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Pyrene-modified guanosine as fluorescent probe for DNA modulated by charge transfer

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Abstract—8-(Pyren-1-yl)-2′-deoxyguanosine (Py-G) was incorporated synthetically as a modified DNA base and optical probe into oligonucleotides. A variety of Py-G-modified DNA duplexes have been investigated by methods of optical spectroscopy. The DNA duplex hybridization can be observed by both fluorescence and absorption spectroscopy since the Py-G group exhibits altered properties in single strands versus double strands for both spectroscopy methods. The fluorescence enhancement upon DNA hybridization can be improved significantly by the presence of 7-deazaguanin as an additional modification and charge acceptor three bases away from the Py-G modification site. Moreover, Py-G in DNA can be applied as a photoinducable donor for charge transfer processes when indol is present as an artificial DNA base and charge acceptor. Correctly base-paired duplexes can be discriminated from mismatched ones by comparison of their fluorescence quenching.

1. Introduction

Routine analytical problems of molecular diagnostics and biomedicine demand powerful and versatile DNA labels for optical spectroscopy. A variety of organic dyes have been investigated as covalently attached optical probes for DNA analytics.[1–6] Recently, the detection of single nucleotide polymorphisms by fluorescent DNA base substitutes has been successfully achieved using ethidium,[7] thiazole orange[8] or pyrene derivatives.[9] Moreover, there is an increasing demand for optical DNA hybridization labels that change not only their emission but also their absorption properties as a result of the duplex formation.[10] One suitable way to create such duplex-sensitive optical properties is to attach organic chromophores covalently to DNA bases. Over the last 5 years, we used this kind of modification strategy for the investigation of DNA-mediated electron transfer processes[11,12] and for the development of versatile and tunable fluorescent probes for DNA.[13,14] We recently described preliminarily 8-(pyren-1-yl)-2′-deoxyguanosine (Py-G) as a duplex-sensitive probe for absorption and fluorescence spectroscopy with DNA.[15] Herein, we want to describe our recent studies showing that charge transfer processes enhance the duplex/single-strand discrimination and are influenced by base mismatches, which is observable by fluorescence spectroscopy.

2. Results and discussion

2.1. Pyrene-modified guanosine as an optical label and charge donor

As previously published, the pyrene chromophore attached to the guanine base can serve as a photoinducable donor for positive charges since excitation of the pyrene group in Py-G results in formation of the guanine radical cation as the product of an intramolecular hole transfer ($Py^{\cdot-}$-$G^{\cdot+}$).[16,17] Based on $E_{00} = 3.25$ eV for Py*,[18] and using $E_0(Py/Py^{\cdot-}) = -1.9$ V[19] and $E_0(G^{\cdot+}/G) = 1.3$ V,[20] the driving force ΔG of this process is max. −0.1 eV. This charge transfer assignment has been proven by picosecond transient absorption experiments using benzopyrenyl-2′-deoxyguanosine conjugates.[21,22] Due to our experience with the photoinduced charge-separation in such pyrene-modified DNA bases[15–17,23] and with respect to literature[24] we expected that the modified guanosine Py-G exhibits an exciplex-type fluorescence due to the strong electronic coupling between the guanine and the pyrene moiety via the

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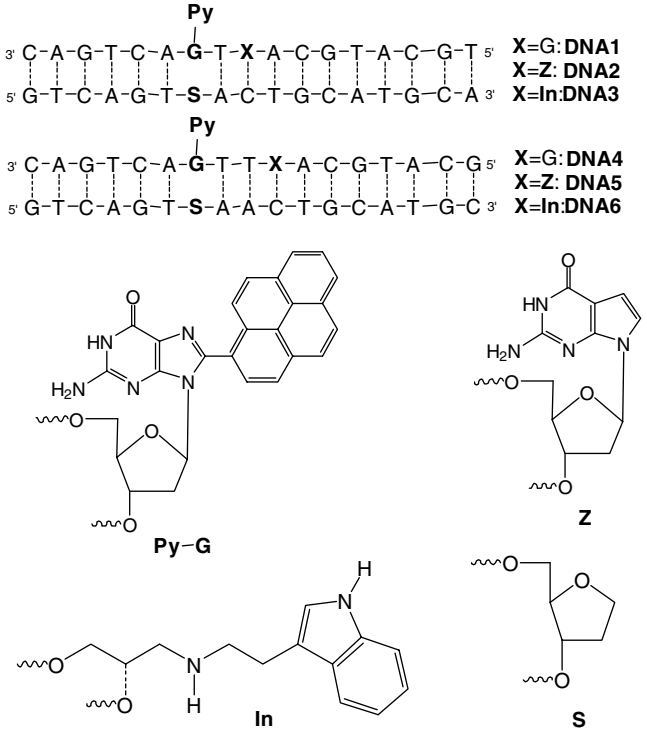
single C–C bond. The exciplex-type emission typically exhibits a strong solvent dependence. Thus, a DNA duplex sensitive emission of Py-G is expected considering the DNA base stack as an aromatic 'solvent' that is different to the aqueous buffer solution outside the helix. The modified nucleoside Py-G was prepared via a Suzuki–Miyaura-type cross-coupling between 8-bromo-2′-deoxyguanosine and 1-pyrenyl boronic acid.[17] We investigated the isolated modified nucleoside Py-G by NMR (NOESY) showing that the presence of the pyrene forces the guanosine into the *syn*-conformation.[17] Therefore, we expected that the local DNA conformation around the Py-G modification site is perturbed in such a way that the pyrene moiety is partially intercalated.

Using the standard phosphoramidite DNA building block strategy and automated DNA synthesis we prepared two sets of Py-G-modified duplexes **DNA1**–**DNA3** and **DNA4**–**DNA6** (Scheme 1). Although it has been shown previously that the counterbase to Py-G in the complementary strand has only a minor influence on the optical properties of the Py-G label, an abasic site analog S has been incorporated in all duplexes to guarantee an optimal intercalation of the Py-G moiety. **DNA1** and **DNA4** represent control duplexes lacking any charge acceptor. In **DNA2** and **DNA5**, 7-deazaguanine (Z) serves as the charge acceptor that has been placed in a distance of one or two intervening T-A base pairs to the charge donor (Py-G). Z exhibits the same base pairing properties as G but is more easily oxidized than G ($E_0(Z^+/Z) = 1.0$ V).[25] As an alternative charge acceptor to Z, an indole (In) group has been incorporated as an artificial DNA base in **DNA3** and **DNA6**. In has similar redox properties as Z.[26] The (S)-amino-2,3-propanediol linker has been applied as an acyclic linker and substitute for the 2′-deoxyribofuranoside moiety of natural nucleosides. We have previously used this linker in order to incorporate chromophores, e.g., ethidium[27] and perylenbisimide,[28] site-specifically into oligonucleotides. Moreover, we have previously shown that the indole base surrogate is intercalated in the DNA base stack and behaves as a universal base analog with only little influence of the counterbase.[29]

2.2. Absorption properties

First, we measured the UV/vis absorption of the single-stranded Py-G-modified oligonucleotides in comparison with the fully hybridized duplexes **DNA1**–**DNA6** (Fig. 1). For the reference duplexes (**DNA1** and **DNA4**) and for the duplexes bearing Z (**DNA2** and **DNA5**), the maximum of the pyrene absorption range (320–430 nm) can be found at ca. 370 nm. This indicates a strong stacking interaction of the pyrene chromophore with the adjacent base pairs inside the DNA duplex. The absorption maximum of the corresponding single strands is shifted by approximately 20 nm to ca. 350 nm as a result of destacking of the pyrene chromophore. Interestingly, the difference of the absorption maximum of the In-containing DNA samples (**DNA3** and **DNA6**) in comparison to the corresponding single strands is not very significant.

In order to elucidate the absorption changes of the Py-G label upon DNA hybridization more clearly, we repre-



Scheme 1. Sequences of the Py-G-modified **DNA1**–**DNA6**.

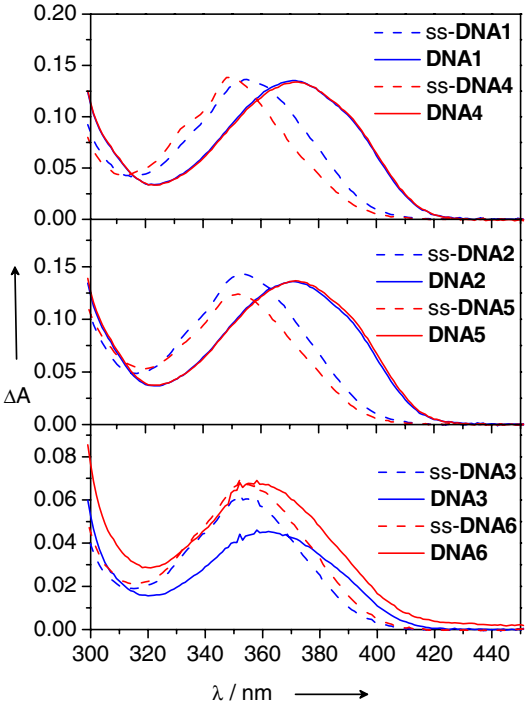


Figure 1. UV/vis spectra of the pyrene absorption range of the single-stranded (ss) and duplex **DNA1**, **DNA2**, **DNA4**, **DNA5** (5 μM) and **DNA3**, **DNA6** (2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0).

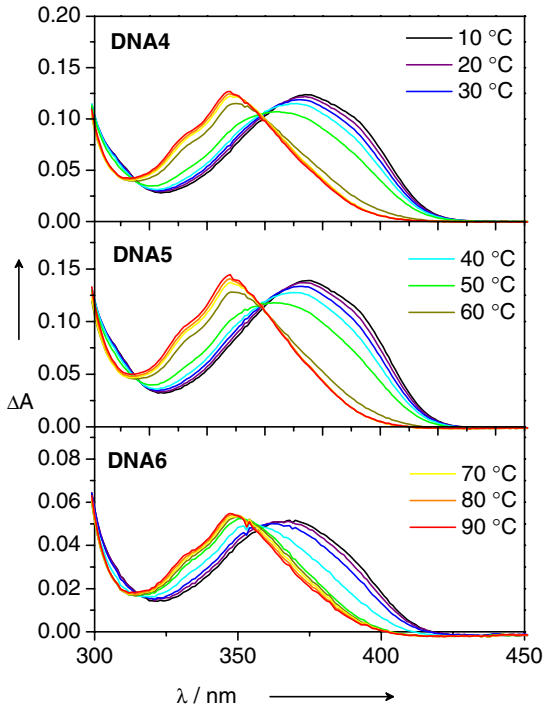


Figure 2. Temperature-dependent UV/vis spectra of **DNA4**, **DNA5** (5 μM) and **DNA6** (2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0).

sentatively measured the absorption of **DNA4**–**DNA6** at eight different temperatures between 20 °C and 90 °C (Fig. 2). At low temperatures the maximum of the pyrene absorption of **DNA4** and **DNA5** is found at ca. 370 nm and at high temperatures the maximum is shifted to ca. 350 nm. The latter absorption is similar to that of the corresponding single-stranded Py-G-modified oligonucleotides at rt. The isosbestic point at ca. 360 nm shows that there is a single transition between the pyrene in the duplex DNA and the dehybridized chromophore in the single strand. According to the pyrene absorption spectra this transition can be assigned to the cooperative thermal dehybridization of the DNA duplexes that occurs between 50 °C and 60 °C and correlates with the DNA melting temperatures (T_m) at 260 nm. As mentioned previously, the *syn*-conformation of Py-G yields the intercalation of the pyrene at least partially and forces the guanine part of Py-G and/or the counterbase C into a perturbed position outside the helix. The Py-G modification decreases the thermal stability of the DNA duplex significantly.[18] In fact, this structural scenario can explain the significant absorption differences of the pyrene which are observed in comparison between the full duplex (at 20 °C), the dehybridized DNA (at 90 °C), and the single-stranded oligonucleotide (at rt). In case of **DNA6**, the observed shift of the absorption maximum upon DNA dehybridization is considerably smaller compared to **DNA4** and **DNA5**. The latter observation indicates that the conformational perturbation of the acyclic linker as the 2′-deoxyribose substitute seems to interfere with the ability of the pyrene chromophore to intercalate into the DNA base stack.

2.3. Fluorescence properties

Subsequently, we compared the pyrene emission of the single-stranded Py-G-modified oligonucleotides with the fully hybridized duplexes **DNA1**–**DNA6** at the excitation wavelength of 360 nm (Fig. 3). As expected, all fluorescence spectra of the Py-G-modified duplexes **DNA1**–**DNA6** display an exciplex-type emission profile. The different fluorescence intensities of the duplexes reflect primarily the different exciplex stabilization by the adjacent base pairs and secondly the influence of charge transfer processes by the presence of either Z or In as artificial DNA bases. The duplex set **DNA3**/**DNA6** shows no significant discrimination between the duplex emission intensity and that of the corresponding single strands. In contrast, for the other duplexes, especially in case of **DNA4** and **DNA5** the emission quantity of the duplex is clearly higher than the amount of emission of the corresponding single-stranded oligonucleotide. Interestingly, **DNA5** exhibits the lowest fluorescence in the single-strand and thus the fluorescence increase upon DNA hybridization is remarkably high (approximately one magnitude of order).

The discrimination of duplexes from single-strands by increased fluorescence intensity makes the Py-G label potentially useful as an optical DNA hybridization label. We have previously shown that the emission intensity enhancement can be increased to 10–23 by shifting the excitation wavelength to 390–400 nm.[15] Within the latter absorption range the duplex extinction is significantly higher than that of the single strand, as discussed above. In order to elucidate the abilities of the Py-G

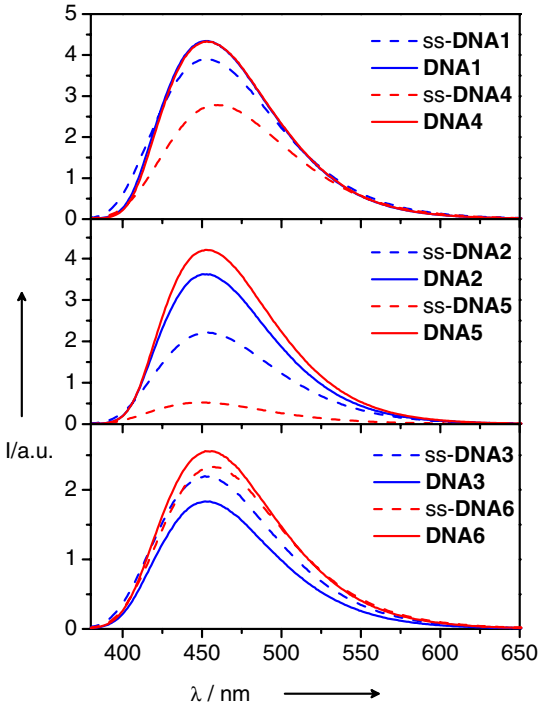


Figure 3. Relative fluorescence intensity of the single-stranded (ss) and duplex **DNA1**, **DNA2**, **DNA4**, **DNA5** (5 μM) and **DNA3**, **DNA6** (2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0).

label as a fluorescent DNA hybridization label more detailed, we representatively measured the relative fluorescence intensity of the duplexes **DNA4**–**DNA6** as a ratio between the integrated emission of the double-stranded DNA and the integrated emission of the single-stranded oligonucleotides I(ds)/I(ss) (Fig. 4). With respect to the absorption spectra (see Fig. 1) we performed these measurements at three different wavelengths: 335 nm (single strand-sensitive), 360 nm (isosbestic point), and 390 nm (duplex-sensitive). It is obvious that for all three duplexes the recorded ratios are highest upon excitation at 390 nm where the absorption differences between duplex DNA and the single-stranded oligonucleotide are highest. Interestingly, the fluorescence ratios measured with **DNA5** bearing the artificial DNA base Z three base pairs away from the Py-G label are higher compared to the reference duplexes **DNA4** and **DNA6**. The comparison of the fluorescence spectra of the single strands of both duplexes reveals that the Z-containing oligonucleotide exhibits significantly diminished emission intensity. This observation can be clearly attributed to the presence of Z and therefore seems to be the result of a fluorescence quenching by charge transfer between the pyrene chromophore and Z. Obviously, this fluorescence quenching occurs most efficiently in the single strand. This is a remarkable result since it shows that the replacement of a normal DNA base by an artificial base as a charge acceptor in a certain distance to a fluorescence label has the ability to improve the optical–analytical properties of a fluorescence label significantly.

2.4. DNA-mediated charge transfer and influence of base mismatches

In recent investigations we used pyrene-modified oligonucleotides to study electron transfer in DNA due to their initial charge-separated character in the excited state.[11,15] Moreover, we applied the fluorescence quenching by charge transfer in order to detect single base mismatches by measurement of the relative fluorescence intensities.[7] For the latter studies, ethidium was incorporated as a fluorescent artificial DNA base and charge donor together with Z as the charge acceptor in oligonucleotides. In order to study the DNA-mediated charge transfer with Py-G as the photoinducable charge donor, 7-deazaguanine (Z) was placed as a charge acceptor in a distance of one (**DNA2**) or two (**DNA5**) intervening T-A base pairs to the charge donor (Py-G). However, the measurement of the relative fluorescence intensities of these duplexes in comparison to the reference duplexes **DNA1** and **DNA4**, respectively, revealed only low fluorescence quenching values (Fq = 28% for **DNA2** and Fq = 4% for **DNA5**, Fig. 5). This result indicates that the DNA-mediated charge transfer between the Py-G and Z group does not occur very efficiently in the duplex over 1–2 intervening base pairs. Thus, this donor–acceptor couple is not suitable for the development of a DNA assay for the fluorescent detection of single base mismatches by charge transfer.

Recently, we studied charge transfer processes between DNA and small interacting peptides.[15,30] The latter studies revealed that a transfer of a positive charge from $Py^{\cdot-}$-$G^{\cdot+}$ to the more easily oxidizable indole side chain of tryptophan can be observed by fluorescence quenching. Therefore, we incorporated the indole side chain as an alternative charge acceptor to Z in a distance of one (**DNA3**) or two (**DNA6**) intervening T-A base pairs to the charge donor (Py-G). As expected, the measurement of the relative fluorescence intensities of these duplexes in comparison to the reference duplexes **DNA1** and **DNA4**, respectively, yielded promising fluorescence quenching values (Fq = 52% for **DNA3** and Fq = 26% for **DNA6**). These Fq values are considerably higher than the previous ones (for **DNA2**/**DNA5**) and

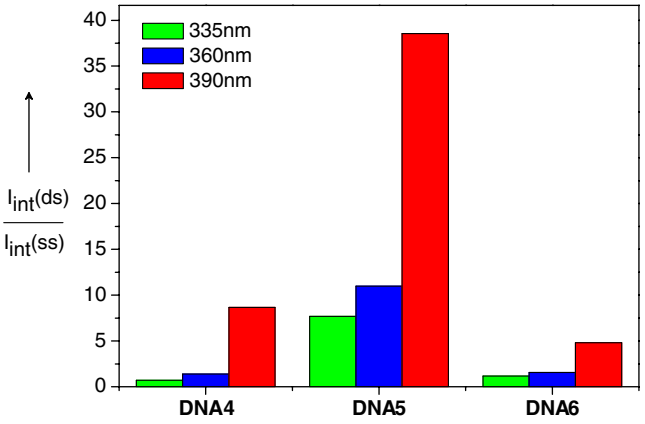


Figure 4. Relative fluorescence intensity (duplex vs single-stranded) of **DNA4**, **DNA5** (5 μM) and **DNA6** (2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0) at different excitation wavelengths (335, 360, and 390 nm).

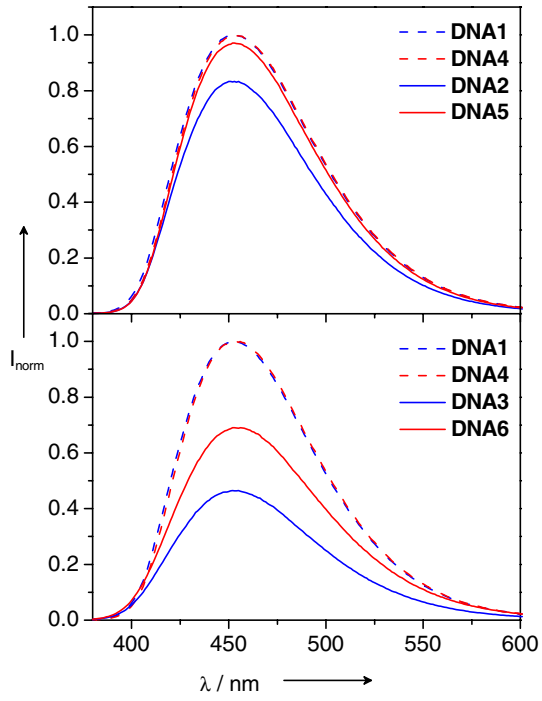


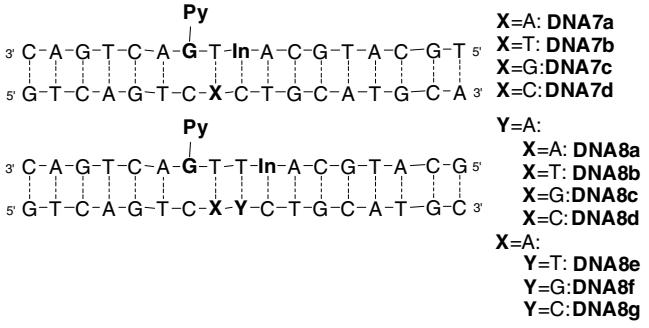
Figure 5. Relative fluorescence intensity of the duplex sets **DNA4**/**DNA5** (5 μM) and **DNA3**/**DNA6** (2.5 μM) each to the normalized reference duplexes **DNA1**/**DNA4** (top: 5 μM, bottom: 2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0).

indicate an efficient charge transfer within these DNA duplexes. Time-resolved measurements are currently performed to further support this interpretation by the characterization of the charge transfer dynamics.

Finally, we investigated the influence of single base mismatches on the fluorescence quenching as a result of a photoinduced DNA-mediated hole transfer between the Py-G group and the indole moiety. For this purpose, we synthesized the duplex sets **DNA7a**–**DNA7d** and **DNA8a**–**DNA8g** (Scheme 2). **DNA7a** and **DNA8a** represent the rightly paired DNA duplexes; all the others contain a single base mismatch. In contrast to **DNA1**–**DNA6**, C has been placed as a counterbase to the Py-G modification site. With respect to a potential DNA assay the Py-G labeled strand would represent the fluorescent probe oligonucleotide and the complementary strand that has to be analyzed for the presence of single nucleotide polymorphisms. Hence the complementary strand would not contain an abasic site. Interestingly, the melting temperatures of these duplexes do not show a significant contribution of the single base mismatches (see Table 1).

We measured the fluorescence quenching (Fq) of the duplex sets **DNA7b**–**DNA7d**, **DNA8b**–**DNA8d**, and **DNA8e**–**DNA8g** in relationship to the fully matched reference duplexes **DNA7a** or **DNA8a**, respectively (Fig. 6). All mismatched duplexes show a diminished fluorescence quenching in comparison to the corresponding fully matched ones. With respect to the similar fluorescence intensities of the reference duplexes it is evident that the observed emission differences have to be attributed to different charge transfer efficiencies. Hence, the differences cannot be solely a result of structural pertubations such as partial versus complete intercalation of the Py-G chromophore.

In case of mismatched duplexes of the set **DNA7**, the observed Fq values are nearly identical with the matched duplex **DNA7a**; except **DNA7d** makes a significant difference. For the duplex set **DNA8**, the Fq values are in the range 10–44%. They can be improved to 27–54% by shifting the absorption wavelength from 360 nm (isosbestic point of the absorption spectra, see Fig. 2) to the more duplex-sensitive excitation wavelength 390 nm. However, even the latter Fq values are still



Scheme 2. Sequences of the Py-G-modified **DNA7a**–**DNA7d** and **DNA8a**–**DNA8g**.

Table 1. Melting temperatures (T_m) of **DNA1–DNA6** (2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 250 mM NaCl, pH 7.0)

Duplex	T_m	Duplex	T_m
DNA1	58.5 °C	**DNA4**	55.0 °C
DNA2	59.5 °C	**DNA5**	55.0 °C
DNA3	46.4 °C	**DNA6**	42.3 °C
DNA7	**a**: 46.5 °C	**DNA8**	**a**: 44.3 °C
	b: 47.2 °C		**b**: 44.5 °C
	c: 47.6 °C		**c**: 53.7 °C
	d: 49.0 °C		**d**: 41.6 °C
			e: 42.3 °C
			f: 45.2 °C
			g: 41.5 °C

too low to be suitable for a potential DNA assay. They are also significantly lower than the previously published Fq values that have been obtained with a DNA system bearing ethidium (E) as an artificial DNA base and fluorescent charge donor together with Z as the charge acceptor (see Fig. 6).[7] Interestingly, the DNA–Py-G system works slightly better if the base mismatches are located next to the In group (**DNA8e**–**DNA8g**) and not directly adjacent to the Py-G chromophore (**DNA8a**–**DNA8c**).

Nevertheless, it is remarkable to note that in this DNA system, base mismatches do enhance the charge transfer efficiency (and therefore yield a stronger fluorescence quenching), similarly to the previously published DNA–ethidium system. It was known from various studies, that DNA mismatches or lesions typically interrupt the charge transfer efficiency.[31–34] Time-resolved studies showed clearly, that the intercalated ethidium that has been incorporated by the (S)-amino-2,3-propanediol linker system, is electronically fixed in a conformational orientation that prohibits a fast and efficient charge transfer through the DNA base stack.[35] It could be that in the Py-G–DNA system the conformational scenario is similar to the ethidium–DNA system. In the presence of base mismatches some amount of conformational flexibility could be regained resulting in increased charge-transfer efficiency.

3. Conclusion

The Py-G modification represents an interesting and promising optical label for DNA analytics and electron transfer studies. Remarkably, the DNA duplex hybridization can be observed by both fluorescence and absorption spectroscopy since the Py-G group exhibits altered properties in single strands versus double strands for both types of spectroscopy methods. The fluorescence enhancement upon DNA hybridization can be improved significantly by placing 7-deazaguanine (Z) as an additional modification and charge acceptor three bases away from the Py-G label. The presence of Z diminishes the fluorescence intensity by charge transfer in the single strand. Moreover, Py-G in DNA can be applied as a photoinducable donor for charge transfer processes when indol is present as an artificial DNA base and charge acceptor. Correctly base-paired duplexes can be

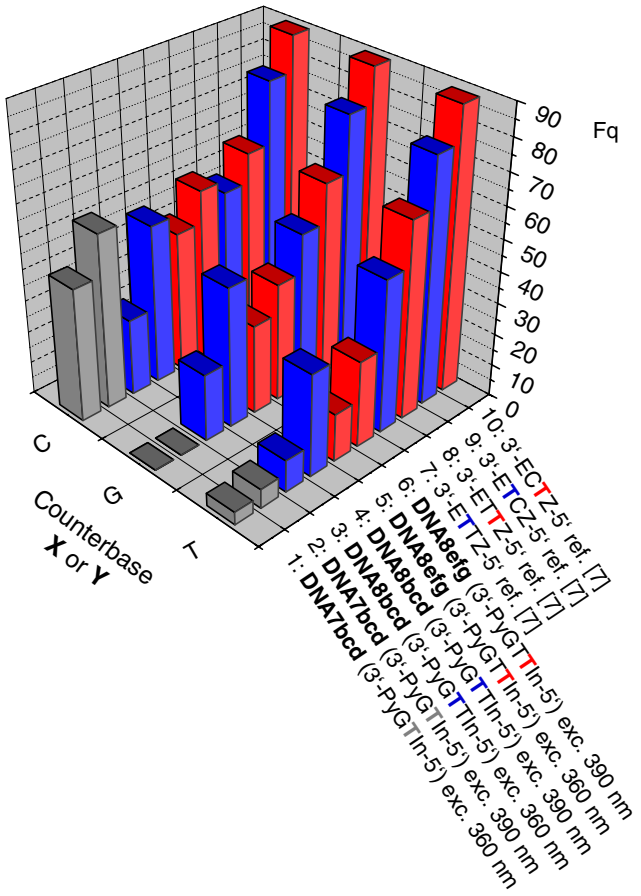


Figure 6. Fluorescence quenching of the mismatched duplexes **DNA7b–DNA7d** and **DNA8b–DNA8g** in relation to the rightly matched duplexes **DNA7a** and **DNA8a** (2.5 μM) in buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0); $Fq = 1 - I_{Int}(mismatch)/I_{Int}$ (match). The bold DNA base in the sequences represents the position of the mismatch with the counterbase X or Y.

discriminated from mismatched ones by comparison of their fluorescence quenching. In conclusion, it can be envisioned that the Py-G group represents an important label for time-resolved studies of DNA dynamics and stacking interactions[36] and could be applied especially for assays in which conformational changes or base-flipping processes are crucial to be observed, such as the investigation of DNA–protein complexes with DNA repair proteins.

4. Experimental

4.1. Materials and methods

Solvents were dried according to standard procedures. All reactions were carried out under argon. Chemicals were purchased from Sigma-Aldrich or Lancaster and used without further purification. ESI-MS analysis was performed on a TSY 7000 (Finnigan). MALDI-TOF analysis was performed in the analytical facility of the department on a Bruker Biflex III spectrometer using 3-hydroxypicolinic acid in aqueous ammonium citrate as the matrix. C18-RP analytical and semipreparative HPLC columns (300 Å) were purchased from Supelco. All spectroscopic measurements were performed in quartz glass cuvettes (1 cm) and using Na-P_i-buffer (10 mM). The melting temperatures (260 nm, 10–90 °C, interval 0.5–1 °C, scan speed 0.7–1 °C/min) were recorded on a Varian Cary Bio 100 spectrometer. The fluorescence spectra were measured on a Fluoromax-3 fluorimeter (Jobin-Yvon) at an excitation wavelength of 360 nm, 335 nm or 390 nm and corrected for Raman emission from the buffer solution. All emission spectra were recorded with a bandpass of 2 nm for both excitation and emission and are intensity corrected.

4.2. Oligonucleotide synthesis

The oligonucleotides were prepared using the standard solid phase phosphoramidite protocol on an Expedite 8909 DNA synthesizer from Applied Biosystems. Phosphoramidites and CPGs (1 μmol) were purchased from Glen Research or ABI, all other synthesizer chemicals from ABI. The DNA building blocks of Py-G and In were synthesized according to previously published protocols.[15,29] The DNA building block of Z was purchase from Glen Research. An extended coupling time of 15 min was used for PydG. For In, a modified coupling protocol was applied.[27] The oligonucleotides were cleaved off the resin by treatment with concd NH_4OH at 55 °C for 10 h and dried on a SpeedVac system (Christ). Purification of the oligonucleotides was achieved on a semipreperative RP-C18 column (300 Å, Supelco) using a linear gradient (0–30% B in A over 45 min, A: NH_4OAc buffer (50 mM, pH 6.5), B: MeCN). The purified oligonucleotides were analyzed by MS (ss-**DNA1** (MALDI): *m/z* calcd: 5409 $[M-H^+]^-$; found: 5410 $[M-H^+]^-$; ss-**DNA2** (MALDI): *m/z* calcd: 5409 $[M-H^+]^-$; found: 5417 $[M-H^+]^-$; ss-**DNA3** (ESI): *m/z* calcd: 5375.8 $[M-H^+]^-$; found: 1791,8 $[M-3H^+]^{3-}$; ss-**DNA4** (MALDI): *m/z* calcd: 5408 $[M-H^+]^-$; found: 5408 $[M-H^+]^-$; ss-**DNA5** (MALDI): *m/z* calcd: 5408 $[M-H^+]^-$; found: 5409 $[M-H^+]^-$; ss-**DNA6** (ESI): *m/z* calcd: 5375.8 $[M-H^+]^-$; found: 1791,8 $[M-3H^+]^{3-}$) and quantified by their absorbance at 260 nm (Py-G: ε_{260} (MeOH) = 17.9 $mM^{-1}\,cm^{-1}$, ε_{360} (MeOH) = 10.3 $mM^{-1}\,cm^{-1}$, In: ε_{260} (MeOH) = 4 $mM^{-1}\,cm^{-1}$).

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