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Specific High-Affinity Binding of Thiazole Orange to Triplex and G-Quadruplex DNA[†]

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ABSTRACT: Interaction of Thiazole Orange (TO) with double-, triple-, and quadruple-stranded forms of DNA was studied. We have demonstrated by UV−vis absorption, circular dichroism (CD), and fluorescence spectroscopy that TO binds with much higher affinity to triplex and G-quadruplex DNA structures compared to double-stranded (ds) DNA. Complexes of the dye with DNA triplexes and G-quadruplexes are very stable and do not dissociate during chromatography and gel electrophoresis. TO binding to either triple- or quadruple-stranded DNA structures results in a >1000-fold increase in dye fluorescence. The fluorescence titration data showed that TO to triad and tetrad ratios, in tight complexes with the triplex and the G-quadruplex, are equal to 0.5 and 1, respectively. Preferential binding of TO to triplexes and G-quadruplexes enables selective detection of only these DNA forms in gels in the absence of free TO in electrophoresis running buffer. We have also demonstrated that incubation of U2OS cells with submicromolar concentrations of TO results in preferential staining of certain areas in the nucleus in contrast to DAPI which binds to dsDNA and efficiently stains regions that are unstained with TO. We suggest that TO staining may be useful for the detection of noncanonical structural motifs in genomic DNA.

Cyanine dyes have been shown to interact with various types of nucleic acids, i.e., double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and RNA. Thiazole Orange {TO,[1] 4-[3-methyl-2,3-dihydro(benzo-1,3-thiazole)-2-methylidene]quinolinium iodide} is among the oldest synthetic cyanine dyes, today widely used in reticulocyte analysis. The dye was shown to permeate live cell membranes and efficiently stain residual RNA in reticulocytes (*1*). It has been demonstrated that TO also binds to dsDNA with a stoichiometry of one dye residue per two base pairs of DNA (*1*). Binding to dsDNA results in a stock shift in the absorption spectrum, results in an induced CD signal, and leads to a large increase in the fluorescence of the dye. The dye fluorescence in the aqueous solution is extremely weak; formation of a complex with dsDNA and RNA produces a very large increase in the fluorescence yield (*1*−*3*). Binding of TO to dsDNA and ssDNA homopolymers is relatively weak (*2*) and readily reversible (*4*). A dimeric form of the TO dye, TOTO, has much higher affinity for dsDNA compared to that of the monomeric form. Complexes of TOTO with dsDNA are almost irreversible and, unlike the DNA−TO complex, do not dissociate during gel electrophoresis (see ref *5* for a review). This property of the homodimeric cyanine dyes, along with a >1000-fold increase in their fluorescence quantum yield upon binding to dsDNA, allowed detection and quantitation of the DNA fragments in gels with very high sensitivity.

Although the double-stranded B-DNA is most common in cells, a growing number of theoretical and in vitro studies indicate that alternative structural motifs like triplexes and G-quadruplexes might coexist with the double-stranded DNA (*6*−*11*). The vast majority of TO binding studies have been focused so far on the interaction of the dye with RNA or single- and double-stranded DNA (*1*−*3*). Recently, Teulade-Fichou and colleagues reported binding of TO to G4-forming intramolecular and tetramolecular oligonucleotides that mimic telomeric sequences (*12*−*14*). The estimated affinity of TO for these G-quadruplex structures was in a low micromolar range and was similar to those reported for double- and single-stranded DNA (*2*).

We have recently developed methods for the synthesis of novel long (hundreds of nanometers) noncanonical DNA nanostructures, i.e., poly(dG-dG)-poly(dC) triplexes and continuous mono- and tetramolecular G-quadruplexes (*15*, *16*) composed of hundreds of stacked triads and tetrads, respectively. The long G-quadruplexes (G4 wires), in contrast to short telomeric G4 DNA structures composed of a few G-tetrads only (*9*, *17*−*21*), are stable in the absence of K and Na ions (*15*, *16*, *21*). The G4 wires lacking the cations in the core can be transferred to a K (Na) form by addition of the cation (*16*). We have shown that K (Na) ions strongly affect the mode of binding of meso-tetrakis-(4-*N*-methylpyridyl)porphyrin (TMPyP) to the G4 wires (*22*); the dye binds strongly to the G4 wires by an intercalative mechanism in the absence of the cations and binds much more weakly to the K form of the wires via outside, nonintercalative modes. This led us to suggest that the presence of K^+ in the space between the guanine planes prevents intercalation of aromatic molecules into G-guadruplexes and that a competition between the cation and the TMPyP takes place.

Here we investigate the interaction of TO with long (hundreds of nanometers) double-, triple-, and quadruple-stranded DNA molecules using UV−vis absorption, circular dichroism (CD), and fluorescence spectroscopy. We have demonstrated that TO binds very tightly to the triplex and the G-quadruplex (G4)

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[1]Abbreviations: TO, Thiazole Orange; AFM, atomic force microscopy; CD, circular dichroism.

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DNA. Highly fluorescent complexes of TO with these structures are stable and do not dissociate during chromatography or gel electrophoresis. The affinity of G4 DNA for the dye is strongly reduced in the presence of K and Na ions. The dsDNA binds TO more weakly compared to the triple- and quadruple-stranded DNA forms; complexes of TO with dsDNA easily dissociate during electrophoresis. This enables the selective fluorescence detection of the noncanonical DNA structures in gels, in the absence of free TO in electrophoresis running buffer. In contrast to TO, a dimeric form of the dye, TOTO, binds tightly to all the DNA types mentioned above. We have also shown that incubation of U2OS cells with submicromolar concentrations of TO leads to preferential staining of nucleoli in the cell nucleus.

MATERIALS AND METHODS

Materials. Unless otherwise stated, reagents were purchased from Sigma-Aldrich and were used without further purification. TOTO was purchased from Invitrogen. The concentration of TO was determined using an extinction coefficient of 63000 mM^{-1} cm^{-1} at 500 nm (*2*).

DNA Samples. The DNA samples, poly(dG)-poly(dC) duplexes, intramolecular poly(dG-dG)-poly(dC) triplexes, and monomolecular G-quadruplexes (G4 wires) containing 700 or 2K bp, 700 triads, and 1000 tetrads, respectively, were prepared essentially as described in our recent publications (*16*, *23*, *24*). The G4 wires were prepared in the absence of stabilizing ions (K^+ and Na^+) and thus will be termed the "K (Na)-free" G4 wires. The K (Na)-free G4 wires were converted to the K form during a 30 min incubation with 10 mM KCl in 100 mM Tris-Ac buffer (pH 8.0) at 25 °C. Concentrations of double-, triple-, and quadruple-stranded DNA polymers were estimated using extinction coefficients of 14.8, 22.2 (*23*), and 36 mM^{-1} cm^{-1} (*22*) at 260 nm for the GC base pair, GGC triad, and G-tetrad, respectively. TO−DNA complexes were prepared by incubation of poly(dG)-poly(dC) duplexes, intramolecular poly(dG-dG)-poly(dC) triplexes, and monomolecular K (Na)-free G4 wires with an amount of TO equal to that of base pairs, triads, and G-tetrads, respectively, in 100 mM Tris-Ac buffer (pH 8.0) for 30 min at 25 °C. The complexes were subsequently passed through a Sephadex G-25 column (1 cm × 5 cm) equilibrated with 100 mM Tris-Ac (pH 8.0). The amount of TO eluted in the void volume of the column in complex with DNA was measured by absorption spectroscopy (see below).

Absorption, CD, and Fluorescence Spectroscopy. Absorption spectra of G4 wires were recorded with a Jasco V-630 spectrophotometer. CD spectra were recorded at 25 °C with an Aviv model 202 series (Aviv Instruments Inc.) circular dichroism spectrometer. Each spectrum was recorded from 220 to 600 nm and was an average of four measurements. Recording specifications were as follows: wavelength step, 1 nm; settling time, 0.333 s; average time, 1.0 s; bandwidth, 1.0 nm; path length, 1 cm. 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 taken in 100 mM Tris-Ac (pH 8.0); the absorption of all samples was ≤0.3.

Gel Electrophoresis. The DNA samples were loaded onto a 1% agarose gel and then electrophoresed at room temperature and 130 V for 25 min. TEA buffer, in addition to being used for the agarose gel preparation, also served as the running buffer. The gel was stained with either ethidium bromide (0.5 μg/mL), visualized with a Bio Imaging System 202D instrument, or with TO (0.5 μg/mL) and scanned on a FLA-2000 fluorescent-image analyzer (Fujifilm) using excitation at 473 nm (Y520 filter).

Atomic Force Microscopy (*AFM*). Atomic force microscopy was performed on molecules adsorbed onto Muscovite mica surfaces. DNA samples (10−20 μL) in 2 mM Tris-acetate (pH 8) containing 2 mM $MgCl_2$ were deposited on freshly cleaved mica plates for 5 min, then washed with distilled water, and dried with a nitrogen blow. AFM imaging was performed on a Solver PRO AFM system (NT-MDT), in a noncontact (tapping) mode, using 130 μm long Si-gold-coated cantilevers (NT-MDT) with a resonance frequency of 119−180 kHz. The images were "flattened" (each line of the image was fitted to a second-order polynomial, and the polynomial was then subtracted from the image line) with Nova image processing software (NT-MDT). The images were analyzed and visualized using Nanotec Electronica S.L (Madrid, Spain) WSxM imaging software (*25*).

Cell Growth and Preparations. U2OS cells, an osteosarcoma cell line, were grown in DMEM with 10% bovine serum, supplemented with antibiotics and 10 μg/mL L-glutamine. For the purpose of staining and imaging, cells were seeded on 20 mm round coverslips and grew overnight. Cell fixation was conducted with 4% paraformaldehyde for 10 min. Fixed cells were incubated with 0.1 μM TO for 30 min at room temperature, after which the excess of color was removed as the cells were washed with PBS. The cells were subsequently incubated with 0.2 μM DAPI for 20 min at room temperature. The coverslips with cell specimens were mounted on glass slides using an antifade gel.

Microscopy. The U2OS cell samples stained with TO and DAPI were visualized on a Leica TCS SP5 confocal microscope, using excitation wavelengths of 405 and 488 nm for DAPI and TO, respectively. The images were acquired using 4PO ×63 1.4 NA optical lenses and processed with LAS AF image analyzer software.

RESULTS

The interaction of TO with triplexes and K (Na)-free G4 wires results in the formation of stable complexes between the dye and the DNA that do not dissociate during chromatography on a size exclusion column. The complexes were prepared by incubation of 7 μM (expressed in triad or tetrad) DNA with 9 μM TO and subsequently passed through a Sephadex G-25 column. Chromatography on the Sephadex G-25 column results in complete separation of the DNA polymers from loosely bound and nonbound dye. The dye was eluted from the column as two well-separated peaks; the fraction that was eluted in the void volume of the column corresponds to TO tightly bound to DNA, and the second fraction that was eluted much later corresponds to free TO. Figure 1A shows absorption spectra of the dye that co-eluted with the DNA in the void volume. As seen in the figure, binding to the triplex results in an ~14 nm red shift of the absorption maximum, while binding of the dye to the quadruplex causes a 15 nm blue shift (compare the solid and dashed lines in Figure 1A). Chromatography of poly(dG)-poly(dC), random sequence plasmid DNA (pUC19), and the K^+ form of G4 DNA, preincubated with TO on a Sephadex G-25 column under the same conditions as the triplexes or K (Na)-free G4 wires, results in a separate elution of the dye and the DNA. The void volume fractions eluted from the column showed no absorption in 450−550 nm range of the spectrum (data not shown). This chromatographic behavior is consistent with weak binding of the dye to dsDNA and to the

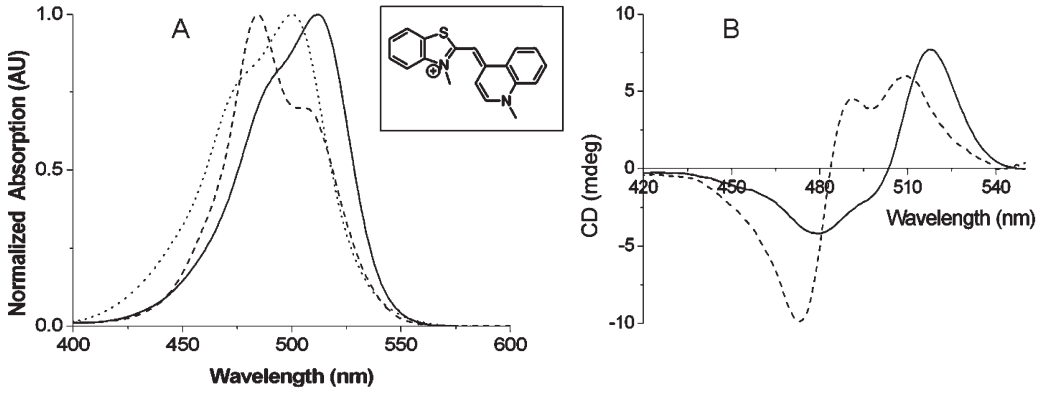

FIGURE 1: Visible absorption (A) and CD (B) spectra of TO (· · ·) in a complex with triplex (—) and the K (Na)-free form of the G-quadruplex (---). TO (9 μM) was added to 7 μM triplexes or 7 μM G-quadruplexes (concentrations expressed in triads or tetrads, respectively) in 100 mM Tris-Ac (pH 8.0) from a 1 mM stock solution in 50% DMSO. The samples were incubated for 30 min at room temperature and were subsequently passed through a Sephadex G-25 column in 100 mM Tris-Ac (pH 8.0) as described in Materials and Methods. Spectra of the DNA samples (dashed and solid curves in panel A) were normalized to that of free TO (dotted line in panel A) to account for the dilution of the samples during chromatography. The inset shows the chemical structure of TO.

K^+ form of G4 wires and complete dissociation of the dye from the DNA during the chromatography.

We have shown that TO exhibits chirality in complex with either triplex or G-quadruplex forms of DNA. Although TO is achiral, CD signals are induced once the dye is bound to the chiral DNA template. Figure 1B shows the induced CD spectra of stable complexes of TO with triplexes and G-quadruplexes eluted from the Sephadex G-25 column. As seen in Figure 1B, TO shows a strong negative peak at 473 nm and a broad positive signal in a 485−520 nm range when bound to the G-quadruplex (dashed line) and a negative signal around 480 nm and a positive peak at 520 nm when bound to the triplex (solid line).

The fluorescence intensity of the dye as a function of the amount of TO added to 0.2 μM solutions of the triplex or G-quadruplex DNA is shown in Figure 2. As seen in the figure, the emission intensity increases almost linearly with the increase in TO concentration, reaching a maximum at a stoichiometry of one TO per two triads in the triplex and per one G-tetrad in the quadruplex correspondingly (see ▲ and ● in Figure 2). The linear increase in emission intensity is in good agreement with the formation of a tight complex between the dye and both forms of DNA mentioned above. The fluorescence enhancement was noticeably lower with either dsDNA or G4 DNA in the presence of K ions as compared to that with triplexes and the quadruplexes (see Figure 2). This suggests that binding of TO at low concentrations (ranging from 2×10^{-8} to 3×10^{-7} M) to the latter molecules is weaker compared to binding to the triplexes and the quadruplexes in the absence of K ions (see Figure 2) and is consistent with relatively high values of a dissociation constant (10^{-5}−10^{-6} M) estimated for complexes of TO with dsDNA and G-rich telomeric sequences in the presence of K ions (*2*, *12*).

We have demonstrated that the high-affinity complexes of TO with the triplex and K (Na)-free G-quadruplex forms described above are electrophoretically stable and do not dissociate in agarose gels. Figure 3A (lanes 1−4) shows electrophoresis of various DNA samples premixed with stoichiometric amounts of TO. It is clearly evident from the image that only TO complexes with the G-quadruplex (lane 1) and the triplex DNA (lane 2) can be detected with a fluorescent gel scanner in contrast to the double-stranded forms, poly(dG)-poly(dC), and the plasmid

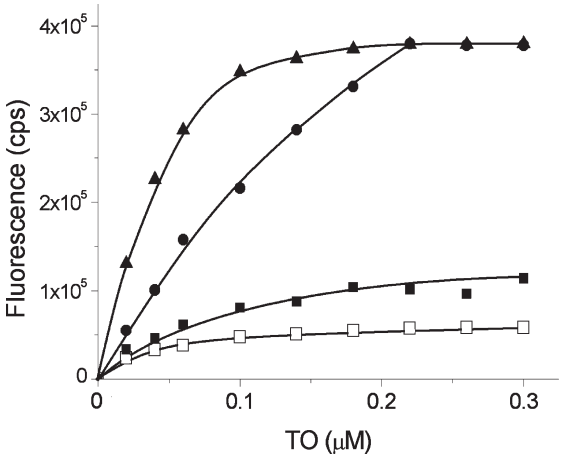

FIGURE 2: Fluorescence titration of pUC19 (■), poly(dG-dG)-poly(dC) triplex (▲), and G4 DNA in the absence (●) and presence of 10 mM K ions (□). The DNA concentration is expressed in base pairs, triads, and tetrads for double-, triple-, and quadruple-stranded forms, respectively, is equal to 0.2 μM. The excitation and emission wavelengths were 500 and 524 nm for the dsDNA (■), 514 and 555 nm for the triplex DNA (▲), and 485 and 550 nm for the G4 DNA (● and □), respectively.

DNA (lanes 3 and 4 in Figure 3A). Poststaining of the gel with 1 μM TO results in the appearance of two new bands corresponding to the dsDNA (lanes 3 and 4 in panels A and B) species. TOTO, in contrast to TO, is tightly bound to all the DNA types mentioned above and can be detected with a fluorescent gel scanner (Figure 3A, lanes 5−8). As seen in panels A and B of Figure 3, the bands corresponding to TOTO complexes with the triplex and G-quadruplex DNA appear smeared in the agarose gel (see lanes 5 and 6). This is most probably due to aggregation of these DNA forms caused by the homodimeric dye. The results presented in panels A and B of Figure 3 are consistent with readily reversible binding of TO and almost irreversible binding of TOTO to dsDNA (*26*, *27*). We have shown previously (*23*) that triple- or quadruple-stranded forms of DNA are poorly stained with ethidium bromide (EtBr), which is typically used to stain dsDNA in gels. The results presented in Figure 3D confirm our earlier observation and show that EtBr staining enables the detection of only dsDNA species (lanes 1−3). The triplex and

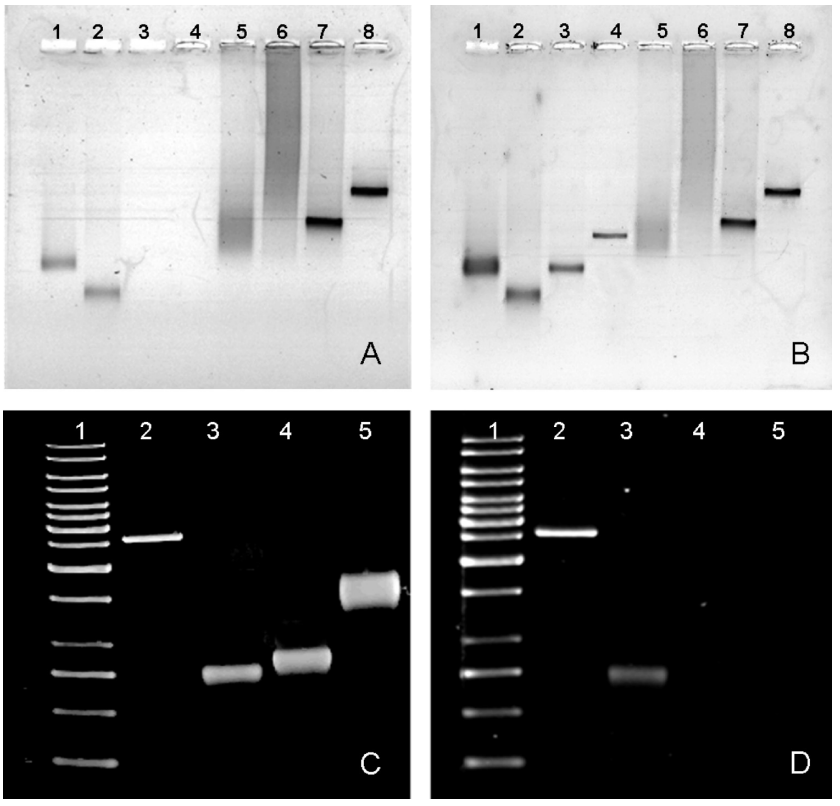

FIGURE 3: Detection of various DNA forms in gels using TO, TOTO, and EtBr. (A) The G-quadruplex (4.5 μM) (lanes 1 and 5), 5.5 μM poly(dG-dG)-poly(dC) triplex (lanes 2 and 6), 6.7 μM poly(dG)-poly(dC) duplex (lanes 3 and 7), and 6.7 μM pUC19 plasmid (lanes 4 and 8) were each mixed with stoichiometric amounts of TO (lanes 1−4) or TOTO (lanes 5−8) and incubated for 30 min at room temperature prior to being loaded onto a 1% agarose gel. Concentrations are expressed in base pairs, triads, and tetrads for double-, triple-, and quadruple-stranded DNA forms, respectively; the concentration of TOTO is expressed in TO monomers. Separation was conducted at 130 V for 25 min at room temperature in TEA buffer. The gel was visualized using a fluorescent image analyzer as described in Materials and Methods. (B) The same gel (as in panel A) was immersed in TEA containing 1 μM TO, incubated for 20 min at room temperature while being shaken constantly, and imaged. (C and D) A 1 kb ladder (lane 1), pUC19 plasmid (lane 2), poly(dG)-poly(dC) duplex (lane 3), poly(dG-dG)-poly(dC) triplex (lane 4), and G-quadruplex (lane 5) were loaded onto a 1% agarose gel and electrophoresed at 130 V for 25 min at room temperature in TEA buffer. The gel was incubated for 20 min at room temperature with 0.5 μg/mL TO (C) or 0.5 μg/mL EtBr (D) and visualized using a fluorescent image analyzer (C) or a Bio Imaging System (D) as described in Materials and Methods.

quadruplex DNA were not visible in the gel image (lanes 4 and 5). TO staining enables the detection of all tested DNA forms (Figure 3C, lanes 1−5).

To further investigate the binding mechanism, we have directly measured the lengths of individual DNA molecules using AFM. It has been shown that intercalation of organic molecules between base pairs results in lengthening of DNA, whereas there is no increase in DNA length upon binding of the groove binding ligands (*28*−*30*). The average contour lengths of bare triplex molecules and the molecules in complexes with TO were estimated by AFM. The latter complex was prepared by incubation of the triplex DNA with TO as described in Materials and Methods. AFM images of the bare triplex molecules and the molecules in complexes with TO are presented in panels A and B of Figure 4, respectively, along with their statistical contour length analysis (Figure 4C,D). The statistical AFM morphology imaging analysis of 100 individual molecules of each type (see Figure 4C,D) shows that the average contour length of the complex is clearly greater (by ~20%) than the length of the triplex not in a complex with the dye. Interaction of TO with G-quadruplexes also results in lengthening of the DNA (data not presented); the lengthening, however, did not exceed 10% of the original length of the quadruplex. The lengthening may suggest an intercalative mode (*28*−*30*) of TO binding to the DNA forms mentioned above.

Much stronger interaction with the triplex and the quadruplex, compared to that with dsDNA, along with strong enhancement of fluorescence upon binding of the dye to the former DNA types makes TO an appealing candidate for staining of noncanonical DNA structures in the cell. There are many long G-rich sequences in the genome of mammals in general and humans in particular (*31*) that may fold into configurations different from classical double-helical ones (*32*). Temporal association of double- and single-stranded fragments might also take place in the cell (*33*, *34*). We have shown (see Figure 2) that highly fluorescent DNA−TO complexes are being formed in vitro upon incubation of the triple- and quadruple-stranded DNA with submicromolar concentrations of the dye. The complexes are stable and do not dissociate for hours in contrast to the much weaker complex of the dye with a dsDNA which dissociates readily into its constituents. This could lead to selective binding of TO to noncanonical DNA structures in living cells. As seen in Figure 5A, treatment of U2OS cells with a low (0.1 μM) concentration of TO for 30 min results in the enhanced staining of certain areas of the nucleus (seen as bright green spots in the image). The increase in the concentration of the dye above 1 μM produces staining of the entire nucleus (not shown). The incubation of cells with DAPI (4′,6-diamidino-2-phenylindole), which preferentially targets dsDNA by binding to the minor groove (*35*), produces intensive staining of the entire nucleus with the exception of certain nuclear

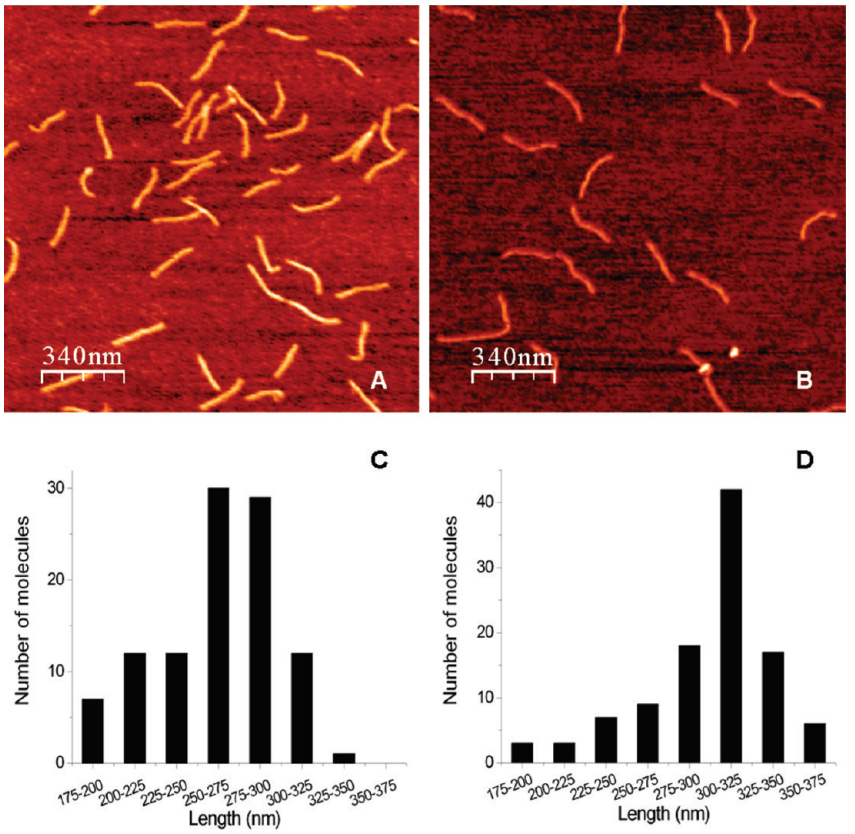

FIGURE 4: AFM images of 700 triad poly(dG-dG)-poly(dC) triplex molecules (A) in complexes with TO (B). The complex was prepared by incubation of the DNA with TO and subsequent chromatography of the mixture on a Sephadex G-25 column as described in Materials and Methods. The molecules were deposited on a mica surface and imaged as described in Materials and Methods. (C and D) More than 100 molecules of each type were subjected to the statistical contour length analysis. The length values are corrected for the finite tip radius by subtraction of the molecule's apparent width (a good approximation for the tip diameter) from the measured length and yield an average value of 259 nm (standard deviation of 36.2 nm) for the triplex (C) and 307 nm (standard deviation of 39.6 nm) for the DNA−TO complex (D).

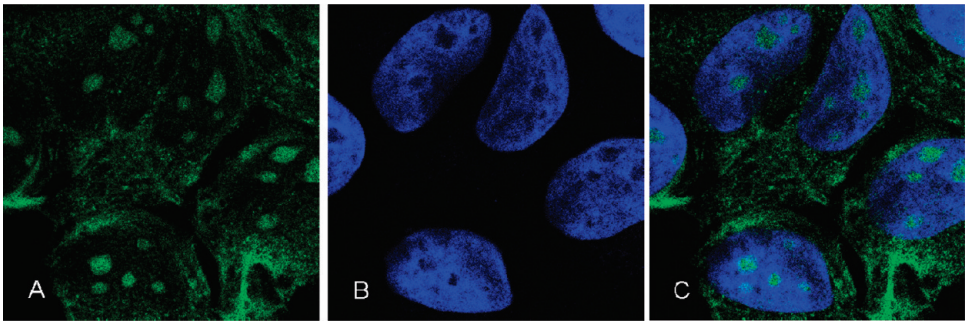

FIGURE 5: TO staining in cells. The U2OS cells were fixed and incubated with 0.1 μM TO (A) and 0.2 μM DAPI (B) at room temperature as described in Materials and Methods. The imaging was performed on a Leica TCS SP5 confocal microscope, using excitation wavelengths of 405 and 488 nm for DAPI and TO, respectively. (C) Superposition of images presented in panels A and B.

regions (Figure 5B). It is clearly seen (compare panels A and B of Figure 5) that DAPI staining revealed a pattern almost opposite to that of TO.

DISCUSSION

We have shown that TO binds strongly and preferentially to triple- and quadruple-stranded DNA forms, as compared to dsDNA. Incubation of the dye at submicromolar concentrations with the former DNA structures results in a strong enhancement of its fluorescence. We have shown that the fluorescence efficiency increases approximately 1000-fold upon binding of TO to the DNA. This corresponds to the quantum yield increase from 2×10^{-4} (*2*) to more than 0.1. It has been shown that binding to single- and double-stranded DNA results in a similar increase in the dye quantum yield (*2*).

We have demonstrated that binding of TO to triplex DNA causes a red shift of the absorption spectrum, while binding of the dye to the G-quadruplex causes a blue shift of the spectrum (see Figure 1A). It is well-known that cyanine dyes in general and TO in particular tend to dimerize in aqueous solutions (*2*). The absorption spectrum of the TO monomer is characterized by a maximum intensity at 500 nm and a shoulder at a shorter wavelength, 480 nm. On the other hand, the spectrum of the TO dimer has a maximum at a shorter wavelength, 471 nm, and a shoulder at 495 nm (*2*). The spectra of the dye bound to triplex and to G-quadruplex DNA resemble the spectra of the TO monomer

and dimer, respectively (see Figure 1). These results are consistent with stabilization of the monomeric form of the dye by triplexes and the dimeric form by G-quadruplexes. We have shown that in high-affinity complexes, one dye moiety is bound per two triads and per one G-tetrad in triplex and quadruplex DNA (see Figure 2). One can speculate that in the latter complex two dye molecules are bound to every other tetrad. This corresponds nicely to the estimated TO to G-tetrad stoichiometry of 1 (see Figure 2) and can explain a similarity between the absorption spectra of the bound dye and TO dimer.

It has been shown that the dye is achiral; upon binding to chiral DNA polymers, however, TO acquires chirality and exhibits induced CD. It has been shown that the sign of the induced CD spectra of dyes bound to nucleic acids provides important information about their binding modes. For example, a positive induced CD band in the Soret region is indicative of outside binding of cationic porphyrins, while a negative induced CD band is produced upon intercalation of porphyrin residues into the DNA (*36*−*38*). The correlation between the mode of binding and the shape of the CD spectrum is much less clear for TO and its analogues. It has been hypothesized that a negative CD band centered at 490−500 nm indicates intercalation of asymmetric cyanine dyes into DNA (*39*−*41*). The induced CD signal in the 450−550 nm range (see Figure 1B) is consistent with binding of TO to G-quadruplexes or triplexes and orientation of the dye molecules by these polymers. It is, however, not possible to draw any firm conclusion about the mode of interaction of TO with the DNA based on the analysis of these spectra.

We have demonstrated that the addition of K ions prevents formation of a high-affinity complex between the G4 DNA and TO (see Figure 2). Similar results were reported for the interaction of G4 wires with cationic porphyrin, TMPyP, by us recently (*22*). We have shown that TMPyP binds to the G4 wires via intercalation with high affinity only in the absence of K ions. The presence of the cation in the center space between the adjacent tetrads prevents access of TMPyP to this region, thus enabling only weak external binding of the dye to the wires. The data presented in this study agree nicely with the results obtained on interaction of G4 wires with TMPyP. We have shown that the high-affinity complex between TO and the G4 wires is formed only in the absence of K (Na) ions. Addition of the dye to G4 DNA in the presence of K ions did not lead to the enhancement of fluorescence (see Figure 2), suggesting that no intercalation of the dye into the K form of the wires takes place. These results also correspond with nonintercalative binding of TO to G4-forming oligonucleotides demonstrated previously (*12*). The affinity of the latter G4 structures (composed of three tetrads) for the dye ($K_a \sim 1-2 \times 10^6$ M^{-1}) is only slightly higher than the affinity of single- and double-stranded DNA (*2*). The short G4 structures in contrast to long G4 wires used in this study are stable only in the presence of K (Na) ions. The presence of cations in the core is most likely the reason for relatively low-affinity nonintercalative binding of the dye to G4-forming oligonucleotides (*12*).

It was shown that a number of small aromatic molecules can bind preferentially to triplex DNA (*42*−*45*). Some of these molecules have different binding affinities for the triple helices containing T·AxT and C·GxC+ triplets. Comparative investigation of TO binding to different DNA triplexes can thus provide insights into the base preference and the mode of binding of TO to the DNA. Further investigation of the interaction of TO and its analogues with various types of DNA is underway in our laboratory.

TO is a very attractive dye for staining DNA in gels. The fluorescence quantum yield of the dye is enhanced greatly when it binds to DNA. The disadvantage of the dye is its relatively low affinity ($K_d \sim 10^{-5}-10^{-6}$) for dsDNA and its fast dissociation from the DNA−TO complex. We have shown that TO binds with very high affinity to triplex and G-quadruplex forms of DNA. As in the case of a complex of TOTO and dsDNA, complexes of TO with the triplex and the quadruplex are stable to electrophoresis and can be selectively detected in gels. As clearly demonstrated in Figure 3A, only bands corresponding to these forms can be detected in the agarose gel (see lanes 1 and 2 in Figure 3A).

The triple- and quadruple-stranded DNA, in contrast to dsDNA, cannot be detected on an ethidium bromide-stained gel (see lanes 4 and 5 in Figure 3D). We have demonstrated earlier that the inability to detect DNA triplexes (*23*) and quadruplexes is due to relatively weak enhancement of EtBr fluorescence upon binding to these structures. We have shown that TO, in contrast to EtBr, is a highly sensitive and effective stain, allowing detection of triple- and quadruple-stranded DNA in gels. The complexes of these DNA forms with TO are stable and do not dissociate during electrophoresis. This is a significant advantage that enables the detection of low concentrations of these noncanonical DNA structures in gels without any background fluorescence. It might be particularly useful for the detection of noncanonical structural motifs in genomic DNA or in RNA.

Noncanonical DNA structures have gained substantial attention in the past decade because of their potential involvement in the regulation of gene transcription and in the maintenance of genomic stability. It has been suggested that G-rich sequences (*47*) in eukaryotic genomes may adopt noncanonical conformations in vivo and exist naturally in chromosomes of living cells (*32*−*34*, *46*). Specific staining of triplex and G-quadruplex structures with TO demonstrated in this work may provide a useful tool for the selective detection of these structural motifs in the genome of living cells. We have observed in pilot experiments (data not shown) that products of enzymatic digestion of chromosomal DNA with the *Bum*HI restriction enzyme interact with high affinity with TO and can be readily detected in gels without poststaining with TO. This suggests the possible presence of triple- and quadruple-helical structures in these fragments.

We have demonstrated that TO preferentially stains certain regions in the cell nucleus. These regions (seen as green spots in Figure 5A) are probably associated with nucleoli, organelles that are responsible for synthesis of rRNA (*48*, *49*). One can speculate that a high density of nucleic acids, DNA, and RNA in the nucleoli and the high transcriptional activity of these organelles (*50*) could lead to the temporal formation of noncanonical triplex and/or quadruplex DNA structures that can be efficiently stained by TO. This suggestion is supported by the finding that the nucleolus-associated DNA has a higher GC content than the bulk of the nuclear DNA (ref *51* and references cited therein) and may account for the increased probability of noncanonical structure formation, leading to preferential staining of these regions with TO. We cannot, however, exclude the possibility that preferential staining of these regions is due to the interaction of the dye with nuclear RNA. As seen in Figure 5, TO stains the cytoplasm of the cells. The cytosolic labeling is probably due to the interaction of TO either with cytosolic proteins or with tRNA. We have studied the interaction of TO with bovine serum albumin and find that no high-affinity binding of the dye to the

protein takes place. We cannot, however, exclude the possibility that certain proteins in the cytoplasm exhibit much stronger binding affinity toward TO compared to the albumin, leading to protein-mediated cytoplasmic labeling. In preliminary experiments, we found that incubation of TO with tRNA results in the formation of a fluorescent complex between the dye and the RNA. Therefore, the cytoplasmic staining seen in the image (see Figure 5) might be attributed to the interaction of the dye with tRNA.

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