Fluorescence of DNA Duplexes: From Model Helices to Natural DNA

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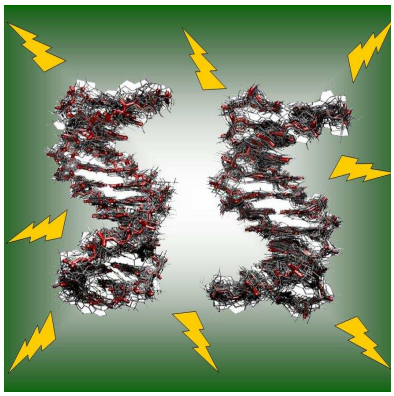
Abstract

Recent fluorescence studies of DNA duplexes with simple repetitive base sequence have revealed important collective effects which are very sensitive to conformational disorder. In contrast to the monomeric chromophores, whose fluorescence lifetimes are shorter than 1 ps, the fluorescence decays of duplexes span several decades of time. The occurrence of excitation energy transfer, evidenced by the decay of the fluorescence anisotropy on the femtosecond time-scale, is a common feature of all the examined systems. It is explained by the existence of Franck-Condon exciton states, in line with the UV absorption spectra. Understanding the nature of the long-lived excited states, whose emission dominates the steady-state fluorescence spectrum of natural DNA, will be one of the challenges for the years to come.



Keywords: DNA fluorescence, time-resolved spectroscopy, DNA excitons, conformational disorder, biophotonics

In biology and genetics the term “DNA fluorescence” is related to fluorescent probes, largely used for following structural and functional aspects of nucleic acids. Yet, nucleic acids do emit fluorescence albeit with a very low fluorescence quantum yield, on the order of 10^{-4} at room temperature. Fluorescence spectroscopy, which provides information about both the energy and the relaxation of the singlet electronic excited states, is a precious tool for the understanding of the primary processes induced by UV radiation, ultimately leading to carcinogenic mutations.[1] Natural DNA is a huge multichromophoric system composed of four different monomeric units (nucleotides: dAMP: 2’-deoxyadenosine 5'-monophosphate; TMP: thymidine 5'-monophosphate; dGMP: 2’-deoxyguanosine 5'-monophosphate; dCMP: 2’-deoxycytidine 5'-monophosphate) rendering the interpretation of experimental results extremely difficult.[2] Therefore, model duplexes, composed of a single type of base pairs (adenine-thymine: AT or guanine-cytosine: GC), have since many years become a favorite subject for spectroscopic studies.[3-5] Such model DNA duplexes have recently attracted attention for their potential applications in the field of molecular electronics and optoelectronics.[6-9] This perspective greatly increases the interest for the characterization of their optical properties.

Here, we focus on AT and GC duplexes with homopolymeric or alternating sequences. We follow their fluorescence over several decades of time, starting from the directly excited Franck-Condon (FC) states to long-lived states characterized by nanosecond lifetimes. We stress that an understanding of their excited states cannot be reduced to only a sequence effect. One has to realize that many other factors also come into play because of the structural flexibility of the examined systems. All these factors need to be considered in order to disentangle the puzzling behavior of natural DNA.[10]

The very first step in fluorescence studies is actually related to the absorption process leading to the population of bright states. Going from mononucleotides to duplexes, important

changes in the absorption spectra can be observed.[11-14] Different theoretical approaches have tempted to explain this behavior during the past few years. Calculations have been performed for duplexes composed of several base pairs in the frame of the exciton theory but neglecting orbital overlap.[14-17] Such interactions were taken into account in quantum chemical calculations carried out for smaller systems,[18-22] which pointed out the parentage between Frenkel excitons and charge transfer excitons.[21] Despite the different assumptions used, a common feature emerged: the FC excited states of duplexes are delocalized on several chromophores. This delocalization persists even in the presence of conformational disorder but depends on the sequence. It is higher for GC duplexes[16] in which structural fluctuations are weaker due to the existence of three hydrogen bonds instead of two for AT duplexes.[15] This is illustrated in Figure 1 (left panel) where the number of coherently coupled bases, quantified by the participation ratio, is represented for the FC excited states of $(dGdC)_5 \cdot (dGdC)_5$ and $(dA)_{10} \cdot (dT)_{10}$.

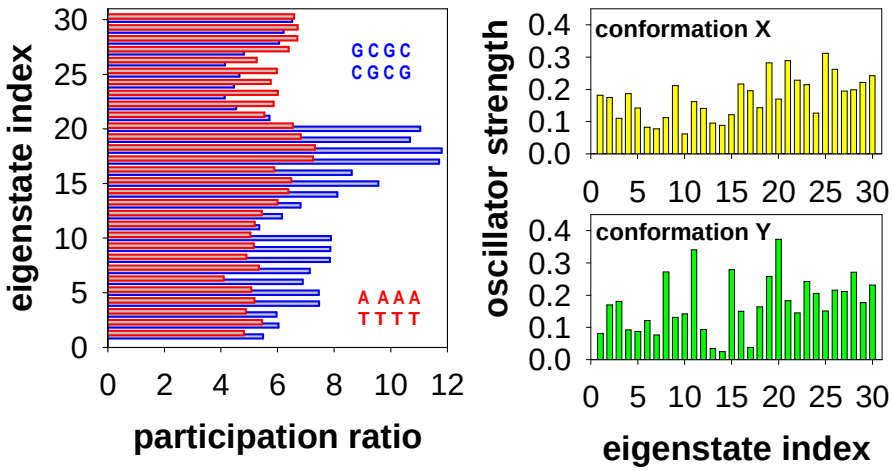


Figure 1. Left: participation ratios corresponding to the thirty Frank-Condon excited states of $(dGdC)_5 \cdot (dGdC)_5$[16] (blue) and $(dA)_{10} \cdot (dT)_{10}$[15] (red) averaged over 100 duplex conformations. Right: distribution of the oscillator strength over the thirty eigenstates associated with two different ground state conformations of $(dGdC)_5 \cdot (dGdC)_5$.[16]

From the above mentioned theoretical studies, it became quite clear that the geometrical arrangement of the bases within duplexes, which governs the electronic coupling, will be a crucial parameter for the properties of their excited states. As DNA duplexes exhibit continuous conformational motions, spectroscopic data collected for bulk solutions correspond to an average of a large number of conformations. An example is shown in Figure 1 (right panel) where the distribution of the oscillator strength over the thirty FC excites states of $(dGdC)_5 \cdot (dGdC)_5$ is plotted for two different conformations.[16] Evidently, conformational motions of a given duplex will affect the relaxation of its excited states and, consequently, its fluorescence properties. The corollary is that any factor having an impact on the conformational motions of the duplex will impinge on its fluorescence. A striking demonstration of this is the role of the duplex size whose increase enhances the collective behavior. As shown in Figure 2, the steady-state fluorescence spectra of polymeric duplexes are narrower and their average fluorescence decays, recorded either by fluorescence upconversion (FU) or time-correlated single photon counting (TCSPC), are slower than those of the oligomeric analogues.[23-25] Therefore, we focus below on polymeric systems in order to outline the sequence effect. But before discussing this aspect, we insist on some "experimental details" which are important for the study of DNA fluorescence.

Conformational motions of DNA duplexes play a key role in the collective behavior of their excited states and affect their fluorescence properties.

As mentioned above, fluorescence of DNA duplexes is very sensitive to other factors. Thus, the presence of various chemicals (added salts, alcohols….) in the solution[26] as well as the photodamage provoked during the measurements,[27] can seriously alter the results. We emphasize that, although the formation of photoproducts is restricted to one or two bases,

subsequent structural changes may be more extended,[28] affecting the electronic coupling and the relaxation of the excited states. In order to avoid contamination of the fluorescence signals with photons emitted from damaged helices it is important to keep the laser intensity as low as possible, use a sufficiently large ratio of molecules compared to that of the photons absorbed during the measurement and avoid local accumulation of photoproducts. However, the ultimate test is to check if successive signals recorded with the same solution are reproducible.[29]

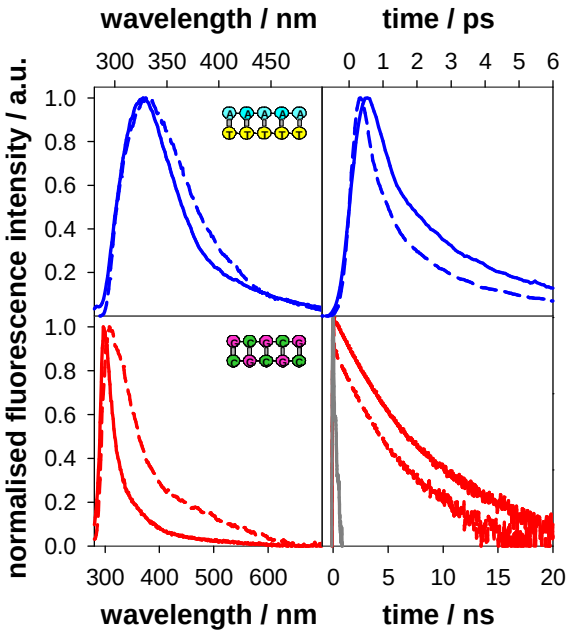


Figure 2. Size effect on the steady state fluorescence spectra (left) and the fluorescence decays (right) determined for $(dA)_n \cdot (dT)_n$[30] (blue: solid line: n = 1000, dashed line: n = 20) and $(dGdC)_n \cdot (dGdC)_n$[25] (red: solid line: n = 1000, dashed line: n = 10) by fluorescence upconversion (emission wavelength: 330 nm) and time-correlated single photon counting (emission wavelength: 305 nm), respectively.

By applying such experimental protocols, we have examined how the base sequence affects the emission from model duplexes dissolved in 0.1 M phosphate buffer containing 0.25 M NaCl and compared them to natural DNA studied under exactly the same conditions. The time-resolved fluorescence was measured using a common laser excitation source (150 fs; 267 nm) and two different detection techniques, FU and TCSPC.

A common characteristic of various model duplexes concerns their fluorescence quantum yield. Although higher than that of mono-nucleotides, it remains very low, on the

order of 10^{-4}. This is shown in Figure 3 (left panel) where the steady-state emission spectra of polymeric duplexes are presented[13,25,31] together with that of TMP, whose fluorescence quantum yield is 1.54×10^{-4}.[32] As in the case of monomeric chromophores, non-radiative routes are the dominant deactivation processes for the excited states of duplexes. The question arises whether the non-radiative deactivation paths operating in polymeric duplexes composed of hundreds of base pairs and in the much longer natural DNA[10] are the same as those described for the monomeric building blocks.[33] In this respect, the time dependence of the fluorescence could bring some insight.

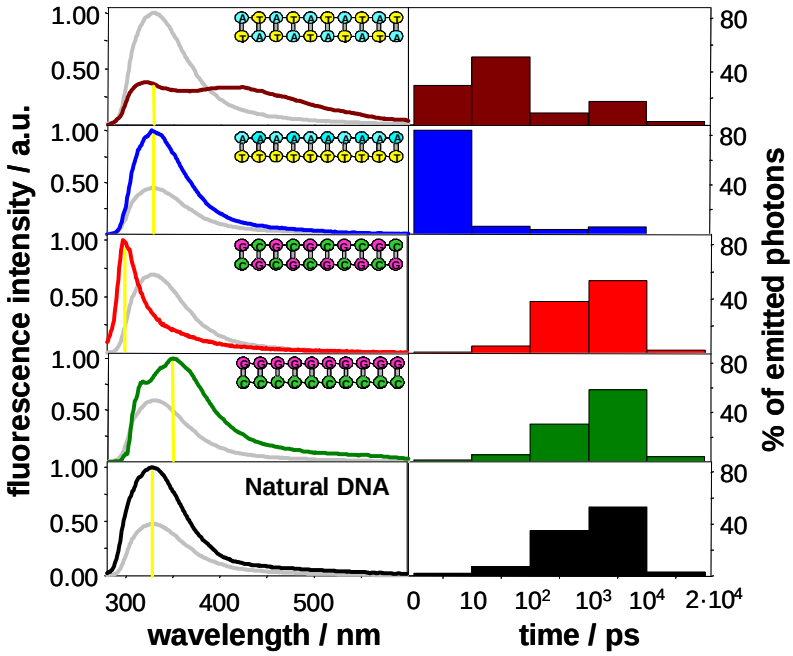


Figure 3. Steady-state fluorescence spectra (left) and distribution of the emitted photons per decade of time (right) determined for the polymeric model duplexes, poly(dAdT)·poly(dAdT) (dark red), poly(dA)·poly(dT) (blue), poly(dGdC)·poly(dGdC) (red) and poly(dG)·poly(dC) (green), and purified genomic calf thymus DNA (black), following excitation at 267 nm. Arrows denote the emission wavelength. The spectral area is representative of the fluorescence quantum yield ϕ; for comparison the TMP spectrum ($\phi = 1.54 \times 10^{-4}$) is shown in grey.

An important outcome of the recent fluorescence studies is that excitation energy transfer takes place among bases in all the examined model duplexes.[29,31,34] This was evidenced by probing the fluorescence anisotropy on the femtosecond time-scale, much

before any important structural motions can occur (Figure 4, left panel). The fluorescence anisotropy of the duplexes was found to be lower and to decay more rapidly than that of the stoichiometric mixture of monomeric chromophores. Taking into account the time-resolution of the experimental set-up, the onset of the energy transfer process was evaluated to be shorter than 100 fs. It is highly unlikely that such an ultrafast energy transfer proceeds via a Förster mechanism considering, in particular, the very large Stokes shift associated with the monomeric chromophores.[32] In contrast, the existence of delocalized excited states allows ultrafast energy transfer via intraband scattering, as evidenced, for example, for photosynthetic antennas.[35-37]

Excitation energy transfer, involving Franck-Condon excited states delocalized over several bases, takes place in model duplexes on the femtosecond time-scale.

The fluorescence decays of the bright excited states measured by FU depend strongly on the base sequence. The slowest ones are observed for homopolymeric AT duplexes and the fastest for alternating GC, the alternating AT exhibiting an intermediate behavior (Figure 4, right panel). The average lifetimes of these duplexes are respectively longer,[34] shorter[12] or equal[24] to those of the stoichiometric mixture of nucleotides. But fluorescence decays alone do not inform about the spatial extent of the emitting states. Such evidence is provided by the dependence of the fluorescence anisotropy decays on the emission wavelength (Figure 4, left panel) as described above. The rapid decay observed contrasts with the behavior of the monomeric chromophores, which proves, that, at least partially, emission from exciton states occurs.[23,24]

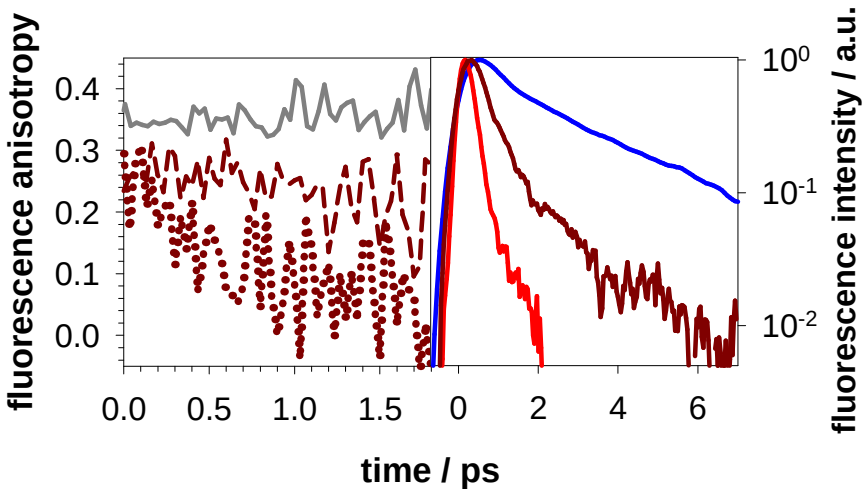


Figure 4. Left: fluorescence anisotropy decays determined for poly(dAdT)·poly(dAdT)[24] at 330 nm (dashed line) and 420 nm (dotted line) determined by fluorescence upconversion; the anisotropy corresponding to an equimolar mixture of dAMP and TMP is shown in grey. Right: Fluorescence decays recorded by fluorescence upconversion at 330 nm for poly(dA)·poly(dT)[31] (blue), poly(dAdT)·poly(dAdT)[24] (dark red) and poly(dGdC)·poly(dGdC)[12] (red).

The fluorescence decays of polymeric duplexes recorded by TCSPC[25,31,38] show just the opposite trend than their counterpart detected by FU. They become slower and slower going from homopolymeric AT, to alternating AT and further to GC sequences (Figure 3, right panel). It is tempting to link the fluorescence quenching occurring on the femtosecond time-scale to the appearance of the emission at longer times. In other words, it is as though the bright states were trapped by weakly emitting "dark" states.

The fluorescence decays of duplexes span several decades of time, the nanosecond components being dominant for those composed of guanine-cytosine pairs as well as for natural DNA.

Let us now consider the steady-state fluorescence spectra in the light of the time-resolved measurements. Those of alternating duplexes are clearly different from the corresponding spectra recorded on the femtosecond time-scale.[24,25] But this is not true for homopolymeric AT duplexes for which the major part of the photons are emitted within the

first 10 ps.[23,31] The steady-state spectra of the other examined sequences surprisingly contain an emission band located around 300 nm, *eg.* at higher energy than that of the bright states recorded by FU and peaking at *ca.* 330 nm. In the case of GC duplexes, this high energy peak corresponds to an excited state decaying on the nanosecond time-scale.[13,25] In addition to the 300 nm emission band, fluorescence from excited states emitting at longer wavelengths than those of the monomeric chromophores has been detected for homopolymeric GC[13] and alternating AT duplexes.[39] For the latter sequence, the low energy band was assigned to exciplex emission.[39,40]

Exciplexes were evoked in the early studies of natural DNA[2,41] and associated with fluorescence components decaying on the nanosecond time-scale. But no exciplex/excimer band is present in the steady-state fluorescence spectrum of purified calf thymus DNA (Figure 3) which is similar to that of the stoichiometric mixture of the constitutive nucleotides[10] as well as to that of poly(dA)·poly(dT), peaking at 327 nm. Yet, in the case of poly(dA)·poly(dT), only 16% of the photons are emitted at times longer than 10 ps, whereas this part amounts to 98 % for natural DNA, close to the behavior of GC duplexes[13,25] (Figure 3). We have thus the puzzling situation that the fluorescence of natural DNA is monomer-like but decays on the nanosecond time-scale.

Before looking toward the future, we remark that, since the first study performed for a DNA model duplex with femtosecond resolution,[42] many efforts were dedicated by us and other groups[40,43] to explore the behavior of short-lived excited states. The characterization of the sequences examined here is almost complete by now even if these measurements cannot yet give the whole picture about all the deactivation routes.

Regarding the long-lived excited states, the situation is more obscure. One could think that the high energy long-lived components determined for GC duplexes are associated to a very small fraction of bright states which escaped the non-radiative processes leading to

ground state recovery. Yet, considering their energy, they cannot be correlated to excited states localized on single chromophores. It would be also surprising if excitons extending over several bases keep their coherence over several ns. Most probably, they correspond to excited states with very small oscillator strength. Consequently, their population should not be negligible. Taking into account the fluorescence quantum yield and the fluorescence lifetime of alternating GC polymers and assuming a radiative lifetime of 1 µs, their "dark" population can be estimated to 20%. Our understanding of the long-lived emitting states of model duplexes, as well as of their connection with those of natural DNA, would benefit from the determination of time resolved spectra on the time-scale of hundreds of ps and ns and the effect of the emission wavelength on the fluorescence anisotropy.

So far, fluorescence measurements on DNA duplexes were conducted by using a unique excitation wavelength, close to the absorption maximum. Studying systematically the dependence of the fluorescence properties as a function of the energy deposited on the system would allow detecting different paths that contribute to the excited state relaxation, as reported already for mono-nucleotides.[44]

From an experimental point of view, the "Holy Grail" regarding DNA fluorescence would be the detection of emission from individual duplexes. Measurements on single macromolecules are expected to reveal conformational effects and the possible interplay between emitting and dark excited states, as already achieved for other biological systems.[45] The realization of such experiments for DNA is a real challenge.

On the theoretical side, the description of the excited state relaxation to the nanosecond time-scale is also a difficult task. In order to obtain a realistic picture which can be directly compared to experimental results, calculations have to take into account the various factors that affect the fluorescence properties. Although some attempts in that direction have been accomplished,[19,46-48] many obstacles still have to be overcome. Adapting

and combining various computational methods will be necessary in order to incorporate both dipolar and orbital overlap interactions, to consider the role of water molecules and counter-ions in the relaxation process while following conformational motions. At this point it is important to underline that, in order to be meaningful, this task must be carried out for sufficiently large systems. The question is what is the minimum size that has to be considered in the calculations to be relevant of the experimental data? A feedback from experiments is needed at this point. We have mentioned above that noticeable differences are observed in the fluorescence properties of the examined model duplexes when going from eicosamers to polymers composed of hundreds of base pairs (Figure 2). The enhanced collective behavior of the polymeric duplexes was attributed to reduced conformational disorder.[23] However, it is important to stress that polymers are produced by biochemical methods ensuring efficient base-pairing. In contrast, oligomers are prepared by annealing of complementary single strands; for simple repetitive sequences, slipping of the two single strands may lead to imperfect base pairing. In addition, fraying at the edges, further decreases the "double-stranded nature" of the system. Therefore, it would be interesting to investigate the size dependence of spectroscopic properties for oligomers produced by enzymatic methods. Another attractive perspective would be to use hairpins which, tailored at different sizes, have already provided valuable insight about exciton coupling,[14] charge transport and charge recombination.[49-51] Moreover, the study of such oligomers with complex base sequence will certainly help to elucidate the paradox of monomer-like fluorescence of natural DNA decaying on the nanosecond time-scale.

The great complexity of the fluorescence properties determined for model duplexes[38] and associated to conformational disorder contrasts with the relatively simple picture derived from transient absorption measurements.[52-54] According to the latter studies, initially populated $\pi\pi^*$ states relax to energetically low-lying excimers or exciplexes with well defined

time constants: 150 ps, 50 ps and 7 ps for homopolymeric AT,[55] alternating AT[55] and GC[56,57] duplexes, respectively. It would not be astonishing if these constants were longer than those determined by fluorescence measurements, because transient absorption experiments may probe completely dark states. Yet, emission from "dark" states decays at much longer times compared to transient absorption signals. Recording transient absorption spectra, in particular for polymeric duplexes, would greatly help to elucidate this discrepancy. It is worth-noticing that the first step toward that direction allowed detecting the fingerprint of Frenkel excitons.[58] In this way one may obtain a global picture of the excited state relaxation in duplexes, where Frenkel excitons, charge transfer excitons or polarons may evolve and interact,[59,60] possibly giving rise to delayed fluorescence.[10] Therefore it would be also important to combine the results of optical spectroscopy obtained upon direct excitation of DNA with the considerable amount of information accumulated on charge transport, trapping and recombination in DNA.[49-51,61-63]

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Biographies

Dimitra Markovitsi studied chemical engineering at the National Technical University of Athens (Greece). She received her PhD in Chemistry from the “Louis Pasteur” University (Strasbourg, France). She joined the CNRS in 1981 where she is currently research director and Head of the Francis Perrin Laboratory (http://www-lfp.cea.fr/). She has an expertise in photophysics and photochemistry of organized molecular systems. At present her research projects are focused on the interaction of UV radiation with DNA.

Thomas Gustavsson received his PhD in Physics at the University of Stockholm (Sweden) in 1988. He joined CNRS in 1991 where he is presently research director. He developed and applied various techniques of ultrafast laser spectroscopy for studies of solution phase chemistry. He is currently focusing his activity on non-radiative processes in photoexcited DNA.

Ignacio Vayá received his PhD in Chemistry at the Polytechnical University of Valencia (Spain) in 2007. Since 2008 started working at the Francis Perrin Laboratory as post-doctoral fellow supported by French National Research Agency and continues as a laureate of a grant from the Consellería de Educación-Generalitat Valenciana. His studies are focused on fluorescence of DNA helices.

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