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Ensemble hybridisation – a new method for exploring sequence dependent fluorescence of dye–nucleic acid conjugates

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The fluorescence of thiazole orange as artificial base in PNA was investigated in a nearest neighbour analysis; library-to-library hybridisation allowed the identification of probe sequences suitable for homogeneous DNA detection.

Base-modified oligonucleotides have become increasingly popular as probes of DNA–DNA and DNA–protein recognition.[1] Fluorescent nucleobases such as 2-aminopurine[2,3] and many others[4] are invaluable tools due to their ability to sense localized structural alterations, which is difficult to achieve by employing spacer-linked fluorophores. Recently, functional properties of DNA and the DNA-analogous PNA have been significantly expanded by replacing entire nucleobases with artificial fluorophores such as pyrene,[5] acridine,[6] thiazole orange,[7,8] and phenanthridinium.[9] The intimate contact to adjacent nucleobases inevitably gives rise to a pronounced sequence dependency of the fluorescence properties. However, knowledge about the optimal and suboptimal sequence contexts is essential for designing suitable probes. A full investigation of nearest neighbour influences requires the synthesis of 16 different oligomer–dye conjugates. Moreover, hybridisation experiments with 64 different complement strands would be needed in order to analyse for the interaction of intra-strand stacking and inter-strand pairing partners with the fluorescent base under scrutiny. In the following a rapid and convenient means of screening for preferred sequence contexts of base analogues is introduced. The new method draws upon library-to-library hybridisation and requires only four probe libraries and four target libraries.

We recently introduced thiazole orange (TO) as fluorescent base in PNA (Fig. 1).[8] It was observed that binding to complementary DNA strands was accompanied by strong increases in fluorescent emission. In addition, the fluorescence of TO proved responsive to changes in the immediate environment such as those imposed by mismatched base pairs. Such probes that fluoresce upon binding to perfectly complementary targets but show attenuated fluorescence upon binding to single-mismatched targets could prove useful for the homogeneous analysis of single base mutations. The interesting fluorescence properties of the 'thiazole orange base' called for a more detailed analysis of fluorescence in different sequence contexts.

Positional-scanning peptide libraries are useful to define the specificity of receptors and enzymes for peptide ligands and substrates.[10] Likewise, oligonucleotide libraries have been demonstrated to facilitate screening studies such as the identification of accessible hybridisation sites in folded nucleic acids.[11] We reckoned that the ability of nucleic acids to self-organise by Watson–Crick base pairing would allow for the design of a library-to-library hybridisation format. In applying this concept to the identification of optimal thiazole orange stacking and pairing partners, four PNA-probe libraries **1** were produced by split-mix synthesis (Fig. 2). In the PNA-ensembles **1**, the bases (**n**) on the *C*-terminal side of thiazole orange were varied. The *N*-terminal stacking partner [red in Fig. 2(a)] was fixed and contained adenine in library **1a**, cytosine in **1c** and guanine and thymine in **1g** and **1t**. These ensembles were hybridised with four 16-membered DNA libraries **2** such that TO was paired to a discrete base **Y** [blue in Fig. 2(a)] of the target strand and flanked by randomized base pairs **n·N** on the *C*-terminal side. The fixed stacking partner was allowed to pair against randomized bases **Z**. It was assumed that hybridisation of probe libraries **1a**, **1c**, **1g** and **1t** with an excess of target libraries **2A**, **2C**, **2G** and **2T** (A, C, G and T as TO pairing partner) would primarily result in the formation of matched duplexes. For example, addition of probe library **1a** to an excess of target library **2A** was expected to furnish duplexes **1a·2A** in which the *N*-terminal stacking partner **a** [red in Fig. 2(a)] would be paired to **Z** = T and in which base **n** would be complementary to base **N**.

Fig. 2(b) shows the increase in TO emission of probe libraries **1a**, **1c**, **1g** and **1t** in response to the addition of target libraries **2A**, **2C**, **2G** and **2T**. In total, 16 ensemble hybridisation experiments were performed. The strongest fluorescent increases (11–16-fold) were

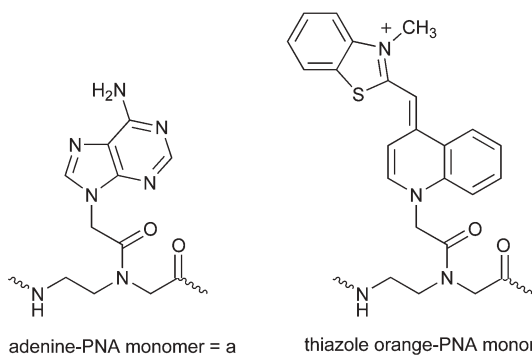

Fig. 1 Structure of an adenine– and the thiazole orange–PNA monomer.

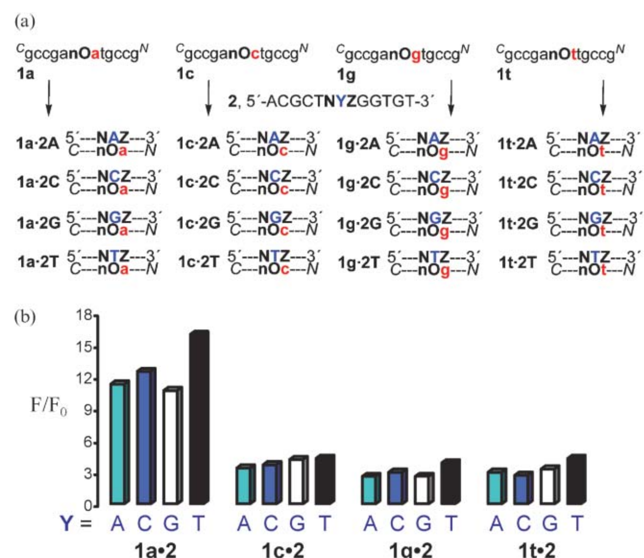

Fig. 2 (a) PNA-probe libraries **1a**, **1c**, **1g** and **1t** were allowed to bind to four DNA libraries **2A**, **2C**, **2G** and **2T** differing by bases marked in red and blue. (b) Relative fluorescence of matched duplexes containing PNA libraries **1** and their DNA-complement **2**. ('**O**' in bold face denotes the thiazole orange monomer; F_0,F = fluorescence at 529 nm before and after addition of target DNA; **N**,**n**,**Z** = mixture of A, G, C and T.)

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obtained with adenine as the fixed TO stacking partner in duplex ensembles containing probes **1a**. Hybridisation with C, T or G as fixed TO stacking partner in **1c**, **1t** and **1g**, respectively, resulted in rather modest fluorescence intensifications (3–4-fold). The influence of the pairing partner **Y** was less pronounced although a preference for thymine was noticeable. For example, formation of duplex ensembles **1a·2** in which TO was stacked against **a** and paired against T (**1a·2T**) was accompanied by a 16-fold fluorescence increase as compared to 12-, 13- and 11-fold intensifications measured with TO pairing partners **a** (**1a·2A**), C (**1a·2C**) and G (**1a·2G**), respectively. There seemed to be only little interdependence between stacking and pairing partners since a small but measurable preference for thymine occurred in all stacking sets. These experiments represent, to the best of our knowledge, the first combinatorial evaluation of the sequence context of base surrogates and suggest adenine as optimal TO stacking partner.

It was assumed that self-organisation would result in the formation of **1·2** duplexes in which DNA base **Z** would be complementary to the defined TO stacking partner (red in **1**). To test this hypothesis, PNA-libraries **1a**, **1c**, **1g** and **1t** were hybridised with DNA-libraries **2TT**, **2TG**, **2TC** and **2TA**, respectively, containing only one randomized position **N** (Table 1, top). The resulting duplex ensembles **1a·2TT**, **1c·2TG**, **1g·2TC** and **1t·2TA** featured TO (**O**) paired to T and flanked by discrete base pairs and thereby represented a sub-set of duplex ensembles **1a·2T**, **1c·2T**, **1g·2T** and **1t·2T**. Also in this more defined environment, the sequence dependence of fluorescence enhancements was virtually unchanged. The strongest fluorescence intensification (20-fold) was again measured for the formation of a discrete a·T base-pair next to TO (**1a·2TT**).

The ensemble measurements suggested that highest fluorescence enhancements should be observed with adenine as the TO neighbour. For further examination, eight model duplexes were explored, in which TO was embedded in a defined sequence of contexts (Table 1). It became apparent that hybridisation of PNAs that contained adenine next to TO (**3a·4T**, **3c·4G**, **3g·4C**, **3t·4A** and **5a·7T**) resulted in strong fluorescence enhancements (12- to 24-fold). Without adenine as the TO stacking partner in PNA-probes **5c**, **5g** and **5t** led to rather modest enhancements (4- to 6-fold). The highest fluorescence enhancement was observed when TO was 'sandwiched' between two adenines (**3a**). In contrast to most other fluorescent bases, the presence of guanine (**3g**, **5a**) did not result in quenching of fluorescence.

Table 1 Fluorescence data of thiazole orange containing PNA–DNA duplexes

5' -CGGCT**N**T**Y**ACGGC-3' Y=A, **2TA**; C, **2TC**; G, **2TG**; T, **2TT**
^{C}Gly-gccga**nOx**tgccg$^{N\text{-}Ac}$ x=A, **1a**; C, **1c**; G, **1g**; T, **1t**

x–Y	a–T	c–G	g–C	t–A
F/F_0 [a]	20	6	5	5

5' -CGGCTA**Y**T**T**ACGGC-3' Y=A, **4A**; C, **4C**; G, **4G**; T, **4T**
^{C}Gly-gccgat**xOa**tgccg$^{N\text{-}Ac}$ x=A, **3a**; C, **3c**; G, **3g**; T, **3t**

x–Y	a–T	c–G	g–C	t–A
F/F_0 [a] (F_0)	24 (3.1)	20 (2.8)	15 (7.3)	14 (2.8)

5' -CGGCTA**Y**T**C**ACGGC-3' Y=A, **7A**; C, **7C**; G, **7G**; T, **7T**
^{C}Gly-gccgat**xOg**tgccg$^{N\text{-}Ac}$ x=A, **5a**; C, **5c**; G, **5g**; T, **5t**

x–Y	a–T	c–G	g–C	t–A
F/F_0 [a] (F_0)	12 (4.5)	6 (4.8)	4 (14.9)	5 (6.1)

[a] F_0 = fluorescence intensity of the PNA single strand; F = fluorescence intensity after addition of DNA. Measurement conditions: 1 μM concentration in degassed buffer (100 mM NaCl, 10 mM NaH_2PO_4, pH 7); excitation: 510 nm; excitation slit: 4 nm; emission: 525 nm; emission slit: 2.5 nm, 25 °C.

The fluorescence properties of PNA conjugates **1**, **3** and **5** are governed by the TO chromophore. Intercalative stacking results in coplanarisation of the two heterocycles, a necessary requirement for fluorescence.[12] For a 'positive' responsiveness single strand fluorescence F_0 must be low while duplex fluorescence F has to be high. In the majority of the PNA–TO conjugates that exhibited high fluorescence increases F/F_0 was characterised by relatively small F_0 values, with **3g** being the only exception. It hence appears plausible to assume that the performance of a given PNA–TO conjugate is governed to a large extent by the fluorescence properties (F_0) of the single strand. This finding is in agreement with results obtained with end-labelled PNA–TO conjugates (Light-Up probes).[13] In contrast to these probes, TO in PNA conjugates such as **1**, **3** and **5** is forced to intercalate at a specific site rather than being appended by a flexible tether. The single strand fluorescence F_0 should hence be predominantly influenced by the intramolecular TO stacking neighbour. Indeed, the hybridisation experiments uncovered the particular role of the stacking partner. While adenine seemed to confer a low fluorescence F_0 in the single stranded form and a high fluorescence F in the duplex form (and thus high fluorescence enhancements F/F_0), both F_0 and F were high (and thus F/F_0 was low) when TO was flanked by two guanine bases (**5g**). Interestingly, G–C pairs in duplex DNA have been reported as preferred binding sites for the dimeric TO dye TOTO.[14] It may be of importance to prevent undesired stacking in the single strand by choosing 'suboptimal' stacking environments.

In conclusion, we have investigated ensemble hybridisations as a new approach to explore sequence dependent fluorescence properties of PNA conjugates containing TO as artificial base. The collected data from ensemble measurements and sequence-defined hybridisation are in agreement and allow us to draw the conclusion that the fluorescence of the thiazole orange base surrogate is most responsive to hybridisation when TO is flanked by adenine. At ambient temperature hybridisation led to up to 24-fold increases in TO emission. Even higher fluorescence enhancements were recorded at elevated temperature (data not shown). It is expected that the described ensemble hybridisation approach should hold applicable for the rapid and cost-efficient nearest neighbour analysis of any kind of fluorescent base in PNA, DNA and other nucleic acid type oligomers.

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