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Fundamentals of the Intrinsic DNA Fluorescence

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CONSPECTUS. The intrinsic fluorescence of nucleic acids is extremely weak compared to that of the fluorescent labels used to probe their structural and functional behavior. Thus, for technical reasons, the investigation of the intrinsic DNA fluorescence remained for long limited. But with the improvement of spectroscopic techniques, the situation started to change around the turn of the century. During the past two decades, various factors modulating the static and dynamical properties of the DNA fluorescence have been determined; it was shown that, under certain conditions, quantum yields may be up 100 times higher than what was known so far. The ensemble of these studies opened-up new paths for the development of “label-free” DNA fluorescence for biochemical applications. In parallel, these studies have shed new light on the primary processes leading to photoreactions that damage DNA when it absorbs UV radiation.

We have been studying a variety of DNA systems, ranging from the monomeric nucleobases to double-stranded and four-stranded structures using fluorescence spectroscopy. The specificity of our work resides in the quantitative association of the steady-state fluorescence spectra with time-resolved data recorded from the femtosecond to the nanosecond time-scales, made possible by the development of specific methodologies.

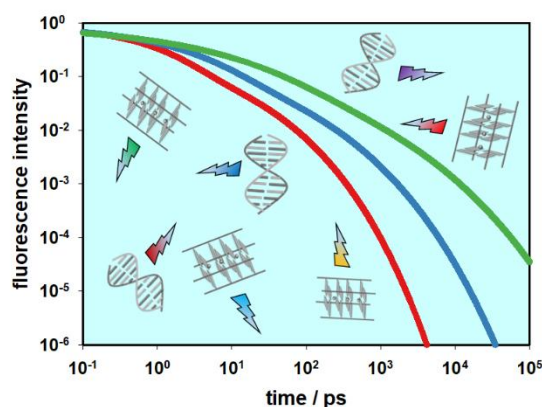
Among others, our fluorescence studies provide information on the energy and the polarization of electronic transitions. These are precious indicators for the evolution of electronic excitations in complex systems, where the electronic coupling between chromophores plays a key role. Highlighting collective effects that originate from electronic interactions in DNA multimers is the objective of the present Account.

In contrast to the monomeric chromophores, whose fluorescence decays within a few picoseconds, that of DNA multimers persists on the nanosecond time-scale. Even if long-lived states represent only a small fraction of electronic excitations, they may be crucial to the DNA

photoreactivity because the probability to reach reactive conformations increases in time, owing to the incessant structural dynamics of nucleic acids.

Our femtosecond studies have revealed that an ultrafast excitation energy transfer takes place among the nucleobases within duplexes and G-quadruplexes. Such an ultrafast process is possible when collective states are populated directly upon photon absorption. On much longer times, we discovered an unexpected long-lived high-energy emission stemming from what was coined “HELM excitons”. These collective states, whose emission increases with the duplex size, could be responsible for the delayed fluorescence of $\pi\pi^*$ states observed for genomic DNA.

Most studies dealing with excited state relaxation in DNA were carried out with excitation in the absorption band peaking around 260 nm. We went beyond and also performed the first time-resolved study exciting in the UVA spectral range, where a very weak absorption tail is present. The resulting fluorescence decays are much slower, and the fluorescence quantum yields are much higher, compared to UVC excitation. We showed that base-pairing of DNA strands enhances the UVA fluorescence and, in parallel, increases the photoreactivity because it modifies the nature of the involved collective excited states.



KEY REFERENCES

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- Vayá, I.; Brazard, J.; Huix-Rotllant, M.; Thazhathveetil, A.; Lewis, F.; Gustavsson, T.; Burghardt, I.; Improta, R.; Markovitsi, D. High energy long-lived mixed Frenkel – charge transfer excitons: from double-stranded (AT)_n to natural DNA. *Chem. Eur. J.* **2016**, *22*, 4904-4914.² *The absorption and fluorescence spectra as well as the fluorescence decays of alternating adenine-thymine duplexes strongly depend on their size. An increase in the number of base-pairs enhances a high-energy long-lived emission correlated with mixed $\pi\pi^*/CT$ excitons. As their fingerprint is also detected for genomic DNA, these excitons could operate as a doorway for the delayed $\pi\pi^*$ fluorescence observed.*
- Banyasz, A.; Vayá, I.; Changenet-Barret, P.; Gustavsson, T.; Douki, T.; Markovitsi, D. Base-pairing enhances fluorescence and favors cyclobutane dimer formation induced upon absorption of UVA radiation by DNA. *J. Am. Chem. Soc.* **2011**, *133*, 5163-5165.³ *This is the first study of DNA fluorescence with UVA excitation. It is shown that under UVA absorption, the fluorescence quantum yield and the fluorescence lifetime increase upon base-pairing.*

INTRODUCTION

Nucleic acids are often considered as non-fluorescent. Yet, they do emit light, but, for most of them, the quantum yield at room temperature is very low, on the order of 10^{-4} . Spectroscopists who tried to measure the intrinsic DNA fluorescence in the latter half of the 20th century found that, not only that its fluorescence is very weak, but also that its extremely short decay rendered the studies particularly difficult.⁴⁻⁷ The situation changed with the development of femtosecond spectroscopy that literally boosted experimental and theoretical studies on the dynamics of electronically excited states of nucleic acids. One important objective of these studies was to shed light on the primary processes leading to DNA damage upon direct absorption of UV radiation.

A first finding of the “new era” investigations was that the fluorescence of DNA monomeric chromophores (major nucleobases, nucleosides and nucleotides), stemming from $\pi\pi^*$ states, decays within, at most, a few picoseconds,^{8,9} in line with results from transient absorption spectroscopy.¹⁰ Quantum chemistry calculations attributed this ultrafast deactivation to the existence of conical intersections connecting the first excited state with the ground state in a non-radiative way.^{11,12}

The situation changes dramatically when going from monomers to multimers, to which the present Account is dedicated. While their fluorescence starts decaying on the femtosecond time-scale,^{1,13-19} it is still detectable on the nanosecond time-scale.^{1,3,20-24} The reason is that in these systems, electronic interactions between nucleobases modify the nature of the excited states, rendering them “collective”, in the sense that they involve more than one nucleobase.

Figure 1 presents a general picture that emerged from numerous transient absorption studies on the excited state relaxation in DNA multimers following excitation around 260 nm, *i. e.* within the first absorption band. These measurements showed that an important part of the initially excited states evolves very rapidly toward charge transfer (CT) states which decay

with lifetimes ranging from ~ 30 to ~ 300 ps, depending on the base sequence and the secondary structure.^{15,19,25-30}

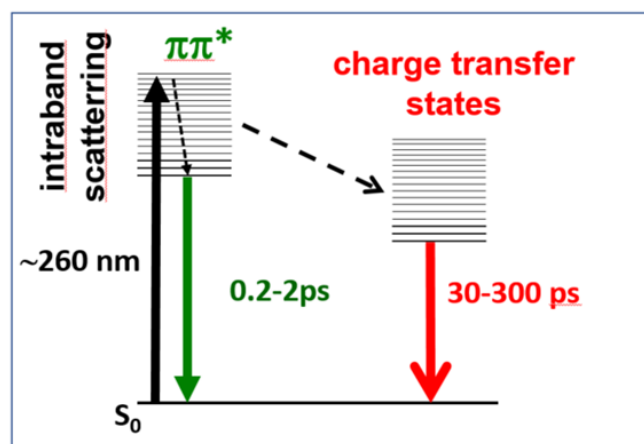


Figure 1. Simplified scheme illustrating the general lines of the excited state relaxation in DNA multimers based on transient absorption experiments.

Fluorescence spectroscopy provides information about the energy and the polarization of electronic transitions, allowing discrimination about various types of collective states, as will be discussed below. In our group, we studied DNA fluorescence over six orders of magnitude in time, combining two detection techniques, fluorescence upconversion (FU) and time-correlated single photon counting (TCSPC), using the same femtosecond laser as excitation source; in most experiments, the excitation wavelength was set at 267 nm. In addition, we associated, in a quantitative way, time-resolved data with the steady-state emission spectra, seeking the type of excited states responsible for the band maxima in the fluorescence spectra. Such information is potentially useful for analytical purposes.

After stressing some experimental and conceptual difficulties inherent in the study of DNA fluorescence, and discussing excitation transfer among nucleobases, we describe the complex image of DNA fluorescence that emerged from recent experimental and theoretical studies. Subsequently, we highlight two specific findings of our work: HELM (High-energy Emitting Long-lived Mixed) excitons and UVA fluorescence. Finally, we refer to potential technological issues as well as implications in the DNA reactivity.

Below we use the notations $(A)_n \bullet T_n$, $(AT)_n \bullet (AT)_n$ and $(GC)_n \bullet (GC)_n$ to designate duplexes with homopolymeric adenine-thymine, alternating adenine-thymine and alternating guanine-cytosine sequences (Figure 2a). The studied G-quadruplexes were formed either by folding of single strands $[GGG(TTAGGG)_3]$; polyguanine strands with ~ 3000 -4000 bases] or by association of four TG_nT strands; they are abbreviated as $TEL21/M^+$, $G\text{-wires}/M^+$ and $(TG_nT)_4/M^+$, respectively; M^+ indicates the type of metal cations (Na^+ , K^+) located in their central cavity (Figure 2b).

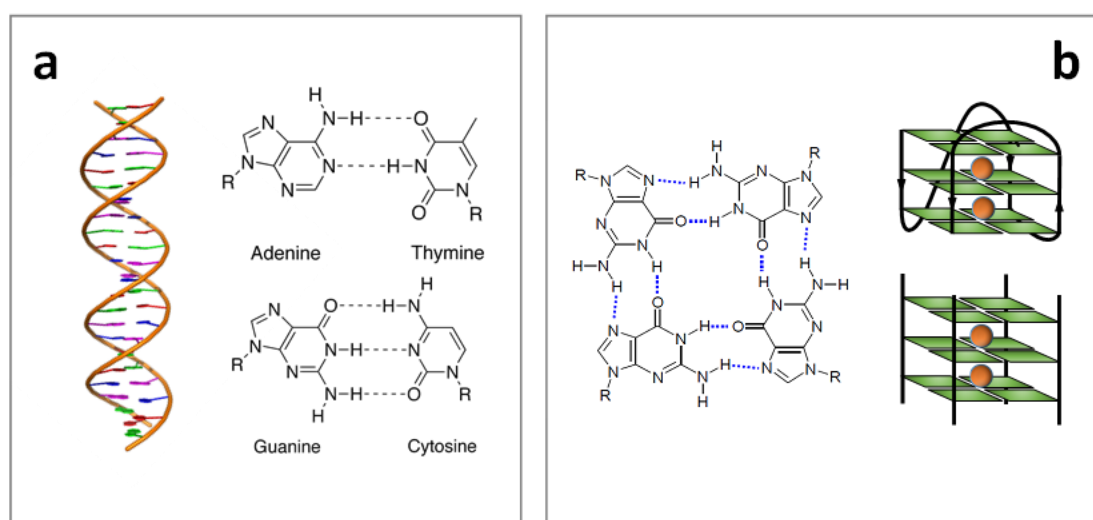


Figure 2. Schematic representation of the studied systems. (a) Duplexes composed of adenine-thymine and guanine-cytosine Watson-Crick pairs. (b) Monomolecular and tetramolecular G-quadruplexes formed by, respectively, folding of a single strand or association of four identical strands in the presence of metal ions; their core is composed of tetrads in which guanines are connected via Hoogsteen hydrogen bonds.

EXPERIMENTAL AND CONCEPTUAL PRERQUISITES

Fluorescence studies of systems with extremely low quantum yield are not trivial. The purity of DNA, the water and salts necessary for the preparation of the solutions, as well as the glassware used in the experiments, is crucial so that to avoid detecting emission from impurities. We performed control checks by comparing results obtained for different DNA batches, purified in different ways (polyacrylamide gel electrophoresis, high performance

liquid chromatography), provided by different suppliers or prepared via different synthetic methods (solid state synthesis, PCR). Only samples having consistent spectral properties were retained. In most of our experiments, DNA was dissolved in phosphate buffer $0.1 \text{ molL}^{-1} \text{ M}_2\text{HPO}_4$, $0.1 \text{ molL}^{-1} \text{ M}_2\text{PO}_4$ and $0.25 \text{ molL}^{-1} \text{ MCl}$ ($\text{M} = \text{Na}^+$ or K^+). A relatively high salt concentration was necessary to ensure efficient base-pairing of oligomeric duplexes. In both steady-state and time-resolved fluorescence experiments, we systematically subtracted the solvent signal from that recorded for DNA. We note that a phosphate buffer (purity of salts 99.99%) gave a much smaller background signal compared to other commonly used buffers (cacodylate, Tris-HCl) and the background was even lower when only MCl was used. We also tested that replacing the type of added salts, while keeping the same ionic strength, did not alter our results.

Additional problems arise from the photo-damage occurring during the experiments. Some of the photoproducts, such as (6-4) photo-adducts, are much stronger emitters than DNA.³¹ Furthermore, due to the plasticity of DNA helices, the presence of certain lesions, such as cyclobutane pyrimidine dimers (CPDs), modifies the DNA structure beyond the reactive sites.³² Consequently, the electronic coupling and, hence, the properties of the collective states are modified. To overcome these complications, we developed specific experimental protocols.³³

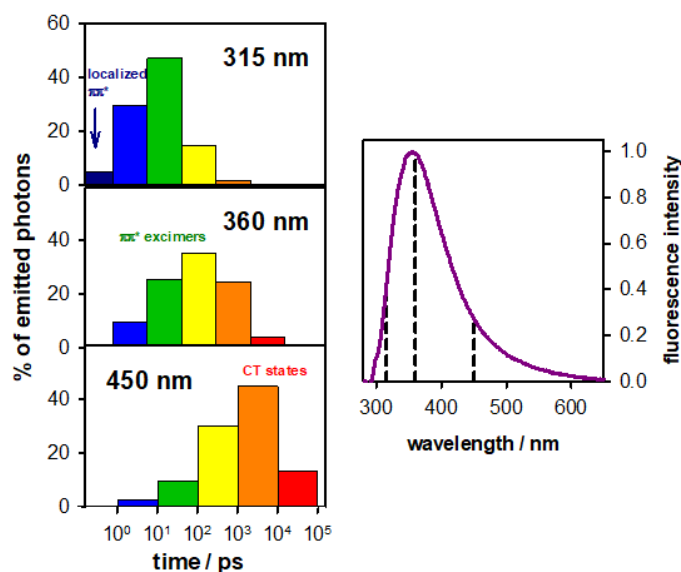


Figure 3. TEL21/Na⁺ G-quadruplexes.¹ Left panel: photons emitted per decade of time at different wavelengths; they were determined from multiscale decays obtained by analyzing FU and TCSPC signals; the excited states dominating the emission at various time domains are indicated. Right panel: steady-state fluorescence spectrum; vertical dashes denote the emission wavelengths corresponding to the histograms in the left panel.

On the conceptual side, the difficulty resides in the data treatment. Usually time-resolved signals are fitted with multi-exponential functions and each time-constant is associated with a specific excited state (or species). Although this analysis provides a quantitative description of the decays, the underlying kinetic models are only valid under certain conditions. One of them is that the chromophores evolve in an isotropic three-dimensional space, which is certainly not fulfilled for excited nucleobases in DNA multimers.²⁰ The description of dynamical processes in complex anisotropic systems requires specific theoretical models and/or simulations.^{34,35}

To avoid a misinterpretation of time constants, we determined the distribution of photons emitted per decade of time at various wavelengths, as represented in the form of histograms in Figure 3. To this end, we analyzed separately the FU and TCSPC decays recorded at a given emission wavelength following a non-linear fitting/deconvolution procedure. We used as

many exponential functions as needed to obtain an “as-good-as-possible” fit. In the case of the FU signals, we fixed the long time constants to the values derived from the TCSPC signals. In the case of the TCSPC signals, the ultrafast components, only measurable with FU, were represented by a component limited by the instrumental response function. Finally, using the fitted functions we constructed composite multiscale (FU+TCSPC) model curves and integrated them per decade of time.³⁶

Combining this information with fluorescence anisotropies and quantum chemistry calculations, we explored the nature of the excited states that govern the steady-state emission spectra, as well as the main time-intervals over which their emission occurs. For example, the fluorescence spectrum of TEL21/Na⁺ peaks at 360 nm. At this wavelength, the largest number of emitted photons is found in the interval 10 - 1000 ps (Figure 3) where the fluorescence anisotropy is ~0.05.¹ In the light of the computed energy and the polarization of the various electronic transitions corresponding to minima of excited state potential energy surfaces, the experimental observations indicate that the emission maximum is dominated by $\pi\pi^*$ excimers (for further discussion see below). The excited states dominating the fluorescence spectra of representative polymeric duplexes and G-quadruplexes are shown in Table 1.

Table 1. Fluorescence quantum yields ($\phi \times 10^4$) determined for long polymeric duplexes and G-quadruplexes with excitation at 255-285 nm. In italics: excited states whose emission is predominant in the steady-state fluorescence spectrum.

	calf thymus DNA ^{a,18}	(GC) _n •(GC) _n ³⁷	(AT) _n •(AT) _n ²	(A) _n •(T) _n ¹⁴
duplexes	3.1 <i>$\pi\pi^*$</i>	1.3 <i>HELM</i>	1.4 <i>HELM; $\pi\pi^*$; CT</i>	3.0 <i>$\pi\pi^*$</i>
monomolecular G-quadruplexes	TEL21/K ⁺³⁸	TEL21/Na ⁺¹	G-wires/K ⁺³⁹	G-wires/Na ^{+b,39}
	6.8 <i>$\pi\pi^*$</i>	8.7 <i>$\pi\pi^*$ excimers</i>	9.2 <i>$\pi\pi^*$</i>	9.5 <i>$\pi\pi^*$; CT</i>
tetramolecular G-quadruplexes	(TG ₃ T) ₄ /K ⁺⁴⁰	(TG ₄ T) ₄ /K ⁺³⁹	(TG ₅ T) ₄ /K ⁺⁴⁰	(TG ₄ T) ₄ /Na ³⁹
	3.2 <i>$\pi\pi^*$</i>	4.0 <i>$\pi\pi^*$</i>	5.1 <i>$\pi\pi^*$</i>	3.0 <i>$\pi\pi^*$; CT</i>

^{a)} 58% AT pairs; 42% GC pairs; ^{b)} ~900 tetrads

EXCITATION ENERGY TRANSFER

We showed that excitation energy transfer takes place among nucleobases in DNA multimers both on the femtosecond time scale and on longer times. It was evidenced by probing the fluorescence anisotropy at wavelengths corresponding to the $\pi\pi^*$ emission. The FU anisotropy was found to be lower and to decay much faster than that corresponding to stoichiometric mixtures of mono-nucleotides^{1,14,22,23} (Figure 4a). Such an ultrafast transfer is possible only if exciton states are directly populated by photon absorption. Theoretical calculations rationalized the existence of Franck-Condon collective states for various DNA model systems.^{12,41-45} In this case, energy transfer proceeds through intraband scattering (Figure 1), that is internal conversion among the states of the exciton band, each one having a specific polarization and topology.⁴⁶ It is also possible that localization of the excitation on single nucleobases occurs.

The dynamics of intraband scattering is too fast for the time-resolution of our fluorescence setup (~330 fs). It was recently resolved by transient absorption measurements in the case of adenine single strands, in which it was found to occur within 100 fs.⁴⁷

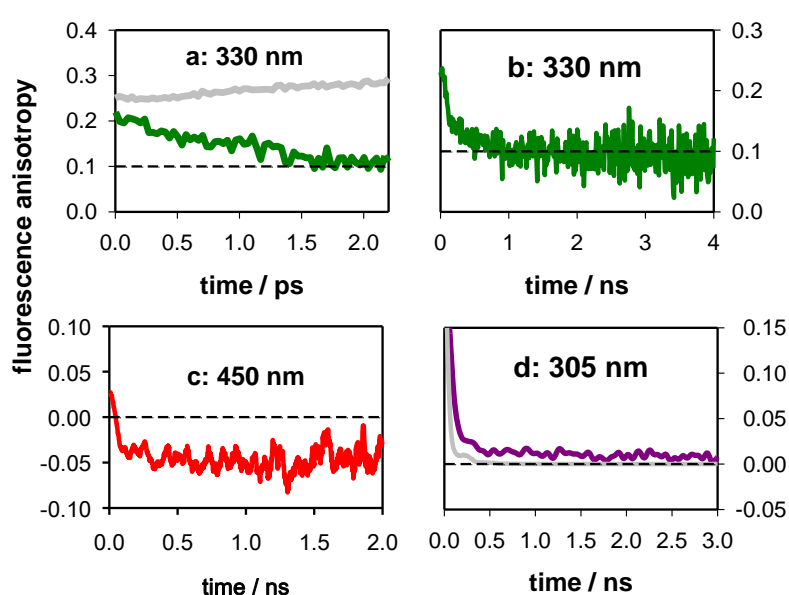


Figure 4. Fluorescence anisotropy corresponding to $\pi\pi^*$ states (green), CT states (red) and HELM excitons (violet); (a) TEL21/ Na^+ G-quadruplexes (FU),¹ (b) calf thymus DNA (TCSPC),⁴⁸ (c) $(\text{TG}_4\text{T})_4/\text{Na}^+$ G-quadruplexes (TCSPC),³⁹ (d) polymeric $(\text{GC})_n\bullet(\text{GC})_n$ duplexes (TCSPC).⁴⁹ Also shown in (a) is the fluorescence anisotropy of a stoichiometric mixture of mono-nucleotides (grey).

For long-lived $\pi\pi^*$ emitting systems, the fluorescence anisotropy acquires a constant value of 0.1^{18,23} indicating an in-plane depolarization. This results from randomization of the emission transition dipoles, polarized within the aromatic plane of the nucleobases, unravelling an efficient energy transfer process. In the case of calf thymus DNA (Figure 4b), the fluorescence anisotropy, after an initial ultrafast decay (similar to that of TEL21 in Figure 4a), continues to decrease at longer times, reaching the value of 0.1 within ~ 800 ps.⁴⁸ This shows, that, in addition to ultrafast intraband scattering, a second slower energy transfer process is operative in native DNA. The underlying mechanism will be discussed in a next section.

FROM DUAL FLUORESCENCE TO A MULTITUDE OF EMITTING STATES

Early studies described the fluorescence of nucleic acids in terms of monomer-like and excimer/exciple emission. Excimers and exciplexes involve two chromophores, of the same or different type, respectively, that do not interact in their ground state. To a large extent, this condition is not fulfilled in duplexes and G-quadruplexes, due to delocalization of Franck-Condon states. Excimer/exciple emission, corresponding to a minimum of the excited state potential energy surface, appears as broad band peaking at wavelengths longer than the monomer emission. In agreement with older observations,⁵⁻⁷ we detected such red-shifted emission peaks for adenine single strands,⁵⁰ $(\text{AT})_n\bullet(\text{AT})_n$ duplexes.² Interestingly, we remarked that, if necessary precautions are not taken during the experiments, a red-shifted emission from damaged DNA may be wrongly attributed to excimers/exciple emission (see SI in reference 18). This is very probably the reason for the discrepancies between calf thymus DNA fluorescence spectra reported by different groups.⁶

On the basis of recent fluorescence studies and the progress made in computational methods the fairly simple picture of a dual fluorescence, constituted of UV monomer-like and red-shifted excimer/excimer fluorescence described above, has evolved into a more complex one. Contemporary quantum chemistry calculations address excited state relaxation in relatively large DNA systems (exceeding 10 nucleobases), including the backbone, counterions and water molecules.^{1,12,44} Quite recently, such studies also integrated a surface hopping approach or molecular dynamics simulations, considering up to 10 base-pairs and 300 different conformations of long DNA strands.^{51,52} Even if the explored time-range corresponds to only a small portion of the total fluorescence decay, their statistical representation of the excited states facilitates conceptually the interpretation of the fluorescence stemming from collective excited states.

We can distinguish two limiting cases of collective states: Frenkel excitons that are linear combinations of two or more monomeric $\pi\pi^*$ states, and CT states, for which positive and negative charges are located on neighboring nucleobases. Strictly speaking, “pure” CT states, in which an entire atomic charge ($q = 1$ a.u.) has been transferred from one nucleobase to the other, are devoid of oscillator strength¹² and are not expected to emit at all. Probably this a reason for the different excited state dynamics of DNA multimers determined by fluorescence and transient absorption measurements. However, the formation of the CT states is not instantaneous, but follows the gradual approach of the involved nucleobases in a continuous manner.^{15,19,47,52} Moreover, conformational motions occurring over a large time-window lead to fluctuations of the electronic coupling between nucleobases⁴¹ and, hence, to the properties of the collective states. Consequently, emission from a continuum of mixed states, with various degrees of CT character and/or delocalization can be observed, as attested by the time-evolution of the fluorescence spectra.^{15,19,27,50} Upon increasing the duplex size the amplitude of the conformational motions decreases, as shown by X-ray diffraction studies in

solution⁵³ and infrared spectroscopy,⁵⁴ rendering them “stiffer”. This is reflected in the absorption and fluorescence spectra, as well as in the fluorescence decays of all the examined model duplexes.^{2,16,21,55} Example of the duplex size effect on the optical properties are presented in Figure 5 for various base sequences.

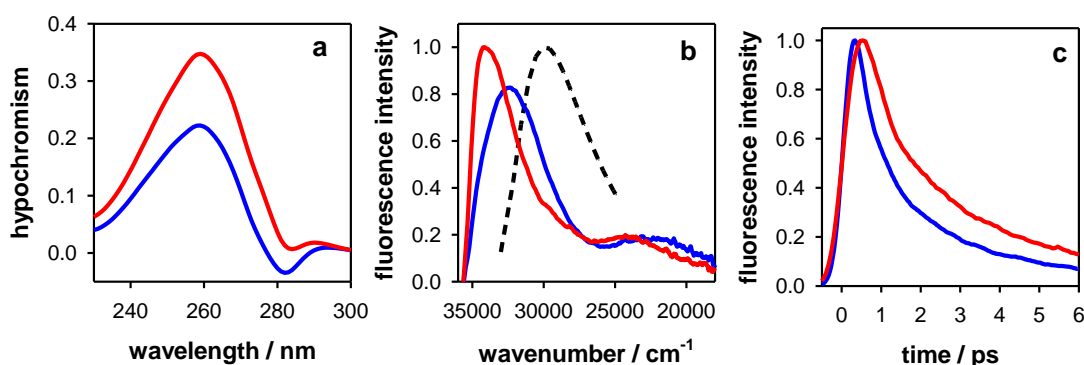


Figure 5. Optical properties observed for short (blue) and long (red) duplexes with various base sequences. (a) Thermal differential absorption spectra of $(AT)_n \bullet (AT)_n$; $n = 3$ (hairpin) and ~ 1000 ; they were obtained by subtracting the spectrum at 23°C from that at 96°C , both divided by the corresponding maximum value of the spectrum at 23°C .² (b) Steady-state fluorescence spectra of $(GC)_n \bullet (GC)_n$; $n = 5$ (hairpin)²¹ and ~ 500 ,³⁷ the dashed line corresponds to $\pi\pi^*$ emission, recorded by FU at 0.1 ps;⁵⁶ a wavenumber scale is used to better appreciate the spectral widths. (c) Fluorescence decays at 330 nm for $(A)_n \bullet (T)_n$; $n = 20$ and ~ 1000 .⁵⁷

Quantum chemistry studies on DNA refer to various types of excimers (bonded, neutral, CT..) and CT states.^{12,45} Fluorescence measurements do not allow the determination of neither the amount of charge transfer nor the number of nucleobases associated with it. Therefore, we have chosen to ascribe the term “CT fluorescence” to emission associated with a negative fluorescence anisotropy, known to indicate an important out-of-plane component of the emission transition dipole moment (Figure 4c).

Focusing on $\pi\pi^*$ fluorescence, we found that it clearly dominates the steady-state spectra of $(A)_n \bullet (T)_n$,¹⁶ calf thymus DNA¹⁸ (Figure 6a), TEL21/ K^{+38} , $(\text{TG}_n\text{T})_4/\text{K}^{+}$ and G-wires/ K^{+} .³⁹ For the model systems, the main fluorescence bands do not only overlap with, but are even

narrower than those of the corresponding stoichiometric mixtures of mono-nucleotides. For example, the spectral width (fwhm) of G-wires/ K^+ is 5000 cm^{-1} versus 8600 cm^{-1} for dGMP.²³ This spectral narrowing indicates that their fluorescence emanates from more stable excited state minima than for the monomeric chromophores.¹¹ Likewise, while for mononucleotides, the majority of photons at 330 nm is emitted before 1 ps, for these model duplexes and G-quadruplexes, the corresponding maxima (determined from histograms as that shown in Figure 3) are located at much longer times. This observation, associated with the spectral narrowing and the ultrafast energy transfer, shows that Frenkel excitons contribute significantly to the fluorescence of these systems and their contribution increases with increasing the size of the system^{2,16,21,39,40,55,57} (Figure 5c). It is also worth-noticing that, although the $\pi\pi^*$ fluorescence of $(GC)_n\bullet(GC)_n$ duplexes is readily probed by FU and found to be faster than that of the corresponding mononucleotides,^{17,56} it represents only a minor part of their steady-state emission spectra.^{37,49}

The steady-state fluorescence maxima of adenine single strands^{50,58} and TEL21/ Na^+ ,^{1,19} located around 360 nm, were assigned to a special type of $\pi\pi^*$ states ($\pi\pi^*$ “excimers”), different than the bonded and CT excimers mentioned above. Quantum chemistry calculations on these systems identified several low-energy minima reached during the excited state relaxation; some of them correspond to excitons that are delocalized on two stacked purines, stabilized by small geometrical rearrangements and exhibiting a very weak CT character.^{1,50}

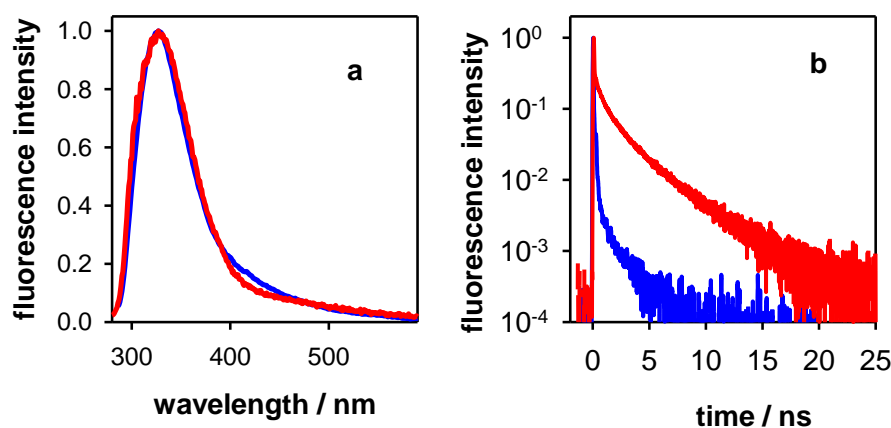


Figure 6. Calf thymus DNA (red) compared to polymeric $(A)_n\bullet(T)_n$ (blue): normalized steady-state fluorescence spectra (a) and fluorescence decays recorded at 330 nm by TCSPC (b).^{14,18}

G-quadruplexes are stiffer systems than duplexes, because, within a tetrad, each guanine is involved in four hydrogen bonds (Figure 2). Thus, it is not surprising that the highest quantum yields reported for DNA fluorescence have been determined for four-stranded structures (Tables 1 and 2), outshining those of duplexes by one order of magnitude.^{24,39,59} However, their fluorescence is sensitive versus structural factors whose effects have been examined both experimentally and theoretically.^{19,23,24,44,45,59-64}

As in the case of duplexes, local motions within G-quadruplexes modulate the excited state relaxation and, hence, the nature of the emitting states. These effects concern the guanine core, the loops joining the tetrads and the dangling ends. The presence in the central cavity of Na^+ cations, smaller and more mobile than K^+ cations, allows weak geometrical rearrangement of the stacked guanines.⁴⁵ Such motions are necessary for the stabilization of $\pi\pi^*$ “excimers” and CT states involving guanines within the tetrads and explain the red shift of the fluorescence spectra observed for TEL21/ Na^+ , $(TG_4T)_4/Na^+$ and G-wires/ Na^+ compared to their K^+ analogs.^{1,38,39}

In addition to CT states involving guanines within the quadruplex core, exciplexes may be formed also between one guanine of an outer tetrad and a nucleobase in the loops or the dangling ends. The type of this nucleobase (adenine, thymine, cytosine) and its position (5' or 3') play a key role in the fluorescence of G-quadruplexes.^{24,59,61} Excited state relaxation towards a CT state formed between a guanine and a thymine in TEL21/ Na^+ was rationalized by quantum chemistry calculations,¹ which also revealed the different properties of 5'-GA-3' and 5'-GA-3' exciplexes.⁶⁵ Longer loops exhibit additional degrees of freedom which are expected to favor the decay toward localized $\pi\pi^*$ states and/or “pure” CT states. While the

former are less fluorescent compared to the G-quadruplex core, the latter are completely dark, explaining the observed fluorescence quenching of the G-quadruplexes.⁶¹

The formation of the above mentioned “pure” CT states may lead to charge separation,⁶⁶ favored by the coexistence of a rigid core (trapping the positive charge), and a mobile loop/ending group, remaining negatively charged and eventually ejecting an electron to the bulk. This scenario constitutes currently the working hypothesis for DNA photoionization by low-energy photons.⁶⁷ Such a non-vertical electron detachment explains the fact that the photoionization quantum yields observed for certain G-quadruplexes are one order of magnitude higher ($\sim 10^{-2}$) compared to those of duplexes (Table 1 in reference 67).

HELM EXCITONS, THE UNEXPECTED PLAYERS

Our fluorescence studies evidenced a hitherto unreported emission at wavelengths shorter than that from the $\pi\pi^*$ states and lasting for several nanoseconds. This particular emission has been studied for various duplexes and hairpins with alternating AT and GC sequences. It is clearly enhanced in longer structures (Figure 5b) but disappears when going from the B-form to the Z-BNA.^{2,21,37,49} Moreover, its spectral width decreases with increasing number of base-pairs, reaching 2700 cm^{-1} (fwhm) for polymeric $(\text{GC})_n\bullet(\text{GC})_n$,³⁷ half of that corresponding to $\pi\pi^*$ emission determined for the same system by FU (Figure 5b).⁵⁶ In parallel, an increase in the duplex size results in an increase of the hypochromism of the absorption around 260 nm (Figure 5a), stemming from coupling between $\pi\pi^*$ and CT states.⁶⁸ The associated fluorescence anisotropy (Figure 4d) is slightly positive (0.02-0.03), which is between those of $\pi\pi^*$ emission (≥ 0.1) and CT states (< 0), suggesting a weak CT character.

The ensemble of these observations indicate that we deal with delocalized states with a mixed $\pi\pi^*/\text{CT}$ character, and, hence, they were designated HELM (for High-energy Emitting Long-lived Mixed) excitons. As HELM excitons are not detected by FU,^{56,57} we estimate that they represent less than 10% of the excited state population.

Given that this particular emission becomes detectable only for duplexes with at least ten base-pairs,²¹ a proper theoretical description of HELM excitons can only be expected for a system of the same size. It is, therefore, not surprising that they have eluded any attention in recent theoretical studies performed on 2-3 stacked AT or GC base-pairs.⁶⁹⁻⁷¹ If the necessary calculations, including a combination of quantum chemistry methods and molecular dynamics simulations, were out of reach a few years ago, they may become feasible in a near future.⁵² Nevertheless, a few exploratory attempts, based on only quantum chemistry calculations, have indicated the existence of relaxed mixed exciton states, involving four bases across both strands.^{2,49} These mixed states account well for the experimental properties of the HELM excitons described above. The CT component involves a weak ($q < 0.2$ a.u.) intrastrand, purine-to-pyrimidine, charge transfer ($G^{+\delta} \rightarrow C^{-\delta}$; $A^{+\delta} \rightarrow T^{-\delta}$). This picture was obtained by imposing a geometrical constraint, a C_2 -symmetry axis in respect to the base-pair. This symmetry characterizes both the initial Frank-Condon states and the HELM excitons, for which, however, the stacking distance is shorter; its disruption leads to evolution of the HELM excitons toward either localized $\pi\pi^*$ states or CT states. The experimentally observed long lifetimes are explained by the fact that reorganization of the backbone in large duplexes may require several nanoseconds.

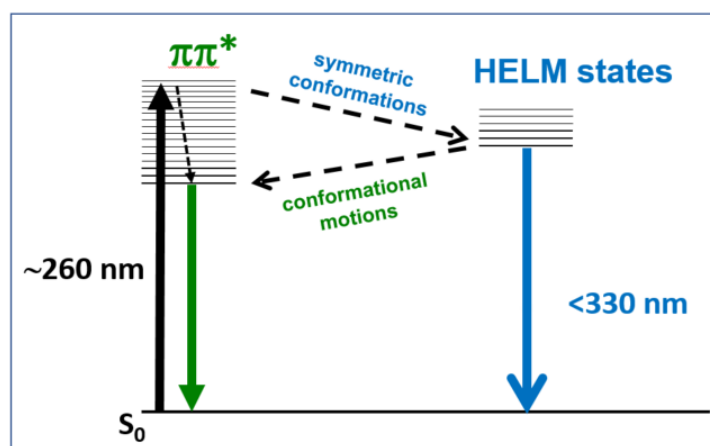


Figure 7. Simplified scheme illustrating generation of delayed $\pi\pi^*$ fluorescence in calf thymus DNA mediated by HELM excitons.

Going from model duplexes to native DNA, which contains GC and AT steps, its large size should favor emission from HELM excitons. Their fingerprint was indeed detected in the fluorescence anisotropy determined for calf thymus DNA: it is lower (0.05) at 305 nm compared to 330 nm (0.1), where $\pi\pi^*$ emission states is dominant.² Thus, the extremely long-lived $\pi\pi^*$ fluorescence observed for this system (Figure 6)⁴⁸ could arise from a slow repopulation mediated by HELM excitons (Figure 7).² This delayed fluorescence was in the past tentatively attributed to charge separation and charge recombination to a $\pi\pi^*$ state.⁴⁸ Notwithstanding the important energy barriers needed to overcome, such a process does not explain the low fluorescence anisotropy at the high energy part of the emission spectrum.² Moreover, as mentioned previously, charge separation is considered to be key step in DNA photoionization by low-energy UV radiation.⁶⁷ But the photoionization quantum yield of calf thymus DNA is, for example, the same as that of (A)₂₀•(T)₂₀ (Table 1 in reference 67), which does not exhibit any delayed fluorescence.²⁰ Thus, at the present stage of knowledge, the scheme in Figure 7 provides a more consistent interpretation.

UVA FLUORESCENCE

All the spectroscopic studies mentioned so far were performed with excitation in the absorption band peaking at ~260 nm. A past publication reported that the absorption spectrum of genomic DNA exhibits a weak tail at wavelengths longer than 320 nm, suspected to originate from guanines.⁷² We found that, despite the absence of guanines, the duplex (A)₂₀•(T)₂₀ (Figure 8a) and its two parent single strands, absorb weakly in the UVA spectral domain.^{3,50,73} Such absorption is not observed for the corresponding mono-nucleotides, pointing toward the collective nature of the excited states. Quantum chemistry studies assigned the UVA absorption to mixed $\pi\pi^*/CT$ states involving bases in the same strand.⁷⁴ This is in line with our observation that the molar absorption coefficient per base increases

upon base-pairing which also improves base-stacking, thus favoring the coupling between these two types of electronic states.

With UVA excitation, the fluorescence spectra peak at longer wavelengths and the fluorescence decays are much slower (Figure 8b) compared to UVC counterparts. Moreover, upon base-pairing, the fluorescence spectrum shifts towards shorter wavelengths, the fluorescence quantum yield increases, reaching 0.02 for duplexes (Table 2), while the decay becomes significantly faster.³ The average radiative lifetime determined for the duplex with UVA excitation is 8 times longer compared to that with UVC excitation, confirming the mixed $\pi\pi^*/CT$ nature of the emitting states. These mixed states probably do not evolve along the same path on the excited state potential energy surface as the $\pi\pi^*$ states, populated by UVC. However, a theoretical description of the relaxation of UVA excited states remains to be done.

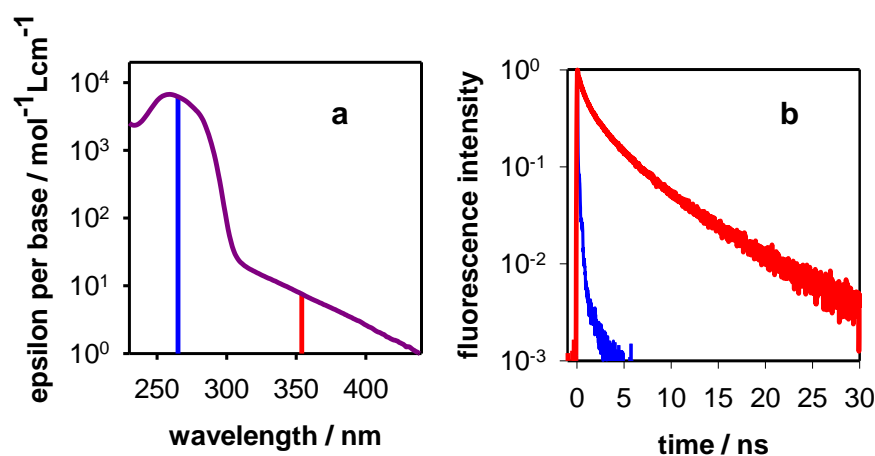


Figure 8. UVA versus UVC excited states of $(A)_{20}\bullet(T)_{20}$:³ (a) absorption spectrum and (b) fluorescence decays recorded by TCSPC at 420 nm (red) and 330 nm (blue); vertical lines in (a) indicate the corresponding excitation wavelengths.

Table 2. Fluorescence quantum yields ϕ and brightness ($\epsilon \times \phi$) determined for selected DNA oligomers; ϵ is the molar absorption coefficient of the entire oligomer at the excitation wavelength λ_{exc} .

system	λ_{exc} (nm)	$\phi \times 10^4$	$\epsilon \times 10^{-4}$ ($\text{mol}^{-1}\text{Lcm}^{-1}$)	$\epsilon \times \phi$ ($\text{mol}^{-1}\text{Lcm}^{-1}$)	ref.
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(A) ₂₀	255/330	6/50	17.2/0.011 ^a	103/0.5	3
(T) ₂₀	255/330	2/20	13.6/0.004 ^a	27/0.08	3
(A) ₂₀ •(AT) ₂₀	255/330	3/200	26/0.052 ^a	78/10	3
(CGGACAAGAAG) ₂ duplex	267	0.8	34 ^b	27	55
TEL21/Na ⁺	267	8.7	16 ^b	140	1
(TG ₄ T) ₄ /K ⁺	267	4	18 ^b	72	41
(G ₃ T) ₃ G ₃ /K ⁺	260	19	15	280	24

ϵ : ^{a)} from the Beer-Lambert law after determination of the oligomer concentration via titration of the PO₄⁻ groups in NaCl solution; ^{b)} from that of the single strand, corrected for the hypochromism of the duplex/G-quadruplex structure.

CONCLUSIONS AND PERSPECTIVES

The various types of excited states whose fingerprints have been detected in the fluorescence of DNA multimers are summarized in Figure 9; they are represented in spectro-temporal domains corresponding to observations of all the examined systems. It should be kept in mind that, for a given system, each specific “emission band” is built up of contributions from a large number of states with different delocalization lengths and/or amount of charge transfer. Finally, both their spectral position and their relative weight depend on the size of the system.

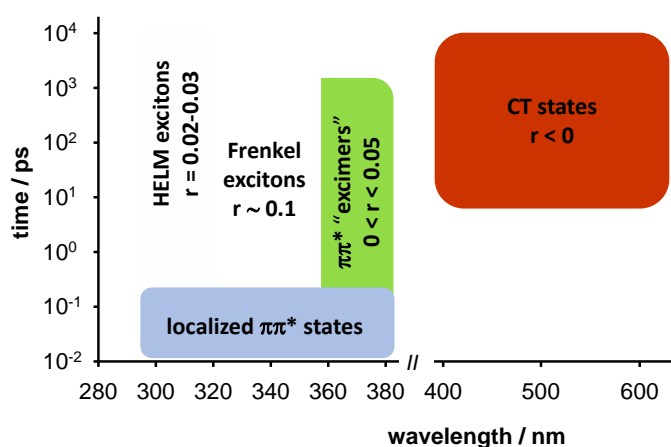


Figure 9. Types of excited states underlying the fluorescence of the DNA model systems discussed in this Account. Only the spectro-temporal domain in which a given type may be dominant is illustrated; “r” denotes the fluorescence anisotropy.

An important outcome of our work is that DNA fluorescence, is governed not only by the base sequence and secondary structure, but also by conformational motions. The corollary is that the study of single-sized duplexes fails to provide a full picture. Furthermore, the “paradigm” of a DNA fluorescence quantum yield on the order of $\sim 10^{-4}$ has been challenged: with UVA excitation it may be as high as 0.02.³ UVA fluorescence was studied only for homopolymeric AT duplexes. As native DNA also exhibits UVA absorption, it would be interesting to explore UVA absorption and the corresponding fluorescence for duplexes for other sequences and G-quadruplexes.

The fluorescence brightness, which is relevant for practical applications, is shown in Table 2 for some oligomeric structures. The brightness of duplexes amounts to only tens of $\text{mol}^{-1}\text{Lcm}^{-1}$ but it can be improved by shifting the excitation wavelength and/or increasing the duplex size, thereby reaching, for example, $120 \text{ mol}^{-1}\text{Lcm}^{-1}$ for $(\text{A})_{30}\bullet(\text{A})_{30}$, with excitation 260 nm. Depending on their structural parameters, the fluorescence brightness of G-quadruplexes may be twice as high as that of duplexes. The values in Table 2 are at least one order of magnitude lower than those reported recently for duplexes containing a modified base,⁷⁵ which, however, require specific synthetic procedures. Recent advances in high-power UV-emitting diodes⁷⁶ should render the use of the intrinsic fluorescence of nucleic acids possible for array screening. Along this direction, a multivariate analysis was performed recently for the intrinsic fluorescence of a large set of sequences.⁶³ Moreover, the first example of a biosensor exploiting the fluorescence of G-quadruplexes has been reported.⁷⁷

Thanks to their sensitivity, fluorescence measurements revealed that electronic excitations survive in DNA on the nanosecond time-scale. Even if long-lived excited states represent only a small fraction of those initially populated, they may play a crucial role in the DNA

photoreactivity. For example, the formation of CPDs, which are major UV-induced lesions,⁷⁸ involves $\pi\pi^*$ states.⁷⁹ Transient absorption studies on thymine single strands showed that the dimerization reaction is completed with 1-2 ps.⁸⁰ The discovery of delayed $\pi\pi^*$ fluorescence in duplex calf thymus DNA^{2,48} suggests that this ultrafast reaction may also take place at much longer times, as far as reactive $\pi\pi^*$ states are populated via the destruction of HELM excitons. Furthermore, quite recently, it was also reported that CPD formation in duplexes may occur 105 Å away from the excitation sites, due to energy transfer,⁸¹ a process that was indeed evidenced by our fluorescence studies. Our finding that base-pairing increases the $\pi\pi^*$ character of the UVA excited states explains the higher quantum yield of CPDs induced in duplexes by UVA radiation compared to single strands, while the opposite is observed for UVC excitation.³ Finally, long-lived CT states may underlie low-energy photo-ionization in DNA, generating guanine radicals responsible for oxidative damage.⁶⁷

Up to this point, DNA fluorescence induced by optical (UVC, UVB, UVA) excitation has been considered. But a quite recent study showed that DNA fluorescence can also be triggered by low energy electrons.⁸² This opens up a new field to probe the very complex excited state topology of DNA and potentially use its intrinsic fluorescence to detect harmful high-energy ionizing radiations.

ASSOCIATED CONTENT

Supporting Information: Materials and DNA Handling; Steady-state Spectroscopy; Time-resolved Measurements; Additional Figures.

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Biographies

Dr. Thomas Gustavsson studied physics and received his PhD at the University of Stockholm (Sweden), specializing in the field of high-resolution molecular spectroscopy. He moved to France in 1988 and joined the CNRS, where he is currently Research Director. He has developed and applied various laser-based spectroscopic setups for the study of molecular excited state dynamics, notably DNA and related biomolecules.

Dr. Dimitra Markovitsi studied chemical engineering at the National Technical University of Athens (Greece). Subsequently, she moved to France and received her PhD from the “Louis Pasteur” University (Strasbourg). She joined the CNRS in 1981, where she is currently Emeritus Research Director. She has expertise in photophysics and photochemistry, with emphasis on interchromophore interactions. During the past two decades she focused on the study of UV-induced processes in DNA, from photon absorption to excited state relaxation and reaction dynamics.

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