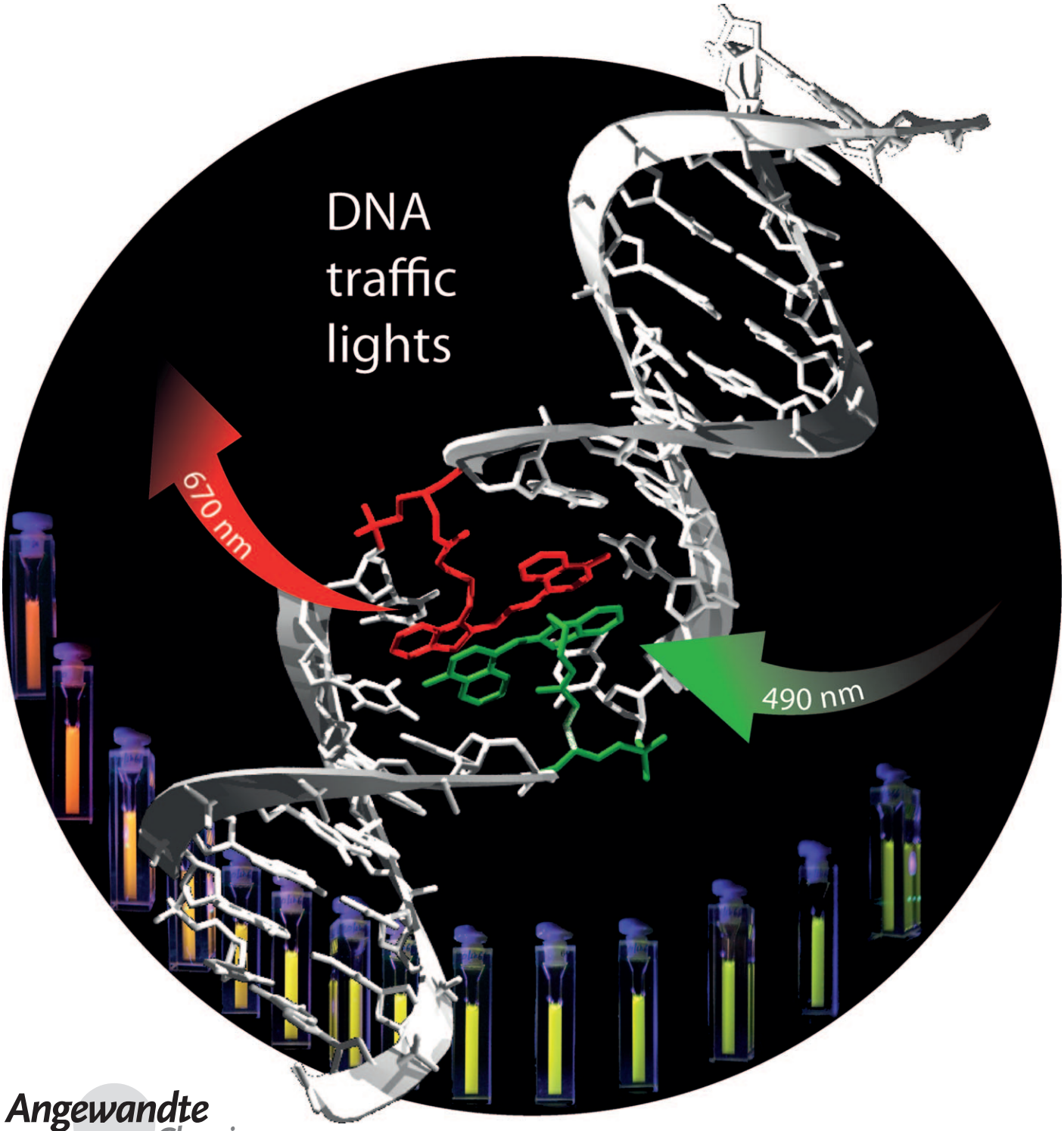
Molecular Beacons

DOI: 10.1002/anie.201101968

In-Stem-Labeled Molecular Beacons for Distinct Fluorescent Color Readout**

*Carolin Holzhauser and Hans-Achim Wagenknecht**



Molecular beacons (MBs) are widely used tools in fluorescence bioanalytical studies of nucleic acids.[1,2] Currently, the biggest challenges in this area are to monitor DNA/RNA uptake and/or sequence-specific hybridization in living cells, and to reliably detect single-nucleotide polymorphism (SNP) by real-time polymerase chain reaction (PCR).[2] If hybridization assays are carried out in vivo, the application of single emission wavelengths bears the risk of wrong positive or wrong negative readout as a result of the autofluorescence of intracellular components or undesired fluorescence quenching. Hence, better MBs are needed. The most important developments in this area have been quencher-free MBs,[3] low-noise stemless PNA MBs,[4] wavelength-shifting MBs,[5,6] and MBs based on excimer fluorescence color readout.[7–11] Recently, we reported that two thiazole orange (TO) chromophores as artificial nucleobases in DNA form a hydrophobically interacting interstrand dimer which results in a distinct change in fluorescence color upon DNA hybridization.[7] Herein we present an advanced design of in-stem-labeled, wavelength-shifting MBs based on the combination of TO and thiazole red (TR) as an interstrand chromophore pair for energy transfer (ET).

Using our published DNA building blocks,[12] we prepared four MBs (**DNA1**–**DNA4**, Figure 1, Table 1), which vary in stem length from 11 down to 5 base pairs (including the dyes as artificial bases). In addition **DNA5** was prepared to elucidate the role of the orientation of the diagonal TO/TR pair (5′–3′ vs. 3′–5′). In all MBs, the TO and TR dyes were embedded in an identical DNA base environment in the stem in order to provide comparable structural scenarios for the chromophore interactions. This includes one A–T base pair on each side of the chromophore pair and thymines as the bases opposite to each dye. Except for this consistent central part, the sequence of the stem is random.

The absorption spectra of all MBs clearly show the presence of both chromophores with well-separated signals at 510 nm (TO) and 640 nm (TR) (see the Supporting Information). The functional characterization of the TO/TR MBs was performed mainly by steady-state fluorescence spectroscopy using the TO-selective excitation at 490 nm. Additionally the melting temperatures (T_m) of the hairpins were compared with those of the duplexes formed in the presence of 1.2 equiv of counterstrands. It proved to be optimal when these counterstrands were complementary not only to the loop region but also to the "inner" parts of the stem. This result was elucidated in representative experiments with **DNA2** and counterstrands of different lengths (see the Supporting Information). With counterstrands covering only the complementary part of the loop region, the chromophores of the stem sequence were insufficiently isolated and nonspecific chromophore aggregation between the remaining "sticky ends" could occur.

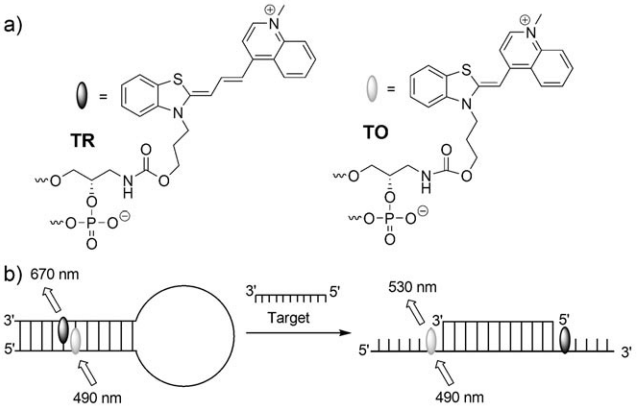


Figure 1. a) Structure of thiazole orange (TO) and thiazole red (TR) as DNA base surrogates. b) Schematic representation illustrating the switching of emission color from red to green when a MB modified with TO and TR binds to the target oligonucleotide.

Table 1: Sequences of the MBs **DNA1**–**DNA6**[a] and counterstrands.

	3′→5′ for MBs, 5′→3′ for counterstrands
DNA1	GTCA**TR**TTGACCGTACGTCAGTTGACTGGTCA**TO**TTGAC
	ACTGGCATGCAGTCAACTGACCAG
DNA2	GTCA**TR**TTGACTGTACGTCAGTTGACTGGTCA**TO**TTGAC
	ACTGACATGCAGTCAACTGACCAG
DNA3	TCA**TR**TTGACTGTACGTCAGTTGACTGATCA**TO**TTGA
	ACTGACATGCAGTCAACTGACTAG
DNA4	CA**TR**TTCGCTGTACGTCAGTTGACTGATCA**TO**TTG
	GCGACATGCAGTCAACTGACTAG
DNA5	GTCAT**TO**TGACCGTACGTCAGTTGACTGGTCAA**TR**TGAC
	CTGGCATGCAGTCAACTGACCAGT
DNA6[b]	**X**-GTCAATTGACCGTACGTCAGTTGACTGGTCAATTGAC-**Y**
	ACTGGCATGCAGTCAACTGACCAG

[a] The underlined bases indicate the stem sequences of the MBs. [b] **DNA6:** Terminally labeled with **X** = rhodamine (TAMRA, 5′) and **Y** = fluorescein (FAM, 3′); see the Supporting Information.

In order to compare the fluorescence readouts of the different TO/TR MBs presented herein and also commercially available MBs, the enhancement factor f was applied.[14,15] f represents the fluorescence ratio I_{530}/I_{670} of the duplex relative to that of the hairpin form (Table 2); I_{530} and I_{670} are the fluorescence intensities at the TO- and TR-typical wavelengths 530 nm and 670 nm, respectively. First, the optimal orientation of TO and TR was elucidated by comparing **DNA1** with **DNA5** (Figure 2, left). The T_m difference between hairpins and duplexes is nearly the same (10.6 vs. 10.7 °C). The fluorescence readout, however, shows significant differences: Although the more intense red TR fluorescence of hairpin **DNA5** indicates a better ET efficiency, the recovery of the green TO fluorescence after the hairpin in opened is much better in case of **DNA1**.

[*] Dipl.-Chem. C. Holzhauser, Prof. H.-A. Wagenknecht
Institute for Organic Chemistry
Karlsruhe Institute of Technology (KIT)
76131 Karlsruhe (Germany)
Fax: (+49) 721-6084-4825
E-mail: wagenknecht@kit.edu
Homepage: http://www.ioc.kit.edu/wagenknecht/

[**] Financial support by the Deutsche Forschungsgemeinschaft (Wa 1386/9-4) and KIT is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201101968.

Table 2: Enhancement factors f and melting temperatures (T_m) for **DNA1**–**DNA6**.

	f[a]	T_m (duplex)[b] [°C]	T_m (hairpin)[c] [°C]	ΔT_m[d] [°C]
DNA1	34.2 ± 0.5	79.9	69.3	10.6
DNA2	13.0 ± 0.5	77.3	66.4	11.0
DNA3	39.7 ± 2.4	76.8	63.9	12.9
DNA4	22.3 ± 0.9	78.6	58.5	20.1
DNA5	13.4 ± 2.0	79.1	68.4	10.7
DNA6	3.9 ± 0.4	78.4	72.8	5.6

[a] Enhancement factor $f = (I_{530}/I_{670})_{duplex}/(I_{530}/I_{670})_{hairpin}$. [b] Melting temperature T_m of **DNA1**–**DNA6** measured at 260 nm when annealed with 1.2 equivalents target strand. [c] Melting temperature of **DNA1**–**DNA6** at 260 nm in hairpin form. [d] $\Delta T_m = T_m(\text{duplex}) - T_m(\text{hairpin})$.

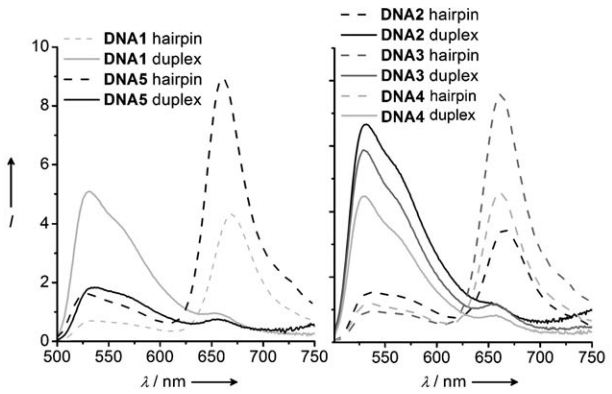


Figure 2. Fluorescence spectra of duplexes **DNA1** and **DNA5** (left) and **DNA2**–**DNA4** (right), each in duplex and hairpin form, 2.5 μM in 10 mM sodium phosphate buffer, 250 mM NaCl, pH 7, 20 °C, excitation at 490 nm.

Hence, **DNA1** gives a much better enhancement factor f (34.2) between the two colors, green and red, than **DNA5** (13.4). We currently cannot explain this observation adequately. From our recent studies with diagonal TO/TO and TO/TR pairs in DNA it was evident that these dyes can undergo significant interstrand excitonic interactions,[7,12,13] which interfere with ET from an excited monomer (TO) to a ground-state monomer (TR). If such ground-state dimers were excited as a conformational subensemble of the DNA sample, ET efficiency would drop significantly. Differences in the absorption spectra between **DNA1** and **DNA5** possibly indicate excitonic interactions for the latter DNA hairpin (see the Supporting Information). In conclusion, the diagonal arrangement of the two dyes in the sequential context of **DNA1** is clearly the better one for the application in TO/TR-modified MBs.

In a second set of experiments the optimal stem length was elucidated (Figure 2, right). As a result of stem shortening, the T_m values decrease from **DNA1** (69.3 °C) to **DNA4** (58.5 °C) and the T_m differences between hairpins and duplexes increases from 10.6 °C to 20.1 °C. In this row of different stem lengths, the critical f value shows two remarkably high maxima, which are 34.2 (**DNA1**) and 39.7 (**DNA3**). Finally, the kinetics of the hairpin opening was studied with **DNA1**–**DNA5** after addition of 0.5 equiv of the corresponding counterstrands. The time-dependent measurement of the f values reveals that at low micromolar concentrations of DNA the maximum plateau is reached within 12 min for **DNA2**–**DNA5** and within 30 min for **DNA1** (see the Supporting Information).

Figure 3a shows a complete titration of **DNA1** by stepwise addition of aliquots containing 0.1 equiv of the counterstrand. A delay time of 60 min was applied between each titration step to ensure complete opening, although the kinetic experiments discussed above revealed a much shorter time. Remarkably, the dominant red TR fluorescence of the

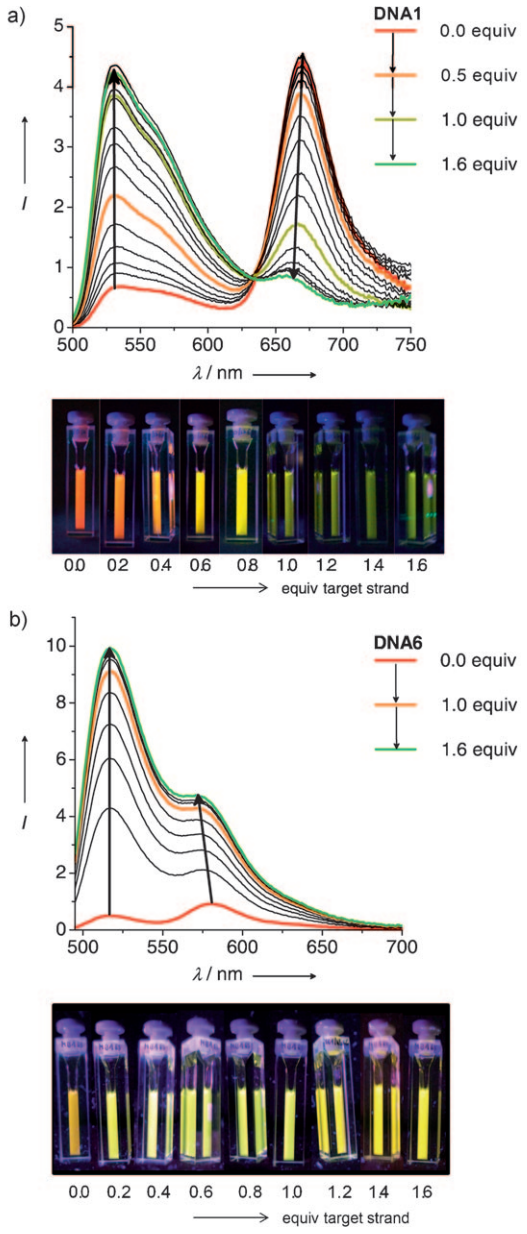


Figure 3. a) Fluorescence spectra of the titration of hairpin **DNA1** (2.5 μM) with up to 1.6 equiv of the target strand, 10 mM sodium phosphate buffer, 250 mM NaCl, pH 7, 20 °C, excitation at 490 nm. b) Fluorescence spectra of the titration of hairpin **DNA6** (2.5 μM) duplex with up to 1.6 equiv of the target strand, 10 mM sodium phosphate buffer, 250 mM NaCl, pH 7, 20 °C, excitation at 488 nm.

MB changes gradually to the green TO fluorescence because both emissions are very well separated by a large shift of 140 nm. The complete opening requires 1.6 equiv of counterstrand because of the relatively small ΔT_m.

Remarkably, quenching of the red TR emission occurs concomitantly with the recovery of the green TO emission. This color change can be observed visually if the cuvette is held under the UV lamp. Since the wavelength shift was also observed in titration experiments with **DNA2**, **DNA3**, and **DNA4** (see the Supporting Information), we expect that the TO/TR system can be used in a large number of different MBs varying in stem length and loop size.

Finally, the MB **DNA1** was compared to a commercially available, so-called wavelength-shifting MB **DNA6** which is terminally labeled with fluoresceine as the ET donor (FAM, 5′-end) and rhodamine as the acceptor (TAMRA, 3′-end).[5] The titration experiment (Figure 3b) provided spectra similar to those published and reveals an enhancement factor f of only 3.9 for **DNA6** (Figure 4). This value indicates a contrast

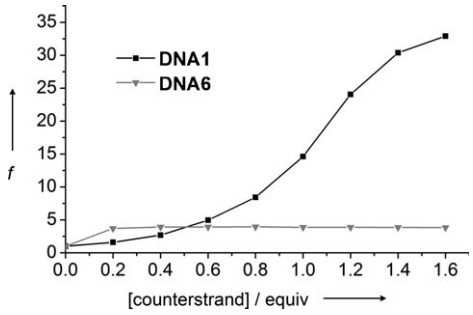


Figure 4. Enhancement factor f of **DNA1** and **DNA6** vs. amount of the corresponding target strand.

that is nearly one magnitude of order lower than that of **DNA1**. This is not surprising since the spectrum shows clearly that the two emission wavelengths of **DNA6** are not very well separated. The TAMRA fluorescence is significantly less intense and unfortunately overlaps with the side band of the FAM emission. This is the reason for the observation that **DNA6** reaches the f maximum already with 0.2 equiv of counterstrand. Although f turned out to be useful for comparing the fluorescence readout of MBs, reports of f values in the literature are rare. For instance, a beacon labeled with Alexa and RedX dyes has gained an f value of 10.5.[15]

From these results it becomes evident that the design of the chromophore attachment to the oligonucleotides makes the significant difference between **DNA1** and **DNA6**.[16] In **DNA6**, both dyes are attached by flexible and long alkyl chain linkers. ET can occur only inefficiently by collisional quenching. In contrast, in **DNA1** the DNA architecture around TO and TR forces the two dyes into close proximity and thereby enhances the ET efficiency by static quenching. During the titration the corresponding counterstrand is delivered step by step; hence the architectural force of the DNA double helix is released by opening the hairpin conformation also step by step, and the resulting separation of the dyes gives the characteristic change in fluorescence color from red to green. In comparison with conventional MBs like **DNA6** and other recently published MBs[3–11] our approach has two major advantages: 1) The fluorescence readout allows a clear and distinct discrimination simply by the emission color (140 nm shift). 2) As a result of the well-separated emission bands, the remarkably high contrast f between duplex and hairpin form enhances the signal-to-noise ratio. Both properties make our newly developed MBs powerful tools for a variety of different applications in fluorescent bioanalytics, real-time PCR, molecular diagnostics, and cell imaging by confocal fluorescence microscopy. Moreover, it is important to point out that we present here a new concept that is promising for the design of bioanalytical tools. If two chromophores as DNA base substitutions in a diagonal interstrand arrangement are forced into close vicinity by the surrounding DNA framework, significantly enhanced ET efficiency results.

Received: March 20, 2011
Published online: June 29, 2011

Keywords: DNA · energy transfer · molecular beacons · thiazole orange · wavelength shift

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