Forced Intercalation Probes (FIT Probes): Thiazole Orange as a Fluorescent Base in Peptide Nucleic Acids for Homogeneous Single-Nucleotide-Polymorphism Detection

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Fluorescent base analogues in DNA are versatile probes of nucleic acid–nucleic acid and nucleic acid–protein interactions. New peptide nucleic acid (PNA) based probes are described in which the intercalator dye thiazole orange (TO) serves as a base surrogate. The investigation of six TO derivatives revealed that the linker length and the conjugation site decided whether a base surrogate conveys sequence-selective DNA binding and whether fluorescence is increased or decreased upon single-mismatched hybridization. One TO derivative conferred universal PNA–DNA base pairing while maintaining duplex stability and hybridization selectivity. TO fluorescence increased up to 26-fold upon hybridization. In contrast to most other probes, in which fluorescence is invariant once hybridization had occurred, the emission of TO-containing PNA probes is attenuated when forced to intercalate next to a mismatched base pair. The specificity of DNA detection is therefore not limited by the selectivity of probe–target binding and a DNA target can be distinguished from its single-base mutant under nonstringent hybridization conditions. This property should be of advantage for real-time quantitative PCR and nucleic acid detection within living cells.

Introduction

Genetic disorders present a threat to human health. There are various diseases, such as Tay Sachs disease, Huntington's disease, Alzheimer's disease, cystic fibrosis, familial hypercholesterolemia, and even cancer, that can be caused by gene mutations.[1] A point mutation can be sufficient to introduce the disease state. DNA-targeted diagnostic methods play an important role in various clinical settings and may offer the possibility of detecting a developing disease such as cancer before symptoms begin to appear. Homogenous hybridization assays have become very popular. In these assays, the need to separate unbound probe molecules from bound ones is avoided and this provides significant advantages such as convenience, speed, and accuracy of DNA analysis.[2–5] Most of the current specific detection systems rely on the use of labeled oligonucleotide-based probe molecules that bind to their DNA target in a sequence-specific manner. Most commonly, bound probes are distinguished from unbound probes by hybridization-induced spatial convergence or separation of two markers that interact through fluorescence resonance energy transfer (FRET). Typical and powerful examples include adjacent probes,[6,7] molecular beacons[8] or TaqMan probes.[9] Usually, the emission intensities are invariant once a probe–target duplex has formed. Hence, the sequence specificity of DNA-detection is solely governed by the selectivity of probe hybridization. We have recently provided preliminary results to suggest that peptide nucleic acid (PNA) probes (or FIT probes, forced intercalation of thiazole orange probes) that contain thiazole orange as a fluorescent base surrogate are endowed with the advantage of being able to distinguish between matched and single-mismatched hybridization (Figure 1b).[10] In contrast to previous probes (Figure 1a), in which intercalator dyes were conjugated to probe molecules through a flexible linker, thiazole orange was forced to intercalate at a specific site.[11–14]

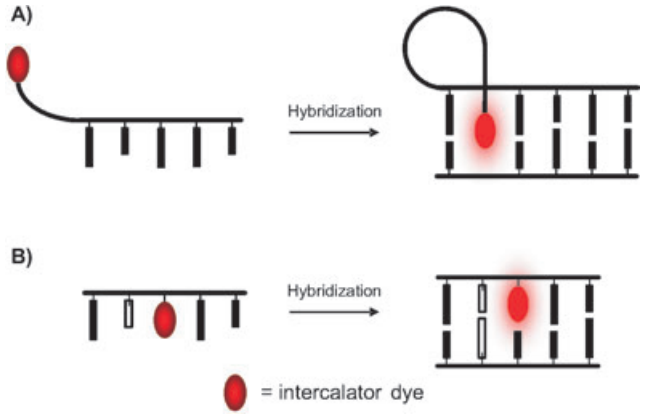

Figure 1. *a) An intercalator dye is attached through a linker to one end of the probe. Upon double-strand formation the fluorophore can fold back and intercalate between the formed Watson–Crick base pairs. b) The intercalator dye serves as a base surrogate that is forced to intercalate adjacent to the expected mutation site. The specific positioning renders the fluorophore responsive to mismatched hybridization.*

When an intercalator is being attached to the backbone of the nucleic acid strand care has to be taken with regard to the conjugation mode and the nature of the linker used. Inappropriate conjugation would interfere with the intercalation process and result in low stabilities of probe–target complexes and/or poor fluorescence properties. It was the aim of this in-

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vestigation to find a fluorescent base surrogate that is able to signal hybridization in a single-nucleotide-specific manner, even under nonstringent hybridization conditions in which both matched and single-mismatched hybridizations occur. We present here full details of the evaluation of varied thiazole orange base surrogates that have not been investigated before. A rapid and automatable means of accessing the probes was provided by a divergent solid-phase synthesis protocol which is applicable to the synthesis of any type of base-modified PNA molecule. It is shown that the linker length and conjugation site decide whether a base surrogate conveys sequence-selective DNA binding and whether probe fluorescence is increased or decreased upon formation of single-mismatched duplexes. This study therefore allowed the identification of probe molecules that strongly fluoresced only upon binding to matched DNA and showed attenuated fluorescence upon binding to single-mismatched DNA.

Design principles

Most common fluorescent labels are attached to nucleic acids by linking them through a flexible tether. The linker unit is designed to not interfere with the hybridization process. As a result local alterations of duplex structure, such as those imposed upon formation of a mismatched base pair, show little effect on the fluorophore's emission properties. We reckoned that the incorporation of a fluorophore as a base surrogate should render probe fluorescence responsive to adjacent base mismatches (Figure 1 b). Such probes could prove useful for the homogeneous analysis of single-base mutations in cases where it is difficult to discriminate matched from single-mismatched targets by hybridization alone. For example, real-time quantitative PCR, array technology, and live-cell analysis could profit from probes in which the detection of single-base mutations were possible by applying nonstringent hybridization conditions.

In choosing a fluorophore to be used as the base surrogate, we considered two major obstacles. First, it was desired that probe hybridization would result in a positive signal, that is, in enhancement of fluorescence rather than quenching of fluorescence. Second, the possible interference with the probe's ability to hybridize had to be avoided. Optimally, the fluorescent base surrogate would pair equally well to all four canonical nucleobases. However, it was deemed important that probe–target binding still proceeded in a sequence-selective manner without detriment to hybridization fidelity. Hence, the properties demanded correspond to those desired for a universal base that fluoresces upon hybridization.[15,16]

We surmised that intercalators should possess the required properties. Intercalators are endowed with a high base-stacking ability which was envisaged to provide compensation for the losses of duplex stability that would be induced upon omission of hydrogen-bonding interactions. In addition, there are intercalator dyes used in DNA staining that fluoresce upon intercalation in double-stranded DNA, a property that provides the basis for positive signaling of hybridization.[5]

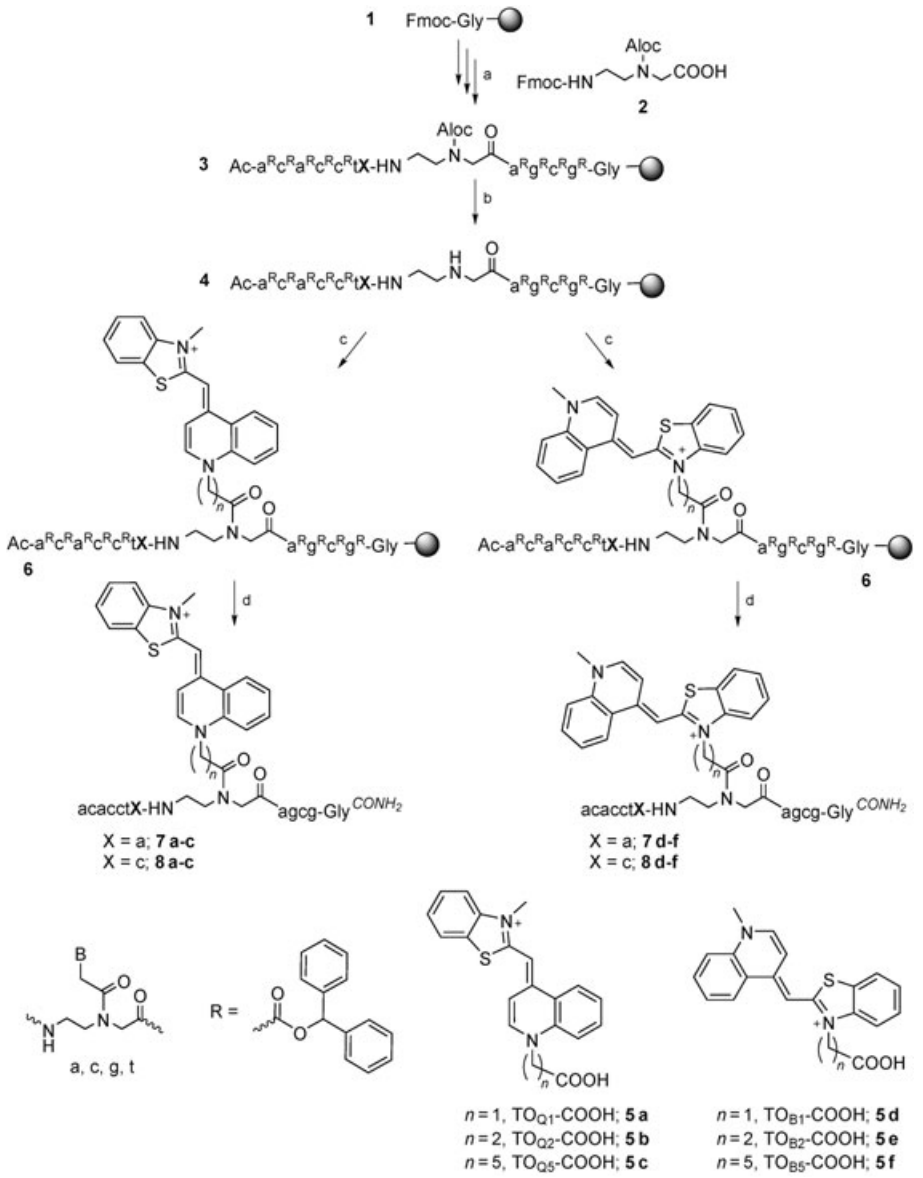
Previous work in the field of artificial DNA bases has been focused largely on introducing structural probes for studying DNA–DNA[17–22] and DNA–protein recognition,[23–26] expanding the genetic alphabet,[27–32] and fashioning metal-based hybridization systems.[33–38] Only few studies have been reported in which intercalators were incorporated as base surrogates. For example, the introduction of C-nucleotides containing coumarine[39,40] and pyrene[17,18] residues into DNA led to decreases in duplex stability or quenching of fluorescence. Acridine,[41,42] phenanthridinium,[43,44] and pyrene[45–47] were incorporated into DNA by means of flexible open-chain 2-deoxyribose analogues. However, fluorescence enhancements in response to matched (or single-mismatched hybridization) were lower than fivefold or have not been reported. The opposite to universal fluorescent bases, namely base-discriminating fluorescent bases, have been obtained by coupling nucleotides bearing benzo- and naphthopyridopyrimidines,[48,49] 5-phenanthrolinylethinyluridine,[50] and benzodeazainosines.[51]

The relative ease of introducing base analogues into PNA, a DNA mimic that binds complementary DNA with increased affinity and selectivity, has been exploited in many studies aiming for enhancements of hybridization properties.[52–58] Aromatics,[59] heteroaromatics,[60,61] and fluoroaromatics[62] have been incorporated in order to confer universal base pairing, albeit at the cost of compromising duplex stability by $\Delta T_M = 9$–$29\,^{\circ}C$ (ΔT_M = change in the duplex melting temperature). Fluorescent base analogues such as 2-aminopurine,[63] psoralen,[64] flavin,[65,66] and 3,5-diaza-4-oxo-phenothiazine[67] have been placed in the interior of PNA–DNA duplexes. None of these artificial bases had been designed to fulfill the demands desired for a universal base that fluoresces upon hybridization. We reckoned that positively charged intercalator dyes such as ethidium[68] or thiazole orange[69] (TO) should provide the necessary properties.[70] Both dyes are paradigms of intercalators and it can therefore be assumed that their strong preference for base stacking will allow nondiscriminate binding to the four natural nucleobases. Both dyes fluoresce upon intercalation, but fluorescence intensification is higher with thiazole orange than with ethidium bromide. We therefore chose to evaluate thiazole orange derivatives as fluorescent base surrogates (see Scheme 1).[71] It was unclear in which binding geometry thiazole orange would intercalate in PNA–DNA duplexes. For this reason, both linkages of the benzothiazole (TO_B) and quinoline (TO_Q) ring to a peptide nucleic acid scaffold were investigated.

Results

Synthesis

The TO fluorophore (see **5** in Scheme 1) was equipped with carboxyalkyl spacers of varying length in order to enable coupling with the PNA backbone. Rather than preparing preformed monomer building blocks for each of the six TO derivatives **5a**–**f** to be tested, it was preferable to gain a divergent access to PNA–dye conjugates. As the central building block, an orthogonally protected backbone module such as the Fmoc/Aloc-protected aminoethylglycine **2** was incorporated



Scheme 1. *a) Cycle of 1) piperidine/DMF (1:4); 2) Fmoc-X(Bhoc)-OH (for X = a, c, or g) or Fmoc-t-OH B is used in the scheme for the bases on their own and t doesn't need the protecting group or* ***2****, NMM, PyBop, NMP; 3) Ac_2O/lutidine, DMF; b) 2 × [$Pd(PPh_3)_4$], dimethyl amine borane complex, CH_2Cl_2; c)* ***5****, PyBOP, PPTS, NMM, DMF (double coupling); d) TFA,* m*-cresol, H_2O, H-Cys-OMe. Aloc = allyloxycarbonyl, B = base A, C, G, or T as appropriate, Bhoc = R = benzhydryloxycarbonyl (protecting group for NH_2 group on base), DMF =* N,N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, NMM =* N*-methylmorpholine, NMP =* N*-methylpyrrolidine, PPTS = pyridinium* p*-toluenesulfonate, PyBop = (Benzotriazol-1-yloxy)tripyrrolidonophosphonium hexafluorophosphate, TFA = trifluoroacetic acid.*

during the Fmoc-based PNA solid-phase synthesis. After completion of the PNA assembly the Aloc group was removed by Pd^0-mediated allyl transfer. Prior to subsequent dye coupling, the full-length PNA–resin conjugate **4** was divided into six portions. The low solubility of the thiazole orange derivatives in commonly used peptide-synthesis solvents along with the poor reactivity of the *N*-alkyl amino acid structure in **4** rendered the acylation of the liberated backbone amino group more difficult than expected. Ultimately, it was found that the addition of pyridinium *p*-toluenesulfonate increased the thiazole orange solubility and enabled its coupling to the supported PNA oligomer **4**. Standard acidolysis liberated the unprotected PNA–TO conjugates **7a–f**, which were subsequently purified by reversed-phase (RP) HPLC. MALDI-TOF MS along with UV/Vis and fluorescence spectrometry confirmed the molecular masses of **7a–f** and the integrity of the chromophores. The single-base mutants **8a–f** were synthesized by utilizing the same approach.

T_M measurements

In PNA–TO conjugates **7** and **8** the bicyclic fluorophores were linked through C_2, C_3, and C_6 tethers to the quinoline ring (TO_{Q1}, TO_{Q2}, TO_{Q5}) or the benzothiazole ring (TO_{B1}, TO_{B2}, TO_{B5}). In order to assess the ability of the TO derivatives to confer strong and sequence-selective binding to complementary DNA, melting analyses were performed. Probes **7** and **8** were hybridized with oligodeoxynucleotides **9** and **10** which resembled wild-type and mutant sequences, respectively, with respect to the known G12V mutation in the *ras* gene.[72] The melting curves showed sigmoid behavior in all cases, a result which suggests cooperative base pairing. However, the duplexes containing TO_{B5}–PNA, **7f** and **8f**, had different denaturation and renaturation curves that indicated hysteresis. Melting analyses revealed that the T_M values of duplexes that contained quinoline-linked TO (TO_Q) showed a pronounced dependence on the spacer length (Table 1). For example, a decrease of the spacer length by 4 carbon atoms (TO_{Q5} versus TO_{Q1}) led to an increase in the T_M value of 16 °C for matched duplex **7·10** (T_M = 42 °C with TO_{Q5} and T_M = 58 °C with TO_{Q1}) and of 25 °C for matched duplex **8·9** (T_M = 41 °C with TO_{Q5} and T_M = 66 °C with TO_{Q1}). Duplexes in which the TO chromophores were anchored through the benzothiazole ring (TO_B) were less dependent on the spacer length and varied in thermal stability by 6 °C and 15 °C for matched duplexes **7·10** and **8·9**, respectively. The highest duplex stability was measured for duplexes **7a·10** and **8a·9** in which TO was anchored through an acetyl tether to the quinoline ring (TO_{Q1}).

The hybridization selectivity of probes **7** and **8** was investigated by studying mismatched duplexes **7·9** and **8·10**. It was

Table 1. Melting temperatures of PNA–DNA duplexes containing TO derivatives as base surrogates.

5′-CGCTG**Y**AGGTGT-3′
^{C}Gly-gcga**OX**tccaca^{N}

O		X–Y = a–T T_M(**7·10**) [°C]	X–Y = a–G T_M(**7·9**) [°C]	ΔT_M [°C]		X-Y = c–G T_M(**8·9**) [°C]	X-Y = c–T T_M(**8·10**) [°C]	ΔT_M [°C]
TO_{Q1}	**7a**	58	50	8	**8a**	66	54	12
TO_{Q2}	**7b**	41	45	−4	**8b**	42	41	1
TO_{Q5}	**7c**	42	42	0	**8c**	41	40	1
TO_{B1}	**7d**	46	56	−10	**8d**	57	47	10
TO_{B2}	**7e**	48	43	6	**8e**	52	46	6
TO_{B5}	**7f**	42	52	−10	**8f**	42	40	2

observed that the incorporation of TO_{Q2} and TO_{Q5} in mismatched duplexes **7c·9** (T_M = 42 °C) and **8b,c·10** (T_M = 41, 40 °C) had little effect on duplex stability when compared to matched duplexes **7c·10** (T_M = 42 °C) and **8b,c·9** (T_M = 42, 41 °C). Interestingly, in a few cases single-mismatched duplexes were rendered even more stable than perfect duplexes, for example, TO_{Q2} and TO_{B1} in duplexes **7b·9** and **7d·9** led to an increase of 4 and 10 °C, respectively, in the T_M values. Hybridization with the acetyl-linked TO_{Q1} behaved "normally", the presence of the A–G mismatch in **7a·9** and of the C–T mismatch in **8a·10** decreased the melting temperature by 8 and 12 °C, respectively. The data suggested that TO_{Q1} conferred higher DNA affinities and hybridization selectivities than the other tested TO derivatives. We therefore chose PNA conjugates bearing TO_{Q1} as the fluorescent base surrogate for further evaluation of hybridization properties.

Melting studies with duplexes **11·12** were performed in order to explore the role of nucleobases opposite to TO_{Q1}. In PNA–DNA duplexes **11·12** TO_{Q1} was paired against each of the four natural DNA bases. Melting analyses suggested that each of the four nucleobases was tolerated well by TO_{Q1} (Table 2). The T_M values were within a range of 1 °C, with an average value of 68 °C. Most interestingly, a comparison with the T_M value of 69 °C provided by an A–T base pair (**11A·12AT**) revealed that the replacement of adenine by TO reduced duplex stability by only 1 °C. This suggests that, within the studied sequence context, pairing TO against A, C, G, or T is almost as efficient in duplex stabilization as pairing A against T. A comparison of TO base pairing with the more stable C–G base pairing was the next study performed. It became apparent that replacement of a C–G base pair by a TO–G pair reduced duplex stability by ΔT_M = 3 °C (data not shown).[10]

The decreased T_M values (ΔT_M = 9, 14, 11, and 11 °C, respectively) observed with duplexes **11·12CA, 11·12CC, 11·12CG**, and **11·12CT** (Y = C) containing a mismatched cytosine adjacent to TO_{Q1} revealed that probe hybridization was sequence selective irrespective of the identity of the TO_{Q1} pairing partner (Table 2). The ΔT_M value of 15 °C determined when comparing the thermal stability of matched and mismatched duplexes **11A·12AT** and **11A·12CT** suggested that hybridization of unchanged PNA **11A** is slightly more selective than hybridization of TO_{Q1}-containing PNA **11**. It was concluded that thiazole orange, when linked through an acetyl tether to the quinoline ring, has the potential to serve as universal PNA base conveying a "pairing strength" that can resemble that of an A–T base pair.

Fluorescence studies

PNA probes **7a** and **8a** bearing TO_{Q1} as an artificial base met the demanded hybridization criteria, in that TO_{Q1} paired well to all four natural nucleobases with an unimpaired DNA affinity and high hybridization selectivity. The fluorescence properties of PNA–TO conjugates, such as **7a–f** and **8a–f**, were studied next. Thiazole orange has low fluorescence in its free form but is rendered highly fluorescent upon intercalation into DNA. We presumed that hybridization of PNA probes **7** and **8** would also result in TO fluorescence enhancements that would enable homogeneous DNA detection. Figure 2 shows fluorescence spectra of PNA–TO conjugates **11** as measured before and after hybridization to oligodeoxynucleotides **9** and **10.** The analysis of quinoline-linked PNA–TO_Q conjugates **7a–c** showed that the fluorescence emission of propionyl- and caproyl-linked TO (TO_{Q2} and TO_{Q5}, respectively) was enhanced by a factor of 2–3, relative to the single-strand emissions, upon matched hybridization (Figures 2B and C, respectively). Similar fluorescence enhancements were obtained after addition of mismatched DNA **9**. The emission properties of TO_{Q1} proved

Table 2. Melting temperatures of PNA–DNA duplexes containing TO_{Q1} as a base surrogate.

5′-CGGCT**YZ**TACGGC-3′
^{C}Gly-gccgat**O**atgccg^{N}

YZ = AA, **12AA**; AC, **12AC**; AG, **12AG**; AT, **12AT**; etc.
O = TO_{Q1}, **11**; A, **11A**

Y	**O**–**Z** = TO_{Q1}–A T_M [°C]	**O**–**Z** = TO_{Q1}–C T_M [°C]	**O**–**Z** = TO_{Q1}–G T_M [°C]	**O**–**Z** = TO_{Q1}–T T_M [°C]	**O**–**Z** = A–T T_M [°C]
A	67	68	68	68	69
C	58 (9)[a]	54 (14)[a]	57 (11)[a]	57 (11)[a]	54 (15)[a]

[a] The ΔT_M values between the matched and mismatched duplexes are given in brackets.

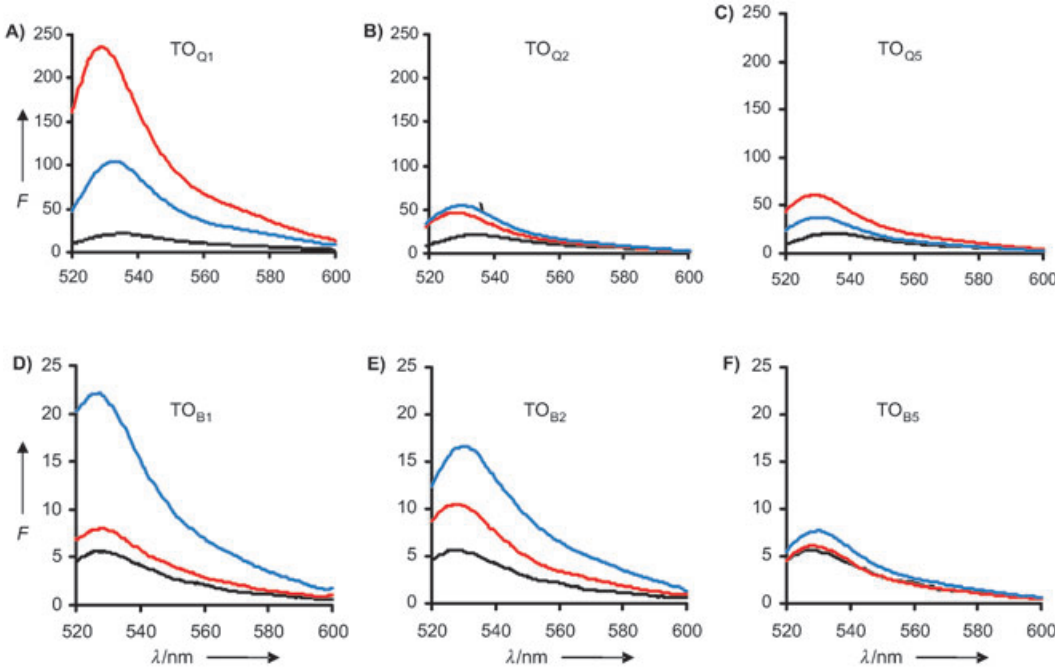


Figure 2. *Fluorescence spectra (arbitrary units, calibration based on fluorescence of single-stranded* ***7c*** *for the upper panel and single-stranded* ***7d*** *for the lower panel) of single strands (black), matched duplexes (red), and mismatched duplexes (blue) containing PNA probes A)* ***7a****, B)* ***7b****, C)* ***7c****, D)* ***7d****, E)* ***7e****, and F)* ***7f****. Measurement conditions: 1 μM probes and DNA in buffer at 25°C (100 mM NaCl, 10 mM NaH_2PO_4, pH 7), excitation: 510 nm, emission: 520–600 nm.*

significantly more responsive (Figure 2A). Binding of **7a** to the matched target **10** resulted in pronounced increases in fluorescence intensity (15-fold at 525 nm). Interestingly, the fluorescence intensification obtained after addition of mismatched DNA **9** was significantly lower (5-fold at 525 nm).

PNA probes **7d–f**, in which TO was linked through the benzothiazole ring, performed differently, in that fluorescence of mismatched duplexes was higher than fluorescence of matched duplexes (Figure 2D–F). Fluorescence enhancements upon matched or mismatched hybridization can both be of use in developing DNA-detection chemistry. However, the sensitivity of TO_B fluorescence in response to hybridization was lower than that of TO_Q fluorescence. For example, the emission of TO_{B1} in **7d** was enhanced by a factor of 4 upon mismatched hybridization (Figure 2D) in contrast to the enhancement factor of 15 observed upon matched hybridization of TO_{Q1}-containing probe **7a**.

In summary, PNA in which thiazole orange was linked as a base surrogate through an acetyl tether to the quinoline ring exhibited a superior fluorescence behavior when compared with TO_{Q2}, TO_{Q5}, TO_{B1}, TO_{B2}, and TO_{B5}. Both hybridization-induced fluorescence enhancements and responsiveness to adjacent mismatched base pairs were higher. To exclude the possibility of nonspecific intercalation of TO_{Q1} in PNA conjugates such as **11**, sequence-unrelated duplex DNA was added (Figure 3). However, a change in emission intensity was not observable, a result indicating that changes in TO_{Q1} fluorescence are due to interactions with specific targets. Moreover, TO_{Q1} conferred higher DNA affinities and hybridization selectivities than the other tested TO base surrogates. Subsequent fluorescence studies were therefore focused on PNA bearing TO_{Q1} as the artificial base.

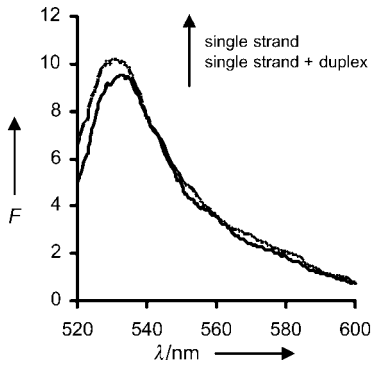


Figure 3. *Fluorescence spectra of PNA probe* ***11*** *before and after addition of unrelated duplex DNA (5′-CACACCTCCAGCGCCC-3′:5′-GGGCGCTGGAGGTGTG-3′). For measurement conditions, see the legend of Figure 2.*

The previous melting studies attested to the ability of TO_{Q1} to pair equally well to A, T, G, and C. To test the fluorescence response upon universal base pairing, more detailed hybridization studies were performed. In PNA–DNA duplexes **11·12** TO_{Q1} was paired against eight different DNA strands offering four different TO_{Q1} pairing partners (Z) and four different TO_{Q1} interstrand stacking partners (Y in Figure 4A). It became apparent that the duplex formation was accompanied by a shift of the emission maximum from 533 to 527 nm and enhancements of fluorescence intensity, properties that are typically observed upon TO intercalation.[69,70] The highest fluorescence enhancements were measured upon addition of perfectly complemen-

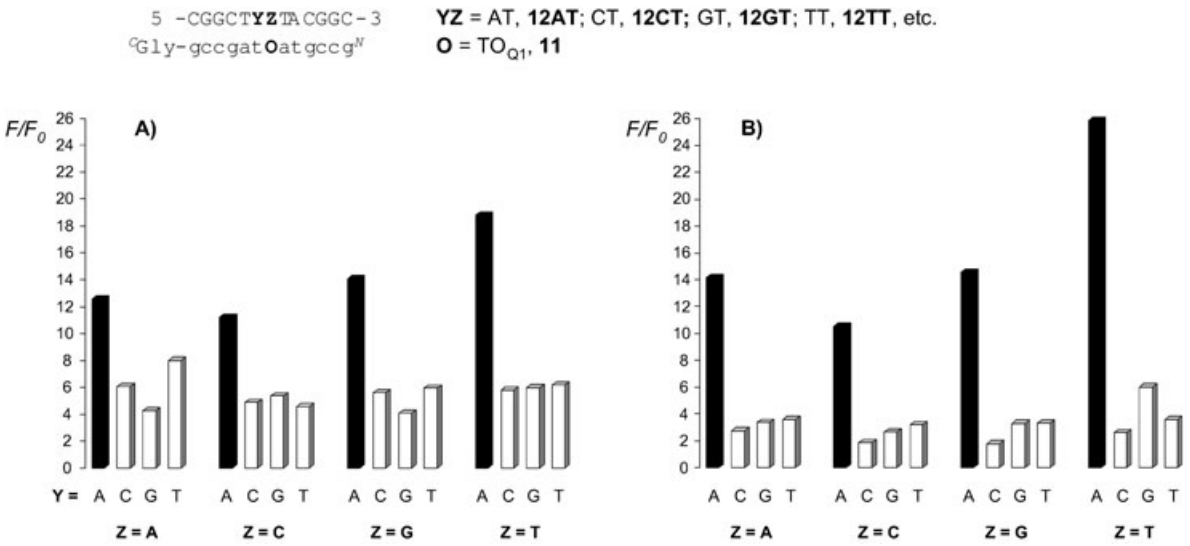


Figure 4. *Fluorescence enhancement F/F$_0$ of PNA probe **11** measured at 530 nm after formation of match duplexes (black columns) and mismatch duplexes (white columns) at A) 25 °C and B) 60 °C. F$_0$ = fluorescence intensity of the PNA single strand **11**, F = fluorescence intensity after addition of DNA **12**. For measurement conditions, see the legend of Figure 2.*

tary DNA (black columns in Figure 4A). For example, the emissions of the matched duplexes **11·12AG** and **11·12AT**, in which TO was paired against guanine and thymine were 14 and 19 times higher, than the emission of the single-stranded probe **11**. Binding opposite to adenine (**11·12AA**) and cytosine (**11·12AC**) increased TO fluorescence by factors of 13 and 11, respectively. The lowest fluorescence enhancements were obtained upon addition of single-mismatched DNA (white columns in Figure 4A). It has to be noted that the fluorescence measurements depicted in Figure 4A were performed at 25 °C. Both matched and single-mismatched duplexes were present. Nevertheless, fluorescence intensities of single-mismatched duplexes containing PNA **11** were always lower than those of matched duplexes. This attenuation of fluorescence in response to mismatched hybridization is expected to be useful for the analysis of single-base mutations.

In real-time quantitative PCR analysis, probe hybridization and hence DNA detection is performed in the annealing cycle at temperatures of 50–70 °C. Figure 4B shows the results of fluorescence measurements at 60 °C. Matched hybridization at 60 °C was accompanied by almost unchanged (Z = C, G) or even increased fluorescence enhancements (Z = A, T) in comparison to the results obtained for hybridization at 25 °C. At 60 °C hybridization to **12AT** increased the fluorescence of **11** by a factor of 26 as opposed to the 19-fold fluorescence enhancement at 25 °C. In contrast, the increase of temperature led to reduced fluorescence enhancements upon mismatched hybridization. As a result, hybridization at elevated temperature allowed better discrimination between matched and single-mismatched targets. For example, the emission of probe **11** in the presence of matched target **12AT** was tenfold higher than that observed in presence of the single-mismatched target **12CT** (Figure 4B), as opposed to a threefold stronger fluorescence enhancement upon matched hybridization at 25 °C (Figure 4A). This enhanced match/mismatch discrimination can be explained by the reduced thermal stability of the mismatched duplex which is 11 °C lower when compared with the T_M value of the matched duplex (see Table 2). The ability to discriminate at a given temperature between matched and mismatched targets without concomitantly affecting the fluorescence intensification upon matched hybridization could prove useful if PNA–TO probes such as **11** were to be used in real-time PCR analysis.

A commonly observed problem of fluorescent nucleobases and base surrogates concerns fluorescence quenching by flanking G–C base pairs.[73] To explore the effect of adjacent G–C base pairs on TO$_{Q1}$ fluorescence, hybridization studies with probes **13** and **14** providing C or G as intrastrand stacking partners, respectively, were performed (Figure 5). Again, hybridization was found to result in strong fluorescence enhancements, which reached factors of 20 and 15 for **13** and **14**, respectively.

Discussion

Duplex stability

The dramatically different thermal stabilities that were measured for duplexes that contained thiazole orange base surrogates linked through different tethers are unprecedented. However, in previous studies, intercalators have been most commonly linked into DNA helices by means of flexible open-chain ribose analogues such as threoninol.[41–47] Linking base surrogates to flexible open-chain sugar analogues may result in an attenuation of linker effects, which, in contrast, may become apparent when anchoring base surrogates to the rigid 2′-deoxyribose phosphate backbone or its aminoethylglycine equivalent in PNA. Of the six TO base surrogates that were evaluated, TO$_{Q1}$ proved to be most efficient in stabilizing a PNA–DNA duplex and in conferring selective base pairing of

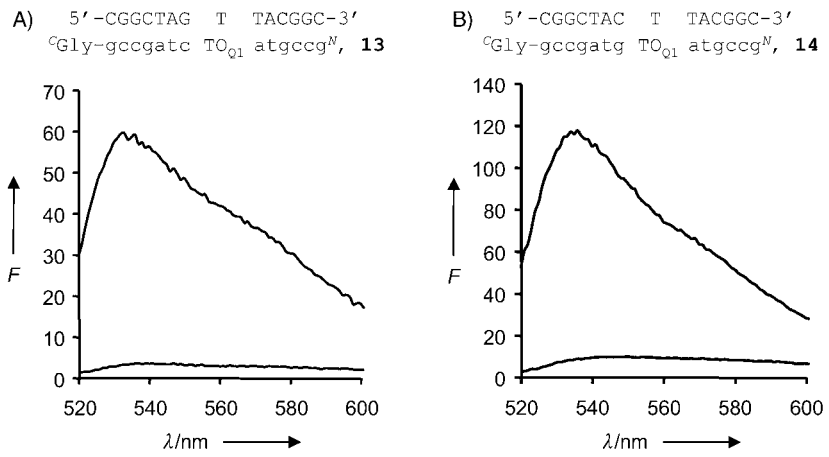


Figure 5. *Fluorescence spectra (arbitrary units) measured before (lower curve) and after (upper curve) matched hybridization of A) PNA probe* ***13*** *containing cytosine adjacent to TO and B) PNA probe* ***14*** *containing guanine adjacent to TO.*

adjacent nucleobases. Presumably, TO_{Q1} is best at duplex stabilization and hybridization selectivity because the acetate tether most closely resembles DNA or PNA in linker length.

TO_{Q1} was found to pair equally well (within a 1 °C range of T_M values) with each of the four natural DNA bases and to destabilize the duplex by only 1 °C (relative to an A–T base pair; see Table 2). Although similar abilities in nondiscriminative PNA–DNA pairing have been described before,[16] none of the previously reported universal bases, to best of our knowledge, was as efficient in maintaining duplex stability. Given the almost unchanged duplex stability of TO_{Q1}-containing PNA–DNA duplexes, it appears plausible to assume that TO can compensate for the losses in thermal stability induced by the lack of hydrogen-bonding interactions by means of its extraordinary base-stacking ability. Similar but less pronounced effects have been observed when typical intercalators such as pyrene, acridine, and phenanthridinium were incorporated as artificial bases in DNA. The ability to nondiscriminatively pair to each of the four natural bases is surprising at first glance since it is difficult to imagine how both TO_{Q1} and the opposing base can fit into the interior of a nucleic acid duplex. However, steric clashes can be avoided by forming a bulged structure or by flipping the TO_{Q1} pairing partner into an extrahelical position.

Fluorescence properties

TO-based chromophores become fluorescent when a binding event such as intercalation forces the two ring systems into a coplanar conformation. The six tested TO derivatives are characterized by an identical cyanine chromophore. The different fluorescence properties observed for PNA probes containing TO derivatives as base surrogates are therefore attributable to changes in the fluorophore's microenvironment. Remarkably, TO fluorescence showed characteristics that varied from virtually nonresponsive to hybridization (TO_{Q2}, TO_{Q5}, TO_{B5}) to match-specific (TO_{Q1}) and single-mismatch-specific (TO_{B1}, TO_{B2}) fluorescence enhancements. These results highlight the importance of performing linker studies. TO_{Q1} brought about the highest responsiveness to the presence of target DNA (Figure 2), a result which provides further support for the notion that the acetate linkage to the quinoline ring best allowed intercalation and hence coplanarization of the TO ring systems.

Despite exhibiting nondiscriminative base pairing, the fluorescence of TO responded to changes in the immediate environment. For example, within the studied sequences, thymine appeared to be the optimal pairing partner when aiming for a high fluorescence enhancement (Figure 4). In contrast to results obtained with most other fluorescent base analogues, the presence of guanine, either as pairing partner or as a stacking partner, did not result in quenching of fluorescence (Figure 5). The hybridization induced fluorescence enhancement reached a factor of 26, which is in the range of fluorescence intensifications obtained with commonly used dual-labeled probes such as adjacent probes and molecular beacons. Typically, structured dual-labeled probes such as molecular beacons exhibit the highest fluorescence enhancements at low temperatures.[8] In contrast, PNA–TO conjugates such as **11** exhibit their highest performance at the elevated temperatures applied in real-time PCR analysis (Figure 4B).

End-labeled PNA–TO conjugates (light-up probes) have been used in real-time PCR analysis.[13,14] In these probes, TO is linked through a flexible spacer that allows the fluorophore to intercalate between base pairs of the formed duplex. The fluorescence enhancements measured upon hybridization of PNA–TO conjugates such as **7** and **11** were comparable to or higher than those obtained with mixed-sequence light-up probes. We therefore expect that probes such as **11** will be similarly effective in real-time analysis. There is, however, a marked difference as to the responsiveness to single-base mismatches. In PNA probe **11** the TO is forced to intercalate at a chosen site within the sequence rather than hanging by a flexible tether. This intercalation mode rendered TO fluorescence sensitive to an adjacent base mismatch, as opposed to the situation in dual-labeled and end-labeled probes in which fluorescence is usually invariant once a matched or single-mismatched duplex has formed. The low emission in the presence of adjacent mismatches can be attributed to a locally increased flexibility of mismatched duplexes and less efficient planarization of TO. Remarkably, fluorescence of mismatched duplexes proved lower than fluorescence of matched duplexes at any temperature (data not shown). Match/mismatch discrimination was highest at elevated temperatures and reached factors of tenfold, which compare well with the discriminative power of dual-labeled hybridization probes.

Less pronounced abilities in distinguishing a target sequence from its single-base mutant at temperatures below the T_M value of mismatched probe–target complexes have been reported for HyBeacons[74] and 1,10-phenanthroline-modified bases.[50] Recently, fluorescent nucleotides comprised of benzo- and naphtho-annulated nucleobases and pyrene-modified pyrimidine bases have been described that sometimes show even higher match/mismatch selectivities (2–20-fold).[48–51,75] However, fluorescence enhancements in response to matched hybridization appear lower than those observed with TO as the fluorescent base, particularly in the presence of adjacent G–C base pairs. We envision that PNA probes such as **11** containing TO as an artificial base might be of use in applications where it is difficult to select stringent hybridization conditions, such as in real-time PCR and self-reporting arrays. Moreover, the unique fluorescence properties and known resistance of PNA to biological degradation suggests the use of these probes in live-cell analysis. However, prior to such ventures, a detailed analysis of TO fluorescence in different sequence contexts is needed.

Conclusion

It was the aim of this investigation to find a fluorescent base surrogate that is able to signal hybridization in a single-nucleotide specific manner, even under nonstringent hybridization conditions in which both matched and single-mismatched hybridization occur. Of the six thiazole orange base surrogates that were tested, the one linked by an acetate tether to the quinoline ring (TO_{Q1}) proved to be the best suited for the purpose. TO_{Q1} was most efficient in stabilizing a PNA–DNA duplex and conferring selectivity as far as pairing of the adjacent nucleobase was concerned. The hybridization-induced fluorescence enhancement of forced intercalation of thiazole orange probes (FIT probes) reached a factor of 26, which is in the range of the fluorescence intensifications obtained with commonly used dual-labeled probes. In contrast to common probes, FIT probes containing thiazole orange as a base surrogate show attenuated fluorescence enhancements upon formation of single-mismatched duplexes. This is an important result because it provides two levels of sequence discrimination. First, hybridization requires lower temperatures for the mismatched sequence than for the fully matched sequence, a typical feature of "conventional" hybridization probes. However, because the fluorescence enhancement of FIT probes is lower for mismatched sequences, this strategy might allow mismatched duplexes to form but can still discriminate them from fully matched duplexes. It hence appears possible to distinguish a target sequence from its single-base mutant even at temperatures below the T_M value of the mismatched probe–target complexes. Such properties could prove useful in real-time quantitative PCR analysis, array technology, and live-cell analysis.

Experimental Section

General methods and materials: Fluorescence spectroscopy was performed with a Varian Cary Eclipse fluorescence spectrophotometer. A Varian Cary 100 Bio-UV/Visible spectrophotometer was used for optical and melting analyses. DNA was purchased from MWG-Biotech in HPSF quality. Fmoc/Bhoc-protected PNA monomers were purchased from Applied Biosystems. Water was purified with a Milli-Q Ultrapure water purification system (Millipore Corporation).

T_M measurements: UV melting curves were measured at 260 nm. A degassed aqueous solution of 100 mM NaCl and 10 mM NaH_2PO_4 adjusted to pH 7.0 with 2 M NaOH or 2 M HCl (as required) was used as the buffer. The DNA and PNA oligomers were mixed in 1:1 stochiometry and the solutions were adjusted to a final duplex concentration of 1 μM. Prior to analysis, the samples were heated to 85 °C and cooled within 1 h to the starting temperature of 20 °C. The samples were heated to 85 °C at a rate of 1 °C min^{-1}. T_M values were defined as the maximum of the first derivative of the melting curve.

Fluorescence measurements: Stock solutions of the PNA probes (125–500 pmol μL^{-1} in H_2O) and the DNA targets (200–250 pmol μL^{-1} in H_2O) were prepared. PNA-probe solution was added into a fluorescence quartz cuvette (4×10 mm) and diluted with aqueous degassed buffer (100 mM NaCl, 10 mM NaH_2PO_4 at pH 7.0; 987–994 μL). The fluorescence spectrum (excitation: 510 nm; excitation slit width: 5 nm; emission slit width: 2.5 nm) was recorded at the specified temperature. DNA-target solution (1 nmol) was added to give a total volume of 1 mL and the fluorescence spectrum was recorded after 20 min.

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Keywords: fluorescence spectroscopy · fluorescent probes · peptide nucleic acids · single-nucleotide polymorphisms · solid-phase synthesis

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