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ORIGINAL PAPER

Mono and Trimethine Cyanines Cyan 40 and Cyan 2 as Probes for Highly Selective Fluorescent Detection of Non-canonical DNA Structures

Vladyslava B. Kovalska · Mykhaylo Yu. Losytskyy · Sergiy M. Yarmoluk · Irit Lubitz · Alexander B. Kotlyar

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Abstract Two of earlier reported dsDNA sensitive cyanine dyes—monomethine Cyan 40 and meso-substituted trimethine Cyan 2 were studied for their ability to interact with non-canonical DNA conformations. These dyes were characterized by spectral-luminescent methods in the presence of G-quadruplex, triplex and dsDNA motifs. We have demonstrated that Cyan 2 binds strongly and preferentially to triple- and quadruple-stranded DNA forms that results in a strong enhancement of the dye fluorescence, as compared to dsDNA, while Cyan 40 form fluorescent complexes preferentially only with the triplex form. Highly fluorescent complexes of Cyan 2 with DNA triplexes and G-quadruplexes and Cyan 40 with DNA triplexes are very stable and do not dissociate during gel electrophoresis, leading to preferential staining of the above DNA forms in gels. The data presented point to the intercalation mechanism of the Cyan 2 binding to G4-DNA, while the complexes of Cyan 40 and Cyan 2 with triplex DNA are believed to be formed via groove binding mode. The Cyan dyes can provide a highly sensitive method for detection and quantification of non-canonical structures in genome.

V. B. Kovalska (✉) · M. Y. Losytskyy · S. M. Yarmoluk
Department of Combinatorial Chemistry,
Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine,
150 Zabolotnogo St.,
03143 Kyiv, Ukraine
e-mail: v.b.kovalska@imbg.org.ua

I. Lubitz · A. B. Kotlyar (✉)
Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University,
Ramat Aviv 69978, Israel
e-mail: s2shak@post.tau.ac.il

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Introduction

In living organisms mostly genomic DNAs exist in a double-stranded (ds) helical form, but occurrence of some other conformations also seems possible. The guanine-rich nucleic acid sequences are known to fold into four-stranded (G-quadruplexes, G4-DNA) or triple-stranded (triplex) structures in which the nucleotide bases are connected by means of Hoogsteen base-pairing bonding (Fig. 1). It was shown that in the human genome, more than 300,000 distinct sites can potentially form G4-DNA structures [1, 2]. This attracted significant attention of the research community to the problem of the possible role of triplex and quadruplex DNA motifs in gene regulation and genomic stability [3–6]. Therefore, development of a specific probe for distinguishing quadruplex or triplex conformations from a canonical ds one may have a significant scientific and practical importance.

Among the dyes proposed for the detection of G4-DNA regions, porphyrin dyes have been found to give much stronger fluorescent response in the presence of G-quadruplexes compared to dsDNA [7–9].

Since the 1990s cyanine dyes have been intensively applied for DNA and RNA detection and quantification [10]. SYBR Green I [10], which belongs to the family of monomethine cyanine dyes [11], is considered to be one of the most sensitive dsDNA probes up so far. DNA-binding dye, Thiazole Orange (TO) has been shown to strongly and selectively bind to G-quadruplexes and triplexes [12, 13]. Chemical conjugation of TO with Bisquinolinium, a G-quadruplex specific ligand, yielded selective fluorescent

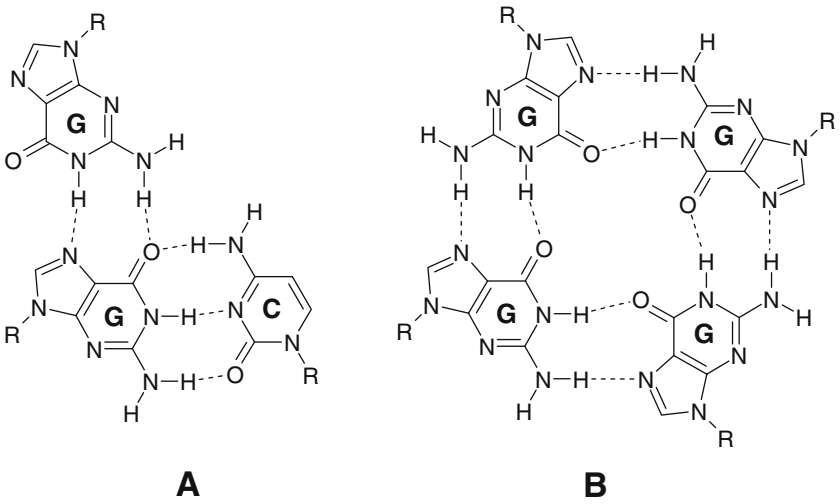

Fig. 1 Structures of G-G-C triad in poly(dG-dG)-poly(dC) (**a**) and G-tetrad in G4- DNA. (**b**)

probe for the detection of G4-DNA [12, 14]. Triphenylmethane dyes have also been shown to preferentially bind to G4-DNA as compared to single and double stranded DNA [15].

Cyanine dyes with a long polymethine chain have been employed for the fluorescent DNA detection [14, 16]. Long wavelength absorption and fluorescence maxima position of trimethine dyes permit using of non expensive semiconductor lasers for excitation of these probes [10]. Earlier we have shown that the emission intensity of meso-substituted trimethine cyanines increased almost hundred times upon interaction with DNA [17]. The symmetric tri- and pentamethine cyanine dyes were also utilized in fluorescence method of mix and measure screening of ligand binding to G-quadruplex DNAs of diverse sequence and structure [18].

The present paper is aimed at studying the interaction of DNA sensitive cyanine dyes—monomethine Cyan 40 [19] and meso-substituted trimethine Cyan 2 [17, 20] (see Fig. 2) with non-canonical DNA structures. For this purpose, absorption and fluorescent properties of these dyes are studied in the presence of G-quadruplex, triplex and ds DNA motifs. We have shown that Cyan 2 binds very tightly to the triplex and the G-quadruplex DNA, while Cyan 40 binds as fluorescent monomer preferentially only to the triplex motif. A strong complex of the latter dye with the triplex, in contrast to complexes of Cyan 40 with other DNA forms, is stable to electrophoresis and can be selectively detected in gels.

Materials and Methods

Materials

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (USA) and were used without further purification. Cyanine dyes were dissolved in DMSO. Cyan 40 and Cyan 2 were synthesized as described [21, 22]. The dyes concentration was determined by absorption spectroscopy using the extinction coefficient of 47 $mM^{-1}cm^{-1}$ at 543 nm for Cyan 2 [20] and 54 $mM^{-1}cm^{-1}$ at 435 nm for Cyan 40 [21].

The DNA samples: poly(dG)-poly(dC) duplexes, intramolecular poly(dG-dG)-poly(dC) triplexes and monomolecular G-quadruplexes containing 1,500 base pairs, 1,000 triads and 750 tetrads respectively were prepared essentially as described in our recent publications [23–25]. The G-quadruplexes (G4-DNA) were prepared in the absence of stabilizing ions (K^+ and Na^+) and thus will be termed the "K-free" G4-DNA throughout this paper. The K-free G-quadruplex was converted to the K-form during 30 min incubation of the DNA in 10 mM KCl, 100 mM Tris-Ac buffer (pH 8.0) at 25°C.

Concentrations of the double-, triple- and four-stranded DNA polymers are given in base pairs, triads and tetrads respectively and were estimated using the extinction coefficients of 14.8, 22.2 [23, 24] and 36 $mM^{-1}cm^{-1}$ [8, 25] at 260 nm for the GC-base pair,

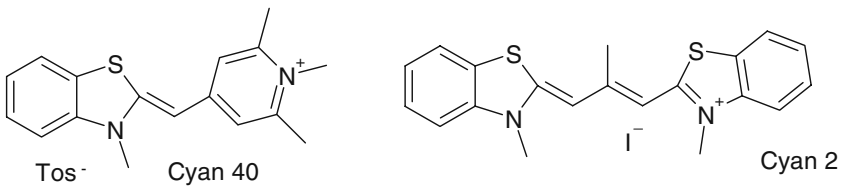

Fig. 2 Chemical structures of Cyan 40 and Cyan 2

GGC-tirade and G-tetrad respectively. Cyan 40- and Cyan 2-DNA complexes were prepared by incubation of poly (dG)-poly(dC) duplexes, poly(dG-dG)-poly(dC) triplexes and K-free G4-DNA with an amount of each of the dyes equal to that of base pairs, triads and G-tetrads respectively in 100 mM Tris-Ac buffer (pH 8.0) for 1 h at 25°C. The complexes were subsequently passed through a Sephadex G-25 column (1×5 cm) equilibrated with 100 mM Tris-Ac buffer (pH 8.0). The amount of the dyes eluted in the void volume of the column in complex with DNA was measured by absorption spectroscopy (see below).

Absorption and Fluorescent Spectroscopy

Absorption spectra were recorded with a Jasco (Japan) spectrophotometer. Fluorescence spectra were recorded with a FluoroMax 3 (Jobin Yvon) spectrofluorometer using a 150 W arc Xenon lamp as a light source. The spectra were recorded in a 0.4 cm×1 cm quartz cell with a band-pass of 2 nm at the excitation side and 2 nm at the emission side. The contribution of buffer to the measured fluorescence was subtracted. All measurements were conducted in 100 mM Tris-Ac buffer (pH 8.0); the absorption of all samples was 0.3 or less.

Analysis of Dye-DNA Binding Parameters

Binding of the dye to DNA may be described in terms of the mass action low:

$$\frac{C_{bd}}{C_{fd} \times C_{fDNA}} = K \tag{1}$$

C_{bd}, C_{fd}, correspond to concentrations of bound and free dye, C_{fDNA}, to concentration of non-occupied binding sites on DNA and K is the binding constant. If the concentration of the dye binding sites (C_{DNA}) is much higher than that of the dye, $C_{DNA} >> C_d$, than $C_{fDNA} \approx C_{DNA}$, and using $C_{fd} = C_d - C_{bd}$ the expression (1) can be presented as follows:

$$C_{bd} = \frac{K \times C_{DNA} \times C_d}{1 + K \times C_{DNA}} \tag{2}$$

Further if we titrate the DNA with the dye, than the saturation of the fluorescence intensity is achieved when all the DNA is occupied with the dye, so that $C_{bd} = C_{DNA}/n$, n being the average number of DNA triads/tetrads occupied by one dye molecule. Since the dye concentrations are very low, the fluorescence intensity is proportional to C_{bd} and the ratio of the fluorescence intensity of the dye (at concentration C_d) in DNA presence to this upon reaching saturation is:

$$\frac{I}{I_{\max}} = \frac{n \times C_{bd}}{C_{DNA}} \tag{3}$$

Substitution of (3) into (2) gives:

$$\frac{I}{I_{\max}} = \frac{n \times K \times C_d}{1 + K \times C_{DNA}} = A \times C_d \tag{4}$$

Thus in the case of DNA titration with the dye for $C_{DNA} >> C_d$, dependence of I/I_{max} on C_d is expected to be linear. Hence if we approximate the titration curve normalized to 1 unity (i.e. the dependence of I/I_{max} on C_d) in the region of small C_d values with the linear dependence, its slope will be equal to A from expression (4). Besides, from (4) we have:

$$K = \frac{A}{n - A \times C_{DNA}} \tag{5}$$

Since K>0, $n > A \times C_{DNA}$. Thus, $A \times C_{DNA}$ allows to estimate the lowest limit of the possible n value.

Gel Electrophoresis

The DNA samples were loaded onto 1% agarose gel and then electrophoresed at room temperature at 130 V for 25 min. TEA buffer, in addition to being used to prepare the agarose, also served as the running buffer. The gel was stained with either Cyan 2 or Cyan 40 dyes (1 μM) in TEA buffer and scanned visualized on a FLA-2000 fluorescent-image analyzer (Fujifilm, Japan) using excitation at 473 nm (510 filter) and 532 nm (575 filter) for Cyan 40 and Cyan 2 respectively.

Results and Discussion

Absorption and Fluorescent Properties of Cyan Dyes Bound to Double-, Triple- and Quadruple-stranded DNA

Absorption and fluorescent spectra of Cyan 2 and Cyan 40 in the presence of various DNA forms are presented in Figs. 3 and 4 correspondingly. The absorption spectrum of Cyan 2, free in solution, is characterized by a main 535 nm band and a side band at 470 nm (Fig. 3a, black line). The former band is believed to be characteristic of the dye monomer while the latter of the aggregated form of the dye [22]. The absorption spectrum of Cyan 40 is characterized by a single 435 nm band

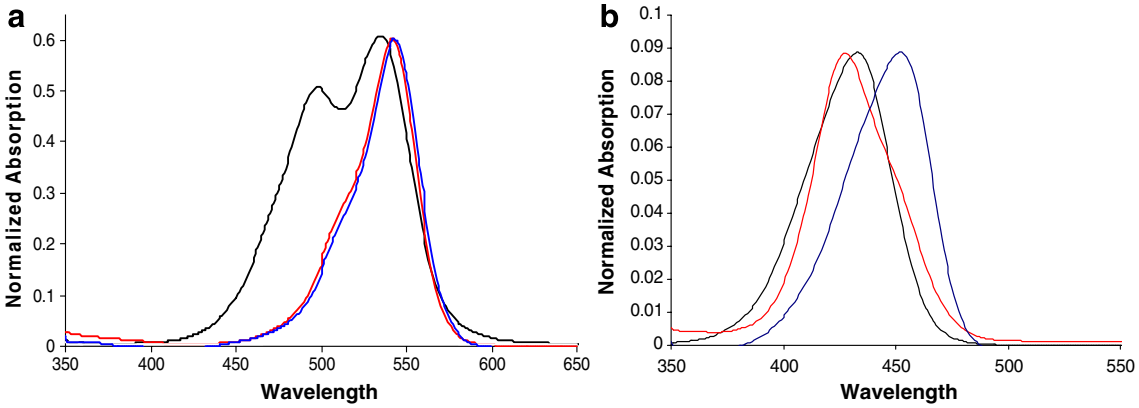

Fig. 3 Visible absorption spectra of Cyan 2 (**a**) and Cyan 40 (**b**) in free form (*black lines*), in the presence of triplex- (*blue line*) and G4-DNA (*red line*). 4 μM Cyan 40 was added to 4 μM poly(dG–dG)–poly(dC) or 3.8 μM G-DNA (concentrations expressed in triads or tetrads respectively) in 100 mM Tris-Ac buffer (pH 8.0). The samples were incubated for 30 min at room temperature and were subsequently passed through a G-25 Sephadex column in 100 mM Tris-Ac buffer (pH 8.0) as described in Materials and Methods. Spectra of the dye in DNA presence (*red and blue lines*) were normalized to that of the free dye (*black line*) in order to account for the samples dilution during chromatography

(Fig. 3b, black line) which corresponds to the dye monomer [26].

Prior to spectral-luminescent measurements, complexes of cyanine dyes with poly(dG)-poly(dC) duplexes, poly (dG-dG)-poly(dC) triplexes and K-free G4-DNA were passed through a Sephadex G-25 column. The chromatography of poly(dG)-poly(dC) complexes with Cyan 40 or Cyan 2 resulted in a separate elution of the dyes and the DNA. The void volume fractions eluted from the column showed no absorption in 400–550 nm range of the spectrum (data not shown). This chromatographic behavior is consistent with a weak binding of the dyes to dsDNA and complete dissociation of the dye from the dsDNA during the chromatography.

In contrast to poly(dG)-poly(dC), complexes between the dyes with the triplex and a K-free form of the quadruplexes are stable and do not dissociate during chromatography on a size exclusion column. Binding of Cyan 2 to the triplex and a K-free G4-DNA (Fig. 3a blue and red line correspondingly) results in a strong change of the absorption spectrum of the dye. The absorption maximum of Cyan 2 in complex with G4-DNA or triplex DNA shifts from 535 to 542 nm along with the disappearance of the 470 nm band. These changes are associated with dissociation of Cyan 2 aggregates into monomers that are efficiently bound to DNA. The absorption spectrum of Cyan 40 is also strongly affected by the triplex; a strong red shift to

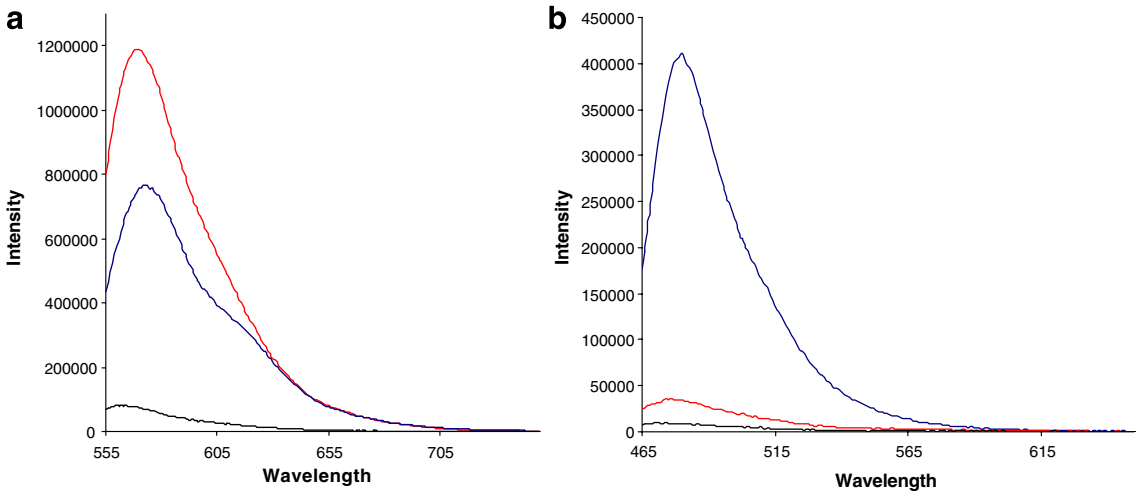

Fig. 4 Fluorescent spectra of Cyan 2 (**a**) and Cyan 40 (**b**) in the presence of poly(dG)–poly(dC) (*black line*), poly(dG–dG)–poly(dC) (*blue line*) and G4-DNA (*red line*). 0.2 μM DNA (concentrations expressed in bp, triads or tetrads respectively) was mixed with 0.1 μM Cyan 2 (**a**) or 0.1 μM Cyan 40 (**b**) in 100 mM Tris-Ac buffer (pH 8.0) The excitation wavelengths were: 435 nm (**a**, *black and red curves*), 452 nm (**a**, *blue curve*) and 543 nm (**b**). Both excitation and emission slits were set to 2 nm

452 nm due to the dye monomer binding to DNA is observed (Fig. 3b, blue line). Binding to K-free G4-DNA results in (see Fig. 3b, red line) slight blue shift of the absorption maximum of the dye and the appearance of a shoulder on the long-wavelength edge of the spectrum. This spectrum is characteristic for cyanine dyes aggregates [26], suggesting that the dye binds to the DNA mainly in aggregated form.

As shown in Fig. 4a, the fluorescence emission of Cyan 2 increases strongly in the presence of G-qudruplexes and triplexes as compared to duplexes; emission intensity of the dye is about 1.5 times higher in the presence of the G4-DNA than in the presence of the triplex form. Cyan 40 demonstrates relatively weak emission with maximum at about 470 nm in the presence of ds- and G4-DNA. The weak emission in complex with G4-DNA might be due to stabilization of low dimeric or even polymeric forms of Cyan 40 characterized by relatively week fluorescence. A significant enhancement of the dye fluorescence is however observed in the presence of triplexes. These data show that Cyan 40 is specific with respect to triplexes and binds at low (ranging from 2×10^{-8} to 2×10^{-7} M) concentrations in monomeric form only to this DNA form while Cyan 2 interacts with high affinity with both triplex and G-quadruplex forms of DNA.

The fluorescent titration of different DNA types with increasing dye concentration is shown in Fig. 5. As seen in Fig. 5a the maximum enhancement of the fluorescent emission is achieved at one Cyan 2 to approximately 3–4 triads or to 1.5 G-tetrads ratios (black diamonds and blue squares correspondingly). The maximum enhancement of Cyan 40 fluorescence is achieved at the dye to triad ratio equal to 3–4 (see black diamonds in Fig. 5b).

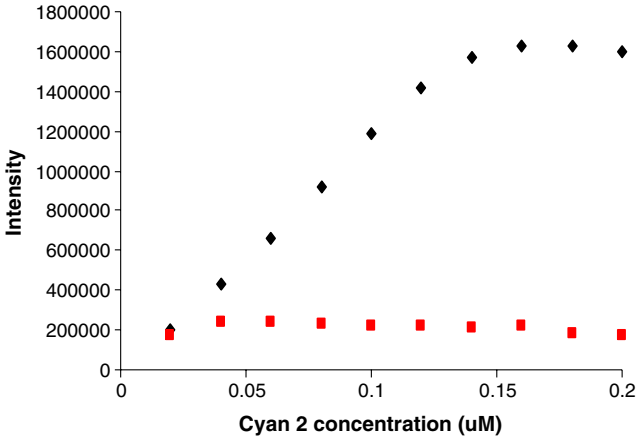

Fig. 6 Fluorescence titration of G4-DNA with Cyan 2 in the absence (*black diamonds*) and the presence (*red squares*) of 10 mM KCl. The concentration of DNA (expressed in G-tetrads) is equal to 0.2 μM. The titration was performed at 25°C in 100 mM This-Ac buffer (pH 8.0). The excitation was at 543 nm, both excitation and emission slits were set to 2 nm

We have recently reported the synthesis of novel continuous monomolecular G-quadruplex DNA nanostructures composed of single self-folded poly(dG) strands and containing hundreds of stacked tetrads [27] and shown that K^+ and Na^+ ions strongly influence the mode of porhpyrin interaction with G-qudruplexes [8]. These cations are located in the DNA core between the adjacent tetrads and prevent intercalation of cationic porphyrins into the molecule [8]. Figure 6 shows that K-ions abolish strong fluorescence enhancement of Cyan 2 induced by interaction with G4-DNA. This suggests that competition of K^+ and the dye for binding to the core of the quadruplex takes place and indicates for the intercalative mechanism of the dye binding to the G4-DNA [8].

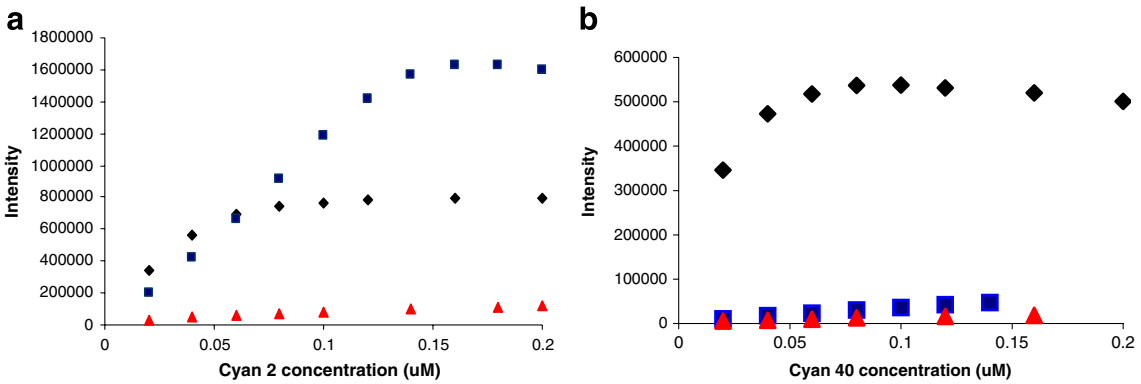

Fig. 5 Fluorescence titration of double- (*red triangles* in panels **a** and **b**), triple- (*black diamonds* in panels **a** and **b**) and quadruple-stranded DNA (*blue squares* in panels **a** and **b**) with Cyan 2 (**a**) and Cyan 40 (**b**). The concentration of Poly(dG)-Poly(dC), Poly(dG-dG)-Poly(dC) and G4-DNA (expressed in bp, triads and tetrads correspondingly) was equal to 0.2 μM. The titration was performed at 25°C in 100 mM This-Ac buffer (pH 8.0). The excitation wavelengths were: 435 nm (**a**, *red triangles* and *blue squares*), 452 nm (**a**, *black diamonds*) and 543 nm (**b**). Both excitation and emission slits were set to 2 nm

Gel Staining Experiments

Cyan 40 and Cyan 2 were studied for their efficiency as stains for pre- and post- electrophoretic visualization of two types of ds DNA, poly(dG)-poly(dC) and random sequenced linear plasmid Puc 19, triple-stranded poly(dG-dG)-poly(dC) and G-quadruplex (in absence and presence of K-ions) in agarose gels. The dyes were mixed with stoichiometrical amounts of DNA and loaded on electrophoresis gel.

As seen in Fig. 7a only complex of the triplex DNA with Cyan 40 can be detected in the gel (lane 2 in Fig. 7a). The post staining of the gel with Cyan 40 results in detection of all above DNA types (see Fig. 7b, lanes 1 through 5). This shows that the complex of Cyan 40 with the triplex in contrast to complexes of the dye with dsDNA and G4-DNA does not dissociate during the electrophoresis.

Figure 7c shows pre staining of different DNA forms with Cyan 2. It is clearly seen in the image that all G-rich forms of DNA can be detected in the gel (lanes 1 through 4) in contrast to native random sequence Puc 19 (Fig. 7c lane 5); post staining of the gel with Cyan 2 results in visualization of Puc 19 as well (see Fig. 7d, lane 5).

The Mechanism of Cyan Dyes Binding to DNA

We have shown that binding of Cyan 2 and Cyan 40 to DNA results in a strong enhancement of their fluorescence intensity; the fluorescence increases approximately 10-fold upon binding of Cyan 2 and Cyan 40 to triplex and 15-fold upon binding of Cyan 2 to G4-DNA. Addition of the dyes at submicromolar concentrations to dsDNA did not result in the fluorescence enhancement.

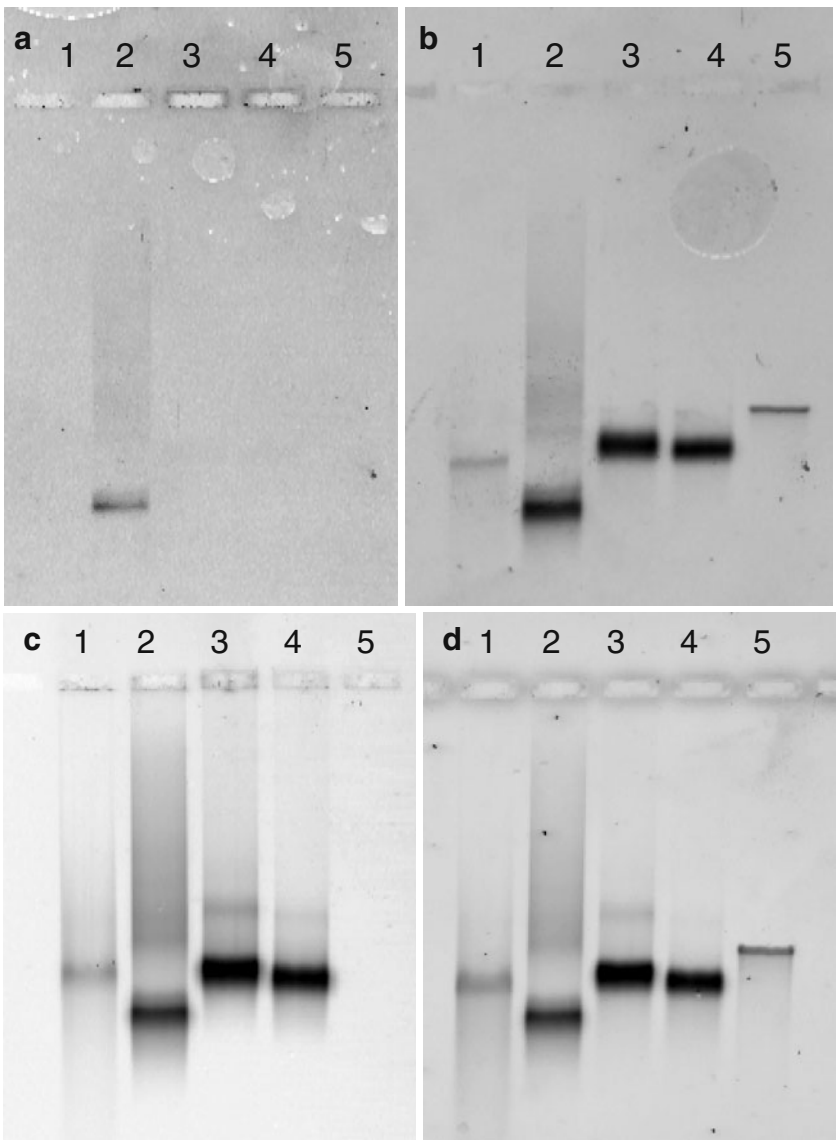

Fig. 7 Electrophoresis of Cyan 2 and Cyan 40 complexes with double-, triple- and quadruple-stranded DNA. 1,500 bp poly(dG)-poly(dC) (lanes 1 on panels **a**, **b**, **c** and **d**); 1,000 triad poly(dG-dG)-poly (dC) (lanes 2 on panels **a**, **b**, **c** and **d**); 750 tetrad G4-DNA in the absence (lanes 3 on panels **a**, **b**, **c** and **d**) and the presence of 10 mM KCl (lanes 4 on panels **a**, **b**, **c** and **d**) and Puc 19 (lanes 5 on panels **a**, **b**, **c** and **d**) were incubated with stoichiometrical amounts of Cyan 40 (**a**) or Cyan 2 (**c**) for 30 min and loaded onto 1% agarose gel. The electrophoresis was conducted for 30 min at 130 V in TEA buffer. The amount of DNA loaded per lane was approximately 30 ng. Gels were visualized on a FLA-2000 fluorescent-image analyzer (Fujifilm, Japan) using excitation at 473 (**a** and **b**) and 532 nm (**c** and **d**). Gels were pos-stained during 20 min incubation in 1 μM Cyan 40 (**b**) or 1 μM Cyan 2 (**d**) solutions at room temperature during constant shaking and visualized as described above

Thus, we can conclude that Cyan 2 binds with high affinity to both triple- and quadruple-stranded DNA structures, while Cyan 40 preferentially binds in fluorescent monomer form only to the triplex. Binding of both above dyes to dsDNA is much weaker compared to triple- and quadruple-stranded structures. We have shown that complexes of the dyes with these non-canonical DNA are stable and do not dissociate during chromatography and gel electrophoresis.

To make some assumptions about the mechanism of dyes binding to triplex and quadruplex DNA, the curves of DNA titration by the dyes (Fig. 5) were analyzed as described in Materials and Methods (section *Analysis of dye-DNA binding parameters*). Basing on the titration results, values of the lowest limit of the average number of DNA base triads/tetrads occupied by one dye molecule (n) was roughly estimated. For the Cyan 40 complexes with triplex the n value was found to be more than 6.6, while for Cyan 2 binding with triplexes and quadruplexes this value is more than 4.1 and 1.4 respectively. At the same time for intercalative binding mode the n value should be close to 2 due to the nearest neighbor exclusion principle. This allows us to assume that the mode of the both dyes interaction with the triplex is the groove-binding, while for the Cyan 2 complex with G4-DNA intercalation might also occur.

It is known that in general GC-sequences do not favor the minor groove-binding. The reason for this is location of a hydrogen bond between the guanine's NH_2 group and a carbonyl group of the cytosine in the minor groove. Therefore, we believe that in poly(dG-dG)-poly(dC) triplex the dyes bind to the groove formed by two guanines (through a Hoogsteen base-pairing). Illustrative drawing of the hypothetical structure of the complex between the triplex and the dyes is presented in Fig. 8.

We have demonstrated that addition of K-ions prevents the formation of high-affinity complex between G-quadruplexes and Cyan 2 (see Fig. 6); the high-affinity complex between Cyan 2 and the DNA is formed only in the absence of K or Na ions. Addition of the dye to G4-DNA in the presence of K ions did not lead to the enhancement of fluorescence intensity. Similar results were reported by us earlier for the interaction of G4-DNA with cationic porphyrin, TMPyP [8]. This result indicates for the intercalative mechanism of Cyan 2 binding to the G4-DNA and for the competition of the cation and the dye for binding to the core of the G4-DNA.

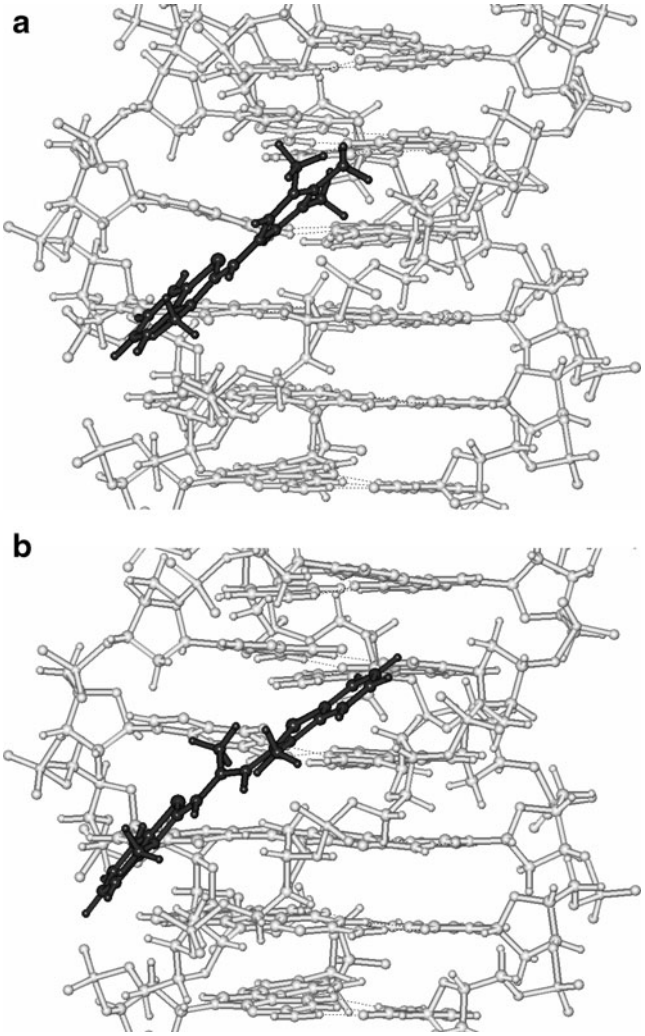

Fig. 8 Schematic drawing showing a hypothetical groove-binding complex between Cyan 40 (**a**) and Cyan 2 (**b**) with poly(dG-dG)-poly (dC) triplex

Conclusions

DNA sensitive dyes, monomethine cyanine Cyan 40 and trimethine cyanine Cyan 2, were studied by spectral-luminescent methods in the presence of dsDNA and non-canonical, triplex and G-quadruplex DNA motifs. Cyan 40 demonstrated strong preference for triplex over duplexes and quadruplex DNA forms; whereas Cyan 2 was less specific with respect to non-cannonical DNA structures and binds with high affinity to either triplex or quadruplex DNA forms. We suggested that the complexes of Cyan 40 and Cyan 2 with triplex DNA are formed via groove binding mode, while Cyan 2 interacts with G-4 DNA by intercalation.

Highly fluorescent complexes of Cyan 2 with the triplex and the quadruplex and Cyan 40 with the triplex DNA were stable under gel-electrophoresis conditions. This enables the use of the dyes for the detection of non-canonical motifs in gels and cell genome.

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