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Electronic excited states of guanine-cytosine hairpins and duplexes studied by fluorescence spectroscopy†

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Guanine-cytosine hairpins, containing a hexaethylene glycol bridge, are studied by steady-state fluorescence spectroscopy and time-correlated single photon counting; their properties are compared to those of duplexes with the same sequence. It is shown that, both in hairpins and in duplexes, base pairing induces quenching of the ππ* fluorescence, the quantum yield decreasing by at least two orders of magnitude. When the size of the systems increases from two to ten base pairs, a fluorescent component decaying on the nanosecond time-scale appears at energy higher than that stemming from the bright states of non-interacting mono-nucleotides (*ca.* 330 nm). For ten base pairs, this new fluorescence forms a well-defined band peaking at 305 nm. Its intensity is about 20% higher for the hairpin compared to the duplex. Its position (red-shifted by 1600 cm^{-1}) and width (broader by 1800 cm^{-1} FWHM) differ from those observed for large duplexes containing 1000 base pairs, suggesting the involvement of electronic coupling. Fluorescence anisotropy reveals that the excited states responsible for high energy emission are not populated directly upon photon absorption but are reached during a relaxation process. They are assigned to charge transfer states. According to the emerging picture, the amplitude of conformational motions determines whether instantaneous deactivation to the ground state or emission from charge transfer states will take place, while ππ* fluorescence is associated to imperfect base-pairing.

Introduction

The way that the energy of a UV photon absorbed by DNA is redistributed within the double helix may affect its photoreactivity. The redistribution process evolves along complex paths, opened up *via* the action of electronic coupling which depends on the base sequence and the conformation of the double helix.[1,2] Thus, ultrafast energy transfer, related to Franck–Condon excited states delocalized over several bases, was evidenced both in model helices[3] and in genomic DNA.[4] Subsequently, the excitation energy may be trapped by dark excited states,[5] some of them surviving on the nanosecond time-scale.[6] The characterization of such long-lived excited states resulting from the specific molecular organisation of the chromophores in a double-stranded structure is thus important for elucidating the DNA photoreactivity.

Fluorescence spectroscopy, providing information not only about the lifetime but also about the energy and polarization of the electronic transitions, is a valuable tool for the study of excited state relaxation in DNA. Despite its low quantum yield, of the order of 10^{-4},[7] refined measurements on DNA became possible thanks to technical improvements and a better control of the experimental conditions so as to avoid detecting emission from damaged helices.[8] As a result of these developments, the fluorescence spectra of some duplexes were found to be located at higher energy than had been reported in previous studies. The most striking effect was reported for duplexes with alternating guanine-cytosine (GC) base pairs.[9] Their spectra are dominated by emission decaying on the nanosecond time-scale and peaking at shorter wavelengths than the spectra of the corresponding non-interacting mononucleotides (330 nm).

High energy long lived fluorescence was detected for GC duplexes with 20 (d20) and 1000 (d1000) base pairs[9] but the nature of the emitting excited states remained unclear. Despite their similarity, the emission bands of the two systems present important differences: that of the oligomer is much broader (8800 *vs.* 3400 cm^{-1} FWHM) and five times less intense than that of the polymer. The question arises whether the difference in the behaviour of the two systems is due to the number of GC pairs or to imperfect base pairing of the oligomer.

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It should be noted that polymeric duplexes are prepared by biochemical methods, whereas oligomeric ones are prepared by annealing of two single strands. It is possible that annealing gives rise to a variety of structures formed by slipping between the two self-complementary strands and/or undesired hairpin formation.

Another question regarding the peculiar fluorescence of GC duplexes concerns its connection to the quenching of $\pi\pi^*$ emission. In addition to nearly complete disappearance of the $\pi\pi^*$ band from the steady-state fluorescence spectrum of d1000,[9] significant shortening of the corresponding lifetime was detected by femtosecond measurements.[10] Similar quenching was observed for individual Watson–Crick GC pairs in organic solvents.[11] This was attributed on the basis of theoretical calculations to coupled electron–proton transfer that leads to a conical intersection between the excited and the ground states.[12,13] However, as no steady-state fluorescence spectra were reported for the individual Watson–Crick GC pairs, the presence of a short wavelength emission band could not be checked.

In order to answer the above questions, we have undertaken a study on shorter systems with well-defined structures: GC hairpins linked by a hexaethylene glycol (Gly) bridge and possessing 2 (hp2), 6 (hp6) and 10 (hp10) base pairs; we also examine the much longer hp20 (Fig. 1). The Gly linker is known to form stable hairpins with normal B-DNA base-pair geometries.[14,15] DNA hairpins with non-natural linkers have been employed in a variety of applications including studies of hairpin stability,[16,17] charge transfer,[18] and thymine photodimerization.[19] Here, their fluorescence properties are compared with those of short duplexes (d2, d6, d10). Hairpins and duplexes are studied by circular dichroism, steady-state fluorescence spectroscopy and time-correlated single photon counting (TCSPC).

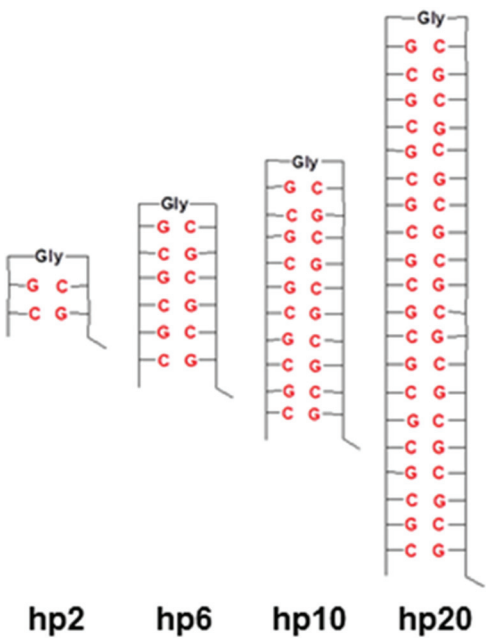

Fig. 1 Structures of the studied hairpins; Gly = hexaethylene glycol.

Experimental section

The hairpins shown in Fig. 1 were prepared as previously described.[20] The oligonucleotides were synthesized on a 1 μmol scale on CPG supports (500 Å) using an Expedite 8909 synthesizer; they were deprotected using 30% ammonium hydroxide at room temperature for 24 h, purified by HPLC and characterized by MALDI-TOF spectroscopy (Table ESI-1 †). The samples were dissolved in phosphate buffer (0.1 mol L^{-1} NaH_2PO_4, 0.1 mol L^{-1} Na_2HPO_4 and 0.25 mol L^{-1} NaCl). Samples were contained in 1 × 1 cm quartz cuvette. Water was delivered by a MILLIPORE (Milli-Q Synthesis) system.

Circular dichroism (CD) spectra were recorded on a Jasco-J-815 CD spectrometer. Steady-state absorption spectra were obtained with a Lambda 900 (Perkin-Elmer) spectrophotometer. Steady-state fluorescence spectra were measured with a fully corrected Fluorolog-3 (Jobin Yvon) spectrofluorometer, equipped on the emission side with two different grating monochromators centred at 330 and 500 nm. Fluorescence spectra were recorded simultaneously from 270 to 700 nm on both detectors. A Schott WG 385 filter was added in front of the grating centred at 500 nm in order to eliminate the contribution of the second order of the scattered excitation beam. The quantum yields of fluorescence were determined by using TMP in water as standard (Φ_F(TMP) = 1.54×10^{-4}).[21]

For the time-correlated single photon counting (TCSPC) experiments, the laser source was a commercial mode-locked Ti:Sapphire (MIRA 900, Coherent), pumped by a cw solid state laser (VERDI V10, Coherent), delivering ~120 fs pulses at 730 nm, at 76 MHz repetition rate. The 267 nm excitation beam was generated by focusing the 532 nm beam, from an optical parametric oscillator (MIRA-OPO, Coherent), into a 5 mm BBO type I crystal. The repetition rate was reduced at 4.75 MHz by diffracting the 532 nm-beam into a pulse picker (Model 9200, Coherent). The fluorescence, filtered by a Schott WG 295 filter, was collected and transported to a monochromator (SPEX) by two parabolic mirrors. The detector was a microchannel plate (R1564 U, Hamamatsu) providing an instrumental response function of ~70 ps (FWHM) as given by the Raman line of water at 295 nm. The TCSPC setup used a SPC630 card (Becker & Hickl). The decays at different emission wavelengths were made either at the magic angle (54.7°) or for parallel (I_{par}) and perpendicular (I_{perp}) excitation/emission configurations by controlling the polarization of the exciting beam with a half-wave plate (267 nm). The excitation energies under parallel and perpendicular conditions were identical, giving a G factor of 1. We checked that the total fluorescence decays, calculated as $F(t) = I_{par}(t) + 2I_{per}(t)$, were identical to those recorded directly at the magic angle. To avoid photodegradation, the samples were continuously stirred. The number of absorbed photons per pulse was approximately 10^9 times lower than the number of bases in the excited volume. Successive measurements gave identical decays. Thus the data sets were finally merged to increase the signal-to-noise ratio.

Results and discussion

Fig. 2A shows the fluorescence spectra of the hairpins together with that of an equimolar mixture of dGMP and dCMP; the intensity of the spectra is representative of the quantum yields. Going from the monomer mixture to hp2, the peak remains at 330 nm but its intensity diminishes by a factor of two. A further decrease in the fluorescence intensity is observed for hp6 with concomitant change in the spectral shape: the ratio of intensities at 305 and 330 nm (I_{305}/I_{330}) increases from 0.7 to 0.9. In the case of hp10, the spectrum is dominated by a well-defined band peaking at 305 nm (I_{305}/I_{330} = 1.7). Its width, determined after conversion on a wavenumber scale, is 5200 cm^{-1} (FWHM). The spectrum of hp20 (Fig. ESI-1†) is nearly flat; two peaks around 315 and 325 are hardly distinguishable.

The spectra obtained for hp2, hp6 and hp10 were unchanged following successive heating/cooling cycles whereas the spectrum of hp20 undergoes important changes. In this case, a band around 305 nm grows in intensity upon repeated annealing cycles (Fig. ESI-1†). This suggests that, following synthesis and removal of protecting groups, hp20 forms a complex mixture of metastable structures whose composition changes upon repeated annealing.

Fig. 2B shows the CD spectra of the hairpins together with that for an equimolar mixture of dGMP and dCMP. The spectra of all hairpins display a positive band at long wavelengths (*ca.* 285 nm) and negative bands at *ca.* 260 and 210 nm. The CD spectra of hp10 and hp20 (Fig. ESI-2A†) closely resemble that of polymeric GC in 10 mmol L^{-1} sodium phosphate buffer at pH 7.0, which has been assigned to a B form structure.[22] The relative intensities of the CD bands change as the number of hairpin base pairs increases, the most notable change being the increased intensity of the 210 nm bands for hp6 and hp10 *vs.* hp2. However, in the case of hp20 the 210 nm band is less intense than that of hp6. The CD spectra of the corresponding duplexes d2, d6, and d10 are shown in Fig. ESI-2B†. The spectra of the longer duplexes d6 and d10 are similar to those of the corresponding hairpins; however, the CD spectrum of d2 is missing the two prominent negative bands observed for the other duplexes and hairpins. Thus, two GC pairs may be sufficient for the formation of a stable base-paired glycol-linked hairpin,[23] but not for the formation of a duplex.

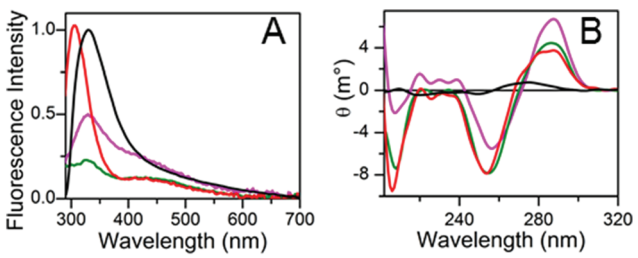

Fig. 2 (A) Fluorescence spectra (λ_{ex} = 255 nm) and (B) CD spectra of hp2 (pink), hp6 (green), hp10 (red) and an equimolar mixture of dGMP and dCMP (black). The intensity of the fluorescence spectra is proportional to the quantum yield.

The fluorescence decays recorded for the hairpins at 305 nm contain a fast component, which cannot be resolved by the TCSPC technique. A slower component, decaying on the nanosecond time-scale, is observed for hp6 and hp10 (Fig. 3A). The value of the longest time constant is 3 and 3.4 ns for hp6 and hp10, respectively. The amplitude of the slow component is 0.05 for hp10 corresponding to 98% of the emitted photons. In the case of hp2, the amplitude of the slow component is 5×10^{-4}, below the experimental precision.

It is important to stress that the fluorescence decays at 420 nm, where typically long-lived excimers of nucleobases are expected to emit,[24] are faster than those at 305 nm (Fig. 3B). The quantum yield of the band around 420 nm, determined by fitting the fluorescence spectrum of hp10 (Fig. 2A) with two log-normal curves, is 10^{-5}. This is within the limit of the experimental error, including weak emission from impurities. In any case, the variation of the fluorescence decays with the emission wavelength (Fig. 3B) shows clearly that the 3.4 ns component is not associated with the emission at 420 nm. In contrast to the long wavelength band, the short wavelength emission is not due to impurities, as largely discussed in ref. 9.

In the case of hp6, the fraction of photons emitted at 330 nm within the apparatus function is about 0.03. From this

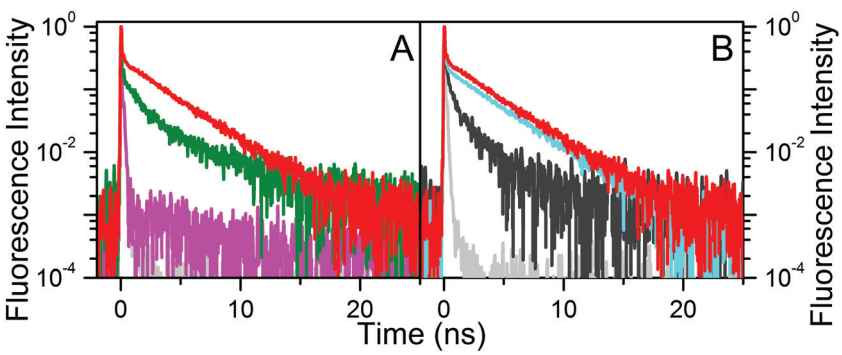

Fig. 3 Fluorescence decays of (A) hp2 (pink), hp6 (green) and hp10 (red) at 305 nm and (B) hp10 at 305 nm (red), 330 nm (cyan) and 420 nm (dark grey); λ_{ex} = 267 nm. In grey: instrumental response function.

value and the overall fluorescence quantum yield, we estimate that the quantum yield of the "monomer like" ππ* emission is of the order of 10^{-6}. This corresponds to a quenching by two orders of magnitude.

At this point we can draw several conclusions. (i) The quenching of ππ* fluorescence upon base pairing takes place also in hairpins. The fact that this quenching is not complete for hp2 is certainly due to imperfect base pairing as suggested by comparison of the CD spectra of the three hairpins (Fig. 2B). (ii) The quenching of the ππ* fluorescence is not necessarily accompanied by the appearance of the short wavelength long-lived fluorescence. (iii) The latter is observed from hairpins possessing six or more base pairs.

The size dependence of the fluorescence properties may arise from a combination of structural and electronic factors. X-ray diffraction measurements on AT duplexes in aqueous solution showed that an increase in size reduces the amplitude of conformational motions.[25] In line with this observation are molecular dynamics simulations performed for short hairpins.[2] Reducing the conformational disorder increases the degree of delocalization of Frenkel excitons. The same effect may be produced by simply increasing the number of interacting chromophores with no change in the structural disorder. For example, in the case of columnar aggregates, the pallier regarding the number of coherently coupled chromophores is reached for an aggregation number of *ca.* 20.[26]

In order to disentangle the above factors, a comparison between hairpins and duplexes with the same number of base pairs may be informative. As a matter of fact, the existence of a Gly bridge linking the two single strands is reported to reduce the amplitude of conformational motions in the base pair stem, with the exception of the base pair adjacent to the Gly linker which displays enhanced mobility.[14,15] The stability of the hairpins compared to duplexes is attested by the variation of the absorbance at 260 nm as a function of temperature. In contrast to d6 and d10, which exhibit melting curves typical of duplexes with melting temperatures of *ca.* 52 °C and 76 °C, respectively (Fig. ESI-3B and 4A†), hp6, hp10, and even hp2 (Fig. ESI-3A†) show no evidence of melting up to 96 °C. The difference in the fluorescence spectra of hp6 and d6 is comparable to the experimental error (Fig. ESI-4†) showing that, even for such a short duplex, base paring is nearly as efficient as for the hairpin. In the case of ten base pairs, a clear difference appears between duplex and hairpin (Fig. 4B). The fluorescence spectrum of d10 is characterized by the same emission band as that of hp10 but its intensity is lower by 23%. Accordingly, the fluorescence decay of d10 at 305 nm contains the same long-lived component (3.4 ns) as that of hp10, albeit with smaller amplitude (Fig. 4C).

From the comparison between hp10 and d10 we deduce that conformational disorder of the terminal GC pairs does not affect the properties of the short-wavelength long-lived emission (position and shape of the band, lifetime) but changes the population of the emitting excited states. The fluorescence anisotropy $r(t)$ shows that these excited states are not populated directly by photon absorption. We observe in Fig. 5A that the $r(t)$ signal recorded for hp10 and d1000 on the sub-nanosecond time-scale decays to 0.02 ± 0.01 within the apparatus function. Such a low value is not due to depolarization provoked by rotational diffusion because the same anisotropy signal was found for the much larger system d1000.[9] We stress that the anisotropy of an equimolar mixture of dGMP and dCMP is 0.26, as determined by fluorescence upconversion (because their fluorescence decays on the femtosecond time-scale).[21] Evidently, energy transfer involving ππ* transitions, polarized within the aromatic plane of the bases, leads to a decrease of $r(t)$. The lowest value expected in this case is 0.1,[27] which corresponds to in-plane depolarization of the fluorescence and was observed for natural DNA[4] and G-quadruplexes.[28]

According to the formula that associates the fluorescence anisotropy to the angle θ formed by the transition moments related to photon absorption and photon emission, $r = (3\cos^2\theta - 1)/5$, an anisotropy value of 0.02 corresponds to an angle of 53°. This feature proves that emission of hp10 is connected to an electronic transition polarized out of the aromatic plane of the bases. We note that the presence of alternating GC sequences within double helices is known to provoke deviations from the ideal B-form, but these deviations are far below 53°.[29]

Having ruled out localized or delocalized ππ* states as the possible origin of the short-wavelength long-lived GC

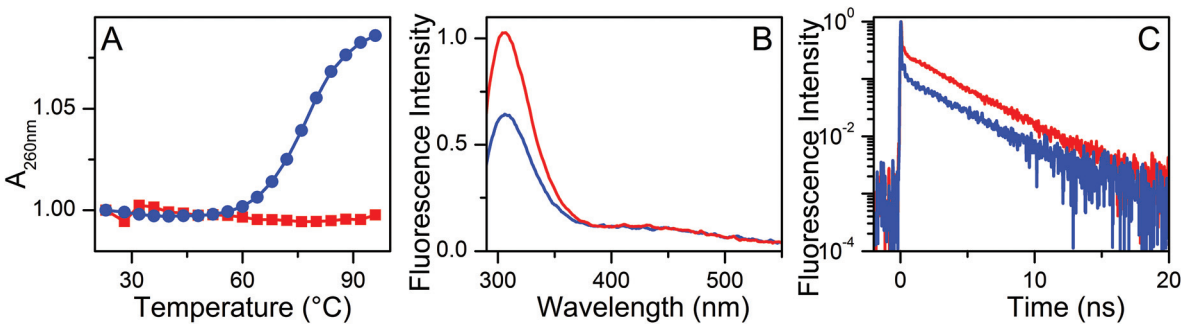

Fig. 4 Comparison between the duplex d10 (blue) and the hairpin hp10 (red). (A) Temperature dependence of the absorbance at 260 nm, (B) fluorescence spectra (λ_{ex} = 255 nm) and (C) fluorescence decays at 305 nm (λ_{ex} = 267 nm).

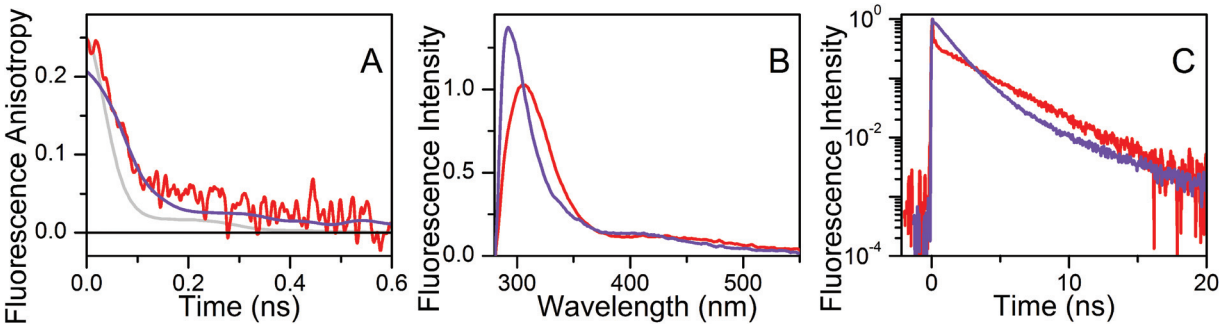

Fig. 5 Comparison between the hairpin hp10 (red) and the polymeric duplex d1000 (purple): (A) fluorescence anisotropy decays at 305 nm (λ_{ex} = 267 nm), (B) fluorescence spectra (λ_{ex} = 255 nm) and (C) fluorescence decays. The d1000 time-resolved signals are taken from ref. 9. In grey: instrumental response function.

fluorescence, we examine whether nπ* states or excited charge transfer (CT) states can account for such emission.

The nπ* states of DNA bases have lower energy than the ππ* states in the gas phase but are greatly destabilized in aqueous solution.[5,30] However, transient absorption experiments established that, even in aqueous solution, such states may be populated during excited state relaxation.[31] Therefore, one could think that the local environment of a double-stranded structure, less exposed to water, favours weak emission from nπ* states. Yet, the dependence of the position and the lineshape of the emission band on the size of the double helix is not compatible with nπ* states, which are localized on single bases due to very weak exciton coupling. We observe in Fig. 5B, that when going from hp10 to d1000, the emission band shifts to shorter wavelengths and becomes narrower. After conversion to a wavenumber scale, the difference in the peak position and the width (FWHM) is 1600 cm^{-1} and 1800 cm^{-1}, respectively. Not only the position and the width but also the lifetime of the short wavelength emission band of hp10 is different from that of d1000 (Fig. 5C). Although the fluorescence decays for both systems are on the nanosecond time-scale, the average decay for d1000 is faster (1.6 ns *vs.* 3.4 ns) and clearly non-exponential. We think that the latter difference arises from the fact that, while only linear base-paired conformations are expected for hp10, this should not be the case for the much longer d1000, for which regions with different conformations may coexist.

In contrast to nπ* states, the experimental observations are consistent with emission from CT states involving different bases. Indeed, these are governed by electronic coupling due to orbital overlap, which is much more sensitive than dipolar coupling to minor conformational changes.[32,33] The helix conformation is expected to affect not only the strength of the coupling due to orbital overlap but also possible mixing between Frenkel excitons with CT states[34] and thus determine whether the latter behave as traps for the excitation energy.

In view of the above described quenching of ππ* fluorescence in the Watson–Crick (WC) pairs without simultaneous appearance of the short-wavelength long-lived emission band (Fig. 2A), it is obvious that the latter does not originate from CT states involving the two bases of a WC pair. In contrast, its attribution to intrastrand CT states is quite plausible. In this respect, it is worth-noting that a recent theoretical study on alternating GC duplexes reports that the lowest CT state in the Franck–Condon region involves a GC pair located in the same strand.[35]

Summary and conclusions

The key point for understanding the fluorescence properties of GC hairpins and duplexes is the modification of their FC excited states with respect to those of non-interacting mononucleotides. When the size of the double-stranded structure increases, the amplitude of conformational motions is reduced, improving base-stacking and enhancing exciton coupling. As a result, the FC states start acquiring a delocalized character, extended on more than one base. However, the degree of delocalization of the resulting collective excited states (Frenkel excitons, charge transfer states and their combination), as well as their ordering, depends on the precise conformation of the system at the moment of photon absorption.[1] Accordingly, various relaxation mechanisms may intervene. We describe below three limiting cases concerning systems with decreasing disorder.

1. Population of excited states localized on single bases leads to fluorescence quenching due to instantaneous proton coupled electron transfer[12,36,37] and thus does not contribute to the fluorescence. Such a relaxation process pertains to WC pairs connected *via* three hydrogen bonds and may be modified when one or more hydrogen bonds are disrupted. In this respect, it is worth noticing that base pair opening is a natural process and plays important biological roles.[38,39] The partial fluorescence quenching of ππ* fluorescence observed for hp2 and d2 is representative of this case.

2. Population of exciton states may be followed by localization of the excitation *via* phonon coupling. Subsequently, instantaneous relaxation to the ground state or emission from structural defects, as described above may occur. This mechanism appears to dominate in the case of hp6 and d6.

3. Exciton states may be trapped by lower lying charge transfer states and emitting around 300 nm. Although the emission

energy is higher than that corresponding to emission from localized ππ* states (330 nm), it is well below the energy of the FC states (267 nm). This trapping process is favoured by well-ordered conformations, like those of hp10 and especially d1000.

All the above mentioned mechanisms contribute to the excited state relaxation of each system at various degrees. Thus, partially paired bases could be responsible for the ππ* fluorescence detected for d1000 by fluorescence upconversion.[10] This conclusion is consistent with the efficiency of quenching of the ππ* emission within the duplex *vs.* the non-interacting mono-nucleotides. The quenching efficiency derived from steady-state and TCSPC measurements, corresponding to the total number of emitted photons, is of three orders of magnitude;[9] whereas the fluorescence lifetime is shortened by only a factor two, as determined by fluorescence upconversion.[10]

Our attribution of the short wavelength fluorescence to charge transfer states is made on the basis of the low fluorescence anisotropy and by ruling out two other types of excited states, ππ* and nπ* states. Transient absorption spectra could bring a direct proof by detecting the bands of a radical cation and a radical anion. Such measurements have yet to be performed for systems exhibiting the short wavelength fluorescence.

In conclusion, the role of GC pairs in respect to the DNA stability *versus* UVC irradiation, as far as this could be related to the lifetime of the excited states, is quite ambiguous. On the one hand, when a few of them are interspersed among AT pairs, they may accelerate the deactivation of ππ* states,[40,41] and affect lesion formation.[42] On the other, extended GC regions, which are known to be present in the human genome and play an important biological role,[43] give rise to particularly long-lived excited states. Even if the fraction of the excitations reaching these states is not high and possibly is connected with specific conformations, it would be interesting to examine the consequences on the formation of GC lesions reported recently.[44]

Acknowledgements

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