Excited-State Intramolecular Proton Transfer Distinguishes Microenvironments in Single- And Double-Stranded DNA

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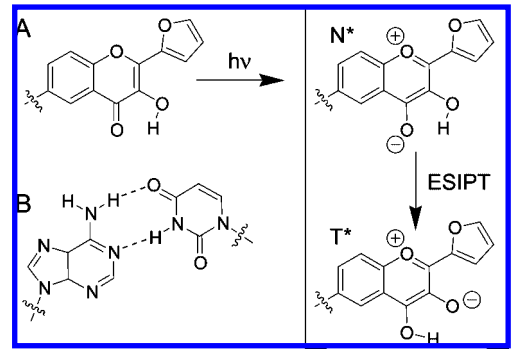
Herein, the efficient interaction of an environment-sensitive fluorophore that undergoes excited-state intramolecular proton transfer (ESIPT) with DNA has been realized by conjugation of a 3-hydroxychromone (3HC) with polycationic spermine. On binding to a double-stranded DNA (dsDNA), the ratio of the two emission bands of the 3HC conjugates changes up to 16-fold, so that emission of the ESIPT product increases dramatically. This suggests an efficient screening of the 3HC fluorophore from the water molecules in the DNA complex, which is probably realized by its intercalation into dsDNA. In sharp contrast, the 3HC conjugates show only moderate changes in the dual emission on binding to a single-stranded DNA (ssDNA), indicating a much higher fluorophore exposure to water at the binding site. Thus, the 3-hydroxychromone fluorophore being conjugated to spermine discriminates the binding of this polycation to dsDNA from that to ssDNA. Consequently, ESIPT-based dyes are promising for monitoring the interaction of polycationic molecules with DNA and probing the microenvironment of their DNA binding sites.

Introduction

Solvatochromic (environment-sensitive) fluorescent dyes are highly useful for obtaining new information about biomolecules and their interactions.[1−3] However, applications of these dyes for DNA research remains a challenge and only few examples are available in the literature. For instance, a dansyl fluorophore has been used to estimate the microenvironment polarity in the major groove of double-stranded DNA (dsDNA).[4,5] Moreover, DNA dynamics has been monitored by the time-resolved solvent relaxation of a coumarin.[6] In recent reports, a PRODAN-based fluorophore was conjugated with DNA bases[7−9] to evidence differences in the microenvironment of the major and minor grooves of A-, B- and Z-DNA. Environment-sensitive fluorophores having cationic groups, can bind DNA and report on the local properties of the DNA binding sites. For instance, the polarity of the minor groove of dsDNA has been estimated using the polycationic bisbenzimide (Hoechst 33258).[10] Similarly, a cationic anthracene derivative[11] and cationic acridizinium salts[12] provide a site-specific fluorescence signal on binding to DNA. Consequently, environment-sensitive fluorophores are highly promising for detecting the interaction of small molecules with DNA and probing the microenvironment of their binding site. In this respect, a prospective approach, which has not been realized so far, is to use a fluorophore undergoing excited-state intramolecular proton transfer (ESIPT) reaction as a building block of a DNA probe. ESIPT results in the formation of a new long-wavelength emission band.[13−16] This reaction is highly sensitive to the environment, so that solvent polarity and H-bonding may strongly modulate the relative intensity of the ESIPT product.[17−19] In this respect, 3-hydroxychromone (3HC) derivatives are probably among the best candidates for DNA applications. They undergo ESIPT between their 3-hydroxyl and 4-carbonyl groups[20] resulting in the emission of both normal excited-state (N*) and phototautomer (T*) species (Scheme 1).

SCHEME 1: 3-Hydroxychromone Derivative and Its Excited State Intramolecular Proton Transfer (ESIPT) Reaction (A) and, for Comparison, the Structure of a Natural Base Pair (dA-dT) (B)



The dual emission of 3HCs is highly sensitive to the environment,[17,18,21−23] since an increase in the polarity and H-bond donor ability of solvents inhibits the ESIPT reaction and thus, decreases the relative intensity of the T* band.[23,24] Moreover, the position of the maximum of the T* band in 3HCs is insensitive to solvent polarity, but exhibits high sensitivity to H-bond donor ability.[24] Thus, 3HCs provide two independent spectroscopic channels allowing a detailed characterization of their environment. These unique properties of 3HCs have already been applied for probing proteins, lipid bilayers and cell membranes.[25−28] However, application of 3HCs for DNA probing has never been shown so far. For this purpose, 2-(2-furanyl)-3-hydroxychromone is probably the most convenient fluorophore among known 3HCs, since it is a flat molecule with a size close to that of a base pair (Scheme 1), and exhibiting dual emission in aqueous media with a satisfactory fluorescence quantum yield.[29] In the present work, we conjugated 2-(2-furanyl)-3HC to an amino group of a natural polyamine, spermine, which is a polycation at neutral pH. In eukaryotic

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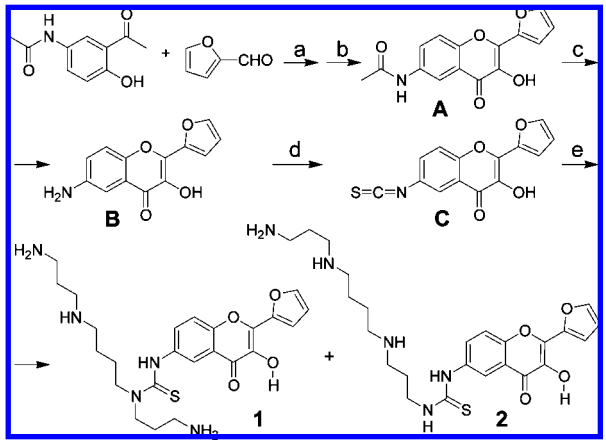
SCHEME 2: Synthesis of Conjugates 1 and 2[a]



[a] Reagents: (a) DMF, MeONa; (b) H_2O_2, EtOH, NaOMe; (c) 10% HCl; (d) DPT, THF; (e) spermine, DMF.

cells, spermine and its shorter analog spermidine form strong complexes with DNA and, thus, stabilize chromatin and prevent DNA damage.[30−32] Moreover, their lipid conjugates are efficient gene delivery vectors.[33] Our results show that binding of 3HC-spermine conjugates to dsDNA strongly increases the relative intensity of the T* state, indicating a low polar surrounding of the fluorophore inside DNA probably due to an intercalation of the dye between the DNA bases. In contrast, in complex with single-stranded DNA (ssDNA), a different dual emission profile is observed, indicating a significantly higher polarity of the fluorophore surrounding. Thus, our environment-sensitive dye conjugated with spermine can distinguish between ssDNA and dsDNA. Application of environment-sensitive dyes undergoing ESIPT constitutes thus a new approach for DNA probing.

Materials and Methods

Reagents and Solvents. All the reagents were purchased from Aldrich-Sigma Chemical Company. Solvents for synthesis were of reagent quality, they were appropriately dried if necessary. For absorption and fluorescence studies the solvents were of spectroscopic grade. Calf thymus DNA (CT-DNA) and single-stranded polydeoxyadenylic acids poly(dA) and poly(dT) were from Sigma.

Synthesis of 3HC Conjugates with Spermine (See Scheme 2). 5′-Acetamido-2′-hydroxyacetophenone and furfural were condensed into the corresponding chalcone in dry DMF in the presence of sodium methoxide (RT, 24 h). The reaction mixture was diluted with several volumes of ethanol and treated with 10 mol excess of hydrogen peroxide and 12 mol excess of sodium methoxide. Refluxing the mixture for 5 min afforded corresponding 3-hydroxychromone (**A**) with yield 55%. ^{1}H NMR (300 MHz, DMSO-d6) δ 10.25 (s, 1H, OH), 9.95 (br.s, 1H, NH), 8.43 (d, J = 2.5 Hz, 1H, ArH), 8.03 (br.s, 1H, HetH), 7.97 (dd, J = 9 Hz, J = 2.5 Hz,1H, ArH), 7.69 (d, J = 9 Hz, 1H, ArH), 7.29 (d, J = 3.5 Hz, 1H, HetH), 6.8 (dd, J = 3.5 Hz, J = 1.5 Hz, 1H, HetH), 2.09 (s, 3H, $COCH_3$); m/z 286.4 (M++H).

The above-obtained acetamide (**A**) was hydrolyzed in 10% HCl under refluxing during 7 h (100 °C). Then water was added and the mixture was neutralized with base to pH 7. The obtained product was filtrated and dried to give the corresponding amine (**B**) with yield 90%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.63 (s, 1H, OH), 7.99 (s, 1H, HetH), 7.44 (d, J = 9 Hz, 1H, ArH), 7,23 (m, 2H, ArH, HetH), 7.08 (dd, J = 9 Hz, J = 2.5 Hz,1H, ArH), 6.77 (dd, J = 3.5 Hz, J = 1.5 Hz, 1H, HetH), 5.47 (br.s, 2H, NH_2); m/z 244.2 (M^+ + H).

A 0.2 g (0.82 mmol) sample of 6-amino-2-furan-2-yl-3-hydroxychromone (**B**) was dissolved in 10 mL of dry THF and 0.35 g (1.5 mmol) of DPT (1,1′-thiocarbonyldi-2(1*H*)pyridone) was added to this solution. The reaction mixture was stirred under Ar at room temperature for 2 h. The solvent was removed in vacuo, and the product was purified by column chromatography (CH_2Cl_2/cyclohexane = 50/50) to give 0.17 g (72%) of pure isothiocyanate (**C**). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.26 (s, 1H, OH), 8.07 (m, 2H, ArH), 7.82 (s, 2H, HetH), 7.32 (d, J = 4 Hz 1H, ArH), 6.82 (dd, J = 3.5 Hz, J = 1.5 Hz, 1H, HetH); m/z 286.3 (M^+ + H).

A solution of 30 mg of **C** (2-furan-2-yl-3-hydroxy-6-isothiocyanato-chromen-4-one) in 1 mL of DMF was added dropwise to 100 μL of spermine with constant stirring for 2 h at rt. Crude mixture of two isomers (**1** and **2**) was applied to a reversed-phase C8 high-pressure liquid chromatography (HPLC) column (5 μm particle size) eluted with (A) aqueous TFA (0.05%, v/v), and (B) 70% of MeCN, 30% of water, with addition of TFA (0.05%, v/v). Linear gradient from 10 to 30% of B in 30 min was used. Fractions were collected at a flow rate of 4 mL/min and the eluant was monitored by adsorption at 360 nm. Fractions containing compounds **1** and **2** were freeze-dried to give lyophilized powders. For compound **1**, ^{1}H NMR (300 MHz, D_2O + drop of TFA) δ 7.65 (m, 2H, ArH), 7.28 (m, 3H, ArH), 6.64 (dd, J = 3.5 Hz, J = 1.5 Hz, 1H, HetH), 3.65 (m, 2H, C**H**$_2$NC=S), 2.84 (m, 10H, C**H**$_2$N), 1.92 (m, 2H, NCH$_2$C**H**$_2$CH$_2$N), 1.76 (m, 2H, NCH$_2$C**H**$_2$CH$_2$N), 1.54 (m, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_2$). For Compound **2**, ^{1}H NMR (300 MHz, D_2O + drop of TFA) δ 7.69 (m, 2H, ArH), 7.38 (m, 3H, ArH), 6.66 (dd, J = 3.5 Hz, J = 1.5 Hz, 1H, HetH), 3.8 (m, 2H, C**H**$_2$NHC=S), 3.6 (m, 2H, C**H**$_2$NHC=S), 2.88 (m, 8H, C**H**$_2$N), 1.99 (m, 2H, NCH$_2$C**H**$_2$CH$_2$N), 1.86 (m, 2H, NCH$_2$C**H**$_2$CH$_2$N), 1.65 (m, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_2$).

Instrumentation. Proton NMR spectra were recorded on a 300 MHz Bruker spectrometer and mass spectra were recorded on a Mariner System 5155 mass spectrometer using the electrospray ionization (ESI) method. All column chromatography experiments were performed on silica gel (Merck, Kieselgel 60H, Art 7736). Purification was carried out on a Shimadzu SPD-20A HPLC using a C8 column (uptisphere 300A, 5 μm; 250 × 10, Interchim, France) with a 10 to 70% linear gradient and monitoring the 3HC dye absorption at 360 nm. Absorption and fluorescence spectra were recorded on a Cary 400 spectrophotometer (Varian) and FluoroMax 3.0 spectrofluorimeter (Jobin Yvon, Horiba), respectively. For fluorescence studies, the dyes were used at 0.1 to 1 μM concentrations. Excitation wavelength was 340 nm. Fluorescence quantum yields were determined using quinine sulfate in 0.5 M sulfuric acid as a reference (quantum yield, φ = 0.577).[34] For the experiments in water, 10 mM phosphate buffer (pH 7.0) was used systematically. Hybridization of poly(dA) with poly(dT) was performed by annealing their equimolar mixture (100 μM, expressed in phosphate groups) in the phosphate buffer containing 100 mM NaCl at 95 °C for 10 min, followed by slow cooling (within *ca* 6 h) to room temperature. For the spectroscopic measurements, the obtained stock solution of the hybridized DNA, poly(dA-dT), was diluted to a final concentration (1 μM) using 10 mM phosphate buffer.

Results and Discussion

Initially, we synthesized a fluorophore building block **A** starting from 2-hydroxyacetonenone and 2-furaldehyde. The **A**

TABLE 1: Spectroscopic Properties of the Parent Dye A, and the Conjugates 1 and 2 in Different Organic Solvents, in Buffer, and as Bound to Different ssDNAs and dsDNAs[a]

media	dye	λ_{N^*}, nm	λ_{T^*}, nm	I_{T^*}/I_{N^*}	Q, %
water ($\alpha = 1.17$,[b] $\epsilon = 78.4$)	**A**	431	506	0.62	2.4
	1	433	510	0.90	0.5
	2	435	510	1.14	0.7
MeOH ($\alpha = 0.43$, $\epsilon = 32.6$)	**A**	421	532	1.14	6.3
	1	423	537	1.01	1.0
	2	425	537	1.32	1.0
EtOH ($\alpha = 0.37$, $\epsilon = 24.9$)	**A**	422	532	2.88	6.2
	1	422	538	2.08	1.0
	2	422	537	2.56	0.94
1-octanol ($\alpha = 0.37$, $\epsilon = 9.86$)	**A**	418	534	4.83	14.6
	1	421	536	5.00	3.0
	2	421	538	5.00	2.8
DMF ($\alpha = 0$, $\epsilon = 37.2$)	**A**	420	540	9.30	8.2
	1	425	543	7.52	1.3
	2	431	543	5.49	2.0
acetone ($\alpha = 0$, $\epsilon = 20.5$)	**A**	411	536	13.9	8.6
	1	419	538	7.4	1.7
	2	420	539	5.8	2.4
EtOAc ($\alpha = 0$, $\epsilon = 5.99$)	**A**	413	535	19.8	12.1
	1	417	537	14.9	3.9
	2	419	537	10.8	5.9
CT-DNA	**1**	435	539	7.70	1.3
	2	439	539	18.2	6.7
poly(dA)	**1**	436	535	1.15	0.7
	2	442	535	1.79	1.2
poly(dT)	**1**	431	532	1.35	0.7
	2	432	535	1.90	0.7
CT-DNA 83 °C	**1**	440	532	0.97	−
	2	444	539	1.88	−
poly (dA-dT)	**1**	430	537	6.35	1.4
	2	433	539	10.4	2.6

[a] Key: Q, fluorescence quantum yield, measured using quinine sulfate in 0.5 M sulfuric acid as a reference; λ_{N^*} and λ_{T^*}, position of the N* and T* emission band maximum, respectively; I_{T^*}/I_{N^*}, intensity ratio of the two emission bands. A 10 mM phosphate buffer was used. α: Abraham's H-bond donor ability (from ref 35). ϵ: dielectric constant. [b] The presented value of α corresponds to the H-bond donor ability introduced by Kamlet−Taft.[36]

compound was further hydrolyzed in hydrochloric acid and the obtained 6-amino derivative was converted into the 6-isothiocyanate derivative of 2-(2-furanyl)-3HC by using DPT (Scheme 2). The resulting derivative was further reacted with spermine giving two products of nearly equal quantities. After isolation with HPLC, we identified compounds **1** and **2**, corresponding to spermine modified with 3HC at the secondary and primary amino groups, respectively. Since the amino group of spermine used for conjugation cannot be protonated, conjugate **2** can be considered as a derivative of spermidine.

Spermine conjugates **1** and **2** as well as compound **A** which corresponds to the fluorophore, were studied in organic solvents of different polarity and H-bond donor ability (Table 1). The compound **A**, similarly to its parent 2-(2-furanyl)-3-hydroxychromone, exhibits dual emission highly sensitive to solvent properties.[24] In aprotic solvents, the I_{T^*}/I_{N^*} ratio is large, so that the emission of the ESIPT product T* dominates. On increase in solvent polarity within aprotic solvents from ethyl acetate to DMF, a decrease in the I_{T^*}/I_{N^*} ratio is observed (Table 1). In protic solvents, the emission of the ESIPT product is strongly decreased, due to the H-bonding perturbation of the ESIPT by the H-bond donor ability of the protic solvents.[17,24] Thus, comparison of protic and aprotic solvents of similar polarity, such as DMF and methanol, shows that in protic solvents, the relative intensity of the ESIPT product T*, the I_{T^*}/I_{N^*} ratio is much lower than in aprotic solvents (where the H-bond donor ability close to 0) (Table 1). Moreover, in protic solvents the dual emission of A varies also as a function of solvent polarity, so that in the polar solvent methanol, the I_{T^*}/I_{N^*} ratio is nearly 5-fold lower than in the less polar 1-octanol. Thus, both the H-bond donor ability and the solvent polarity hamper the ESIPT reaction and thus the emission of the T* form in **A**. As a result, in water, where both parameters are extremely high, the I_{T^*}/I_{N^*} ratio exhibits the lowest values (Table 1). Spectroscopic studies of conjugates **1** and **2** in organic solvents show that the 3HC moiety keeps its dual emission (Figure 1), and thus, undergoes ESIPT. Moreover, the positions of the two bands and the ratio of their intensities, I_{T^*}/I_{N^*} are not strongly modified on conjugation with spermine, as it can be seen by comparison of conjugates **1** and **2** with the parent dye **A** in most of the studied solvents (Table 1, some deviations are observed in water and aprotic solvents, see below). However, **1** and **2** show significantly lower fluorescence quantum yields than **A**, indicating a quenching of 3HC likely by the amine groups of spermine.[37] Similarly to **A**, the nature of the solvent affects strongly the dual emission of **1** and **2**. Indeed, for both conjugates the I_{T^*}/I_{N^*} ratio is much higher in aprotic than in protic solvents. Moreover, an increase in solvent polarity from 1-octanol to methanol also decreases the I_{T^*}/I_{N^*} ratio (Figure 1, Table 1). Noteworthy, the I_{T^*}/I_{N^*} ratio of **1** and **2** varies in a less broad range with solvent properties than that of **A** (its value being higher in water and lower in aprotic solvents, Table 1). This poorer spectroscopic sensitivity of **1** and **2** to solvent could be explained by a screening of the 3HC fluorophore by the spermine backbone. In water, spermine probably decreases the local polarity and H-bond donor ability around the fluorophore, while in aprotic solvents, it may considerably increase the local H-bond donor ability (due to presence of its NH-protons). Thus, similarly to the parent fluorophore A and other 3HC analogs,[17,18,22−24] conjugates **1** and **2** exhibit high sensitivity of their emission to solvent polarity and H-bond donor ability. These two solvent parameters also affect the fluorescence quantum yield of the dyes, so that it is the highest in the apolar aprotic ethyl acetate and the lowest in water.

Since water is a key solvent that modulates the local polarity and H-bond donor ability in biomolecules, we studied the influence of water in the aprotic acetone solvent on the dual emission of conjugates **1** and **2**. The fluorescence spectra of conjugates **1** and **2** exhibit a strong decrease in the I_{T^*}/I_{N^*} ratio on increase in the water content in acetone (Figure 1), so that water hampers the ESIPT process and thus decreases the emission of the ESIPT product. Moreover, the increase in water content shifts considerably the T* band maximum to the blue (Table 1), which according to our previous studies is likely due to the high H-bond donor ability of water.[24,29] Thus, both the ratio of the two emission bands and the position of the tautomer band of 3HC dyes provides information on the water content in their surrounding, so that being bound to biomolecules, 3HC fluorophore can report on its exposure to water.

Addition of dsDNA (calf thymus DNA, CT-DNA) to conjugates **1** and **2** in buffer changes dramatically their fluorescence spectra (Figure 2), while dye **A** does not show any spectroscopic response to dsDNA (data not shown). Thus, conjugation with spermine enables the 3HC fluorophore to bind dsDNA. This binding strongly increases the fluorescence intensity of the T* band and decreases the intensity of the N* band, so that the I_{T^*}/I_{N^*} ratio increases (Table 1). Thus, binding to dsDNA favors the emission of the ESIPT product (T*). Noticeably, the increase in the T* emission on binding to

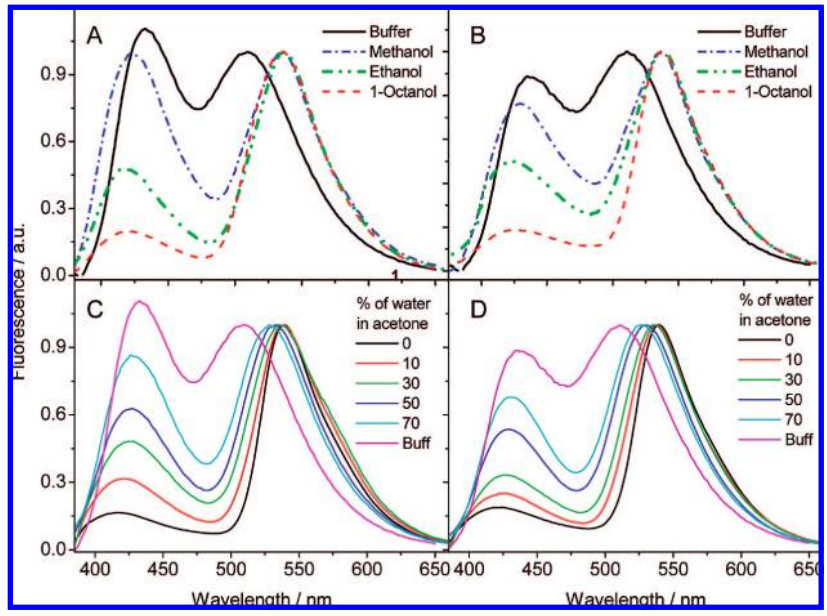


Figure 1. Effect of solvents on the dual emission of conjugates **1** and **2**. A and B: Fluorescence spectra of **1** and **2**, respectively, in different protic solvents. C and D: Fluorescence spectra of **1** and **2**, respectively, in acetone−water mixtures. The spectra were normalized at the maximum of the long wavelength T* band. Excitation wavelength was 340 nm.

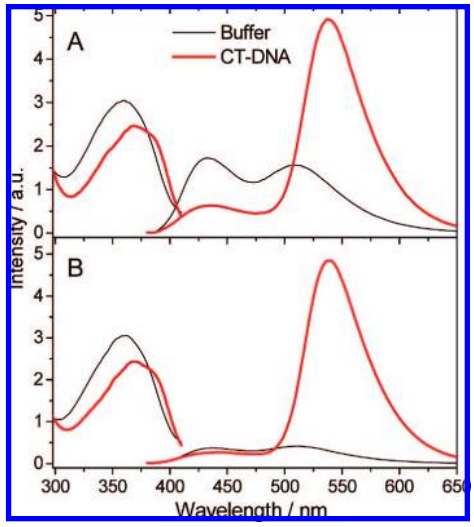


Figure 2. Absorption (left) and fluorescence (right) spectra of **1** (A) and **2** (B) in buffer (thin black curves) and in the presence of CT-DNA (thick red curves). 10 mM phosphate buffer (pH 7.0) was used. Concentration of **1** and **2** was 0.1 μM. The concentration of CT-DNA base pairs was 1 μM. Excitation wavelength was 340 nm.

dsDNA is more pronounced for **2** than for **1** (Figure 2). Moreover, both **1** and **2** exhibit shifts of their T* band to the red (Figure 2, Table 1). According to the data in solvents, the observed spectroscopic effects suggest that on binding to dsDNA, the environment of the 3HC fluorophore changes from a highly polar aqueous one with high H-bond donor ability (high exposure to water) to a less polar one with low H-bond donor ability (low exposure to water). On binding to dsDNA, the absorbance of **1** and **2** decreases by 20−25% (hypochromic effect), and the absorption maximum shifts to the red (from 361 to 369 nm) (Figure 2), strongly suggesting an intercalation of the 3HC fluorophore between the DNA bases. Remarkably, an increase in the fluorescence quantum yield accompanies the binding of **1** and **2** to dsDNA (Table 1), while many fluorescent dyes are quenched by DNA bases.[11,38] This increase in the quantum yield on intercalation of 3HC between the DNA bases can be explained by a screening of the 3HC fluorophore from the bulk aqueous environment and a decreased quenching by the amino groups of spermine. Remarkably, the I_{T*}/I_{N*} ratio in dsDNA for both conjugates is significantly lower than in any protic solvent including the low polar 1-octanol (Table 1), confirming the absence in the probe vicinity of water molecules, which are the major H-bond donors in the DNA surrounding. According to previous studies using other environment sensitive dyes, the polarity of the minor and major grooves of dsDNA is rather high, namely $\varepsilon \approx 20$[10] and 40−60[4] respectively, indicating a significant amount of water molecules in the grooves. The poor water exposure of the 3HC fluorophore of our conjugates in dsDNA declines its possible localization in the DNA grooves, but confirms that the fluorophore is probably intercalated between the DNA bases, where the amount of water molecules is limited. Interestingly, a highly efficient ESIPT was also observed for a 2-(2′-hydroxyphenyl)benzoxazole derivative incorporated in dsDNA opposite an abasic site, in line with a low-polar and aprotic environment between the base pairs.[39]

What should happen then on binding of **1** and **2** to ssDNA, where intercalation is not expected? Addition of the poly(dA) ssDNA also modifies the fluorescence spectra of both conjugates (Figure 3), but the resulting spectra are strongly different from those with dsDNA. Indeed, the I_{T*}/I_{N*} ratio with poly(dA) is 7−10 fold lower than with dsDNA, while the T* band position with ssDNA is slightly shifted to the blue (Table 1). Importantly, on binding to another ssDNA, poly(dT), the conjugates **1** and **2** exhibit very close fluorescence spectra to those in poly(dA) (Figure 3). Similar fluorescence spectra were also obtained when CT-DNA was melted at 83 °C (Table 1).[40] Finally, annealing of poly(dA) with poly(dT) changes dramatically the spectra of **1** and **2**, so that they become very close to the spectrum with double-stranded CT-DNA (Figure 3, Table 1). Thus, irrespective to the DNA sequence, the spectra with ssDNA are strongly different from those with dsDNA, the relative intensity of the ESIPT product (i.e., I_{T*}/I_{N*} ratio) being strongly decreased in ssDNA (Table 1). Moreover, the fluorescence quantum yields of **1** and **2** bound to ssDNA are systematically lower compared to dsDNA (Table 1). Finally, ssDNAs modify the absorption spectra of **1** and **2** to a much lower extent than dsDNAs,

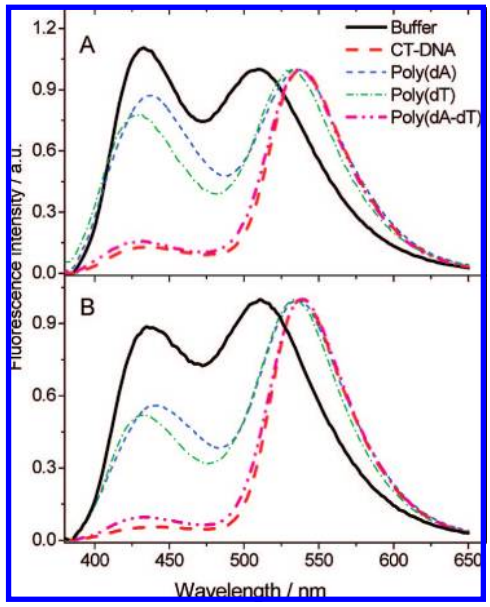


Figure 3. Fluorescence spectra of 0.1 μM of conjugates **1** (A) and **2** (B) in 10 mM phosphate buffer (pH 7) in the absence and in the presence of an excess of dsDNA (CT-DNA and poly(dA-dT) at 20 °C) or ssDNA (poly(dA) and poly(dT)). Concentration of dsDNA (base pairs) and ssDNA (bases) was 1 μM. The spectra were normalized at the T* band maximum. Excitation wavelength was 340 nm.

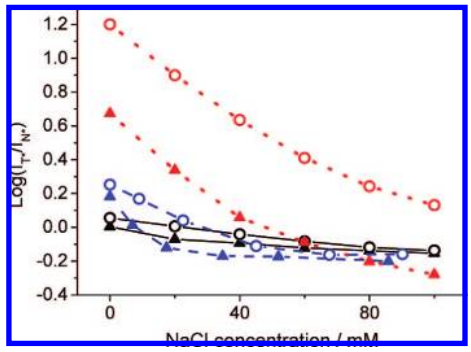


Figure 4. Salt effect on log(I_{T*}/I_{N*}) of conjugates **1** (▲) and **2** (○) in free form (solid curve and symbols in black), bound to CT-DNA (dotted curve and symbols in red) or to poly(dA) (dashed curve and symbols in blue). Conditions were as in Figure 3.

providing only a 5−7% hypochromism and *ca* 5 nm red shift. These data suggest that the 3HC fluorophore of the spermine conjugates is much more exposed to water in ssDNA than in dsDNA, probably because an efficient screening of the fluorophore from the bulk water due to intercalation between the base pairs can only be realized in dsDNAs. Thus, the ESIPT-based fluorophore allows us to clearly distinguish between ssDNAs and dsDNAs, simply by the ratio of the two emission bands.

Due to the polycationic nature of conjugates **1** and **2**, their interaction with DNA should be mainly electrostatic, and therefore, it should strongly depend on salt concentration. In line with our expectations, log(I_{T*}/I_{N*}), which is a function of the I_{T*}/I_{N*} ratio exhibiting a linear correlation with solvent parameters,[23,41] strongly decreases with increasing salt concentrations for **1** and **2** complexed with dsDNA (CT-DNA), while in DNA-free buffer the salt effect is negligible (Figure 4). For both conjugates, the log(I_{T*}/I_{N*}) values at high salt concentrations approach the values in DNA-free buffer, indicating that the complexes of **1** and **2** with dsDNA are probably dissociated. Noticeably, the salt effect is stronger for conjugate **1,** suggesting that it forms weaker complexes with DNA than conjugate **2**. Relatively weak complexes are formed with poly(dA), since the log(I_{T*}/I_{N*}) values of their complexes with **1** and **2** decrease very rapidly with increasing salt concentrations (Figure 4). Titrations in 30 mM NaCl confirm that both conjugates bind dsDNA much stronger than ssDNA, since the binding constants for **1** and **2** with dsDNA are $(2.5 \pm 0.5) \times 10^7$ M^{-1} and $(9.1 \pm 1.4) \times 10^7$ M^{-1}, respectively, while for ssDNA, the corresponding values are $(1.0 \pm 0.5) \times 10^6$ M^{-1} and $(2 \pm 1) \times 10^6$ M^{-1}. The binding constants for dsDNA correspond well to the literature values for spermidine in low-salt buffer.[42] Moreover, the higher affinity of both conjugates to dsDNA as compared to ssDNA is in line with the reported preference of spermine and spermidine for dsDNA.[42] Finally, the higher affinity of **2** compared to **1** can be understood considering that the modification of a primary amino group of spermine (in **2**) perturbs less the binding to DNA than modification of its secondary amino group (in **1**). In case of **2**, the cationic spermidine residue likely binds to the dsDNA minor or major grooves,[43,44] while the long and flexible fluorophore spacer (Figure 1) enables the fluorophore to intercalate between the bases. The appropriate size, the flat aromatic structure (Scheme 1) and the hydrophilicity of the 3HC fluorophore are likely the key factors that favor its intercalation into dsDNA. In case of **1** bound to dsDNA, the 3HC fluorophore is probably locked between the cationic groups, and, therefore, due to insufficient freedom intercalates less efficiently. This reduced intercalation is evidenced by the significantly lower I_{T*}/I_{N*} ratio and thus, the higher water accessibility of **1** compared to **2** in complex with dsDNA. This poorer intercalation could be an additional factor of the lower affinity of **1** compared to **2** for dsDNA.

In conclusion, we have conjugated an environment-sensitive 3HC fluorophore undergoing ESIPT to spermine. On binding to dsDNA and ssDNA, the conjugates show dramatic change in their dual emission. This enables to directly monitor their interaction with DNA by recording the ratio of the two emission bands. In complex with dsDNA, the enhanced emission of the ESIPT product indicates an efficient screening of the 3HC fluorophore from the bulk water, probably due to its intercalation in dsDNA. A very different spectral profile is observed on binding to ssDNA, suggesting that in ssDNA the fluorophore is much less screened from water molecules. Thus, our dyes can clearly distinguish between ssDNA and dsDNA. Consequently, this study shows that labeling of a polycationic molecule (like spermine) with 3HC allows monitoring its interaction with nucleic acids as well as probing the microenvironment of local binding sites.

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