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Fluorescent Hydrophobic Zippers inside Duplex DNA: Interstrand Stacking of Perylene-3,4:9,10-tetracarboxylic Acid Bisimides as Artificial DNA Base Dyes

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Abstract: Perylene-3,4:9,10-tetracarboxylic acid bisimides (PBs) were incorporated synthetically into oligonucleotides by using automated DNA building-block chemistry. The 2′-deoxyribofuranoside of the natural nucleosides was replaced by (*S*)-aminopropan-2,3-diol as an acyclic linker between the phosphodiester bridges that is tethered to one of the imide nitrogen atoms of the PB dye. The *S* configuration of this linker was chosen to mimic the stereochemical situation at the 3′-position of the natural 2′-deoxyribofuranosides. By using this strategy, up to six PB dyes were incorporated in the middle of 18-mer DNA duplexes by using interstrand alternating sequences of PBs with thymines or an abasic site analogue. Both PB dimers and PB hexamers as artificial base substitutions inside the duplexes yield characteristic excimer-type fluorescence. The stacking properties of the PB chromophores are modulated by the presence or absence of thymines opposite the PB modification site in the counterstrand. The interstrand PB dimers can be regarded as hydrophobically interacting base pairs, which display a characteristic fluorescence readout signal. Hence, for the PB hexamers, we proposed a zipperlike recognition motif that is formed inside duplex DNA. The PB zipper shows characteristic excimer-type emission as a fluorescence readout signal for the pairing interaction.

Keywords: excimers · fluorescence · oligonucleotides · self-assembly · stacking interactions

Introduction

Nucleosides, oligonucleotides, and DNA play an increasingly significant role for the design and construction of nanostructures and functional π systems.[1] In a bottom-up approach, these systems are realized by composing small synthetic building blocks with recognition, structuring, and most importantly, self-assembly properties, preferably through hydrogen-bonding or π-stacking interactions.[2,3] Nucleotides fit perfectly into this building-block strategy:

1) Self-assembly: Two oligonucleotides bind sequence selectively through the canonical Watson–Crick pairing into a duplex structure.
2) Regular helix: B-DNA is a predictable topology with a base-pair distance along the helical axis of 3.4 Å that is ideal for hydrophobic and photophysical interactions of chromophores.
3) Synthesis: The well-established automated oligonucleotide chemistry represents a building-block strategy that can be applied for modifications and functionalization.
4) Hierarchical structure: Based on the DNA helix as a regular secondary structure, sequence-specific recognition by DNA-binding proteins and complex tertiary structures can be realized.

π Arrays and clusters of organic chromophores have been of considerable interest because of their properties, which differ significantly from those of the monomeric state and which have potential applicability in molecular devices. The clear structural scaffold of duplex DNA is potentially useful for such π systems because their functional properties depend on the relative orientation and resulting photophysical interactions of each of the molecular components with the others. DNA-based photonic wires have been developed by using multistep electronic exciton migration.[4] Accordingly, DNA has been applied as a template for the helical assembly of noncovalently bound chromophores, for example, cyanine dyes, in the minor groove.[5,6] Over the last few years, a number of publications have appeared that describe

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the application of the structural architecture of oligonucleotides for the self-assembled arrangement of organic chromophores as either 1) DNA base substitutions inside the duplex or as 2) DNA base modifications outside the duplex. In both cases, the arrangements allow the chromophores to interact with each other photophysically.

1) In a combinatorial approach by the group of Kool, four different fluorosides have been used as C-nucleosidic DNA base substitutes in a completely artificial DNA base stack to yield astonishing new fluorescence properties.[7] Similar results were obtained by Inouye and co-workers by using fluorophores that are linked with acetylene bridges to the 2′-deoxyribose moiety.[8] Asanuma and co-workers have incorporated several azobenzene derivatives, for example, the methyl red and naphthyl red dyes, in DNA as base surrogates that form ordered clusters inside the helical duplex.[9] By using the *cis–trans* isomerism of the azo bridge, the zipperlike chromophore clusters act as photoswitchable regulators for DNA hybridization. The group of Häner observed stacked clusters of phenanthrenes, phenanthrolines, and pyrenes in duplex DNA with the corresponding non-nucleosidic base surrogates.[10] Interestingly, the oligopyrenes self-assemble inside duplex DNA according to a strong excitonic CD signal. Leumann and co-workers showed that the interstrand aromatic stacking of bipyridine and biphenyl derivatives as C-nucleosides in DNA can be applied as a zipperlike recognition motif inside DNA.[11]
2) Organic chromophores can be attached to the DNA bases or to the 2′-position of the ribofuranoside moiety of RNA. A helical pyrene array along the outside of duplex RNA that was prepared by using the latter approach was described recently by Nakamura, Yamana, and co-workers. They described a significant pyrene-excimer fluorescence enhancement.[12] In a structurally similar fashion, locked nucleic acids have been functionalized by multiple pyrene derivatives.[13] These locked nucleic acids exhibit promising fluorescence properties and are able to sense full complementarities of the DNA or RNA counterstrands. Recently, we showed that a helical and regularly structured π array of 1-ethynylpyrene groups that have been covalently attached to the 5-position of uridines can only be formed if more than three chromophores are placed adjacent to each other.[14] Similar DNA systems with five adjacent pyrene-modified uridine units showed a remarkably strong fluorescence enhancement, which is sensitive to DNA base mismatches and thermal denaturation of the duplex.[15] DNA duplexes that have been functionalized by a helical stack consisting of five adjacent chromophores with mixed pyrene-modified or phenothiazine-modified uridine sequences show a modulated fluorescence readout.[16]

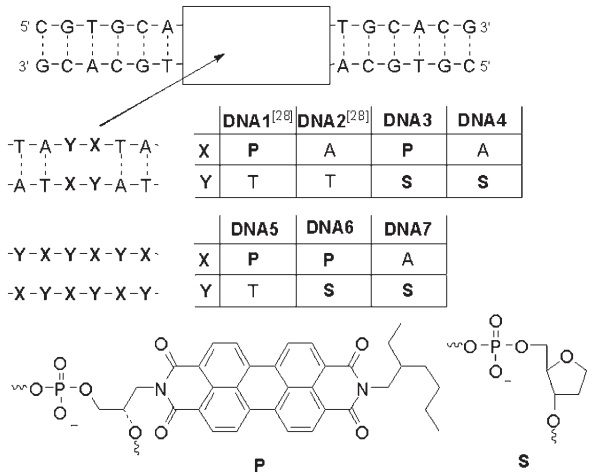
The strong hydrophobic stacking interaction of perylene-3,4:9,10-tetracarboxylic acid bisimide (PB) makes this chromophore an important building block for functional supramolecular architectures and organic multichromophore nanomaterials.[3,17,18] When it was taken into account that PB dyes exhibit excellent photochemical stability, as well as high fluorescence quantum yields, it looked reasonable to apply PB as a chromophore for the construction of a self-assembled and hydrophobically interacting stack inside DNA. The noncovalent DNA-binding interactions were studied with PB derivatives that have been modified with spermine[19] or other amines.[20] An increasing number of publications have become available over the last few years about the covalent modification of oligonucleotides with PB derivatives. In particular, perylene bisimide derivatives have been covalently attached to oligonucleotides for the DNA-directed assembly of this dye[21] in thermophilic foldamers,[22] as caps for DNA hairpins,[23–25] and for the construction of exceptionally stable triplexes.[26]

For the incorporation of PB as an artificial base surrogate at specific sites in DNA, we presented a synthetic route involving the automated phosphoramidite building-block strategy.[27] Moreover, we could show that the incorporation of PB dimers as artificial DNA base substitutions yields a characteristic excimer-type fluorescence inside the DNA duplex.[28] The PB dimer that is formed in an *interstrand* mode can be regarded as a hydrophobically interacting "diagonal" base pair with a fluorescence readout signal for the pairing interaction. The *intrastrand* PB dimer as a clamp around a questionable site of base mismatches or base deletions allows the detection and quantification of the amount of matched counterstrand. Herein, we want to present the extension of the optical DNA functionalization from two PB dyes up to six PB dyes in a row inside the duplex.

Results and Discussion

The synthesis of the PB-modified oligonucleotides was performed according to our published procedure.[27,28] The 2′-deoxyribofuranoside of natural nucleosides was replaced by an acyclic linker that is tethered to one of the imide nitrogen atoms of the PB dye. We showed that this linker allows different chromophores to intercalate in the base stack while facilitating the synthesis of the corresponding phosphoramidites.[27–30] The *S* configuration of this linker was chosen to mimic the stereochemical situation at the 3′-position of natural 2′-deoxyribofuranosides.[31–33] All synthesized duplexes (**DNA1**–**DNA7**) contain palindromic sequences to ensure quantitative duplex formation in the samples for spectroscopic measurements (Scheme 1).

Similar to the previously reported **DNA1**,[28] duplex **DNA3** bears one interstrand PB dimer inside the duplex. However, the thymines opposite the two PB dyes in the counterstrand of **DNA1** were replaced by the abasic site analogue **S** to allow optimal intercalation of the PB dye in **DNA3. S** represents a chemically stable analogue for the natural abasic site.[33] **DNA2**[28] and **DNA4** were synthesized as reference duplexes without the dye. **DNA5** and **DNA6** contain six PB dyes in the middle of the duplex in the form



	DNA1[28]	DNA2[28]	DNA3	DNA4
X	P	A	P	A
Y	T	T	S	S

	DNA5	DNA6	DNA7
X	P	P	A
Y	T	S	S

Scheme 1. Sequences of the duplexes **DNA1**–**DNA7** (**DNA1** and **DNA2** have been reported previously[28]).

of interstrand alternating sequences. In **DNA5**, natural thymines were placed opposite the PB dyes. In **DNA6**, the abasic site analogue **S** was chosen instead of those thymines. **DNA7** represents another reference duplex without the PB dye. In comparison with the fully unmodified duplex **DNA2**,[28] **DNA4** and **DNA7** allow the effects of the PB chromophores to be separated from those of the abasic site analogues **S** with respect to the thermal stabilities of **DNA3** and **DNA6**, respectively.

The UV/Vis spectra of **DNA3**, **DNA5**, and **DNA6** (Figure 1) at low temperatures are different from the corresponding spectra of the PB monomer in oligonucleotides:[27,28] All three absorption bands are shifted bathochromically and the relative ratios of the absorption bands at 506 and 545 nm are significantly changed in comparison with the relative ratio of the 0→1 and 0→0 vibronic transitions of the PB monomer in oligonucleotides. Both observations show the strong π–π excitonic interactions of the two PB dyes inside **DNA3** or the six PB moieties in **DNA5** and **DNA6**, respectively.[22a,34] At higher temperatures, there is a remarkable difference between **DNA3** and the duplexes **DNA5**/**DNA6**. In **DNA3**, the absorption maxima shift to those that are typical for a single PB modification in an oligonucleotide.[27] Additionally, the relative ratio of the absorption maxima resembles that of the monomeric form of PB. It is remarkable that both changes, the absorption shift and the change of peak ratio, occur between 70 and 80 °C, the range of the melting temperature of this duplex (T_m = 78.3 °C, Table 1). The temperature-dependent absorption behavior of **DNA3** is similar to that of the previously reported **DNA1**.[28] When the T_m values are measured at 540 nm, the PB dyes dehybridized at an only slightly higher temperature in the presence of the abasic site **S** (**DNA1**: 75.9 °C; **DNA3**: 77.4 °C). Thus, the absence of the "counterbase" thymine in **DNA3** has only a minor influence on the PB stacking interactions. However in both duplexes, **DNA1** and **DNA3**, the intact helical duplex is required as a framework for the formation of excitonic interactions between the PB dyes.

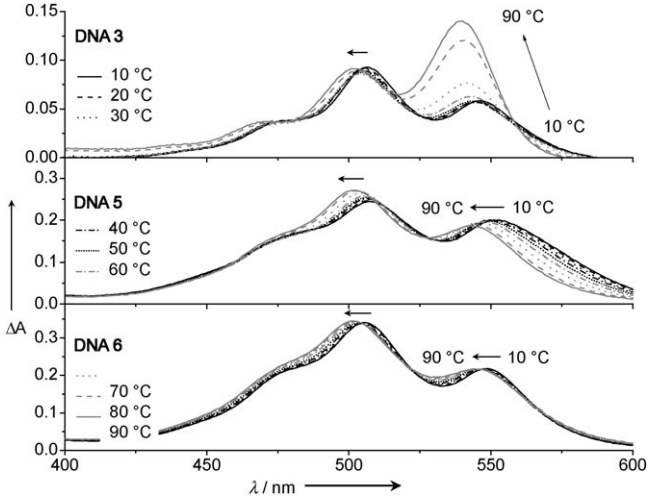


Figure 1. Temperature-dependent UV/Vis spectra of **DNA3** (top), **DNA5** (middle), and **DNA6** (bottom). Conditions: 1.25 μM duplex in 10 mM Na-P_i buffer (pH 7.0) with 250 mM NaCl.

Table 1. Melting temperatures (T_m) of **DNA1**–**DNA7**.[a]

Duplex	T_m [°C]	Duplex	T_m [°C]
DNA1[28]	78.6[28]	**DNA2**	76.2[28]
	75.9 (540 nm)		
DNA3	78.3	**DNA4**	76.9
	77.4 (540 nm)		
DNA5	72.2		
DNA6	66.6	**DNA7**	69.3

[a] Conditions: 2.5 μM duplex, 10 mM Na-P_i buffer (pH 7.0), 250 mM NaCl; 260 nm; 10–90 °C; rate: 0.7 °C min^{-1}.

In contrast to the results for **DNA3**, the absorption spectra of **DNA5** and **DNA6** do not show such significant changes at higher temperatures, although both duplexes exhibit a characteristic cooperative T_m value (72.2 and 66.6 °C, respectively). Only a small hypsochromic shift of the absorption bands is observed (6–8 nm for **DNA5**, 2–5 nm for **DNA6**) but the relative ratios of the peak heights remain nearly unchanged. Obviously, the ground-state interactions between the PB dyes persist even in the dehybridized form. This means that, above the T_m value, the interstrand interactions between the six PB dyes in the duplex change to intrastrand interactions of three PB chromophores in the random-coiled single strand. This interpretation is also supported by the absorption shifts that occur between 10 and 90 °C. These absorption shifts are stronger with **DNA5** than with **DNA6**, which indicates a bigger structural change of the PB chromophores due to dehybridization in the presence of the intervening thymine bases.

A more detailed look into the thermal denaturation studies (Table 1) reveals a stabilization of 1.4 °C in **DNA3** relative to **DNA4**, which also bears two **S** sites but no PB chromophores, and a stabilization of 2.1 °C relative to the fully unmodified **DNA2**. Interestingly, the incorporation of two

abasic site analogues in **DNA4** does not change the T_m value significantly relative to that of **DNA2**. Thus, the observed stabilization of duplex **DNA3** must be mainly the result of interstrand hydrophobic interactions between the two PB chromophores. This observation becomes remarkable only with respect to the fact that a glycol linker as a single substitution for a 2-deoxyribofuranoside typically reduces the duplex stability significantly.[29,32]

The thermal stabilities of **DNA5** and **DNA6**, each bearing six chromophores inside the duplex, are both reduced. In comparison to the unmodified **DNA2**, duplex **DNA5** is strongly destabilized by 4.0 °C. Without any PB chromophores, the incorporation of six **S** moieties instead of thymines in **DNA7** destabilizes the duplex by 6.9 °C; further replacement of the adenines by the PB dyes in **DNA6** destabilizes the duplex by an additional 2.7 °C. In the latter case, no stability is regained by the hydrophobic interaction of the PB chromophores. Obviously, the DNA duplex framework is able to tolerate one interacting PB dimer but not three of them without losing significant thermal stability.

The fluorescence spectra of **DNA3**, **DNA5**, and **DNA6** excited at 505 nm at low temperatures (Figure 2) are dominated by a broad band without fine structure at ≈660 nm; this band represents the excimer-type emission of the PB dye because it has been observed in nanoaggregates of perylene bisimides.[22a,34] Remarkably, **DNA5** and **DNA6** show exclusively the excimer-type fluorescence band and no PB-monomer fluorescence. However, based on the comparison of the steady-state fluorescence spectra, the emission intensity of **DNA6** is reduced to ≈20% relative to that of **DNA5**. This observation indicates a self-quenching of the stacked PB dyes inside duplex **DNA6**. Obviously, the presence of the thymine moieties in **DNA5** restricts the assembly of the PB dyes in such a way that self-quenching is reduced. This scenario will be discussed in more detail with respect to the CD spectra (see below). The thermal denaturation of both duplexes, **DNA5** and **DNA6**, does not regain significant amounts of the monomeric PB emission at ≈550 nm. As already pointed out in the discussion about the absorption spectra, this result indicates that the interstrand interactions between the six PB dyes in the duplex change to intrastrand interactions of three PB chromophores in the random-coiled single strand above the T_m value. However, in comparison with the PB-DNA building block ($\Phi_F = 0.73$), the PB monomer fluorescence is quenched inside single-stranded and duplex DNA ($\Phi_F < 0.01$).[27]

The thermal behavior is different with **DNA3**. At higher temperatures, but still below the T_m value, the excimer-type fluorescence intensity increases. This is an observation that is typical for excited dimers and was used for PB-assisted folding of oligonucleotides.[22] At temperatures above the T_m value, the excimer-type fluorescence band of this duplex vanishes and the monomeric PB fluorescence is regained. This transition occurs cooperatively at a similar temperature (between 70 and 80 °C) to the thermal dehybridization of the whole duplex ($T_m = 78.3$ °C). Obviously, the intact helical duplex is required as a framework for the PB excimer-type fluorescence.

Finally, CD spectroscopy was applied to gain more insight into the excitonic interactions of the PB dyes inside **DNA1**, **DNA3**, **DNA5**, and **DNA6** (Figure 3). The CD spectra of **DNA1**, **DNA3**, and **DNA6** at 20 °C display a strong negative band at ≈500 nm and a strong positive band at ≈560 nm. Remarkably, the CD spectrum of **DNA5** shows a mirror image, that means a strong positive signal at ≈500 nm and a strong negative signal at ≈560 nm. All observed CD bands can be attributed to the exciton coupling between the PB chromophores. According to Lewis and co-workers,[25] the intensity of positive and negative bands of the exciton-coupled CD spectra, $\Delta\varepsilon$, for two identical chromophores with parallel oriented planes is dependent on the angle between the transition dipoles, θ, and follows the function $\sin(2\theta)$. The $\sin(2\theta)$ dependence gives zero intensities for the CD

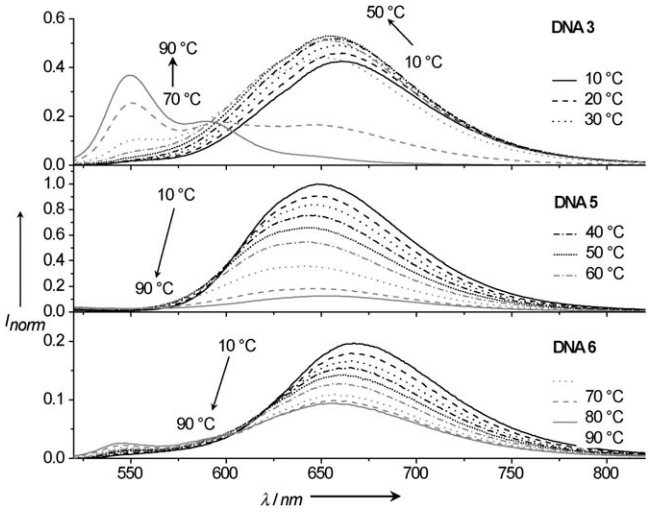


Figure 2. Temperature-dependent fluorescence spectra of **DNA3** (top), **DNA5** (middle), and **DNA6** (bottom). Conditions: 1.25 μM duplex in 10 mM Na-P_i buffer (pH 7.0) with 250 mM NaCl; excitation at 505 nm.

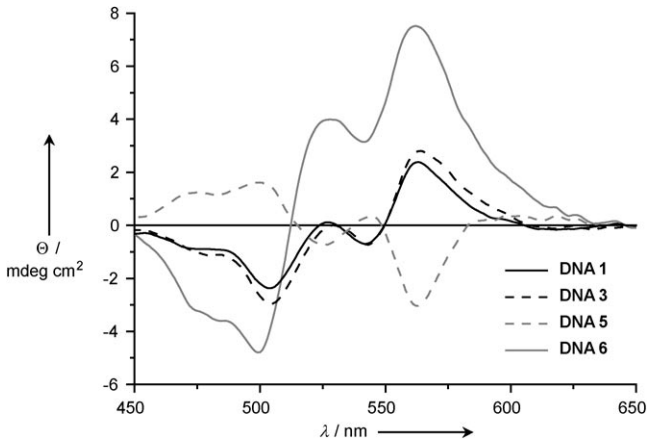


Figure 3. CD spectra of **DNA1**, **DNA3**, **DNA5**, and **DNA6**. Conditions: 1.25 μM duplex in 10 mM Na-P_i buffer (pH 7.0) with 250 mM NaCl at 20 °C.

signals at $\theta = 0$, 90, and 180° and the highest intensities at $\theta = 45$ and 135°. Unconstrained PB dyes in aggregates are typically aligned and give no or just low CD signals. Hence, the significant CD signals that have been obtained for all four PB-modified duplexes (**DNA1**, **DNA3**, **DNA5**, and **DNA6**) reveal a nonaligned (that means helical) arrangement of the PB dyes inside the DNA. Obviously, the structural scaffold of duplex DNA constrains and therefore directs helical PB assembly inside the duplex. The typical CD spectrum of a B-DNA helix shows a strong negative signal below 260 nm and a strong positive signal above 260 nm. We have obtained a similar sequence of CD signals for the assembly of covalently attached pyrenes in the major groove of duplex DNA.[14,15] It is assumed that the right-handed sense of the helical conformation of the DNA around the PB dimers in **DNA1** and **DNA3** forces the PB chromophores in both duplexes to aggregate by following the sense of the right-handed helix. Moreover, the similarities in the shapes of the CD signals for **DNA1**/**DNA3** and **DNA6** indicate a right-handed helical arrangement of the six PB chromophores also in **DNA6**, but not in **DNA5**. Obviously, the absence of sterically hindering thymine bases in the counterstrand of **DNA6** allows this type of helical aggregation.

Although the signal intensities are reduced, the CD spectrum of **DNA5** shows a mirror image of the corresponding spectrum of **DNA6**. Obviously, the presence of thymines as the bases opposite the PB dye in the counterstrand forces the PB chromophores to assemble in a different way to that in **DNA6**, as already indicated by the reduced self-quenching in **DNA5**. If the right-handed helix of B-DNA is taken as the standard, the occurrence of a mirror image to **DNA1**, **DNA3**, and **DNA6** means an at least partial left-handed helical arrangement of the PB chromophores in duplex **DNA5**. The question is how such a structural scenario can be realized in the right-handed duplex framework. We calculated geometries for duplex **DNA5** by using the CHARMM force field. We applied the canonical B-form DNA conformation of duplex **DNA2** and replaced six adenines by the PB chromophores. Indeed, the calculated structure (Figure 4) gives a possible explanation for the mirrored CD signal. If the right-handed helix of this duplex is followed, the angles, θ, between the chromophores follow an alternating sequence of 35–45° (left) and 85–95° (right). As explained above, angles of around 90° do not contribute significantly to the CD spectrum, whereas angles of around 45° do contribute. Hence, the observable CD signal of **DNA5** is only the result of the PB interactions that are following the left-handed orientation.

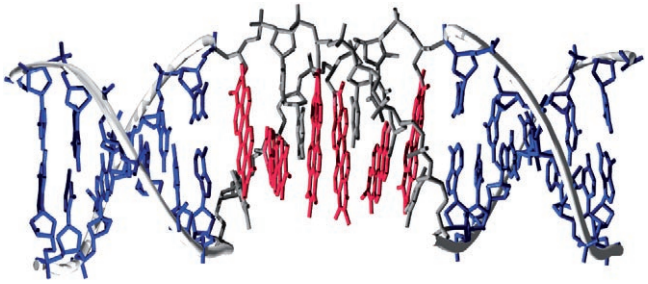


Figure 4. Minimized geometry of **DNA5** with the PB chromophores drawn in red.

Conclusion

PB dimers and hexamers as artificial DNA base substitutions yield a characteristic excimer-type fluorescence inside the DNA duplex. The interstrand PB dimers of **DNA1** and **DNA3** can be regarded as hydrophobically interacting base pairs. Hence, we proposed a zipperlike recognition motif that is formed inside duplex DNA for the PB hexamers in **DNA5** and **DNA6**. The PB zipper shows a characteristic excimer-type emission as a fluorescence readout signal for the pairing interaction. The excitation wavelength of the PB dimer and oligomers (505 nm) is in the range of typical bioanalytical fluorescence readers. In conjunction with the high photostability that is typically observed for the PB chromophores, multichromophoric oligonucleotides that are accompanied by a red-shifted excimer-type fluorescence upon DNA hybridization have significant potential for applications in chemical biology, such as gene or RNAi delivery to cells, as well as in molecular diagnostics, such as the detection of base deletions or mutations.[2]

Experimental Section

Materials and methods: ESIMS spectra were measured in the analytical facility of the institute. All spectroscopic measurements were performed in quartz glass cuvettes (1 cm) and by using Na-P_i buffer (10 mM). Absorption spectra and the melting temperatures (1.25 μM duplex, 250 mM NaCl, 260 nm, 10–90 °C, interval 0.7 °C) were recorded on a Varian Cary 100 spectrometer equipped with a 6×6 cell-changer unit. CD spectroscopy (1.25 μM duplex, 200–650 nm) was performed on a Jasco J-715 spectropolarimeter. The fluorescence spectra (1.25 μM duplex) were recorded on a Fluoromax-3 fluorimeter (Jobin-Yvon) and corrected for Raman emissions from the buffer solution. All emission spectra were recorded with a band pass of 2 nm for both excitation and emission and are intensity corrected. Molecular modeling was performed by using the Hyperchem 7.5 software package from Hypercube, including the CHARMM force field.

Preparation of unmodified and S-modified oligonucleotides (general procedure): The oligonucleotides were prepared on an Expedite 8909 DNA synthesizer from Applied Biosystems by standard phosphoramidite protocols with chemicals and CPG (1 μmol) from Applied Biosystems and Proligo. The phosphoramidite for the abasic site analogue **S** was purchased from Glen Research. After preparation, the trityl-off oligonucleotide was cleaved from the resin and deprotected by treatment with concd NH_4OH at 60 °C for 10 h. The oligonucleotide was dried and purified by HPLC on a semipreparative RP-C18 column (300 Å, Supelco) by using the following conditions: A: NH_4OAc buffer (50 mM), pH 6.5; B: MeCN; gradient: 0–15 % B over 45 min. MS (ESI): single-stranded (ss) **DNA4**: *m*/*z* calcd: 5372.0; found: 1345 [M^{4-}], 1793 [M^{3-}]; ss **DNA7**: *m*/*z* calcd: 5125.1; found: 1283 [M^{4-}], 1710 [M^{3-}]. The oligonucleotides were lyophilized and quantified by their absorbance at 260 nm on a Varian Cary 100 spectrometer: $\varepsilon_{260} = 169\,400\ \mathrm{L\,mol^{-1}\,cm^{-1}}$ for ss **DNA4**; $\varepsilon_{260} = 153\,500\ \mathrm{L\,mol^{-1}\,cm^{-1}}$ for ss **DNA7**. Duplexes were formed by heating to 90 °C (10 min) followed by slow cooling.

Preparation of PB-modified oligonucleotides: These were prepared and purified according to the published procedure.[27] The only difference was the purification of the PB-DNA building block by flash chromatography (SiO_2, CH_2Cl_2:acetone (10:1)) prior to the oligonucleotide synthesis, as already described for **DNA1**.[28] MS (ESI): ss **DNA3**: *m*/*z* calcd: 5697.6; found: 1426 [M^{4-}], 1901 [M^{3-}]; ss **DNA5**: *m*/*z* calcd: 6472.6; found: 1620 [M^{4-}], 2159 [M^{3-}]; ss **DNA6**: *m*/*z* calcd: 6100.8; found: 1526 [M^{4-}], 2035 [M^{3-}]. The PB-modified oligonucleotides were quantified by their absorbance in DMSO on a Varian Cary 100 spectrometer by using $\varepsilon_{528} = 62500\,L\,mol^{-1}\,cm^{-1}$ for ss **DNA3** and $\varepsilon_{500} = 79600\,L\,mol^{-1}\,cm^{-1}$ for ss **DNA5** and ss **DNA6**. Duplexes were formed by heating to 90 °C (10 min) followed by slow cooling.

Acknowledgements

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