efficient on/off signals that can be used as a more sensitive probe.

In conclusion, the hairpin stem stability and the selectivity in sensing target ODNs by MBs described here, arises mainly from π-stacking interaction between the fluorophore and terminal G–C base pair. We have shown that to design efficient G-quenched MBs, at least one G base is required at the 3′-termini. Thus, the G-quenched MBs in which the fluorophores are linked with a readily available six-carbon linker at the 5′-end are capable of sensing the specific target DNA sequence with high selectivity and thus may be used in SNPs typing.

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