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PAPER

Signal control by self-assembly of fluorophores in a molecular beacon—a model study†

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Pyrene excimer fluorescence is efficiently regulated through formation of π-stacked aggregates between dialkynylpyrene (**Y**) and perylenediimide (**E**) residues located in the stem region of a molecular beacon (MB). The building blocks form organized, multichromophoric complexes in the native form. Hybridization to the target results in a conformational reorganization of the chromophores. The nature of the aggregates was investigated by changing the number of chromophores and natural base pairs in the beacon stem. The formation of different types of complexes (**EYEY** → **YEY** → **EY**) is revealed by characteristic spectroscopic changes. The data show that signal control is an intrinsic property of the interacting chromophores. The directed assembly of non-nucleosidic chromophores can be used for the generation of an *on*/*off* switch of a fluorescence signal. The concept may find applications in various types of light-based input/output systems.

Introduction

Molecular beacons (MBs) are hairpin-shaped oligonucleotide probes, in which the loop region contains the target recognition sequence and the stem part enables the generation of a fluorescent diagnostic signal.[1–4] The composition of the stem represents an essential aspect for the successful design of a MB. The stability of the stem has to be balanced to ensure the complete suppression of fluorescence in the closed form and, on the other hand, an efficient formation of the target-beacon complex.[4–8] Fluorescence quenching in hairpin-type MBs is based on the formation of a non-fluorescent ground state complex between fluorophore and quencher or *via* resonance energy transfer (FRET), corresponding to static and dynamic quenching, respectively.[9,10] Incomplete quenching of the signal in the closed form is one of the major drawbacks of MBs for highly sensitive applications. Therefore, the development of new fluorophores and/or quenchers[11–21] as well as innovative fluorophore–quencher systems is in continuous progress.[18,22–31] Improved spectroscopic properties as well as hybridization behaviour were also observed with stem modified MBs.[32–38]

In the course of our work on non-nucleosidic DNA building blocks,[39–47] we have shown that alkynyl- and triazole-substituted pyrenes[48–50] possess excellent fluorescence properties. Large extinction coefficients and quantum yields result in a high brightness of these fluorophores. Excimer fluorescence of these pyrenes is nearly environment independent[50] and may, therefore, be used as a robust output signal in sensor applications.[51,52] Recently, we reported that the placement of two perylenediimide (PDI) units opposite to two pyrenes led to very efficient suppression of the excimer signal.[26,53] The very low background observed with this stem design opens the possibility of using the beacon in a considerable excess over the target, which is often not possible due to incomplete quenching.

This article provides an extended study on the origin of this remarkable signal suppression. The present type of MB possesses a detection system based on the formation of a donor–acceptor (D–A) type complex between 1,4-dialkynylpyrenes and PDI units (Scheme 1).[26] The combination of this chromophore complex with natural base pairs renders this stem a valuable module for fluorogenic detection systems. Control of fluorescence is based on specific interactions between the two types of chromophores. The organization of this multichromophoric complex is the reason for the excellent signal control. Since this type of π-stacked architecture[54–69] can also be applied to other sensor systems, we studied the chromophore organization in more detail. Here, we demonstrate that the supramolecular self-assembly of donor–acceptor π-aggregates serves as a highly reliable and robust system for the control of fluorescence and represents an alternative to the classic MB design.

Results and discussion

For the study of the PDI–pyrene interaction, a set of MBs (**MB1** to **MB5**) varying in the composition of the stem was synthesized (Table 1). Both chromophores possess a strong absorptivity and exhibit a high sensitivity towards stacking interactions which can conveniently be followed by changes in the relative vibronic band intensities ($A^{0\rightarrow0}/A^{0\rightarrow1}$ transitions).[49,70–72] Since the longest wavelength absorption of the two different chromophores appears

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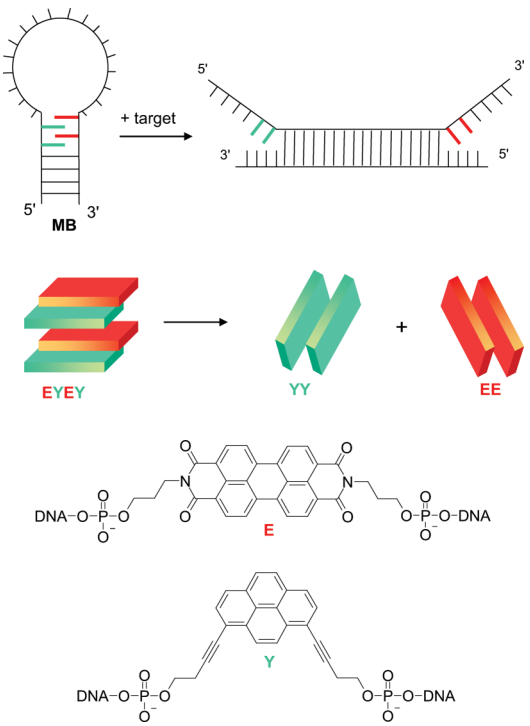
Table 1 Sequences of molecular beacons (**MB1** to **MB5**) and targets (**T1** to **T4**)

	Sequence
MB1	5′ GGT C**YY** CTA GAG GGG TCA GAG GAT **EE**G ACC
MB2	5′ T C**YY** CTA GAG GGG TCA GAG GAT **EE**G A
MB3	5′ **YY** CTA GAG GGG TCA GAG GAT **EE**
MB4	5′ T C**YY** CTA GAG GGG TCA GAG GAT EGA
MB5	5′ GGT C**T**Y CTA GAG GGG TCA GAG GAT EAG ACC
T1	3′ TTT GAT CTC CCC AGT CTC CTA TTT
T2	3′ TTT T<u>A</u>T CTC CCC AGT CTC CTA TTT
T3	3′ TTT GAT CTC <u>A</u>CC AGT CTC CTA TTT
T4	3′ TTT GAT CTC CCC A<u>T</u>T CTC CTA TTT

The chromophores alkynylpyrene (**Y**) and PDI (**E**) are highlighted in bold and the mismatches are underlined.



Scheme 1 Illustration of signal control through self-assembly of aromatic chromophores in a molecular beacon (MB). Generation of the fluorescence signal is regulated by conformational rearrangement of multichromophoric assembly of alkynylpyrene (**Y**) and PDI (**E**) building blocks.

in separate regions of the UV/Vis spectra (dialkynylpyrene, **Y**: 330–420 nm; PDI, **E**: 420–650 nm) conformational changes and aggregation processes can be followed for each type of the chromophores independently.

Fig. 1 shows the changes in vibronic band intensities between open (presence of 1.2 eq. of **T1**) and closed (absence of target) form of **MB1**. A high degree of PDI stacking is revealed by the strong intensity of the 0→1 transition in the open form, in which the PDI units are in close proximity. In the closed form, the PDI–PDI interaction is significantly reduced. This suggests the formation of a different molecular complex. The same pattern is observed in the pyrene area: the vibronic band intensity ratio varies strongly between open and closed form, showing pronounced pyrene–pyrene interactions in the presence of the target. Intensity ratios

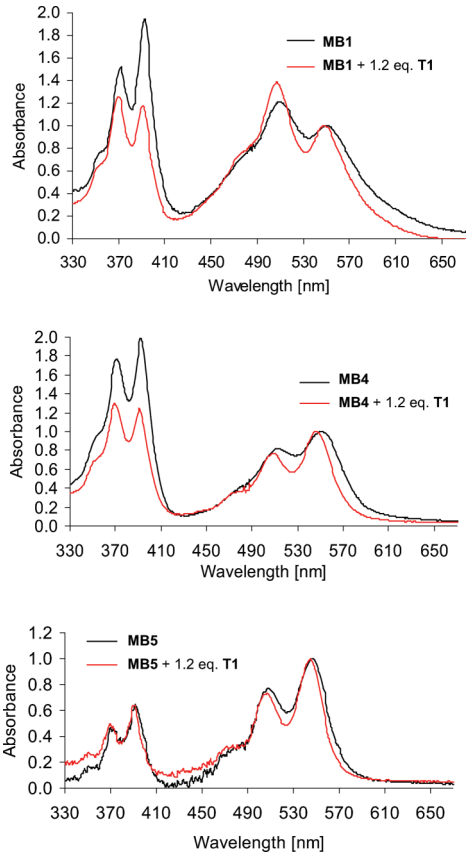


Fig. 1 Normalized UV/Vis absorption spectra at 20 °C of **MB1** (top), **MB4** (middle) and **MB5** (bottom), MB (black line) and with 1.2 equivalents of the target **T1** (red line, normalized at the 0→0 transition band of PDI).

of the vibronic bands are listed in Table 2. These observations are compatible with interstrand stacking interactions between PDI and dialkynylpyrene units in an alternating mode (**EYEY**) in the stem part of the closed beacon. These findings correlate with the well-described effects of hydrophobic stacking interactions between PDI derivatives in a polar environment.[73–76] Furthermore, they are in best agreement with the described distance dependence of vibronic band intensity ratios in DNA–PDI constructs.[77] The same qualitative behaviour was also observed for beacons **MB2** and **MB3** (Table 2 and SI). In **MB4** and **MB5** (Fig. 1), which contain only a single PDI, the ratio of $A^{0\rightarrow0}/A^{0\rightarrow1}$ transitions indicates non-aggregated PDIs in both closed and open form. Therefore, we can conclude that the significant increase of the

Table 2 Absorption ratios of the 0→0 to the 0→1 transition at 20 °C[a]

	$A^{0\rightarrow0}/A^{0\rightarrow1}$ alkynylpyrenes[b]		$A^{0\rightarrow0}/A^{0\rightarrow1}$ PDI[c]	
	No target (closed)	1.2 eq. **T1** (open)	No target (closed)	1.2 eq. **T1** (open)
MB1	1.28	0.94	0.84	0.72
MB2	1.19	0.90	0.74	0.71
MB3	1.19	1.06	0.76	0.73
MB4	1.12	0.96	1.22	1.31
MB5	1.39	1.29	1.30	1.36

[a] Conditions: 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0; [b] 330–420 nm; [c] 420–650 nm.

0→1 transition is primarily due to PDI–PDI and not to PDI–pyrene interactions. Pyrene aggregation is observed for all beacons containing two adjacent pyrenes in the open form. The pyrene vibronic band ratios observed for **MB4** suggest that the same conclusions can also be drawn for the pyrene building block, *i.e.* the pyrene 0→1 transition is most sensitive to pyrene–pyrene interactions, but not for pyrene–PDI interactions (see Fig. 1, **MB5**). The results support the model shown in Scheme 1, in which mixed aggregates (**EYEY**) are present in the closed form and, upon opening of the stem part, self-aggregates (**EE** and **YY**) are formed (see Scheme 1).

Further insight into chromophore organization was obtained by circular dichroism (CD) spectroscopy. Variable temperature spectroscopy of **MB2** (Fig. 2) shows a signal signature for the pyrene units [394 nm (+), 382 nm (–) and 362 nm (–)] and the PDI building blocks [561 nm (+), 499 nm (–)]. The spectra at long wavelengths show a positive bisignate signal characteristic for an exciton coupling[78,79] between the PDI units. The signal of the alkynylpyrene building blocks at shorter wavelengths indicates that exciton coupling also takes place between the pyrene units. However, the CD signal originating from alkynylpyrene interactions is different from the one observed previously in a DNA hybrid in which two alkynylpyrenes were placed in close contact leading to a **YY** interaction.[49] This difference in CD signature can be attributed to the separation of the alkynylpyrenes by PDI units that leads to an alternating **EYEY** π-stack. The CD signatures in the 300–600 nm region gradually disappear with increasing temperature.

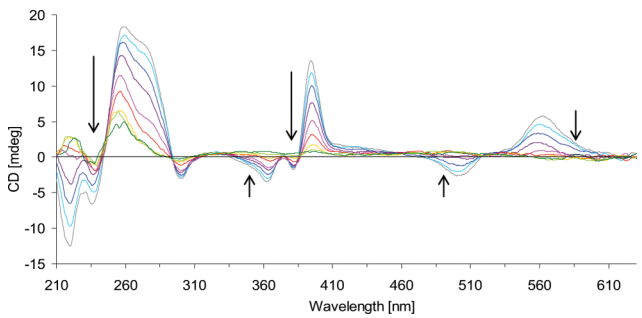


Fig. 2 Variable temperature CD spectroscopy of **MB2** from 10 to 90 °C in 10 °C increments. Arrows indicate increasing temperature. Conditions: 5.0 μM **MB2**, 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0.

The spectra of **MB1** in the presence and the absence of target **T1** are shown in Fig. 3. Addition of **T1** leads to gradual change of this pyrene CD signal (from A = +100 to A = –15), whereas the strength of the PDI couplet is increasing (from A = +26 to A = +64). This finding is explained by the strong hydrophobic PDI interactions in an aqueous environment.

The formation of **EYEY** aggregates follows the well-documented pattern of donor–acceptor π-interactions.[60,80–84] such as arene–perfluoroarene[85–89] or pyrene–naphthalenediimide aromatic interactions.[90–95] The present pyrene–PDI aggregation seems to proceed after this motif.

A worthy goal in the design of MBs consists in the reduction of the stem to a minimal length. The difference between **MB1** and **MB2** is a reduction of the stem length from four to two natural base pairs. Fig. 4 shows the fluorescence curves obtained upon hybridization to the target. The quenching efficiency in the absence of the target is approx. 97% (Table 3), which is excellent in view of the shortness and simplicity of the stem. In addition, **MB2** exhibited a mismatch discrimination comparable to **MB1** (SI) when hybridized to control oligomers **T2–T4**.

Table 3 Quenching efficiency (Q%) values for **MB1–MB5** in presence of 1 eq. target[a]

	MB1[26]	MB2	MB3	MB4	MB5
Q (%)	99.7	97.2	53.1	91.5	36.9

[a] Conditions: 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0; 37 °C, excitation: 370 nm; values were determined at emission maxima.

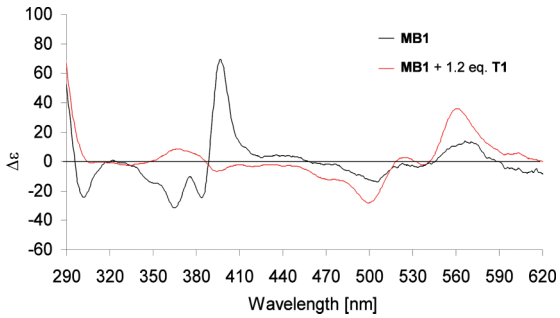


Fig. 3 CD spectra of **MB1** at 20 °C (black line) and with 1.2 equiv. of target **T1** (red line). Conditions: 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0.

Further simplification was tested with **MB3** containing no natural base pairs in the stem. Formation of a stem–loop structure can be expected through intramolecular stacking interactions between the two alkynylpyrene and PDI building blocks that are located at the ends of the oligomer. The UV/Vis spectrum shows PDI and dialkynylpyrene aggregation also for this MB. However, the degree of quenching in the absence of the target is greatly diminished (SI). Furthermore, the CD spectra (Fig. 5) exhibit a remarkable temperature dependent behaviour in the PDI area (410–510 nm). These changes may well be due to PDI-mediated formation of dimers or larger aggregates at low temperature. It is likely that individual molecules associate also *intermolecularly* through stacking interactions between the PDI and/or pyrene residues located at their ends. The formation of interstrand assemblies of DNA conjugates through interaction of sticky ends formed with porphyrine[66] or PDI[67] derivatives was recently demonstrated. The intensity of the broad, unstructured band between 410 nm and 510 nm is significantly reduced on increasing the temperature, which may indicate thermal disaggregation. Therefore, we attribute the broadening of this band to partial intermolecular PDI aggregation.

In the presence of target **T1** (Fig. 6), substantial temperature dependent CD changes are observed in the pyrenyl area of **MB3**. The negative Cotton-effect in the alkynylpyrene signature including an intense negative signal at ~390 nm indicates a change in the aggregation state of the building blocks. These changes may have their origin in competing intra- and intermolecular aggregation of the stemless beacon: at low temperature, interactions between PDI and pyrene sticky ends predominate and at high temperature, after dissociation from the target, the pyrenes adopt the same *EYE*(*Y*) conformation as observed with the beacons containing additional base pairs (**MB1** and **MB2**). In the PDI region, the CD

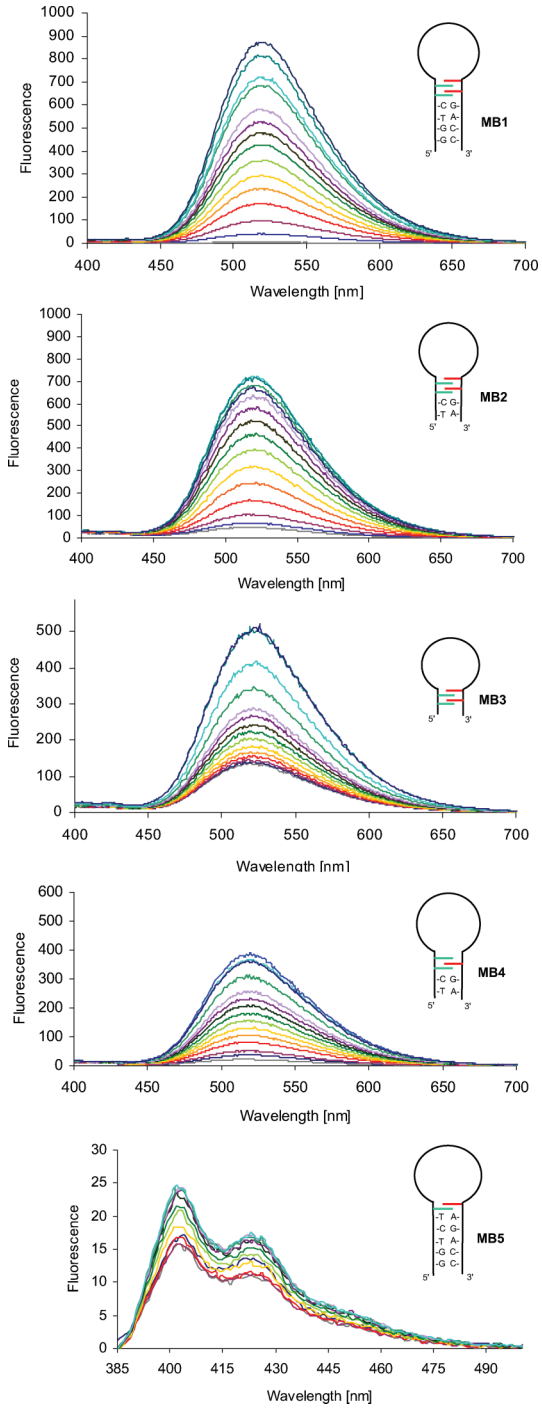


Fig. 4 Fluorescence spectra of **MB1–MB5**. Conditions: **MB1**, **MB2**, **MB3**, **MB4** 1.0 μM, **T1** 0 to 10 equiv., **MB5** 0.1 μM, **T1** 0 to 2 equiv., (lines correspond to: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 5.0, 10 equiv.), 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl. Excitation: 370 nm; Temp.: 37 °C.

spectrum shows a negative Cotton-effect and a broadening of the band at shorter wavelengths. These observations can be due to a dangling PDI unit at the 3′-end of the stem while the PDI closer to the loop region can interact as described above with the two alkynylpyrenes. These findings support the occurrence of donor–acceptor interactions among the chromophores in **MB3** but they also suggest that this type of (stemless) beacon is less suitable for

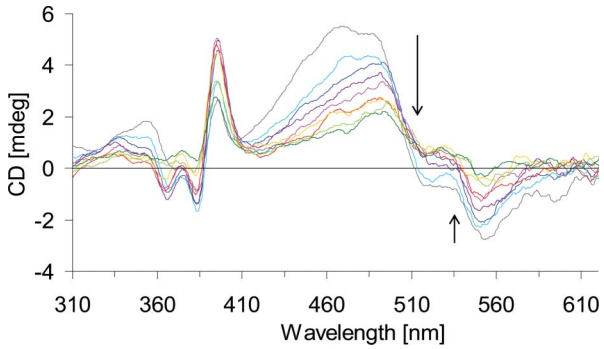


Fig. 5 Variable temperature CD spectroscopy of **MB3** from 10 to 90 °C in 10 °C increments. Arrows indicate increasing temperature. Conditions: 5.0 μM **MB3**, 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0.

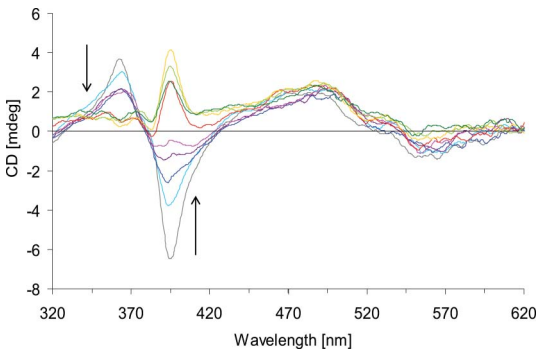


Fig. 6 Temperature-dependent CD spectra for **MB3** in presence of **T1** from 10 to 90 °C in 10 °C increments. Arrows indicate spectral changes with increasing temperature. Conditions: 5.0 μM **MB3**, 6.0 μM **T1**, 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0.

practical use due to the increased competition between inter- and intramolecular stacking of the aromatic chromophores.

The fluorescence data obtained with **MB4** (Fig. 4) are quite remarkable. This MB contains only a single PDI yet the excimer signal is largely suppressed in the closed form (Table 3). The excellent signal control can be attributed to the formation of a *YEY* (donor–acceptor–donor) complex. **MB5** contains only a single pyrene and a single PDI and allows, therefore, a direct comparison between monomer and excimer based sensing systems. The findings reveal the high value of the pyrene excimer read-out. In comparison to beacons **MB1–MB4**, which are all based on excimer formation, **MB5** shows (i) a very weak (monomer) signal (Fig. 4, bottom) and (ii) very poor signal quenching in the absence of the target (Table 3).

The UV/Vis and CD data obtained for **MB1**, **MB2** and **MB3** (see also SI) were quite similar despite significantly differing numbers of natural bases in the stem. This indicates that the organization of the PDI/pyrene complex is an intrinsic property of the chromophoric building blocks and largely independent from the DNA part. Therefore, in the context of supramolecular chemistry, the loop of the MB may be regarded as a flexible linker between the components of the directed assembly. The target sequence serves as an external factor that induces a conformational reorganization of the supramolecular complex under isothermal conditions. Furthermore, it should also be mentioned that this study allows the direct comparison of optical properties and stacking interactions of both types of chromophores (pyrene and

PDI) in a single experiment at equal conditions. The UV/Vis and CD effects are comparable for the two types of compounds. Pyrene interactions lead to pronounced fluorescence signals while the PDI aggregates are basically non-fluorescent.

Conclusions

We have demonstrated that the directed assembly of chromophores can be used as an *on/off* switch for a fluorescence signal. Pyrene excimer fluorescence is efficiently regulated by formation of π-stacked aggregates between non-nucleosidic dialkynylpyrenes and PDI residues located in the stem region of the MB. By varying the numbers of pyrene (**Y**) and PDI (**E**) residues we could show that the two types of chromophores interact in a donor–acceptor fashion in the closed form. The formation of various types of complexes ($EYEY \rightarrow YEY \rightarrow EY$) is revealed by characteristic changes in CD, UV/Vis and fluorescence spectra. Conformational changes induced by target recognition lead to direct pyrene–pyrene interaction and, thus, efficient excimer fluorescence. Highest quenching efficiency was obtained with two PDIs placed opposite two pyrenes in **MB1** and **MB2**. A single PDI, however, showed also surprisingly strong excimer signal inhibition (**MB4**). A longer stem gave better quenching efficiencies (**MB1** *vs.* **MB2** *vs.* **MB3**). Finally, the approach of using aromatic π-stacking works largely better for control of excimer than of monomer suppression (*e.g.* **MB1** *vs.* **MB5**). The data show that signal control is an intrinsic property of the interacting chromophores. Therefore, the concept described herein represents a functional module that may find applications not only in MBs but also in other input/output systems using light as the source of information.[96–98]

Experimental section

Synthetic and analytical procedure

The building blocks alkynylpyrene (**Y**)[49] and PDI (**E**)[99] were synthesized as previously described. The oligonucleotide **T1** was obtained commercially from Microsynth, Balgach, Switzerland. **MB1** to **MB5** were prepared *via* automated oligonucleotide synthesis by an adapted synthetic procedure on a 394-DNA/RNA synthesizer (Applied Biosystems). Cleavage from the solid support and final deprotection was done by treatment with 30% NH_4OH solution at 55 °C overnight. Purification was performed by reverse phase HPLC (LiChrospher 100 RP-18, 5 μm, Merck; Shimadzu LC-20AT and Kontron). Mass spectrometry was done with a Sciex QSTAR pulsar (hybrid quadrupole time-of-flight mass spectrometer, Applied Biosystems); ESI-TOF MS (negative mode, acetonitrile–H_2O–triethylamine). Temperature-dependent UV/Vis spectra were measured on a Varian Cary-100 Bio-UV/Vis spectrophotometer equipped with a Varian Cary-block temperature controller and data were collected with Varian WinUV software over the range of 200–700 nm between 10 and 90 °C. CD spectra were recorded on a JASCO J-715 spectrophotometer using quartz cuvettes with an optic path of 1 cm. Fluorescence spectra were measured on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a Varian Cary-block temperature controller using 1 cm × 1 cm quartz cuvettes. Excitation wavelength: **MB1** to **MB5** 370 nm. Varian Eclipse software was used to investigate the fluorescence data at a wavelength range of 375–700 nm in the temperature range of 20–90 °C. All measurements were performed in 10 mM sodium phosphate buffer pH 7.0 and 100 mM NaCl.

Acknowledgements

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