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Light up G-quadruplex DNA with a [2.2.2]-heptamethinecyanine dye

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The interactions of a triangle-shaped [2.2.2]heptamethinecyanine dye **1**, namely 1,5,7-tris-[3-methylbenzothiazol-2-yl]-[2.2.2]heptamethindiium, with quadruplex DNA were studied with photometric and fluorimetric titrations, thermal DNA denaturation, CD and ^{1}H-NMR spectroscopy. The ligand binds to the quadruplex DNA with moderate affinity ($K = 8 \times 10^5$ M^{-1}), mainly by terminal π stacking. Remarkably, the ligand **1** exhibits a selectivity for quadruplex DNA relative to duplex DNA. Whereas the cyanine dye is very weakly fluorescent in aqueous solution, the emission intensity increases by a factor of >100 upon association with quadruplex DNA. Thus, it is shown that trinuclear cyanine derivatives may be employed as selective probes for the fluorimetric detection of quadruplex DNA.

Introduction

Cyanine dyes are attractive targets for analytical applications in life sciences, such as fluorescent labeling and biomedical imaging.[1] Cyanine derivatives often exhibit very weak fluorescence in solution, which is enhanced drastically upon association with nucleic acids.[2] In this context, the cyanine dye thiazole orange (**TO**) may be considered as a representative example. **TO** exhibits a high affinity for nucleic acids and a strong fluorescence light-up effect upon association with DNA.[3] It has been shown in a recent study that the enhancement of the fluorescence intensity upon intercalation is a result of the suppressed twisting motion of **TO** in the excited state that otherwise leads to radiationless deactivation of the dye in aqueous solution.[4] Because of this property **TO** is applied in fluorescent intercalator displacement (FID) assays that enable the evaluation of putative duplex and quadruplex DNA ligands.[5,6] Nevertheless, **TO** shows only a low selectivity towards different forms of DNA.[7,8]

Intercalation and minor groove binding are common binding modes of cyanine dyes with DNA,[2,9] because the heterocyclic units possess typical properties of intercalators whereas the flexible polymethine unit enables the required structural changes to fit inside the grooves. The actual binding mode depends on the structure of the dye or the sequence of the DNA.[1,2,10] Notably, it has been demonstrated that cyanines may also assemble into DNA-templated aggregates.[11–13] In addition, the interaction of cyanine derivatives with DNA in a PVA matrix has been investigated in detail.[14] Along with studies with duplex DNA as a target, several investigations focus on the interaction of cyanine dyes with non-canonical DNA forms such as triplex and quadruplex structures.[15–18] Along these lines the investigation of ligand–quadruplex interactions has developed into an attractive research area[19] because of the potential relevance of DNA quadruplex structures and the stabilization thereof in physiological processes.[20] Although cyanine dyes have been shown to bind to quadruplex DNA, only a few examples have been reported so far that exhibit a pronounced selectivity towards the quadruplex as compared with duplex DNA.[21–24]

Trinuclear cyanines possess photosensitizing and electrochromic properties.[25] However, to date the DNA-binding properties of this class of compounds have not been reported. We were inspired to examine this particular type of cyanine dyes by the recently published studies on quadruplex DNA binders based on an aromatic three-branched scaffold with C_3-symmetry.[26,27] It has been shown that the triazatruxene derivative **AZATRUX** and the triaza- and triazoniatrinaphthylene derivatives **TrisK** and **TrisQ** constitute promising lead structures for the design of selective quadruplex DNA ligands. Considering that the spatial extension of the known [2.2.2]heptamethinecyanine structure **1**[28] is similar to that of **TrisK** and **TrisQ**, along with the observation that cyanine dyes have the potential to bind to quadruplex DNA,[2,9] we proposed that **1** may bind to the latter DNA form with reasonable selectivity and that this

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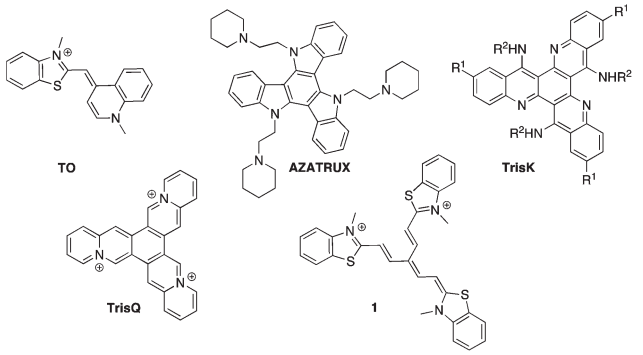
property may be used for the fluorimetric detection of the nucleic acid.



Results

To investigate the aggregation behaviour of the cyanine dye **1** in water, absorption spectra were taken at different temperatures (Fig. 1). With increasing the temperature from 20 °C to 80 °C the absorption maximum is shifted about 50 nm to longer wavelengths and the initial red-shifted shoulder becomes more pronounced at higher temperatures.

Photometric and fluorimetric titrations

A solution of the quadruplex DNA **22AG** [5′-A-$(GGGTTA)_3$GGG-3′] was titrated to a solution of the cyanine **1**, and the changes of the absorption and emission were determined (Fig. 2A and 3A). At low DNA concentrations, *i.e.* when the ligand–DNA ratio (LDR) is >16, the absorption maximum at 577 nm decreases. At smaller LDR, however, the intensity of the absorption increases with a red shift of the absorption maximum ($\Delta\lambda$ = 100 nm at LDR = 0.2). While the cyanine **1** is weakly fluorescent in aqueous solution (Φ_{fl} < 0.005), the addition of DNA leads to the development of a broad emission band with a maximum at 715 nm and a shoulder at 660 nm with a light-up factor of 106 (Fig. 3A). A similar emission band of **1** was detected in a viscous glycerol–water mixture (95 : 5; Fig. 3A, red line). From the fluorimetric data the binding constant $K = 8.2 \times 10^5$ M^{-1} and the number of ligands per quadruplex n = 2.4 were determined according to established curve-fitting procedures.[29]

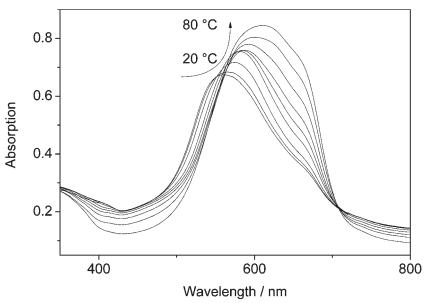


Fig. 1 Absorption spectra of **1** (15 μM) in water at T = 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C.

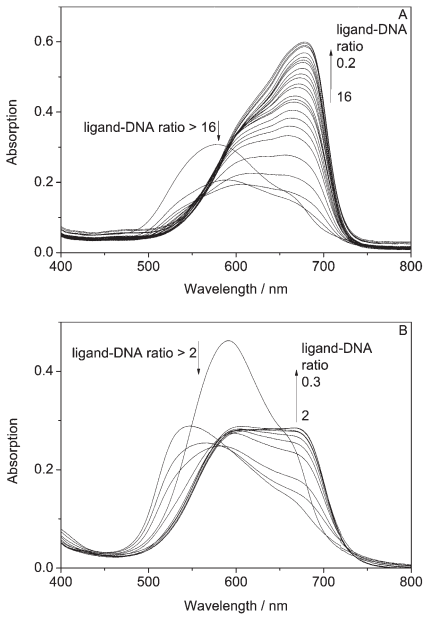


Fig. 2 Photometric titration of **1** (10 μM) with **22AG** (A) in K-phosphate buffer (95 mM, pH 7.0) and with ct DNA (B) in BPE buffer (16 mM, pH 7.0); T = 20 °C.

The titration of double-stranded calf thymus (ct) DNA to a solution of **1** led to similar, but less pronounced changes in the absorption and emission spectra. A bathochromic shift of the absorption maximum ($\Delta\lambda$ = 80 nm) was observed, but the intensity of the long-wavelength band increased to a lesser extent (Fig. 2B). The fluorimetric titration of ct DNA to ligand **1** revealed a similar emission band as in the case of quadruplex DNA with a maximum at 712 nm, but with a smaller light-up factor of 57 (Fig. 3B). The analysis of the fluorimetric titration gave a binding constant of $K = 1.1 \times 10^4$ M^{-1}. Remarkably, the different fluorimetric responses of the ligand **1** to duplex and quadruplex DNA are detectable from a mixture of both DNA forms. Thus, the addition of ct DNA to a mixture of **1** and **22AG** did not induce a change of the fluorescence band, whereas the addition of the quadruplex to a mixture of the ligand **1** and ct DNA led to a strong increase of the emission intensity (Fig. 3D).

To assess the binding stoichiometry between the ligand **1** and quadruplex DNA by the continuous variation method,[30] mixtures of the ligand **1** and **22AG** with different fractional compositions were analyzed by emission spectroscopy and the data were presented as a Job plot (Fig. 4). The intersection of the two linear fitting curves is located at X_{Lig} = 0.71, which corresponds to a binding stoichiometry of 2.4 ligands per quadruplex.

Fluorescence-monitored quadruplex melting

In addition, the influence of the cyanine **1** on the melting temperature of the quadruplex DNA was examined by the established FRET-melting assay with the end-labeled quadruplex-forming oligonucleotide **F21T**, *i.e.* fluorescein-$(GGGTTA)_3$GGG-tetramethylrhodamine (Fig. 5).[31] With

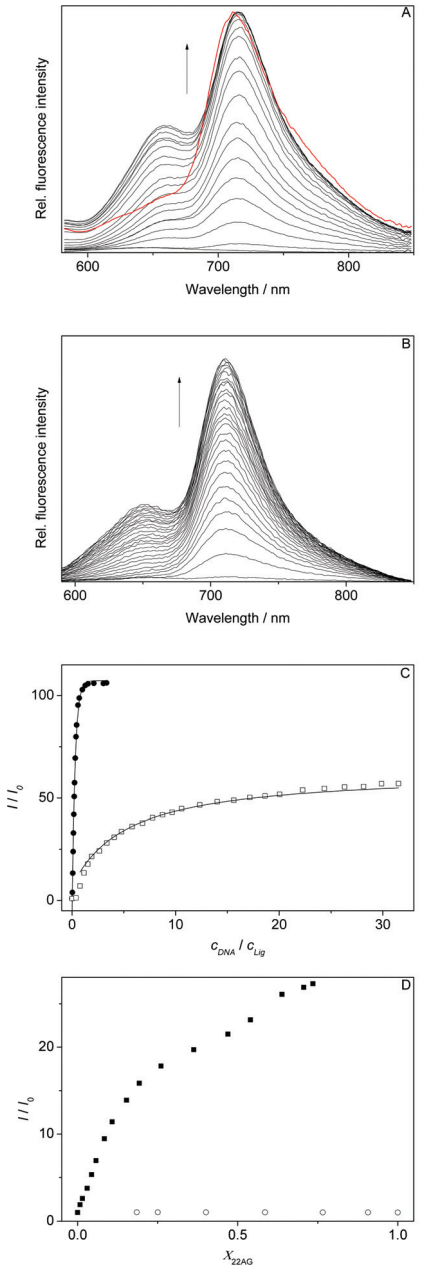


Fig. 3 Fluorimetric titration of **1** (10 μM) with **22AG** (A, C: ●) in K-phosphate buffer (95 mM, pH 7.0) and with ct DNA (B, C: □) in BPE buffer (16 mM, pH 7.0). C: Plot of I/I_0 *versus* the DNA–ligand ratio with the corresponding fitting curves. Red line: normalized emission of **1** (20 μM) in a glycerol–water mixture (95 : 5). D: Plot of the relative fluorescence intensity *versus* molar fraction X_{22AG} of **1** (2.6 μM) as obtained from the addition of **22AG** to a mixture of ct DNA (26 μM) with **1** (■), or the addition of ct DNA to a mixture of **22AG** (26 μM) with **1** (○); K-phosphate buffer (95 mM, pH 7.0). A–D: λ_{ex} = 560 nm or 580 nm; T = 20 °C.

growing LDR the melting temperature of **F21T** increases (1.25: 1.3 °C, 2.5: 3.7 °C, and 5: 8.1 °C), which accounts for a significant stabilization of the quadruplex structure by **1**. To gain more information about the selectivity of **1** towards the quadruplex DNA, the experiment was performed under identical conditions in the presence of the oligonucleotide **ds26** [d-(CAATCGGATCGAATTCGATCCGATTG)] that forms a self-

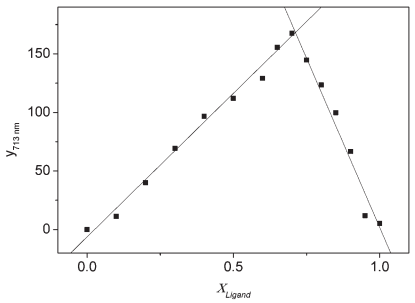


Fig. 4 Job plot obtained from fluorimetric analysis of mixtures of **1** with quadruplex **22AG** (c_1 + c_{DNA} = 10 μM) in K-phosphate buffer (95 mM, pH 7.0); X = mole fraction of the ligand; λ_{ex} = 560 nm; T = 20 °C.

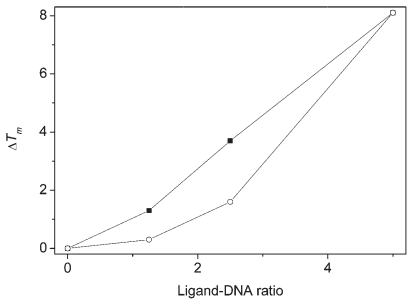


Fig. 5 Induced change of the melting temperature, ΔT_m, of quadruplex DNA **F21T** (0.2 μM) upon addition of ligand **1** in the absence (■) and in the presence (○) of duplex DNA **ds26** (3 μM) in aqueous Na cacodylate buffer solution (10 mM Na^+ and 10 mM K^+, pH 7.2) at different ligand–DNA ratios (0, 1.25, 2.5, 5).

complementary DNA double strand. An excess of **ds26** (15 molar equiv.) results in a slight decrease of the stabilizing effect of **1** on the quadruplex structure; that is, the melting temperature of **F21T** at an LDR of 2.5 decreased about 2 °C as compared to the experiment in the absence of **ds26**. However, the stabilization at a larger LDR of 5 is not affected by the presence of the duplex DNA.

CD spectroscopy

The interaction of the cyanine ligand **1** with quadruplex DNA was examined by CD spectroscopy (Fig. 6). In each of the polarimetric titrations the magnitudes of CD bands at a low LDR of 0.25 change differently as compared to the development of signals at higher LDR. For clarity, the following discussion is mainly focussed on the general trend of spectra at higher LDR (>0.25). The initial spectrum of **22AG** shows the typical signal pattern of a mixture of an anti-parallel quadruplex form and consists of maxima at 210 nm, 250 nm and 295 nm, a weak shoulder at 270 nm, and a negative band at 235 nm (Fig. 6A).[32] Upon addition of **1** the intensity of the minimum at 235 nm increased, whereas the maxima at 210 nm and 250 nm became smaller. The maximum at 295 nm decreased at low LDR (0.3) and enlarged upon further addition of the ligand. At an LDR of 2, a bisignate ICD signal was observed in the absorption range of the cyanine dye **1** that

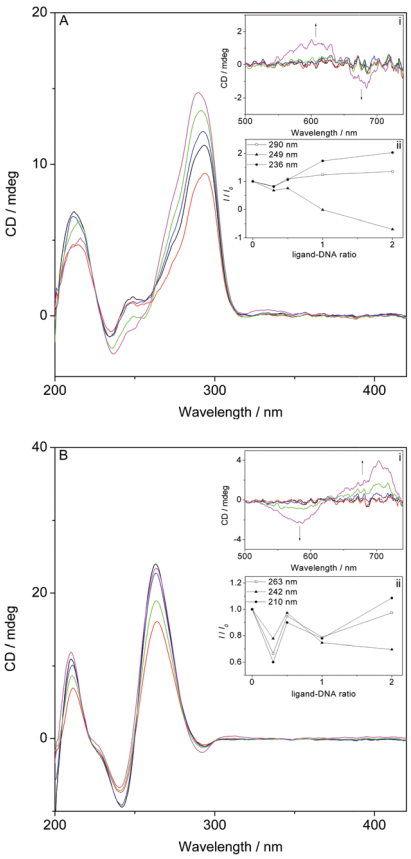


Fig. 6 CD spectra of **22AG** (A) and **c-kit** (B) with **1** at the ligand–DNA ratios of 0 (black), 0.3 (red), 0.5 (blue), 1 (green) and 2 (magenta) in K-phosphate buffer (95 mM, pH 7.0); c_{DNA} = 20 μM; T = 20 °C. Inset: (i) induced CD bands; (ii) plot of the relative intensity CD/CD_0 *versus* the ligand–DNA ratio.

results from coupling between the transition dipoles of the bound ligand and the chiral DNA.[33] In addition, the association of **1** with the quadruplex DNA **c-kit**, *i.e.* d($AG_3AG_3CGCTG_3AG_2AG_3$), was examined, whose all-parallel quadruplex structure results in characteristic positive CD bands at 210 nm and 260 nm, and negative signals at 240 nm and 290 nm (Fig. 6B). Whereas the maximum at 260 nm fluctuates during the titration, the minimum at 240 nm slightly decreased with increasing the ligand–DNA ratio. Furthermore, a bisignate ICD signal was observed at an LDR of 1 in the absorption range of the ligand.

The addition of **1** to a solution of ct DNA also leads to the formation of a bisignate ICD signal in the CD spectrum, even at a low ratio of 0.3, whereas the CD bands of the DNA remain essentially unchanged (Fig. 7A). At the same time the ICD signal disappears upon addition of the quadruplex to the mixture of the ligand **1** and ct DNA, indicating the redistribution of the ligand from the duplex to the quadruplex binding site (Fig. 7B).

^{1}H-NMR spectroscopy

To gain information about the binding site of **1** in the quadruplex DNA, mixtures of the ligand **1** and the quadruplex

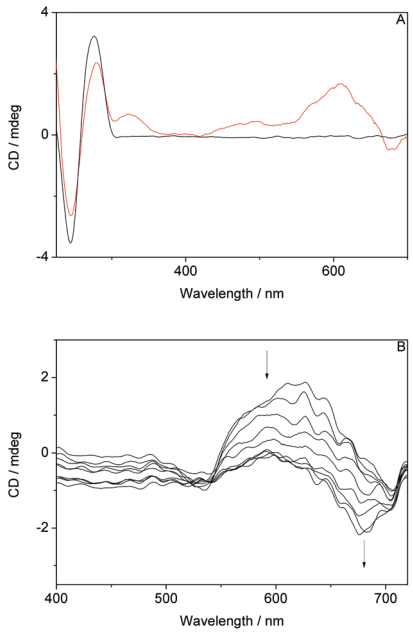


Fig. 7 A: CD spectra of ct DNA (20 μM) in the absence (black) and presence (red) of **1** (6 μM) in BPE buffer (16 mM, pH 7.0); T = 20 °C. B: Development of ICD band of **1** on addition of **22AG** to a mixture of ct DNA (26 μM) with **1** (2.6 μM) in K-phosphate buffer (95 mM, pH 7.0); T = 20 °C.

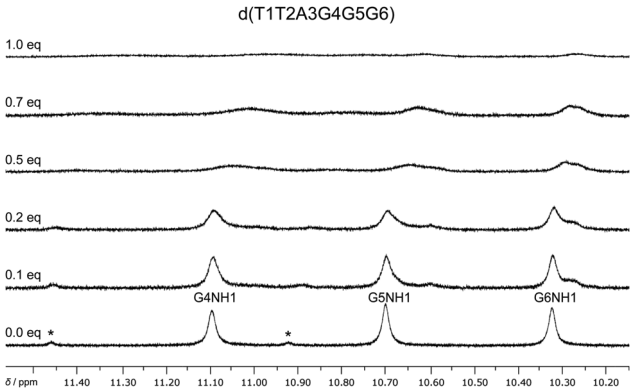


Fig. 8 ^{1}H-NMR spectra of **Tel6** (2 mM in bases) in the range of resonance of the guanine imino protons in the absence and presence of ligand **1**; H_2O–D_2O in the ratio 9 : 1; K-phosphate buffer (95 mM, pH 7.0); T = 25 °C; ★ = monomeric quadruplex.

forming sequence **Tel6**, *i.e.* d(TTAGGG) were investigated by ^{1}H-NMR spectroscopy (Fig. 8 and 9). In aqueous solution **Tel6** forms mainly a dimeric quadruplex structure by π-stacking of the terminal quartets of each intermolecular quadruplex.[34] The dimer and monomer of the quadruplex are in equilibrium and can be distinguished by ^{1}H-NMR spectroscopy (Fig. 8 and 9). In general, the addition of the cyanine **1** leads to significant changes of the ^{1}H-NMR shifts of the DNA. With decreasing LDR, the signals of the imino protons G4NH1 (11.09 ppm), G5NH1 (10.70 ppm) and G6NH1 (10.32 ppm) broaden significantly, and the signals almost disappear in the noise at an LDR of 1. Moreover, a downfield shift of up to 0.09 ppm was

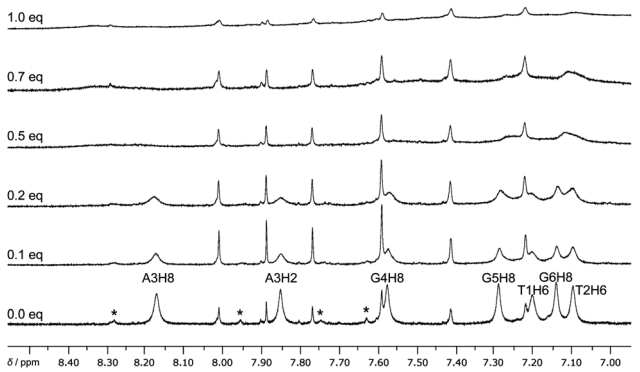


Fig. 9 ^{1}H-NMR spectra of **Tel6** (2 mM in bases) in the range of 7.0–8.5 ppm in the absence and presence of ligand **1**; H_2O–D_2O in the ratio 9 : 1; K-phosphate buffer (95 mM, pH 7.0); T = 25 °C; ★ = monomeric quadruplex.

observed for the signals of the imino protons. At an LDR of 0.1 the formation of two new signals at 10.59 ppm and 10.28 ppm was observed that shift such that they overlap with the signals of G5NH1 and G6NH1 at a larger LDR of 0.7. The signals of the protons A3H8 (8.17 ppm), A3H2 (7.85 ppm), G4H8 (7.57 ppm) and T1H6 (7.20 ppm) broaden with increasing the ligand concentration and are too broad to be detected unambiguously at a ligand–DNA ratio of 0.5. The signals of G5H8 (7.29 ppm), G6H8 (7.14 ppm) and T2H6 (7.09 ppm) get broader as well, but remain detectable up to an LDR of 1. In addition, the signals of G6H8 and T2H6 overlap at 7.11 ppm at an LDR of 0.5. Remarkably, similar changes of the ^{1}H-NMR spectrum of **Tel6** were observed on addition of **TO**.[35] ^{1}H-NMR signals of the ligand could not be detected, which is a commonly observed phenomenon in ligand–quadruplex complexes.[36]

Discussion

In summary all spectroscopic data point to the association of the heptamethine cyanine **1** with quadruplex DNA. Specifically, the photometric titrations reveal a change of the absorption upon addition of the ligand. The broad absorption of the free ligand **1** with maxima at 590 nm in BPE buffer and 577 nm in K-phosphate buffer, respectively, seemingly originates from aggregates, such as commonly observed for cyanine dyes in an aqueous solution.[37] The formation of aggregates is manifested by the blue-shifted absorption maximum compared to the ones observed in methanol ($\lambda_{abs,max}$ = 633, 650 nm)[28] and by the regained monomer absorption of **1** in water at higher temperatures due to thermally induced disassembly of the aggregates. The addition of quadruplex DNA results in a significant red-shift of **1** along with a hyperchromic effect that usually indicates the dissociation of the cyanine aggregates and binding of the monomer to the nucleic acid;[38] however, the lack of an isosbestic point implies the formation of different types of ligand–DNA assemblies. In the case of ct DNA a similar, but less pronounced effect was observed at lower LDR. Moreover, at early stages of the titration, *i.e.* at high LDR, a blue shifted absorption was observed that indicates the formation of aggregates in the grooves[11] or along the phosphate backbone of DNA. With increasing LDR more binding sites become available and the ligand eventually binds as a monomer, eventually leading to a red-shifted absorption. The association of the cyanine **1** with the quadruplex DNA was further confirmed by the significant ICD signal in the presence of **22AG** and **c-kit**. Furthermore, the induced changes of the CD bands of **22AG** indicate the influence of the bound ligand on the quadruplex structure, specifically on the stacking interactions between the G quartets, because the latter have an influence on the shift and intensity of the CD bands.[32b] Unfortunately, the analysis of the CD spectra does not provide an unambiguous identification of one particular stabilized quadruplex form, although it has been proposed that the increasing CD band of **22AG** at 290 nm and the formation of a weak negative band around 250 nm may denote the stabilization of a basket-type or chair-type quadruplex structure by the ligand.[39] In the case of **c-kit** the changes of the CD bands are relatively small. It is concluded that the parallel quadruplex structure remains essentially intact upon association with **1**, because this particular DNA form is already highly stabilized in the presence of physiological K^+-concentration. To be noted is the observation that at low LDR (0.25) the CD bands of the DNA have a significantly different development than at higher ratios. As the observed CD spectra are the combination of bands from different quadruplex forms in solution it appears that at low ligand concentrations particular DNA forms are stabilized to different extent as compared to solutions with higher ligand concentrations.

The association of **1** with quadruplex DNA leads to a moderate stabilization of the nucleic acid as shown by the increasing melting temperature of **F21T** upon addition of **1** (ΔT_m = 8.2 °C at **1 : 22AG** = 5). This value is in the same range as for example the ones of the triazatrinaphthylene derivatives **TrisK** with R^1 = H and R^2 = $-(CH_2)_2NMe_2$ or $-(CH_2)_3NMe_2$ (ΔT_m = 6.2–13.9 °C).[26] The binding affinity of **1** towards quadruplex DNA **22AG** (8.2×10^5 M^{-1}) is only slightly smaller than the one of thiazole orange (**TO**) (2.1×10^6 M^{-1})[40] and falls in the same range as that of **AZATRUX** (10^5 M^{-1}).[27] The binding stoichiometry (**1 : 22AG**) of 2.5 : 1 may not reflect the structure of one distinct ligand–DNA complex, but rather the average of the number of ligands bound to the different quadruplex forms in solution.[41] It has been proposed[36] that independent different binding events take place, namely π stacking of the ligand to the terminal quartet of the quadruplex and additional unselective aggregation of ligands along the DNA backbone. By analogy with this proposal, we suggest a similar binding mode between ligand **1** and the quadruplex DNA. Nonetheless, groove binding may be excluded because with cyanine dyes this particular binding mode typically leads to much stronger ICD bands of the quadruplex-bound ligand.[23]

The ^{1}H-NMR spectroscopic data reveal additional information on the binding mode. Specifically, the shifts of the imino protons of the guanine residues of **Tel6** upon association with **1** provide evidence for terminal π-stacking of the

ligand onto the G-quartets of the quadruplex.[42] Moreover, we propose that the ligand **1** is attached to the G-quartet at position G4 because the proton signals of these nucleobases as well as those of the neighboring adenine and thymine residues are influenced the most upon association of the ligand with the quadruplex. From the results with the intermolecular quadruplex structure **Tel6** and comparison with the data obtained with **TO**,[35] we conclude that the binding mode of **1** with quadruplex DNA resembles the one of **TO** that has been proposed to bind to quadruplex DNA by terminal π stacking.[40] Considering the steric demand of the ligand, however, the terminal π stacking most likely involves just partial overlap of the G quartet with the π system of the ligand. It should be also noted that the results with the intermolecular quadruplex **Tel6** cannot be directly compared with those obtained with the intramolecularly folded **22AG**, as the terminal quartets of the latter are sterically more shielded; however, the former results at least demonstrate the propensity of the ligand to associate with the terminal G quartet of a quadruplex structure.

To be emphasized are the significantly different binding properties of the cyanine **1** with quadruplex and duplex DNA. The ligand **1** binds to ct DNA, as clearly indicated by photometric titrations and the formation of an ICD signal of **1** in the presence of ct DNA. Nevertheless, the binding constant is smaller than the one with the quadruplex by more than one order of magnitude, most likely because of the steric demand of the ligand that hinders a reasonable fit with the intercalation pocket or the grooves of the duplex DNA. The higher selectivity of the cyanine **1** towards quadruplex DNA as compared to ds DNA is further confirmed by the very small effect of the presence of ds DNA on the thermal stabilization of quadruplex DNA by **1**, which is commonly interpreted as a sign of a high binding selectivity to the quadruplex DNA.[31]

The main focus of this study was on the exploration of the emission properties of **1** for the fluorimetric detection of quadruplex DNA. Like most cyanine dyes the derivative **1** exhibits a very low emission quantum yield in aqueous solution, presumably because of radiationless deactivation of the excited state by conformational relaxation or *E–Z* isomerization. This assumption was confirmed by the increased emission intensity in a highly viscous medium; that is, the relaxation processes by structural changes are retarded due to the restricted free volume, and as a result the emission of the excited state becomes competitive.[43] Upon association of the dye with nucleic acids the relaxation pathway is also suppressed within the sterically constrained binding site and the emission quantum yield increases. Remarkably, the light-up effect with quadruplex DNA is almost twice as large as in the case of ct DNA, which is an interesting feature with regard to its potential application as a DNA-sensitive imaging agent.

Conclusions

In summary it was demonstrated for the first time that a trinuclear cyanine dye binds selectively to quadruplex DNA. Although the binding parameters are not ideal and still need optimization, we propose that this class of compounds has a large potential for the development of efficient and selective quadruplex ligands. Specifically, the fluorescence light-up effect upon association of the dye with the DNA may be employed in bioanalytical chemistry for the selective fluorimetric detection of quadruplex DNA.

Experimental

Materials

The [2.2.2]heptamethinecyanine dye **1** (counter ion: BF_4^-) was synthesized according to published procedures.[28] Oligodeoxyribonucleotides (HPLC purified) **F21T** (fluorescein-$G_3T_2AG_3$-$T_2AG_3T_2AG_3$-tetramethylrhodamine), **22AG** [d($AG_3T_2AG_3T_2$-$AG_3T_2AG_3$)], **Tel6** [d(TTAGGG)], **c-kit** [d($AG_3AG_3CGCTG_3A$-G_2AG_3)], and **ds26** [d($CA_2TCG_2ATCGA_2T_2CGATC_2GAT_2G$)] were purchased from Metabion Int. AG (Planegg/Martinsried). Calf thymus DNA was purchased from Sigma-Aldrich (St. Louis, USA). K-phosphate buffer: 25 mM K_2HPO_4, 70 mM KCl, adjusted to pH 7.0 with 25 mM KH_2PO_4; BPE buffer: 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0; Na cacodylate buffer: 10 mM $Na(CH_3)_2AsO_2{\cdot}3H_2O$, 10 mM KCl, 100 mM LiCl, pH 7.2–7.3.

Equipment

Absorption spectroscopy: Varian Cary 100 Bio Spectrophotometer; emission spectroscopy: Varian Cary Eclipse; CD spectroscopy: Chirascan CD-Spectrometer, Applied Photophysics; NMR spectroscopy: Varian VNMR-S 600.

Methods

FRET melting experiments, photometric and fluorimetric titrations, and CD-spectroscopic experiments were performed according to published procedures.[35,44] The fluorescence quantum yield was determined relative to that of rhodamine 6G.[45,46] The binding constants and the binding site size were determined according to established curve-fitting procedures.[29] For the fluorimetric competition experiments (Fig. 3D) a solution of **1** (2.6 μM) and **22AG** or ct DNA (2.6 μM) in K-phosphate buffer (95 mM, pH 7.0) was prepared and a defined portion was transferred into a 10 mm quartz cuvette. Aliquots of the other DNA forms were added, the solution was equilibrated for 2 min at 20 °C and a fluorescence spectrum (λ_{ex} = 560 nm) was recorded after each step. NMR spectra were recorded on a Varian VNMR-S600 with Triple Resonance HCN inverse probehead (3 mm) in 3 mm (150 μL) capillary tubes. Solvent suppression: WET 1d (1D NMR) with 1.5 s relaxation time, 256 scans. Data processing: VNMRJ 2.3A and SpinWorks. Chemical shifts of ^{1}H-spectra are given in ppm (δ) relative to DSS (δ = 0.00 ppm).

Acknowledgements

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