Fluorescent DNA

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Fluorescent Color Readout of DNA Hybridization with Thiazole Orange as an Artificial DNA Base**

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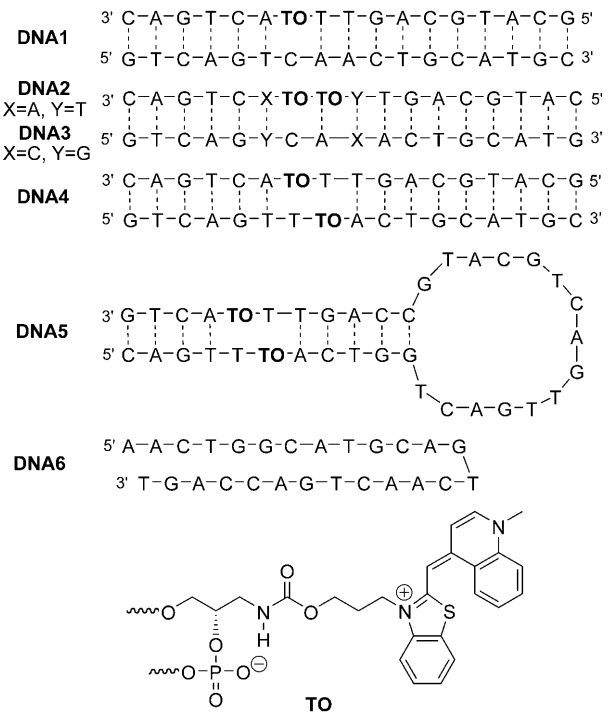
Fluorescent nucleic acid probes often demonstrate the complementary sequential information and single-base variations by enhanced emission intensity or quenching.[1–5] However, such changes could potentially result from side effects causing artifacts in the fluorescence readout. Undesired emission quenching is a significant problem especially in cell biology. Hence, dual labels that change their emission maximum (that is, color) represent superior probes for imaging nucleic acids, an example being wavelength-shifting molecular beacons.[6] DNA with intrastrand and interstrand dimers mainly of pyrene exhibit strong excimer-type fluorescence[7–11] and can also be applied in molecular beacons.[12–14] However, the excitation of pyrene requires high-energy UV light, dramatically limiting the imaging applications. Hence, excimers in DNA with excitation wavelengths >450 nm are highly desirable. Recently, we reported that the excimers of perylene bisimides in DNA shift the fluorescence by approximately 100 nm, which can be used to quantify single-base variations.[15] However, perylene bisimide is able to oxidize guanine and thus low quantum yields are observed when guanines are nearby.[16,17] Thiazole orange (TO) represents a promising alternative as it does not have the potential for photoinduced oxidation of DNA. TO was linked covalently to the phosphodiester[19] or to the 5′-terminus[20] of oligonucleotides. In addition, TO was attached to DNA-binding peptides[21,22] or incorporated as a base surrogate into peptide nucleic acid (PNA) to detect single-base variations.[3,23]

Recently, we presented the optical properties of TO as an artificial DNA base and showed how they could be modulated by short-range electron transfer.[24] However, with this TO base surrogate in the form of interstrand dimers, we could not observe excimer-type fluorescence. Hence, we report herein about an alternative way to use TO as a DNA base surrogate that exhibits the desired properties. As previously, (*S*)-1-aminopropane-2,3-diol serves as an acyclic linker between the phosphodiesters. Similar propanediol linkers have been used by other groups to prepare glycol nucleic acid (GNA),[25] twisted intercalating nucleic acids (TINA),[26] alkyne-modified oligonucleotides for the click-type postsynthetic modification,[27] and by our group for fluorescent DNA base substitutions.[15,28] The linker has been attached to the thiazole ring of the TO dye (Scheme 1). We synthesized the corresponding phosphoramidite as a DNA building block suitable for the preparation of the TO-modified oligonucleotides for **DNA1**–**DNA5** using automated synthesis with extended coupling procedures.



Scheme 1. Sequences of the TO-modified duplexes **DNA1**–**DNA5** and the unmodified **DNA6**.

DNA1 bears a single TO modification and serves as a reference for **DNA2**–**DNA4** in order to study the dimeric TO base substitutions. The TO-modified **DNA5** represents a molecular beacon-like hairpin that was used together with the unmodified counterstrand **DNA6** in a preliminary experiment of fluorescent analytics. The base sequence complementarity forces the two TO chromophores to interact in an intrastrand (**DNA2** and **DNA3**) or interstrand (**DNA4** and **DNA5**) fashion. Accordingly, the UV/Vis spectra of **DNA2**–**DNA5** (Figure 1) at 20 °C exhibit remarkable differences compared to **DNA1**. The absorption of the single TO chromophore in **DNA1** exhibits bands at 486 nm and 507 nm that represent the typical 0→1 and 0→0 vibronic

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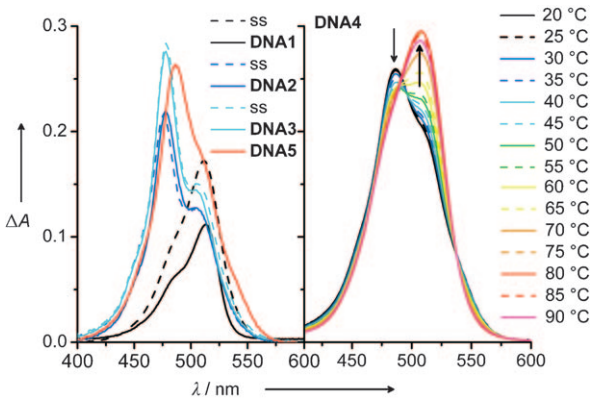

Figure 1. UV/Vis absorption spectra of the single and double-stranded **DNA1**–**DNA3**/**DNA5** (left) and **DNA4** (temperature dependent, right), 2.5 μM in 10 mM Na-P_i buffer, 250 mM NaCl, pH 7; ss = single stranded.

transitions of this dye. The dehybridization of this duplex at higher temperatures decreases the absorbance at 507 nm only slightly (Supporting Information, Figure S1). The two major absorption bands of the TO dimers in **DNA2**–**DNA5** are not significantly shifted but show remarkably different intensities. A similar result has been observed in aggregates of cyanine dyes[29] and with dimeric forms of cyanines (e.g. TOTO, a TO dimer conjugate) that bind noncovalently to DNA.[30–32] The absorption spectra of **DNA2**–**DNA5** show strong excitonic interactions of the two TO chromophores which exist in both the intrastrand dimers of **DNA2**/**DNA3** and the interstrand dimers of **DNA4**/**DNA5**. However it is important to point out that the dye interactions differ slightly, as indicated by the small shifts of the absorption maxima (478 nm for **DNA2**/**DNA3** vs. 486 nm for **DNA4**/**DNA5**). The intrastrand TO chromophore interactions also exist in the single strands of **DNA2** and **DNA3**. In contrast, the thermal dehybridization of the interstrand TO dimers in **DNA4** and **DNA5** is detected at 507 nm and occurs at a very similar temperature to that of the cooperative dehybridization of the whole duplex (Table 1, and Supporting Information, Figure S3). The absorption bands change to values that are characteristic for a single TO dye in an oligonucleotide. This result supports the idea that the interactions of the TO chromophores depend on the framework of the duplex structure.

Table 1: Melting temperatures (T_m), quantum yields (Φ_F), and brightness (B) of **DNA1**–**DNA6**.

Duplex	T_m [°C]		Φ_F	B[c]
	260 nm	507 nm		[$M^{-1}cm^{-1}$]
DNA1	65.5[a]	–	0.217	5800
DNA2	60.5	–	0.041	2400
DNA3	65.0	–	0.054	3800
DNA4	69.4[a]	70.5	0.080	8200
DNA5	77.0	77.0	0.077	7900
DNA5-6	83.0[b]	–	0.073	7000

[a] The corresponding unmodified DNA duplex that contained G instead of TO has $T_m = 67.5$ °C. [b] A second transition at 52.0 °C was observed. [c] $B = \varepsilon_{490nm}\Phi_F$.

When excited at 490 nm, the fluorescence spectrum of the single-labeled **DNA1** shows the typical green TO emission with a maximum at approximately 530 nm (Figure 2). In contrast, the fluorescence of **DNA4** and **DNA5** each containing an interstrand TO dimer is dominated by an orange

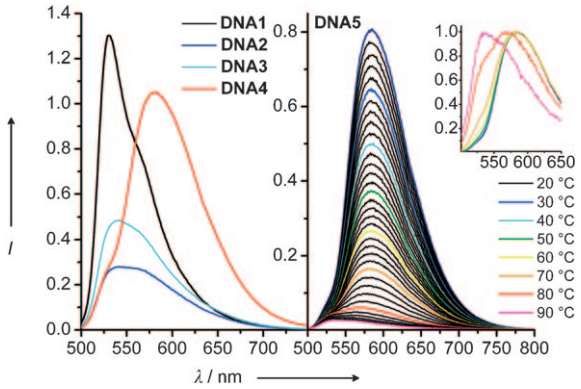

Figure 2. Fluorescence spectra of duplexes **DNA1**–**DNA4** (left) and temperature-dependent spectra of **DNA5** (right), 2.5 μM in 10 mM Na-P_i buffer, 250 mM NaCl, pH 7, excitation at 490 nm. Inset shows normalized temperature-dependent fluorescence of **DNA5**.

emission with a broad band shifted to approximately 580 nm that does not exhibit the TO-typical fine structure. Additionally, the lifetime of the fluorescence of **DNA4** ($\tau = 2.83$ ns) is enhanced significantly compared to **DNA1** ($\tau = 1.55$ ns).[33] Based on these observations we assign the fluorescence of **DNA4** and **DNA5** to an excimer-type emission of the interstrand TO dimers. Concomitantly with the cooperative thermal dehybridization of the whole duplex (Table 1), the excimer-type fluorescence of **DNA4** and **DNA5** vanishes and is replaced by the monomer band (Figure 2). Interestingly, **DNA5** at temperatures around the T_m value, exhibits a dual (yellow) emission arising from the coexistence of the monomeric and the dimeric form of TO. Clearly, the intact helical duplex is required as a framework for the efficient TO excimer-type fluorescence. To our knowledge, excimer-type fluorescence of the TO dye in DNA has not been reported to date, although excitonic interactions were reported several times, for example, in case of noncovalently binding TO conjugates (such as TOTO).[30–32]

In contrast to the interstrand TO dimers in **DNA4** and **DNA5**, the fluorescence spectra of the intrastrand TO dimers in **DNA2** and **DNA3** show mainly quenching arising from the aggregation of the TO dyes, and no excimer formation. There is no significant difference between the A–T environment of the TO dimer in **DNA2** and the G–C neighborhood in **DNA3**. Both duplexes, however, show significant excitonic interactions in the absorption spectra (see above). That means that groundstate interactions of two TO chromophores do not automatically yield excimer-type fluorescence. Otherwise, the excimer-type fluorescence would have been detected previously with TO dimer conjugates (such as TOTO).[31,32]

The destabilization of the duplex caused by the single TO dye in **DNA1** is only 2.0 °C compared to the completely unmodified duplex (Table 1). Even more astonishing is the

observation that the double-modified duplex **DNA4** is stabilized by 3.9 °C compared to **DNA1** owing to the interstrand hydrophobic interactions between the two TO chromophores. This stabilization is remarkable since a single glycol modification typically destabilizes the duplex strongly.[25,28] Clearly, the loss of duplex stability caused by the glycol linker can be regained by stacking interactions between the TO chromophores. That means that the TO dimers in **DNA4** and **DNA5** could be regarded as a hydrophobically and diagonally interacting base pair with a clear fluorescence readout signal as a result of the interstrand interactions. The hypsochromic shift of the absorption maximum to 486 nm together with the red-shift of the excimer-type emission to 580 nm yields a Stokes shift of 94 nm in case of **DNA4** and **DNA5**, a very remarkable value for organic chromophores. Moreover, the brightness of the fluorescent TO pairs is comparable to single TO labels in DNA (Table 1).

In a preliminary analytical experiment, the hairpin **DNA5** was applied for a titration experiment (Figure 3 and Supporting Information Figure S2). The sequence of **DNA5** contains short stretches in the stem that are self-complementary and yield the characteristic orange excimer-type emission. During the titration with the unmodified **DNA6** the hairpin **DNA5** opens. After addition of 4.9 equivalents of **DNA6** the shift of the fluorescence maximum is complete and the fully complementary **DNA5**-**6** is then visualized by the change of the emission color from 580 nm (orange) to 530 nm (green) which is the characteristic emission of the TO-monomer.

In conclusion, the photophysical interaction of two TO chromophores as artificial DNA bases alters their optical properties significantly. The TO dimers are in one strand, as in **DNA2** and **DNA3**, they show strong excitonic interactions that result mainly in fluorescence quenching. The interstrand TO dimers in **DNA4** and **DNA5** exhibit both strong excitonic interactions and red-shifted excimer-type emission. Additionally, the interstrand TO dimer can be regarded as a hydrophobically and diagonally interacting base pair that stabilizes the duplex and shows a clear fluorescence readout signal for DNA hybridization. The large Stokes shift of the TO pairs in **DNA4** and **DNA5** of nearly 100 nm together with a brightness that is comparable to a single TO label in DNA make the TO pair a powerful fluorescent label for applications in molecular diagnostics (e.g. on microarrays) and for imaging in chemical cell biology, such as confocal fluorescence microscopy or single-molecule spectroscopy.

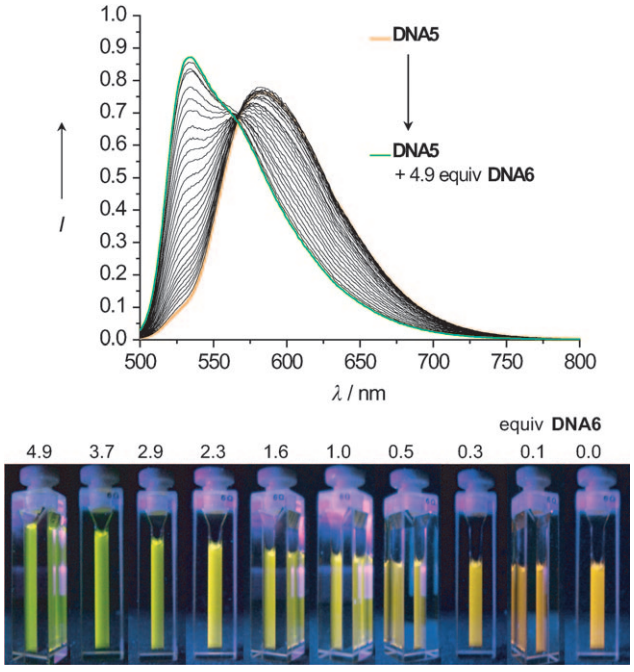

Figure 3. Fluorescence spectra of the titration of **DNA5** (2.5 μM self-hybridized duplex) with up to 4.9 equivalents **DNA6** (relative to single strand) in steps of 0.05–0.4 equivalents, 10 mM Na-P_i buffer, 250 mM NaCl, pH 7, 20 °C, excitation at 490 nm.

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