Red–white–blue emission switching molecular beacons: ratiometric multicolour DNA hybridization probes†

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Molecular beacons with two fluorophores derived from ethynyl pyrene and ethynyl nile red exhibit red emission in the hairpin that changes to blue through white upon binding to the target sequence with a dramatic shift of ≈225 nm.

Molecular beacons (MBs) have become powerful tools in a variety of DNA/RNA based applications such as single nucleotide polymorphism (SNP) detection,[1] real-time polymerase chain reactions (PCR),[2] cellular imaging *etc.*[3] However, undesired emission quenching in the biological systems and the incomplete quenching of fluorescence in the hairpin conformation that reduces the sensitivity (S/B ratio) are the main drawbacks of conventional MBs. Even though several approaches have been developed in recent years to address these issues,[4–13] dual-fluorophore labelled MBs that change their emission maximum (colour) represent a superior probe.[14–16] However, the design of such MBs which (i) can emit distinct fluorescence in the visible spectrum ($\lambda = 400$–750 nm) with a large difference in the emission maxima (>200 nm) between the closed form (hairpin) and the open form (duplex), (ii) bear an energy donor that can be excited selectively, and (iii) exhibit a high FRET efficiency that is a prerequisite for practical applications, remains challenging.

In the majority of MBs, the dye pair is attached to the terminal positions of DNA through flexible linkers. As a result, the FRET efficiency depends mainly on the distance between the fluorophores. On the other hand, attachment of fluorophores through a short and rigid linker to nucleobases offers dual control over the FRET either by the distance dependency or the relative orientation of the fluorophore dipole moments in a stacked ensemble. Moreover, such an incorporation yields unique optical properties which are favourable for DNA based assays.[17–23] Very recently, we showed such a class of donor–acceptor couple composed of pyrene (**Py**, donor) and nile red (**Nr**, acceptor) both covalently attached to the 5-position of 2′-deoxyuridine through an acetylene linker (Fig. 1a). Furthermore, we demonstrated a reversible hybridization induced a red to white emission colour change in a series of doubly labelled DNAs. In these duplexes the energy transfer is controlled by the relative chromophore dipole orientation.[24] In addition, the well separated absorption maxima ($\Delta\lambda = 218$ nm), good spectral overlap integral ($J(\lambda) = 1.5 \times 10^{15}$ M^{-1} cm^{-1} nm^4) and large shift in the emission maxima ($\Delta\lambda = 225$ nm) represent favourable features of this donor–acceptor couple which can potentially be transferred to MBs. Accordingly, we designed and synthesized MBs **DNA1–4** (Table 1) bearing the fluorophores **Py** and **Nr** in the stem region of the MB. Herein, we report the optical characteristics, especially the emission colour switching after addition of the target oligonucleotides (Fig. 1b).

Table 1 MB (**DNA1–4**) and target DNA (**DNA5–7**) sequences[a]

DNA	Sequence (3′→5′)
DNA1	GNrACCTCTTATAGTAGAAACCACAAAGTAAGGPyAC
DNA2	CNrAGCTCTTATAGTAGAAACCACAAAGTAAGCTPyG
DNA3	CNrTGCTCTTATAGTAGAAACCACAAAGTAAGCPyTG
DNA4	CNrAGCTTAATAGAACACAGTAGCPyAG
DNA5	TACTTTGTGGTTTCTACTATAAG
DNA6	TACTTTGTGGTATCTACTATAAG
DNA7	ACTGTGTTCTATTA

[a] The underlined bases indicate the stem sequences of the MBs for **DNA1–4** and a mismatch in the case of **DNA6**.

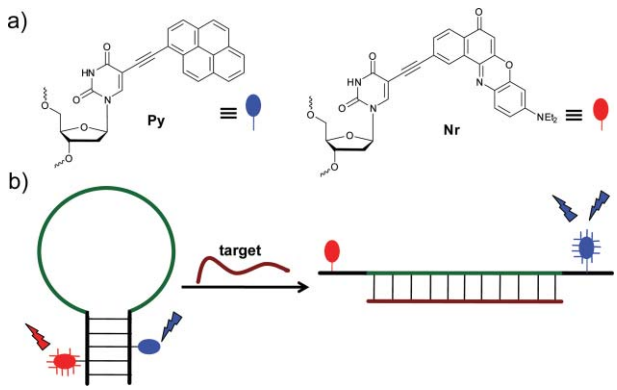

Fig. 1 a) Structure of pyrene (**Py**) and nile red (**Nr**) modified 2′-deoxyuridine. b) A schematic representation illustrating the switching of emission colour from red to blue of a pyrene and nile red modified DNA upon binds to the target.

Synthetic details and characterization of the MBs are given in the Supporting Information (Fig. S1–4). **DNA1–3** are 35 base long oligonucleotides with 23 bases in the loop region and 12 bases in the stem region (underlined, Table 1) whereas **DNA4** contains only 14 bases in the loop region with a length of 26 bases. It is important to note that there are one and two mismatches in the stem regions of **DNA2** and **DNA3**, respectively. The UV/Vis spectra of all modified oligonucleotides, **DNA1–4**, exhibit the characteristic absorption of pyrene and nile red and thus ruled out any interaction of the chromophores in the ground state (Fig. S5). Additionally, the characteristic structural

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change of the probes between the stem-closed hairpin and the stem-open duplex were investigated by temperature dependent absorption and fluorescence spectroscopy. In the absence of target oligonucleotides, MBs should adopt a hairpin conformation (stem closed) due to the complementarity of the bases in the stem region which in turn keeps the fluorophores in close proximity. For instance, in the absence of the target, **DNA1** revealed a melting temperature (T_m) of ≈38 °C for the hairpin. Accordingly, the fluorescence spectrum shows a major peak at 660 nm (λ_{exc} = 380 nm) that revealed an efficient FRET from pyrene to nile red due to the hairpin structure. As expected, when the temperature of the solution of **DNA1** is increased from 20 °C to 80 °C, the intensity of the peak at 660 nm decreases with a concomitant increase of the intensity of the peak at 435 nm (due to the decrease in FRET efficiency). The sigmoidal curve of the temperature-dependent fluorescence intensity of **DNA1** represents a melting temperature of ≈40 °C (Fig. S6). Hence, the decrease in the FRET efficiency at higher temperatures can be attributed to the opening of the hairpin structure. Similar observations were made with **DNA2–4**.

Subsequently, the optical response upon binding of the MBs to the targets was examined by fluorescence analysis at an excitation wavelength of 380 nm where pyrene absorbs predominantly (ε_{380} = 3.4×10^4 M^{-1} cm^{-1}). The extinction coefficient of nile red at 380 nm is extremely low (ε_{380} = 1400 M^{-1} cm^{-1}). As already mentioned the fluorescence spectrum of **DNA1** in the absence of target (stem closed) shows a major peak at 660 nm (I_{660}) due to an efficient FRET (≈75%, k_{ET} = 8.3 × 10^8 s^{-1}) from pyrene to nile red. Interestingly, with the gradual addition of the target (**DNA5**), the intensity of the peak at 660 nm decreases with a concomitant increase of the intensity of the peak at 435 nm (I_{435}) (Fig. 2a). This spectral change can be attributed to the opening of the hairpin conformation of the probe upon binding to the target which drops the FRET efficiency significantly. Noteworthy is the observation that *ca.* 1 equivalent of **DNA5** is enough for the complete switching of the emission maximum, and the addition of excess of the target does not produce any change to the emission spectrum. Moreover, the spectral change can easily be followed up even at very low concentration (down to 1 nM) of the probe and is thus comparable with the sensitivity of the reported FRET based MBs.[7] The S/B ratio of **DNA1** is ≈13 (Fig. 2b). More importantly, a clear visual discrimination is possible which is important for multicolour imaging applications. In the absence of the target, **DNA1** emits red fluorescence that changes to blue upon the addition of one equivalent of the target. Very interestingly, after addition of only 0.5 equivalent of **DNA5**, **DNA1** exhibits an emission spectrum with two major peaks at 660 nm and 435 nm of almost equal intensity yielding a white light emission due to the co-existence of stem closed (hairpin) and duplex structures of **DNA1** (Fig. 2c).

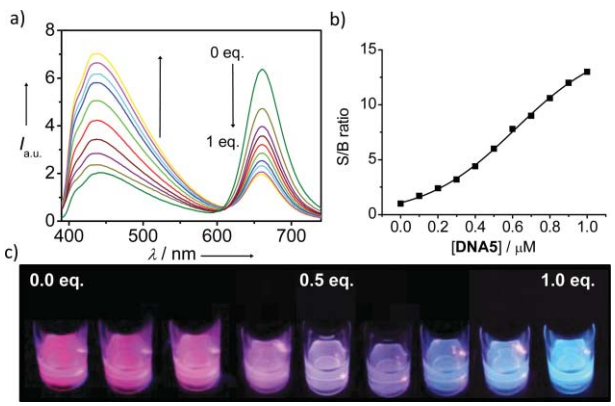

Fig. 2 a) Fluorescence titration spectra of **DNA1** with **DNA5** (0–1 equivalence, c = 1 μM in Na-P*i* buffer, 250 mM NaCl, pH = 7, λ_{exc} = 380 nm, T = 25 °C) and c) the corresponding photographs. b) Plot of S/B ratio $[(I_{435,o}/I_{660,o})/(I_{435,c}/I_{660,c})]$, 'o' indicates stem open (duplex) and 'c' stem closed (hairpin), against the concentration of **DNA5**.

Since the fluorophores are attached to the nucleobase through the rigid acetylene linker, even a single mismatch in the stem region significantly influences the FRET efficiency due to the change in the relative chromophore dipole orientations. Accordingly, **DNA2** (one mismatch) and **DNA3** (two mismatches) exhibit white fluorescence in the stem closed hairpins because only partial FRET occurs in these assemblies. However, the addition of *ca.* 1 equivalent of **DNA5** results in complete opening of the hairpin structure and restores the characteristic blue emission of pyrene. Thus, **DNA2** and **DNA3** show a switching of emission colour from white to blue (Fig. 3a). In contrast, **DNA4** which has a loop length of only 14 bases showed red fluorescence in the hairpin as expected. However, even after the addition of large excess of the target (**DNA7**) the complete recovery of the pyrene fluorescence is not observed. Instead a spectrum with blue and red emitting peaks of almost equal intensity is observed and hence results white emission. This can be understood by considering that the distance between pyrene and nile red even in the completely opened MB is significantly less than the Förster distance (<10 nm). Thus, partial FRET is possible even in the open form of **DNA4** which results in switching of the emission colour from red to white (Fig. 3b). Furthermore, it is important to point out that all these probes require only *ca.* 1 equivalent of the target to open the hairpin conformation owing to the large difference between the stability (T_m) of the hairpin assemblies and the full duplexes (Table S2).

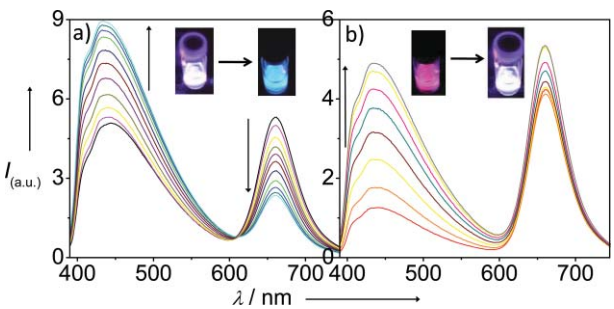

Fig. 3 Emission spectral changes of a) **DNA3** and b) **DNA4** with the addition of **DNA5** and **DNA7**, respectively (0–1 equivalence). Insets show the corresponding photographs of the solutions (c = 1 μM in Na-P*i* buffer, 250 mM NaCl, pH = 7, λ_{exc} = 380, T = 25 °C).

One of the additional features of many MBs is their ability to specifically detect complementary target DNA from nontarget DNA (*e.g.* that contains single base variations). We also investigated the ability of our probes by titrating them with target oligonucleotide containing a single base variation in the middle. Nevertheless, a significant discrimination was not achieved by simply comparing the fluorescence enhancements. Both perfectly matched as well as single mismatched targets show nearly the same fluorescence enhancement upon binding to the corresponding

hairpins. Moreover, about 1 equivalent of the mismatch-inducing target **DNA6** is enough to completely open the hairpin structure for **DNA1**, **DNA2**, and **DNA3** whereas 3 equivalents are required in the case of **DNA4** due to the low stability of the latter oligonucleotide in the duplex compared to hairpin (Table S2). As expected, in the case of **DNA1**, the plot of I_{435} and I_{660} *versus* the concentration of **DNA5** reveals a sigmoidal transition curve indicating a cooperative opening of the hairpin structure. Interestingly, a linear relationship was observed with mismatch-inducing target **DNA6** (Fig. 4). Plot of the ratio I_{435}/I_{660} reveals the same characteristic changes (Fig. S9). Moreover, similar transition from sigmoidal to linear response is observed with other probes **DNA2-4** (Fig. S10–12). From an academic point of view, this observation is highly interesting since the transition of a sigmoidal curve to a linear for the fluorescence readout might be the consequence of the rigid attachment of the fluorophores to the nucleobase through the acetylene linker. This indicates that these chromophore-nucleobase conjugates behave as "optically and electronically coupled fluorescent nucleobases" with base-pairing abilities.

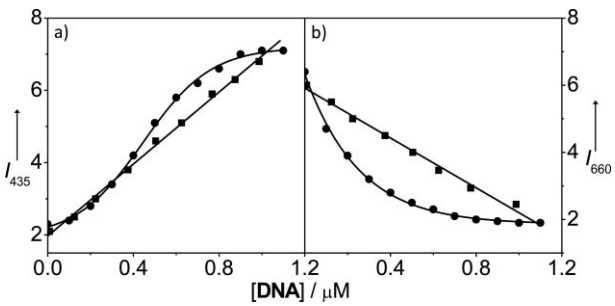

Fig. 4 Plot of a) I_{435} and b) I_{660} of **DNA1** *versus* concentration of **DNA5** (●) and **DNA6** (■). ($c = 1$ μM in Na-P*i* buffer, 250 mM NaCl, pH = 7, $\lambda_{exc} = 380$, $T = 25$ °C).

In conclusion, we have demonstrated a series of dual-fluorophore emission colour switching MBs derived from pyrene and nile red in which fluorophores are attached through a rigid acetylene linker to 2′-deoxyuridine. In the hairpin conformation, **DNA1** exhibits a red fluorescence due to an efficient FRET from pyrene to nile red that changes to blue upon binding to the target. This colour change can further be tuned by modulating the FRET efficiency using either incorporated mismatches in the stem region (**DNA2** and **DNA3**) or shortened loop length (**DNA4**) which results in white to blue and red to white emission switching for **DNA2–3** and **DNA4**, respectively. Moreover, the influence of single base variations in the target sequences has been demonstrated by a linear response of the fluorescence readout whereas a sigmoidal behaviour was observed for perfectly matched targets. Even though several class of dual-fluorophore MBs have been reported, MBs presented herein show a remarkable shift of the emission maximum (225 nm) within the visible spectrum which resulted in three distinct emission colour readouts red, white and blue. Hence, these multicolour DNA hybridization probes may find applications in multicolour cellular imaging.[15]

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