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ARTICLE

Photobehaviour of methyl-pyridinium and quinolinium iodides derivatives, free and complexed with DNA. A case of bisintercalation.

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Excited state dynamics of four azinium salts was studied in buffered water and in the presence of salmon testes DNA. Complexation with DNA changes the photobehaviour of the free ligands lowering photoreactivity and emission in favor of internal conversion. Interaction of these four dyes with DNA was studied with different techniques with the aim to establish the affinity and the type of binding between the ligands and DNA. The results from spectrophotometric and fluorimetric titrations provided evidence of a strong interaction between the azinium salts and the polynucleotide, with binding constant of about 10^6 M^{-1}, making them interesting for therapeutical applications. Dichroic measurements allowed to determine the possible modes of binding for each complex. Short living excited states of the free dyes were detected and characterized by ultrafast absorption spectroscopy. A further decrease of transient lifetimes was observed upon interaction with DNA. The bicationic pyridinium iodide was found to act as a bisintercalative agent, potentially increasing cytotoxicity with low dose and less collateral effects.

Introduction

Interaction between small organic molecules and DNA is acknowledged as determinant for retardation or inhibition of transcription and replication and could be mutagenic for cells. Some methyl-pyridinium derivatives and analogous salts have been reported to efficiently interact with DNA. Planar aromatic molecules interact essentially by intercalation between base pairs, while flexible and large molecules bind on the grooves[1-3].

Recently more attention has been paid to bifunctional or polyfunctional intercalators, with the intent of enhancing ligand-DNA binding constants, slowering dissociation rates and increasing sequence selectivity. Moreover, polyfunctional intercalators could allow the same cytotoxic action with low doses and less collateral effects[4-6].

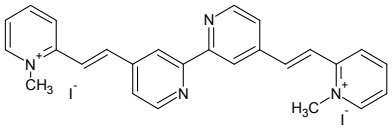
In previous papers[7-11] we investigated the photobehaviour and the interaction with DNA of many styryl dyes, that are of interest as candidates for the therapy of human pathologies because of their good interaction with DNA and their antitumor activity. Some of them present important biomedical applications as fluorescent probes for DNA, RNA, amyloid peptides and other bioanalytes for life-cell imaging, owing to their "light-up" effect when complexed with these biomolecules[12-14]. Recently styryl dyes have also gained attention for their interesting non-linear optical properties and their applications as optical recording media, sensitizers and laser dyes[15,16].

Internal conversion (IC) is the main decay pathway of these substituted cationic azastilbenes, even if the reactive trans → cis (E → Z) process becomes an important deactivation pathway for some of them. Fluorescence and intersystem crossing (ISC) are instead generally weak, as already seen for some analogues[7-9].

The present study deals with four methyl-pyridinium and quinolinium derivatives, both mono- and bi-cationic, expected to show great interaction with DNA. The aim of this work was twofold. Firstly, we wanted to investigate the photobehaviour of the studied systems free and complexed with DNA, to understand the effect of substituents and of an organized media, like the polynucleotide, on the relaxation pathways of the electronic excited states. Moreover, we wanted to investigate the correlation between the structure of the compound and the type of ligand-DNA complex formed. To this extent linear dichroic measurements (LD) have been performed to derive the angles formed between the compounds and the DNA double helix axis. The interesting results obtained suggested that the bicationic styryl dye can act as a bisintercalant agent.

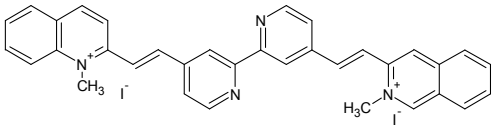
Experimental

The compounds investigated (compounds **1**-**4** in Scheme 1) were synthesized at the Catania laboratory by synthetic methods described elsewhere.[17,18] The photobehaviour of compound **3** in water has been already reported[19], but some data are here shown and duly referenced for comparison with the results obtained in presence of DNA.



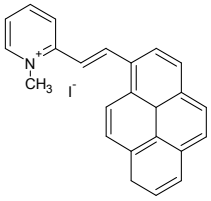
1

1-methyl-2-[(E)-2-{4'-[(E)-2-(1-metylpyridinium-2-yl)vinyl]-2,2'-bipyridin-4-yl}vinyl] iodide



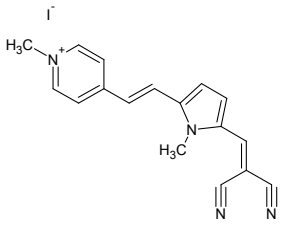
2

1-methyl-2-[(E)-2-{4'-[(E)-2-(1-metylquinolinium-2-yl)vinyl]-2,2'-bipyridin-4-yl}vinyl] iodide



3

1-methyl-2-[(E)-2-pyren-1-yl-vinyl]pyridinium iodide



4

1-methyl-4-{(E)-2-[5-(2,2-dicyanovynil)-1-metyl-1H-pyrrol-2-yl]vinil}pyridinium iodide

Scheme 1- Molecular structures of the investigated compounds

The photophysical and photochemical behaviour was studied in buffered water ETN (1 mM EDTA, 10 mM Tris-HCl, 10 mM NaCl) at pH=7.0. Additions of 2% and 5% of methanol (MeOH) or dimethylsulfoxide (DMSO) (from Sigma-Aldrich) were necessary to increase the solubility of some of the investigated compounds in water (W). Absorption spectra were recorded with Perkin-Elmer Lambda 800 and Varian Cary 4E spectrophotometers.

Fluorescence spectra and quantum yields were measured by a Spex Fluorolog-2 F112AI spectrofluorimeter (mean deviation of three indipendent experiments, ca. 10% for ϕ_F) in air-equilibrated solutions (absorbance <0.1 at the excitation wavelength, λ_{exc}) using 9,10-diphenylanthracene in cyclohexane as fluorimetric standard (ϕ_F = 0.73 in aerated solvent[20]) and neutral density filters with transmittance in the 1-10% range on the excitation line.

Measurements of the azinium salt complexation with DNA were carried out by following the evolution of the absorption or the fluorescence spectrum upon subsequent additions of a ~2×10^{-4} M aqueous buffered solution of salmon testes DNA (from Sigma-Aldrich) to an initial ~2×10^{-5} M concentration of the salt in the same buffer. The complexation constants (K/M^{-1}) were obtained from the absorption/concentration plots using the McGhee and von Hippel (MGH) equation[21] and from emission/concentration plots using a modified Stern-Volmer equation. The experiments, repeated at least two times, had a reproducibility within 30% owing to the rather small changes in absorbance and fluorescence and additional errors associated to corrections for dilution.

Fluorescence lifetimes, τ_F, were measured by use of an Edinburgh Instrument 199S spectrofluorimeter equipped with LED sources (λ_{exc}= 370 or 455 nm) using the single photon counting method.

For photochemical measurements (potassium ferrioxalate in water as actinometer), a 150 W high pressure xenon lamp coupled with a monochromator was used. The trans → cis photoreaction (solute concentrations ~10^{-4} M) was monitored by HPLC using a Waters 600 apparatus equipped with an analytical (Phenomenex Jupiter C18; 250 × 4.6 mm, 5 μm) column and an UV detector. Water/acetonitrile (MeCN) mixtures with some addition of trifluoric acid were used as eluents.

The photoisomerization quantum yields of the DNA-ligand complexes were obtained by adding the same volume of $MgCl_2$ 0.02 M in ethanol to the irradiated solution, and centrifuging for 15 minutes. The supernatant was then filtered with a 0.45 μm filter, concentrated with a stream of compressed air and injected into the HPLC apparatus (to be sure that all the DNA was precipitated and eliminated, to prevent that the polydispersive material interferred with the chromatographic elution)[22]. All measurements were carried out at room temperature in air-equilibrated solutions. The values reported are averages of at least two independent experiments with mean deviations of *ca*. 20%.

A nanosecond laser flash photolysis apparatus[23,24](Nd:YAG Continuum, Surelite II, third harmonics, λ_{exc} = 355 nm, pulse width ca. 7 ns and energy ≤1mJ $pulse^{-1}$) was used to investigate eventual transient formation of triplets or radical ions. Transient spectra were obtained by monitoring the absorbance change at intervals of 10 nm over the 370-850 nm range and averaging at least 10 decays at each wavelenght. All measurements were carried out at room temperature in aereated and nitrogen saturated solutions. The set up was calibrated by an optically matched solution of benzophenone (ϕ_T=1 and ε_T=6500 M^{-1} cm^{-1} at the corresponding absorption maximum, λ_{max}=520 nm) in acetonitrile[25]. The photostability of the compounds under investigation was checked by controlling the absorption spectrum before and after the flash photolysis measurements. To avoid photodegradation of the samples, the solutions (≈25 ml) were flowed through the analysis quartz cell using a continuous-flow system.

The linear flow dichroism spectroscopic (LD) measurements were made at the Department of Pharmaceutical and Pharmacological Sciences of the Padua University by a JASCO500-A spectropolarimeter. The solutions were prepared by successive additions of DNA (3.8×10^{-4} M) to a ligand solution (6×10^{-5} M) to obtain a ligand/DNA ratio of about 0.04 and 0.08. The LD signals for DNA alone in the free and oriented form were measured at an optical density of about 5 in the 230-300 nm region. The signals of the corresponding complexes were measured up to 400-450 nm. Partially oriented (up to 55%) samples of double helix DNA and its complexes with the azinium salts were obtained by producing an hydrodynamic field with the help of a rotating cuvette (700-800 rpm). From the LD signals, the reduced spectrum (LD_r) was obtained by the ratio between the LD signals and the absorbance of the non-oriented sample in isotropic conditions ($LDr = LD/A_{ISO}$)[26,27].

Femtosecond resolved transient absorption measurements were carried out by using Helios, an Ultrafast Systems spectroscopic set up[28,29]. Femtosecond pulses were generated by an amplified titanium-sapphire laser system (Spectra Physics, Mountain View, CA) that produces 1 W pulses of 40 fs centered at 800 nm with a repetition rate of 1kHz. Pump pulses centered at 400 and 266 nm were obtained by second and third harmonic generation in a 500 µm β-barium-borate crystal, respectively. The pump pulses were passed throw a chopper that cut out every second pulse and collimated to the sample in a 2 mm quartz cuvette. Probe pulses for optical measurements were produced by passing a small portion of 800 nm light to an optical delay line with at time window of 3200 ps, and focusing into a 2 mm thick sapphire crystal to generate a white-light continuum in the 450-800 nm range. The white light was focused onto the sample, and the chirp inside the sample cell determined by measuring the laser-induced Kerr signal of the solvent.

Theoretical calculations of the electronic spectra, dipole moments of ground and first excited state and heats of formation of the cations were carried out using the HyperChem computational package (version 7.5). The computed transition energies and oscillator strengths were obtained by ZINDO/S using optimized geometries (according to PM3 method) including 81(9×9) single excited configurations. The overlap weighting factor ($f_{p\pi}$) was set at 0.75 to obtain a satisfactory agreement with the experimental spectra[30].

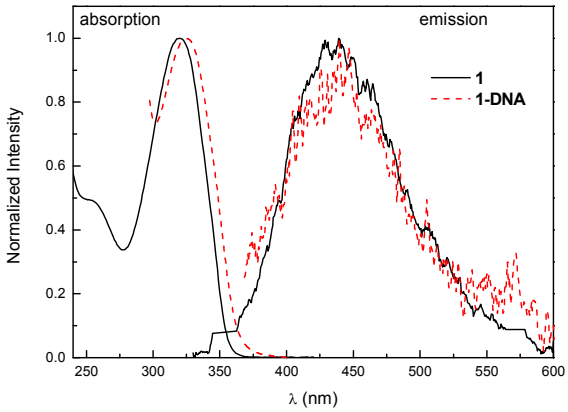
Results and discussion

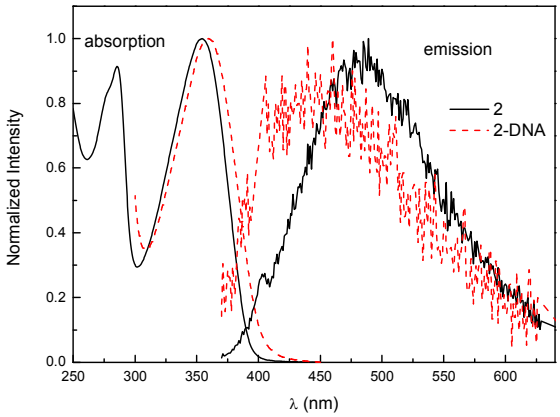
Spectral behaviour of free and complexed compounds

Figure 1 and Table 1 show absorption and emission spectra of compounds **1-4**, free and complexed with salmon testes DNA, in buffered aqueous solution. The main electronic transition of the investigated trans isomers produces a bell-shaped and intense band in the absorption spectrum centred at 320, 355, 420, 468 nm for compound **1, 2, 3** and **4**, respectively. This band is attributed to a π,π^* transition with charge transfer (CT) character (see below, paragraph 3.2). Another absorption band below 250 nm is characteristic of the iodide anion. The emission spectra look-like mirror images of the absorption spectra. The observed large Stokes shifts between the absorption and emission maxima, particularly in the case of **1-3** (see Table 1), indicate that the geometry of the first singlet excited state is quite different from that of the ground state or that an emitting CT singlet state is preferentially stabilized in polar solvents (see below, paragraph 3.2). However, as already reported for analogous compounds[8,10,11], the complexation with DNA causes a bathochromic red-shift of the absorption spectra (very large, of about 20 nm, for compound **3**) with respect to those of the free ligands. The fluorescence spectrum of **4**-DNA complex shows a little blue-shift with respect to that of the free ligand, while the fluorescence spectrum of **1**-DNA and **3**-DNA complexes remains practically unchanged and that of compound **2** shows the biggest blue-shift (more than 40 nm). These effects lead to a general reduction of the Stokes shift (Table 1), which indicates that the molecules are more rigid when they are bound to DNA.

Table 1- Spectral properties of compounds **1-4** in buffered water, free and complexed by DNA.

System	λ^{abs}_{max} (nm)	ε (M^{-1} cm^{-1})	λ^{em}_{max} (nm)	Stokes shift (cm^{-1})
1	320	54000	435	8260
1-DNA	325	42000	438	7940
2	355	32900	481	7380
2-DNA	358	25730	438	5100
3[19]	420	21530	586	6750
3-DNA	447	18950	580	5130
4	468	48490	538	2780
4-DNA	472	42130	530	2320





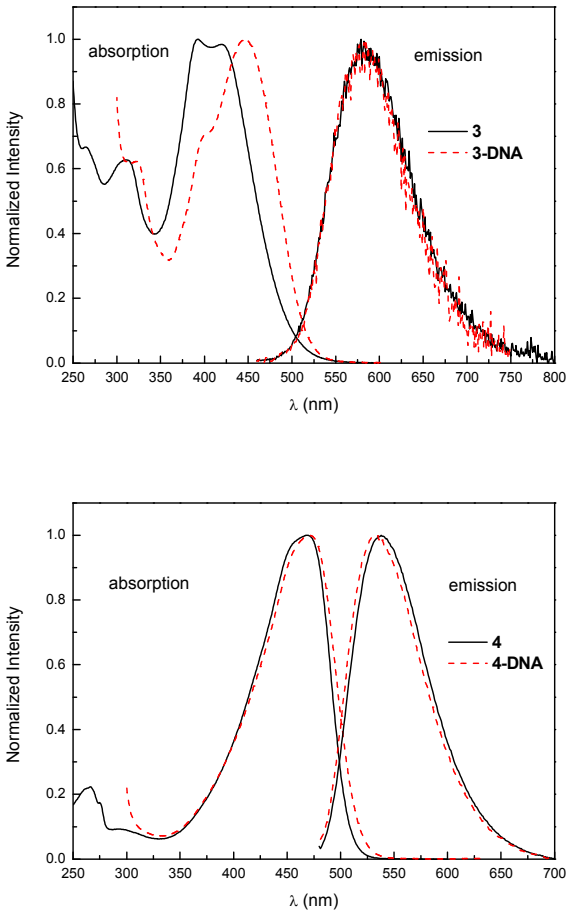


Figure 1- Normalized absorption and emission spectra of **1-4**, free and complexed with DNA, in buffered W.

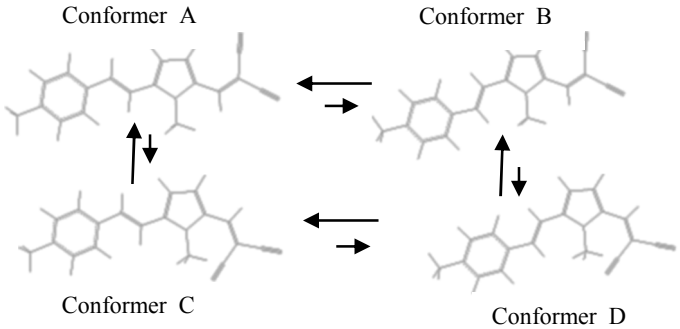
Quantum mechanical calculations

In principle, these compounds can exist as a mixture of conformers originated by the rotation around the quasi-single bonds between the ethene bridges and the aromatic or heteroaromatic rings (see Scheme 2 for compound **4**, as an example)[30]. Also the free rotation around the single bond between the two pyridines in compounds **1** and **2** can produce different conformers with different properties. Theoretical calculations showed that the conformer equilibrium is largely shifted towards the A configurations (see Scheme ESI 1), that are characterized by a lower formation enthalpy (ΔH_f^0), due to reduced steric hindrance. Only compounds **2** and **4** exhibit more than one conformer in solution but there is always the presence of a largely predominant species (see Table 2). The calculated spectra resulted in reasonable agreement with the positions and intensities of the experimental absorption bands. They indicated that for these compounds the first band usually corresponds to a π,π* electronic transition, with a partial charge transfer (CT) from the aromatic rings to the methyl-pyridinium or quinolinium moieties (see Figure ESI 1). The dipole moment of the ground state (μ) is very small for compounds **1** and **2** (because of their internal symmetry that makes them like quadrupolar systems) and does not significantly increase in the excited state. For compounds **3** and **4**, instead, the dipole moment of the excited state (μ^*) is substantially smaller, if compared with the value in the ground state.

Table 2- Calculated electronic spectra (wavelength, λ and oscillator strength, f) and properties (formation enthalpy, ΔH_f°, dipole moment of the ground, μ, and excited, μ*, states, percentage of abundance,%) for the possible conformers of compounds **1-4**[a].

Conformer	ΔH_f° (Kcal/mol)	%	μ (D)	μ* (D)	λ (nm)	f
1-A	476.82	100	1.251	4.557	320 306 240	1.91 0.37 0.27
1-B	479.59	-	2.382	2.303	312 291 240	1.93 0.22 0.24
1-C	479.65	-	3.779	5.620	390 368 306	1.00 0.31 0.21
1-D	479.64	-	3.218	4.696	320 306 297	1.64 0.27 0.22
1-E	478.16	-	0.6375	4.010	319 306 297	1.67 0.26 0.31
2-A	500.63	72	1.024	1.228	355 313 262	2.85 0.15 0.83
2-B	501.20	28	0.003	1.133	353 313 261	2.59 0.19 0.85
2-C	503.24	-	2.187	2.312	350 334 264	2.18 0.34 0.36
2-D	502.57	-	3.317	3.845	350 326 262	1.71 0.64 0.42
2-E	503.63	-	2.124	2.303	350 333 263	1.99 0.38 0.25
4-A	306.04	85.90	23.85	16.92	475 309 278	1.78 0.19 0.06
4-B	306.43	9.40	23.56	17.07	469 309 278	1.79 0.17 0.06
4-C	306.85	3.50	20.75	13.77	451 305 269	1.75 0.11 0.07
4-D	307.11	1.20	20.23	13.14	447 307 269	1.42 0.11 0.04

[a]Data for compound **3** are reported in Ref.19.



Scheme 2- Conformers of compound **4**

Photobehaviour of free and complexed ligands

Table 3 collects the relaxation quantum yields of the free molecules and their complexes with DNA. As already reported for analogous compounds previously studied in our laboratory[8-11], the radiative deactivation decay has a very low yield, which makes its measurement and that of the fluorescence lifetime rather difficult. Just compound **3** shows a fluorescence quantum yield (ϕ_F) of about 2% in W (which allowed us to measure its lifetime (τ_F) by the single photon counting method of 0.5 ns, just the resolution limit of our apparatus). Also the photoisomerization in the singlet manifold is a secondary relaxation pathway for the free compounds. In the case of compounds **1** and **2**, bearing two isomerizable double bonds, only the EE→EZ photoisomerization was observed by irradiatiing the EE isomer. Measurements carried out by nanosecond laser flash photolysis showed a $T_1 \rightarrow T_n$ low intensity absorption at room temperature in de-aereated solutions, with maxima at 480, 530 and 650 nm for compounds **1**, **3** and **4**, respectively (as shown in Figure 2). In all cases a significant quenching of the triplet lifetime was observed in air-equilibrated solution. Compound **2** gave no triplet signal. Considering the modest photoisomerization and the small intensities of the triplet signal, the $S_1 \rightarrow S_0$ internal conversion has to play the major role in the S_1 deactivation of the free ligands.

The binding with DNA affects the relaxation properties of the investigated compounds. The ϕ_F values are smaller than those of the free ligands, probably because of a charge transfer between ligands and DNA base pairs, as already reported for other intercalators[29-31]. Moreover, from our results the occurrence of energy transfer may be excluded considering that the absorption of DNA bases does not overlap the dye emissions. The fluorescence lifetimes resulted undetectable also in this case. The photoisomerization yields ($\phi_{t\rightarrow c}$), measured under irradiation of the pyridinium salts in the presence of DNA, are smaller than those of free complexes of more than one order of magnitude for compounds **1** and **4**. This effect is in agreement with an insertion of the ligand among the base pairs, with a consequent increment of the rigidity of the molecule, that is hindered to isomerize. For **3**-DNA complex there is instead an enhancement of $\phi_{t\rightarrow c}$, that could be explained with the presence of external binding of the ligand in the DNA scaffold, or an intercalation of the pyridine moiety not concerning the double bond which is free to rotate.

For complexes **1** and **3** we measured a $T_1 \rightarrow T_n$ absorption with a maximum of 470 and 550 nm of lower intensity with respect to the corresponding free compounds. No additional transient signals significantly different from those recorded for the free dyes were observed for the DNA complexes of the investigated compounds. In the case of complex **3**-DNA the detection of the bathochromic band at 750 nm, observed in the case of the free compound and assigned to ICT triplet,[19] was rather difficult due to a lower signal-noise ratio. Complex **2**-DNA gave no signal of triplet absorption. It has to be noted that was not possible to perform ns transient absorption measurements for **4**-DNA complex, for solubility problems, since λ_{exc} of the apparatus at our disposal (355 nm) corresponds to a minimum of the absorption spectrum of **4**-DNA complex. In conclusion, considering the low quantum yield for the competitive deactivation pathways, S_1 decays mainly through IC also in the case of DNA-ligand complexes.

Table 3- Fluorescence (F), photoisomerization (t→c), intersystem crossing (T) and quantum yields for **1-4** in buffered W, free and complexed with DNA.

System	ϕ_F	$\phi_{t\rightarrow c}$	ϕ_T
1	0.0075	0.160	0.030
1-DNA	0.0014	0.024	0.047
2	0.0090	negligible	negligible
2-DNA	0.0014	negligible	negligible
3[19]	0.028	0.120	0.016
3-DNA	0.020	0.400	0.010
4	0.006	0.016	0.030
4-DNA	0.003	0.001	-

Femtosecond transient absorption

The singlet excited state dynamics of the compounds was investigated in buffered aqueous solution at the physiological pH by pump–probe absorption spectroscopy upon ultrafast excitation centred at 266 nm for compounds **1** and **2**, and at 400 nm for compounds **3** and **4**, free and complexed with DNA. The time-resolved absorption spectra of the compounds are shown in Fig.3, together with the decay kinetics recorded at noteworthy wavelengths (the latter are also shown in Figure ESI 2). The kinetic traces were analysed at various wavelengths by global analysis: the best fits were obtained by considering three or four decay components, depending on the sample, convoluted with the instrumental response profile with a Gaussian shape. All the obtained results are collected in Table 4.

For compounds **1** and **2** the signals detected were generally broad and positive, corresponding to transient absorption, in the region above 500 nm, with a maximum around 700 and 680 nm, for compounds **1** and **2**, respectively. In these cases the best fitting was obtained by using two components and a 'rest'. The shortest component is consistent with the stabilization of the excited state by vibrational cooling within the first picoseconds after photoexcitation, because of the population of upper vibrational levels of S_1 with the excitation at 266 nm (see Figure 1). The time constant values for this component are of 6.6 ps and 12 ps for compounds **1** and **2,** respectively (Table 4). The second component, characterized by time constant values of 270 and 410 ps, is attributed to the relaxed S_1 state. The longer lifetime value obtained for compound **2** is in agreement with its higher fluorescence quantum yield with respect to compound **1** (Table 3). The absorption spectrum of the transient 'rest' is characterized by a broad band of positive absorption. The 'rest' transient was assigned to the lowest excited triplet state, as detected by nanosecond laser flash photolysis measurements for compound **1**.

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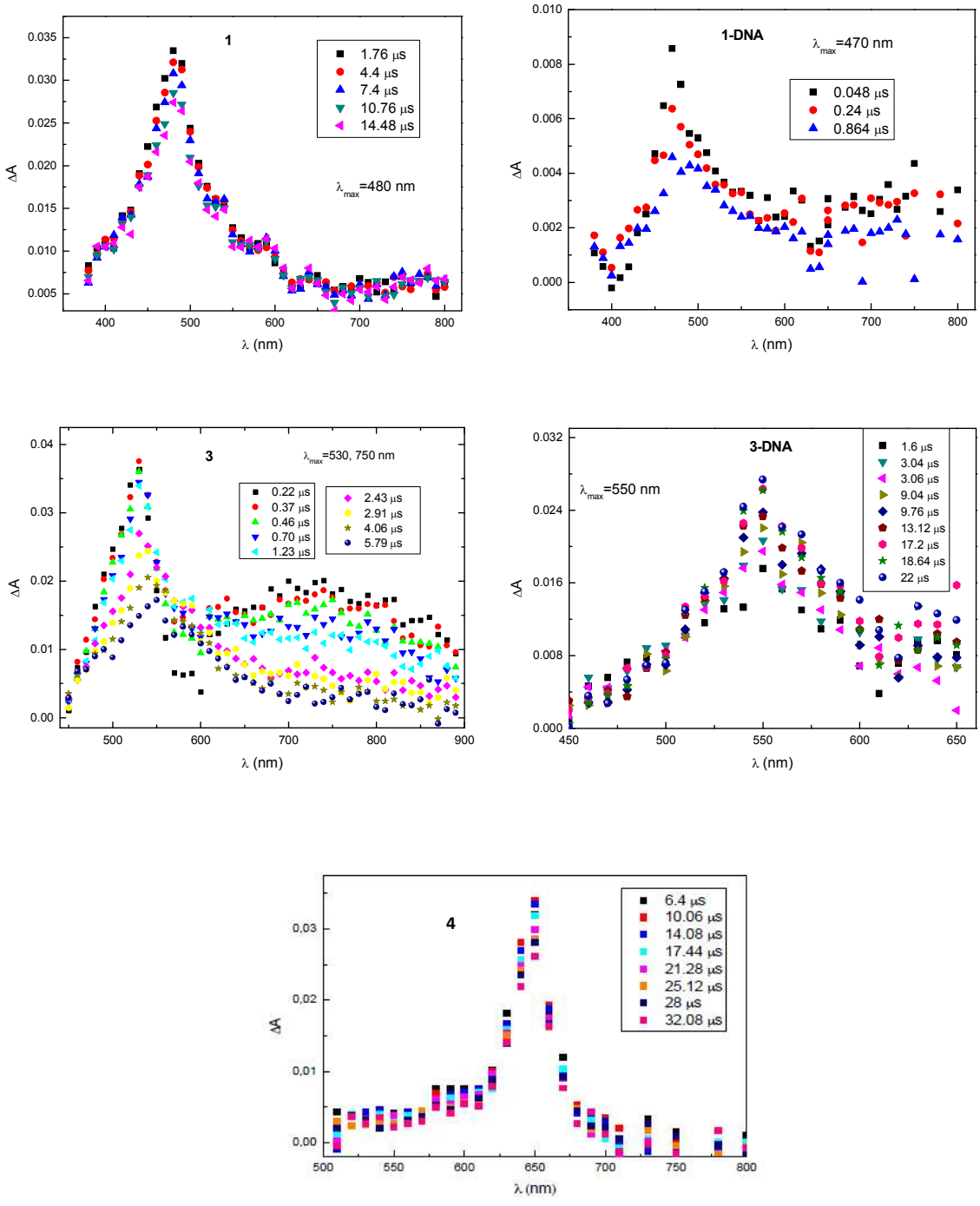

Figure 2 – T_1-T_n absorption spectra of compound **1**, **3** (free and complexed with DNA) and **4** (only free), in buffered W (**3** in W from Ref.19).

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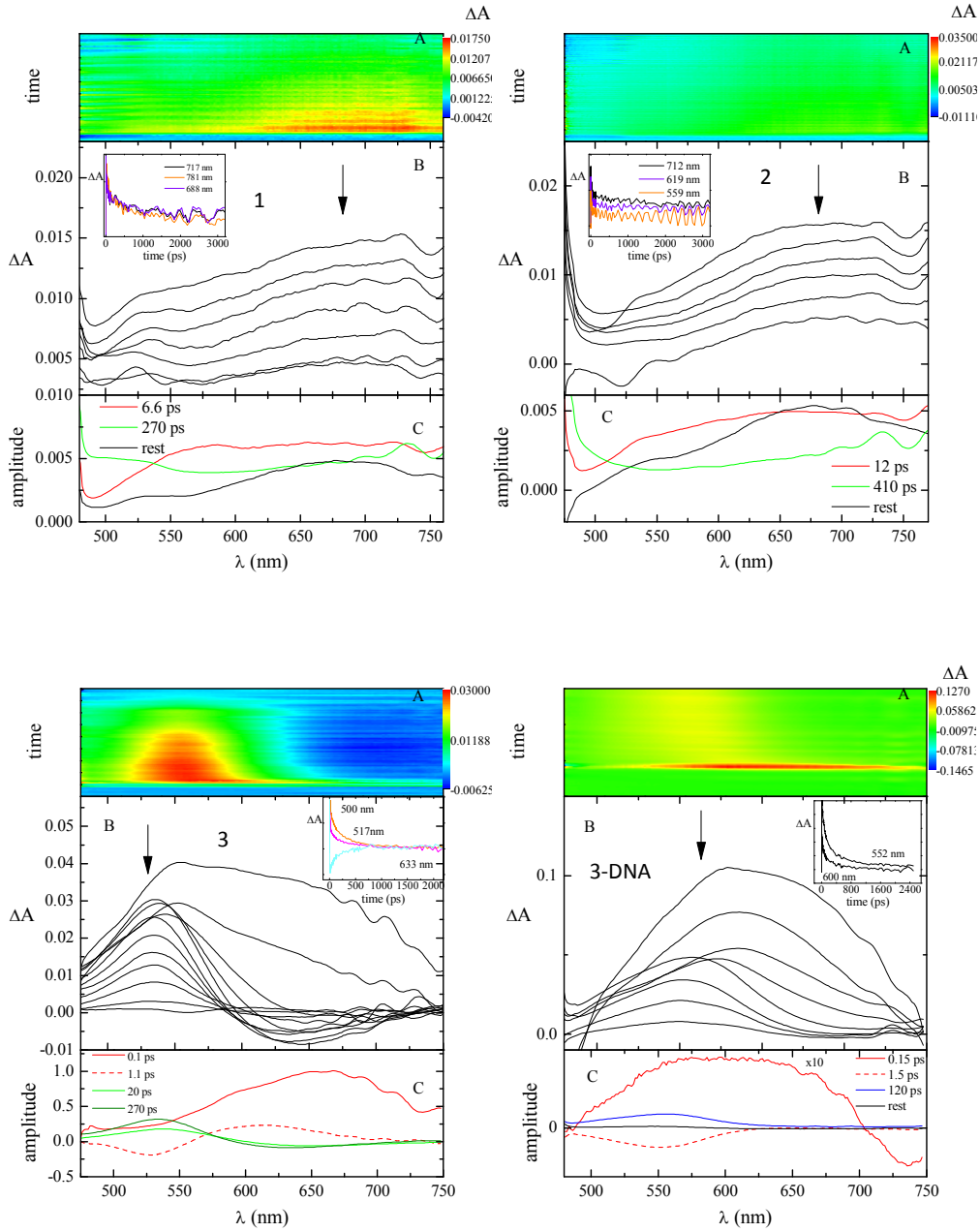
Table 4-Spectral and kinetic properties of the transients of the investigated compounds, free and complexed with DNA, detected in buffered water.

System	λ (nm)	τ (ps)	Transient
1	650 (+)	6.6	VC
	500 (+), 730 (+)	270	S_1
	broad (+)	rest	T_1
2	660 (+)	12	VC
	<520 (+), 740(+)	410	S_1
	<480 (−), 680(+)	rest	T_1
3[19]	650 (+)	0.1	Solv.
	530 (−), 615 (+)	1.1	Solv.
	540 (+), 650(−)	20	S_1 free (LE)
	535(+), 630(−)	270	S_1 free (ICT)
3+DNA (18:1)	610 (+)	0.1	Solv.
	540 (−), 610 (−)	1.0	Solv.
	520 (−), 590 (+)	5.2	S_1 free (LE)
	530 (+)	265	S_1 free (ICT)
	<540 (+)	rest	T_1
3+DNA (1.7:1)	620(+)	0.1	Solv.
	540 (−), 600 (+)	1.9	Solv.
	545 (+)	120	S_1 complexed
	530 (+)	rest	T_1
3+DNA (0.6:1)	630 (+)	0.15	Solv.
	545 (−), 610 (+)	1.5	Solv.
	555 (+)	120	S_1 complexed
	545 (+)	rest	T_1
3+DNA (0.15:1)	620 (+)	0.1	Solv.
	545 (−), 615 (+)	1.8	Solv.
	555 (+)	120	S_1 complexed
	550 (+)	rest	T_1
4	515 (−), 580 (+)	0.72	Solv.
	<530 (+),570 (−)	22	S_1 free
	broad	rest	T_1
4+DNA (20:1)	560 (+)	0.28	Solv.
	505 (+), 570 (−)	25	S_1 free
	broad	rest	T_1
4+DNA (10:1)	515 (−), 560 (+)	0.91	Solv.
	510 (+), 570 (−)	21	S_1 free
	broad	rest	T_1
4+DNA (1:1)	<530 (+), 580(+), 680 (−)	0.30	Solv.
	<530 (−), 670 (+)	1.0	S_1 complexed
	<520 (+), 570 (−)	22	S_1 free
	broad	rest	T_1
4+DNA (0.08:1)	525 (−), 580 (+), 670 (−)	1.0	Solv.
	<530 (−), 580 (−), 670 (+)	2.1	S_1 complexed
	<525 (+), 570 (−)	23	S_1 free
	broad	rest	T_1
4+DNA (0.04:1)	580(+), 670 (−)	1.0	Solv.
	<520 (−), 580 (−), 670 (+)	1.8	S_1 complexed
	<520 (+), 575 (−)	29	S_1 free
	broad	rest	T_1

The excited state dynamics of compound **3** in aqueous solution has been already studied by our group in a previous work[19]. The spectral evolution was well reproduced by considering four decay components. There are two shorter living components (0.1 and 1.1 ps) assigned to inertial and diffusive solvent relaxation processes. The third component characterized by a positive band centered at 540 nm and a short lifetime: τ=20 ps, was assigned to the locally excited state (LE) reached by light absorption. There is a fourth component with a spectrum centered at 535 nm and with the longest lifetime (τ=275 ps), assigned to an intramolecular charge transfer state (ICT). When compound **3** is studied in the presence of increasing amount of DNA, the fitting revealed the presence of three species (when a ratio **3**/DNA=1.7/1 is reached, see Table 4). The first two components (lifetimes of about 0.1 and 2 ps) were assigned to solvent relaxation processes. The observed slowing down of the diffusive solvation could be ascribed to a restricted motion of bulky water ordered at the DNA surface[31]. The longest living component of 120 ps presented a red shifted absorption with respect to that of the free compound, and was assigned to the first excited state of the complexed form. Shorter time constant value for the S_1 of the complex is in agreement with a reduction of its emission quantum yield, with respect to the free species. These findings suggest that the interaction between compound **3** and DNA inhibits the occurrence of intramolecular charge transfer, in favor of CT interactions with the DNA base pairs.

For free compound **4** in buffered water the detected signals were negative in the region between 520 and 700 nm, due to the stimulated emission. In contrast, the signal is positive at shorter wavelengths, below 520 nm. The spectra did not show relevant shifts in time, pointing to the same electronic state observed during the measurement. Global analysis revealed the presence of three components: the shortest with a lifetime of 0.72 ps, a longer one with τ=23 ps, and a component which doesn't decay in the investigated time interval (rest). The shortest component was associated to solvent relaxation, the longest one was assigned to the first singlet excited state S_1. The rest component was assigned to the lowest triplet state of the compound, as confirmed by the flash photolysis measurements. When adding increasing amount of DNA to a solution of compound **4** the presence of a new positive band centered at 670 nm was observed in the transient spectra. The signals are generally negative in the region between 520 and 650 nm and positive in the area above 650 nm. Decay kinetics have been registered in the area of positive absorbance, while growing kinetics can be found in the area of stimulated emission. Global analysis of data revealed the presence of four components: a short living component with τ=1.0 ps assigned to solvent relaxation, a medium living component with lifetime of about 2 ps, a long living one with a lifetime of about 25 ps and a rest. The ~25 ps component presents a spectral shape resembling that of the S_1 of free compound, a negative band of stimulated emission, centered at 575 nm. The presence of S_1 of free compound is consistent with the instauration of an equilibrium between free and complexed forms. In fact its amplitude lowers by adding increasing amount of DNA. The ~2 ps component, characterized by a positive band centred at 670 nm, was instead assigned to the S_1 state of the **4**-DNA complex. The time constant of the latter is smaller than that of the free species, in agreement with the fluorescence quenching observed during

complexation, and its relative amplitude becomes more relevant with adding DNA. In fact, the ratios of the amplitudes (S_1 complexed/ S_1 free, corresponding to their maximum signal at 570 and 670 nm, respectively), derived from the Global Analysis at [**4**]/[DNA]=1/1, 0.08/1 and 0.04/1, were found to be 0.461, 2.28 and 5.09 for the various samples. For compound **4** in the presence of DNA the detection of the S_1 transient of both the free and complexed dye was probably possible because of the difference in the spectral and kinetic properties of the two species. On the contrary the similarity in the excited state spectra and lifetime of **3** and **3**-DNA is the reason that makes difficult their resolution during the ultrafast measurements. Also in the case of **4** the rest component was assigned to the lowest triplet state of the complex.



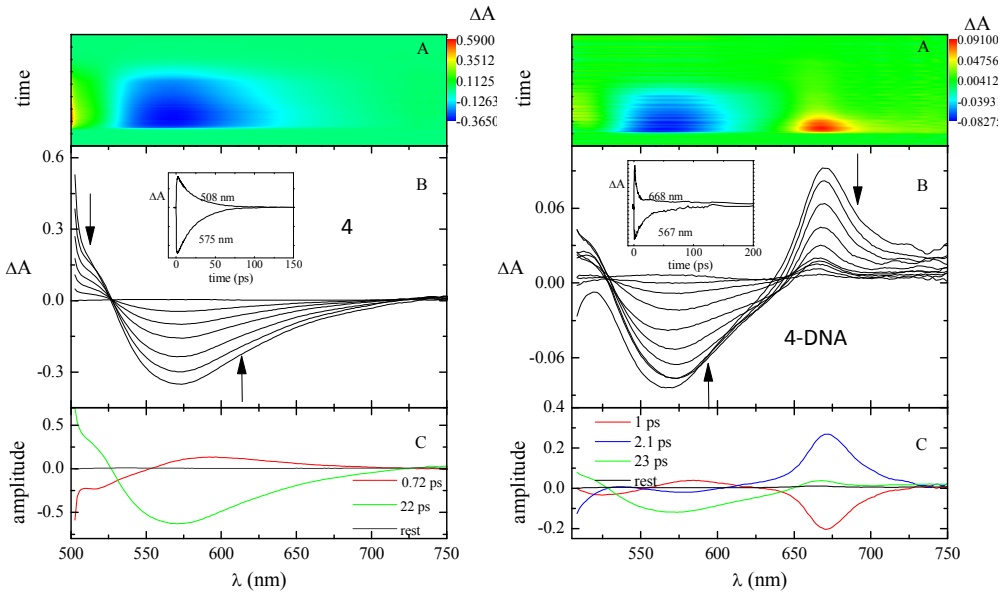


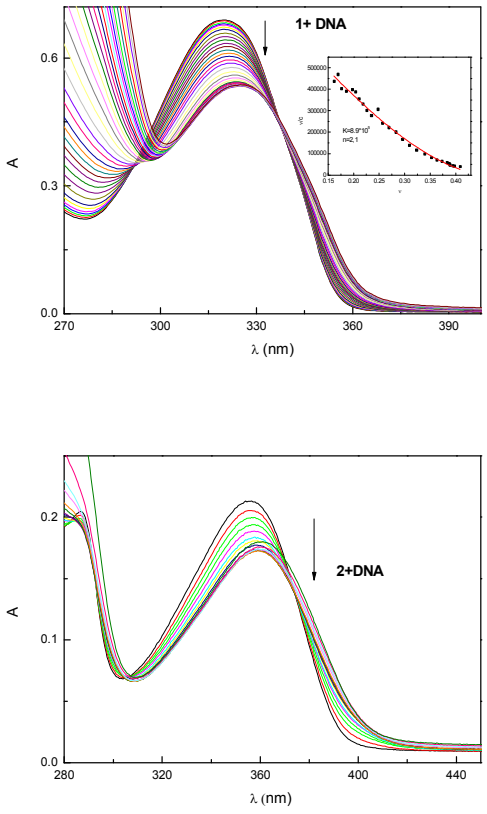
Figure 3- (A) Bidimensional matrix of experimental data. (B) Pump–probe time-resolved absorption spectra of compounds **1**-**4** free (compound **3** from Ref. 19) and complexed with DNA (only for compounds **3** and **4**) in buffered aqueous solution Inset: decay kinetics at meaningful wavelengths (they are shown also in Figure ESI 2). (C) Amplitudes of the decay components in aqueous solution obtained by global analysis.

Binding with DNA

Figure 4 shows spectrophotometric titrations for compounds **1-4**, carried out by adding successive aliquots of a DNA solution ~$2x10^{-4}$ M to a ~$2x10^{-5}$ M ligand solution, up to a [ligand]/[DNA] ratio of 0.01 where the invariance of the absorption spectrum was observed, pointing to an equilibrium shifted towards the complexed form. All compounds showed a red shift of the absorption spectrum with increasing DNA concentration, but only for compounds **1** and **4** an isosbestic point, which indicates the formation of a predominant ligand-DNA complex, was found (see Figure 4). On the contrary, the titration of compound **3** evidenced more than one isosbestic point in the absorption spectrum, this fact pointing to the presence of more species in solution. In the case of compound **2** the presence of scattered light evidenced the scarce solubility of the formed complexes and causes a non-linear behaviour of absorption intensity during the titration. Spectral changes in the absorption were analysed by the McGhee-Von Hippel equation:

$$\frac{\nu}{c} = K(1-n\nu)\left(\frac{1-n\nu}{1-(n-1)\nu}\right)^{n-1} \qquad (1)$$

(where c is the concentration of the free ligand and ν is the binding density, i.e. the ratio between the concentration of bound ligand and DNA) that allowed to estimate the complexation constant K and the number of the excluded binding sites n when the ligand is bound to DNA.



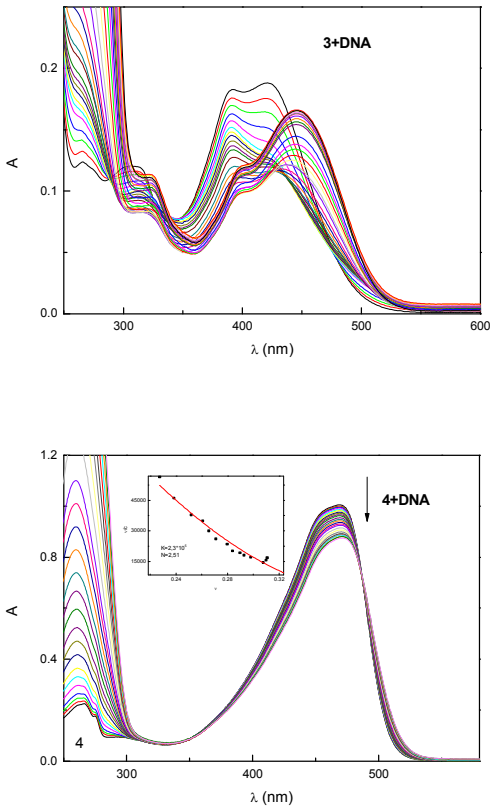


Figure 4- Spectrophotometric titrations of compounds **1-4** with DNA, in buffered W. (Insets: plots of the experimental data according to McGhee von Hippel equation, the latter are also shown in Figure ESI 3).

The absorbance uncertainty for compounds **2** and **3**, due to the scarce solubility of the complexes (or to the presence of different complexes), drived us to perform fluorimetric titrations in more diluite solutions, (see Figure 5), analysed by a modified Stern-Volmer equation[2]:

$$\frac{Area_F}{Area_F^0} = \frac{1 + \Delta Area_F \times K \times [DNA]}{1 + K \times [DNA]} \quad (2)$$

where $Area_F$ and $Area_F^0$ refer to the area under the fluorescence spectrum of the free ligand and after successive additions of DNA, respectively. $\Delta\, Area_F$ is the total variation of fluorescence intensity induced by complexation and K is the association constant for the ligand-DNA complexes.

As shown in Figure 5, complexation of the investigated compounds with DNA caused a quenching of fluorescence probably due to a charge transfer between the compounds and DNA base pairs[32-34]. Complexes **1**-, **3**-, **4**-DNA did not show a significant shift of the emission spectrum, while a modest blue shift was observed for complex **2**-DNA (see also Figure 1).

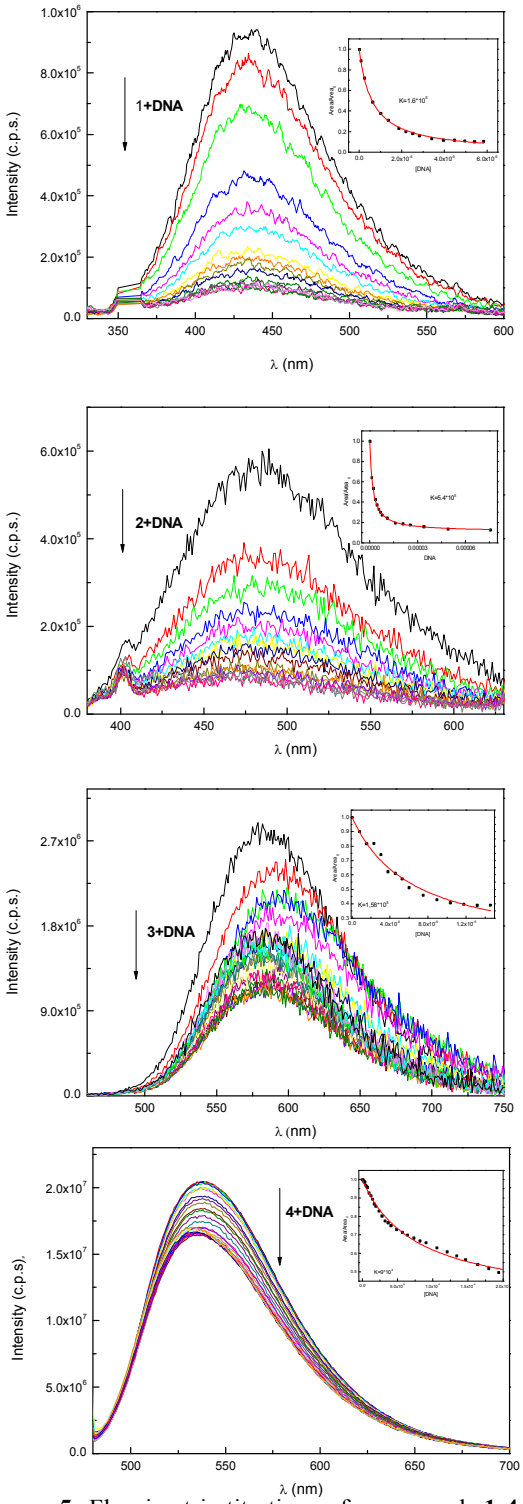


Figure 5- Fluorimetric titrations of compounds **1-4** with DNA, in buffered W. (Insets: plots to estimate the complexation constants by eq. 2, the latter are also shown in Figure ESI 4).

Table 5 collects the binding constants determined from spectrophotometric and fluorimetric titrations (the number of excluded sites estimated by McGhee-Von Hippel treatment was also reported). The ligand-DNA complexation constants obtained were in the range $10^5 \div 10^6\ M^{-1}$, high enough to be of interest for therapeutic

applications, in agreement with preliminary results on in vitro tests[35] and those reported elsewhere for analogous compounds.[17,36-38].

For compounds **1** and **4** the association constants were derived by both spectrophotometric and fluorimetric titrations. The values of K_a and K_f thus obtained are not so far (also considering the complexity of the systems and the different approach of the two methods), both of them indicating a strong ligand-DNA interaction. Just for these two complexes which showed an isosbestic point during the absorption titration, it was possible to calculate the number of excluded sites. For compound **1** that have two charged pyridinium rings, the n value of about 2 is compatible with a double insertion in the double-stranded polynucleotide. This compound could act as a bisintercalant ligand as suggested by dichroic measurements (see below, section 3.7). For compound **4**, a number of excluded sites of about 2.5 is probably due to the presence of a mixed type of interaction with DNA (intercalation and external binding), as confirmed by linear dichroism measurements. The data in Table 5 show higher K values for compounds **1-3** (particularly for **2**) that bear more conjugated aromatic rings, as already reported for analogous compounds[10,11].

Table 5- Binding constants as determined by spectrophotometric (K_{abs}) and fluorimetric (K_f) titrations and number of excluded sites n for complexes of **1-4** with DNA.

Complexes	K_{abs} (M^{-1})	K_f (M^{-1})	n
1-DNA	$(8.9\pm 0.2)\times10^5$	$(1.63\pm0.05)\times10^5$	2.10 ± 0.02
2-DNA	-	$(5.4\pm 0.2)\times10^5$	-
3-DNA	-	$(1.6\pm 0.2)\times10^5$	-
4-DNA	$(2.3\pm0.2)\times10^5$	$(9\pm 1)\times10^4$	2.52 ± 0.04

Linear dichroism measurements

To obtain information about the nature of the binding between the investigated compounds and DNA, we studied the ligand orientation with respect to the double helix by linear dichroism measurements. The absorption spectra and LD and LDr signals for the investigated compound **1** with [ligand]/[DNA] ratios of 0.04 and 0.02, are reported in Fig. 6 as an example (spectra for the other compounds are shown in Figure ESI 5). An intensity increase of the negative signal at 260 nm, corresponding to the DNA absorption, was found for all the compounds investigated, particularly for solutions with the highest ligand concentration. This result indicates a stabilization of the double helix due to an increased stiffening. A negative signal in the region of the ligand absorption (indicating a stronger absorption of the radiation perpendicularly polarized with respect to the axis of the double helix) was found for all compounds. The intensity of the negative band is larger for compounds that show a higher K. Negative signals are typical of complexes where the molecular plane of the ligand is preferentially oriented parallel to the plane of the DNA bases, pointing to the presence of intercalation.

From the signals of the complexes and of DNA alone, the angles of the ligand in the complexed form (a_L) were then obtained by eqn (3):

$$\alpha_L = \arccos\left[\frac{1}{3} - \frac{(LD_r)_L}{3(LD_r)_{DNA}}\right]^{\frac{1}{2}} \qquad (3)$$

and collected in Table 6. It has to be considered that these angles are indicative of an average of the possible orientations of double-stranded DNA the measurements involving only a part of the filaments (little higher than 50%).

Data of Table 6 indicate that compounds **1** and **3** at low compound concentration form a mixed interaction (external and intercalative mode, a_L 60-80°) and intercalate (a_L>80°) in the double helix when reaching saturation. This is in agreement with the good planarity of the aromatic structure of these compounds that can insert in the double helix by π-π stacking interaction with the aromatic bases. In contrast, compounds **2** and **4** show a mixed interaction in all the [lig]/[DNA] investigated. The observed alfa values around 60 degrees (indicating a mixed mode of interaction) are in agreement with more than one isosbestic point observed during the spectrophotometric titration (mainly evident for **3**, see Fig. 4) that points to the presence of more than two absorbing species. A number of excluded site >2 found for compound **4** is also in agreement with a mixed mode of binding. In the case of **1** the coupled information from absorption titration and dichroic measurements assures that the complexation is mainly by bisintercalation, otherwise for **2** the observed α~60-70 degrees points to an only partial intercalation mode accompanied by an external interaction, but the presence of bisintercalation at some extent cannot be excluded also in this case.

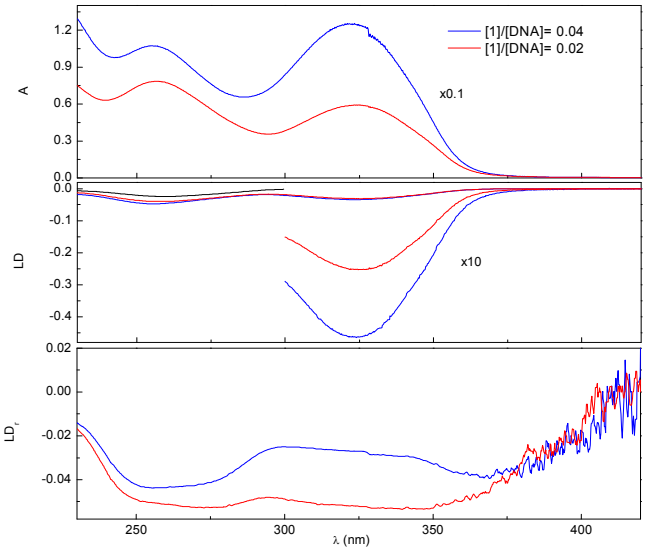


Figure 6- Absorption LD and LDr signals of compound **1** with [ligand]/[DNA] ratio of 0.04 and 0.02

Table 6- Angles between the ligand molecular plane and the double helix axis in the complexed form (a_L) at ligand/ DNA concentration ratio of 0.04 and 0.02.

COMPOUND	[lig]/[DNA]	α (deg)
1	0.04	66
	0.02	85
2	0.04	65
	0.02	67
3	0.04	61
	0.02	82
4	0.04	60
	0.02	69

Conclusions

The collected data from different spectroscopic techniques gave information on the excited states relaxation of the four investigated azinium salts. As observed for analogous compounds previously studied, their excited states prevalently relax by internal conversion and diabatic photoisomerization (the latter mainly for **1** and **3**) with an almost negligible contribution of the radiative pathway. There is also a secondary deactivation process by intersystem crossing with formation of a triplet state.

Complexation with DNA changes the photobehaviour of the free ligands, mainly decreasing their photoreactivity, as previously reported for analogous azinium derivatives[8-11]. The noticeable exception for compound **3** was found, where an increase of photoisomerization was observed probably because only the pyridinium ring is inserted in the DNA helix, leaving the double bond free to rotate. Also the emission quantum yield measured for the present compounds is reduced by complexation with DNA in favour of internal conversion, differently from what previously found, mainly for quinolyl-thienyl-derivatives [10], where an increase of emission accompanied the binding with DNA.

Excited state dynamics of the free dyes was studied by femtosecond transient absorption which revealed the presence of short living S_1 states in all cases. Interaction with DNA caused a further decrease of their lifetimes.

It has been commonly accepted that the interaction of the azinium salts with DNA is driven by the presence of the charged moiety. The parent molecule (iodide of *o*-methylstyrylpiridinium, **R**) showed low affinity with DNA ($K_a \cong 10^3$ M^{-1})[8,9] but the binding constant increased with the molecular complexity (presence of more aromatic rings, two or three branched systems) and presence of electron-donor groups[8,9]. The replacement of the pyridinium with a quinolinium group seemed to favour the ligand-DNA interaction, K_a increasing of four/five times with respect to the analogous pyridinium derivatives[8-11]. The compounds studied in this paper confirmed the previous findings giving high association constants. In fact, compound **3**, bearing a pyrenyl instead of a phenyl ring showed a higher K_a than that of the parent compound **R**. The study of bicationic derivatives is on the line to found systems bearing two DNA interacting moieties. As expected, these systems showed high binding constants supporting the hypothesis that both the azinium moieties are able to interact with DNA sites. The comparison of K_a (derived by the same technique, see Table 5) of **1** and **2** again points to an increased ability to bind DNA of the quinolinium against the pyridinium derivatives.

The derived high association constants of about 10^6 M^{-1} indicate that these compounds interact strongly with DNA, making them interesting candidates for therapeutic applications as antitumor agents. Titration analysis and linear dichroism measurements gave information also on the structure of the complexes. Compounds **2** and **4** form an angle of about 65° with DNA axis, indicating the occurrence of different kinds of interaction (intercalation and external binding), that does not change with ligand/DNA binding ratio. Compounds **1** and **3**, instead, form angles of about 65° indicating a mixed type of binding for low DNA concentration and angles of about 80°, characteristic of intercalative binding, for higher DNA concentration. The behaviour of compound **1** was found to be very interesting. In fact, the number of excluded sites (n=2) calculated from spectrophotometric titration, coupled with the information from linear dichroism measurements that point to a prevalent intercalative binding, indicated the possibility for this compound to act as a bisintercalative binder, the pyridinium moieties probably interacting with the adjacent base pairs. Therefore, compound **1** is expected to have interesting application as antitumor agent, allowing a better activity even with low drug concentration.

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Notes and references

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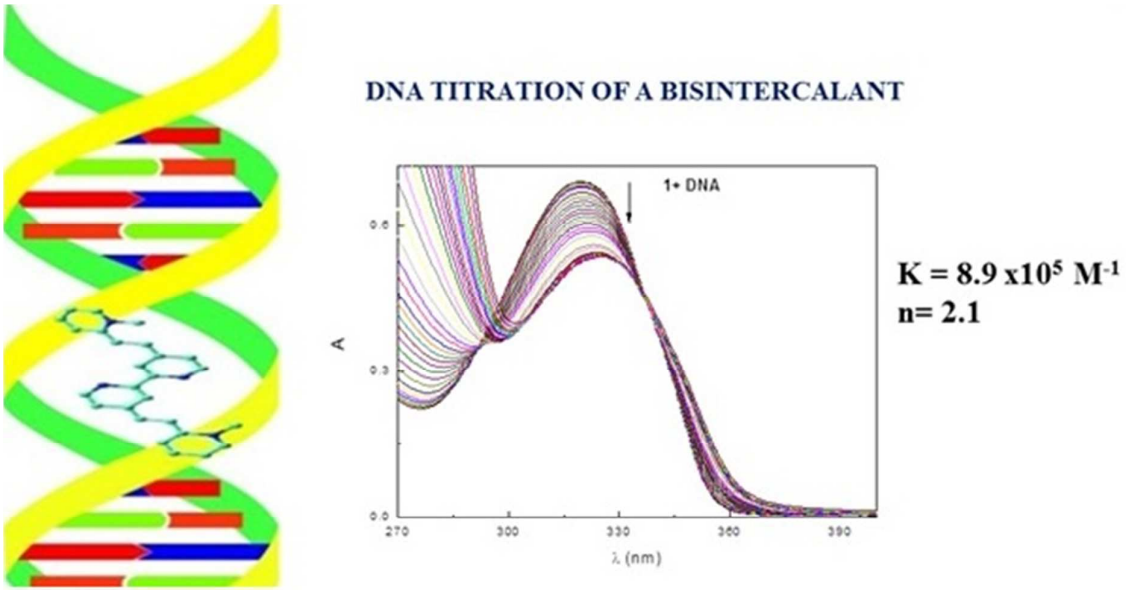

Interaction between azinium iodides and DNA. A bicationic dye acts as bisintercalative agent which could increase cytotoxicity with low dose and less collateral effects.