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Discrimination of G-Quadruplexes from Duplex and Single-Stranded DNAs with Fluorescence and Energy-Transfer Fluorescence Spectra of Crystal Violet

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Abstract: G-rich nucleic acid sequences with the potential to form G-quadruplex structures are common in biologically important regions. Most of these sequences are present with their complementary strands, so the development of a sensitive biosensor to distinguish G-quadruplex and duplex structures and to determine the competitive ability of quadruplex to duplex structures has received a great deal of attention. In this work, the interactions between two triphenylmethane dyes (malachite green (MG) and crystal violet (CV)) and G-quadruplex, duplex, or single-stranded DNAs were studied by fluorescence spectroscopy and energy-transfer fluorescence spectroscopy. Good discrimination between quadruplexes and duplex or single-stranded DNAs can be achieved by using the fluorescence spectrum of CV or the energy-transfer fluorescence spectra of CV and MG. In addition, by using energy-transfer fluorescence titrations of CV with G-quadruplexes, the binding-stoichiometry ratios of CV to G-quadruplexes can be determined. By using the fluorescence titrations of G-quadruplex–CV complexes with C-rich complementary strands, the fraction of G-rich oligonucleotide that engages in G-quadruplex structures in the presence of the complementary sequence can be measured. This study may provide a simple method for discrimination between quadruplexes and duplex or single-stranded DNAs and for measuring G-quadruplex percentages in the presence of the complementary C-rich sequences.

Keywords: biosensors · crystal violet · DNA · fluorescence · quadruplexes

Introduction

G-quadruplexes are unique higher-order structures formed by G-rich nucleic acid sequences and based on stacked arrays of G-quartets connected by Hoogsteen-type base pairing (Scheme 1). These structures are further stabilized by the presence of monovalent cations (especially K^+). G-rich nucleic acid sequences with the potential to form quadruplex structures are common in biologically important regions, such as chromosomal telomeres, gene promoters, and immunoglobulin switch regions.[1] The formation of G-quadruplex structures may be correlated with some biological functions. A good example of this correlation is the human telomeric sequences, in which the formation of G-quadruplex structures can lead to inhibition of telomerase activity and interference with telomere biology.[2] Thus, small molecules that can stabilize telomeric G-quadruplexes may be good candidates in cancer therapy.[3] Similarly, it has been suggested that some quadruplex motifs in gene promoters may be involved in gene transcription.[4]

In these putative G-quadruplex-forming sequences, with the exception of the single-stranded 3′-overhang of telomeres, other sequences are present with their complementary strands.[5] That is to say, competition may have occurred between the formation of G-quadruplex and duplex structures. In order to elucidate the biological importance of these G-quadruplex-forming sequences, their structures in certain circumstances must be clearly identified. Therefore, the development of a sensitive biosensor to distinguish G-quadruplex and duplex structures has received a great deal of attention.[6]

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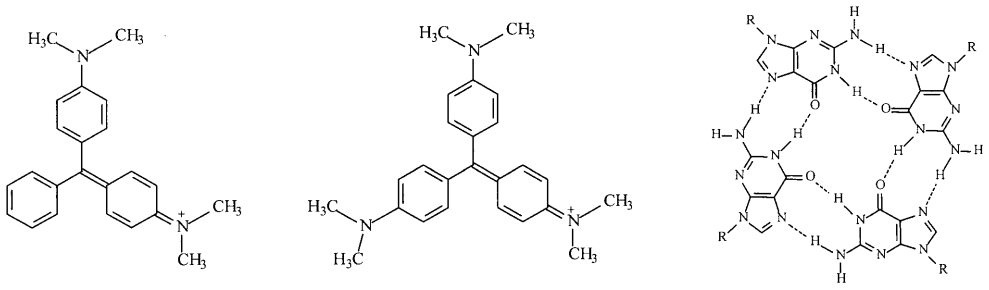

Scheme 1. The structures of malachite green and crystal violet and a schematic representation of a G-quartet.

Malachite green (MG) and crystal violet (CV) are both triphenylmethane dyes (Scheme 1). It has been reported that MG exhibits a strong increase in fluorescence upon binding to intramolecular quadruplexes, which indicates that MG may be used as a sensitive quadruplex biosensor.[7] CV has a similar frame structure to MG; the only difference between the two dyes is the number of dimethylamino substituents. The dimethylamino substituents provide potential for hydrogen-bonding and cation-dipole interactions with the negatively charged phosphate backbone and loops of G-quadruplexes.[8] Thus, the presence of the third dimethylamino substituent in CV may be helpful in the interaction between the dye and G-quadruplexes. However, to our knowledge, the interaction between CV and G-quadruplexes has not been reported. In this study, the interactions between the two dyes and G-quadruplex (both intra- and intermolecular), duplex, or single-stranded DNAs were studied by fluorescence spectroscopy and energy-transfer fluorescence spectroscopy. The abilities of the two dyes to discriminate quadruplexes from duplex and single-stranded DNAs are discussed.

Results and Discussion

Fluorescence spectra: The fluorescence spectra of CV and MG in the presence of different DNAs were measured. In this study, ten different DNAs (Table 1, entries 1–10) were used.[9] Hum21 and Oxy28 can form intramolecular G-quadruplex structures, whereas Hum12 and Oxy12 can form intermolecular G-quadruplex structures (Hum21 and Hum12 have the repeated subunits of human telomeres, Oxy28 and Oxy12 have the repeated subunits of *Oxytricha* telomeres). AT, GC, and LD can form short-stranded duplex structures, calf thymus (Ct) DNA belongs to the long-stranded duplex DNAs, and ssDNA1 and ssDNA2 are single-stranded DNAs with a random DNA sequence. The results showed that the fluorescence of both free MG and free CV was weak, and a significant increase in the emission intensity was observed at a fixed MG or CV concentration in the presence of G-quadruplex DNAs. Similarly, the emission intensities of the dyes also increased in the presence of duplex or single-stranded DNAs, but the extent of fluorescence enhancement was obviously different between CV and MG. It can be seen from Figure 1 that, at similar DNA base concentrations, the fluorescence intensities of CV in the presence of G-quadruplex DNAs were always higher than those in the presence of duplex or single-stranded DNAs; this indicates that CV may be used as a biosensor for discriminating G-quadruplexes from duplex or single-stranded DNAs. By contrast, MG did not discriminate G-quadruplex and duplex DNAs so clearly. The fluorescence enhancement in the presence of Ct DNA was much higher than that in the presence of Oxy28, and the fluorescence enhancement in the presence of AT was higher than that in the presence of Oxy12. These results suggest that CV may be a better candidate for discriminating quadruplexes from duplex or single-stranded DNAs. It is noteworthy that ssDNA2 and Ct DNA also increased the fluorescence of CV to some extent.

Table 1. Oligonucleotides used in this work.

Entry	Abbreviation	Sequence	Extinction coefficient [$L\,mol^{-1}\,cm^{-1}$]	Structure
1	Hum21	5′-$G_3(T_2AG_3)_3$	215000	G-quadruplex (intramolecular)
2	Oxy28	5′-$G_4(T_4G_4)_3$	262000	G-quadruplex (intramolecular)
3	Hum12	5′-$(T_2AG_3)_2$	122400	G-quadruplex (intermolecular)
4	Oxy12	5′-$G_4T_4G_4$	115200	G-quadruplex (intermolecular)
5	AT	5′-$(AT)_6$	133300	duplex
6	GC	5′-$(GC)_6$	101100	duplex
7	LD	5′-$(GC)_2A_2T_2(GC)_2$	295000	duplex
8	Ct DNA			duplex
9	ssDNA1	5′-GAGCTCTCGAAAGAGCT-CCGATTA	235800	single-stranded
10	ssDNA2	5′-TAGAGCACAGCCTGTCCGTG	189400	single-stranded
11	Hum21c	5′-$C_3(TA_2C_3)_3$	185900	
12	GT21	5′-$G_3(T_3G_3)_3$	197300	G-quadruplex (intramolecular)
13	GT21c	5′-$C_3(A_3C_3)_3$	195800	

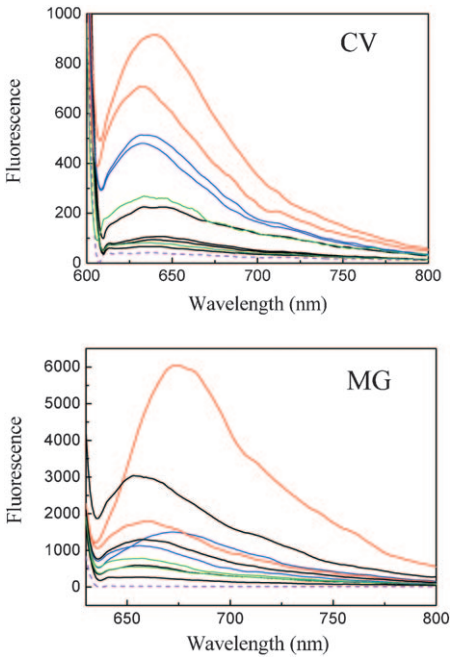

Figure 1. Fluorescence spectra of CV (top) and MG (bottom) in the presence of different DNAs: Free dye (purple, dashed lines), intramolecular G-quadruplexes (red), intermolecular G-quadruplexes (blue), duplex DNAs (black), and single-stranded DNAs (green). The orders (top to bottom) of the DNAs are intramolecular G-quadruplex DNAs: 7.5 μM Hum21 and Oxy28; intermolecular G-quadruplex DNAs: 15 μM Hum12 and Oxy12; duplex DNAs: 360 μM Ct DNA (base concentration), 15 μM AT, LD, and GC; single-stranded DNAs:15 μM ssDNA1 and ssDNA2. [CV] = [MG] = 15 μM.

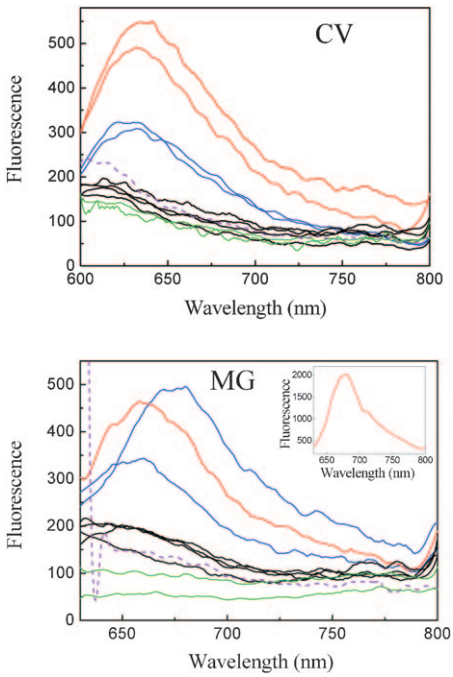

Figure 2. Energy-transfer fluorescence spectra of CV (top) and MG (bottom) in the presence of different DNAs: Free dye (purple, dashed lines), intramolecular G-quadruplexes (red), intermolecular G-quadruplexes (blue), duplex DNAs (black), and single-stranded DNAs (green). The orders (top to bottom) of the DNAs are intramolecular G-quadruplex DNAs: 7.5 μM Hum21 and Oxy28, Hum21 is shown in the inset for MG; intermolecular G-quadruplex DNAs: 15 μM Oxy12 and Hum12 for CV, 15 μM Hum12 and Oxy12 for MG; duplex DNAs: 360 μM Ct DNA (base concentration), 15 μM AT, LD, and GC; single-stranded DNAs:15 μM ssDNA1 and ssDNA2. [CV] = [MG] = 15 μM.

Energy-transfer fluorescence spectra: The energy-transfer fluorescence spectra of MG and CV in the presence of the ten DNAs were also studied. It was previously demonstrated that energy transfer between DNA bases and a bound fluorescent ligand is only possible if the ligand is in close contact with the DNA bases.[10] This happens only when a fluorescent ligand is intercalated between stacked base pairs in duplex DNAs and G-quartets in G-quadruplex DNAs or is stacked onto the two external G-quartets of quadruplexes. When the two dyes are excited at 280 nm, which is in the DNA absorption region of 240–320 nm,[11] their energy-transfer fluorescence spectra were clearly different from their fluorescence spectra. From Figure 2, it can be seen that free CV and MG can also absorb excitation energy and emit weak fluorescence. The addition of G-quadruplexes can increase their fluorescence greatly; this result indicates that the binding modes between the two dyes and the G-quadruplex DNAs were intercalation or external stacking, but not outside binding,[12] and that effective energy transfer occurred between the G-quadruplex DNAs and the dyes. The addition of single-stranded DNAs decreased the fluorescence of the two dyes, which indicates that no effective energy transfer occurred between single-stranded DNAs and the dyes; the competitive absorbance of the excitation energy by single-stranded DNAs decreased the fluorescence emission of the dyes.

The addition of duplex DNAs also decreased the fluorescence of CV to some degree, but the addition of AT, LD, and Ct DNA increased the fluorescence of MG to a small extent, which indicates that partial intercalation may occur between these duplex DNAs and MG. These results indicate that the energy-transfer fluorescence spectra are more suitable than the fluorescence spectra for discriminating between G-quadruplexes and duplex or single-stranded DNAs (especially for MG). Again, CV gave better results than MG.

In order to further investigate the energy transfer between G-quadruplex DNAs and CV, the excitation spectra of free CV and the mixtures of CV with DNAs were studied. As shown in Figure 3, free CV can only emit weak fluorescence when the excitation wavelength was set in the range 240–290 nm, and the fluorescence intensity decreased when duplex and single-stranded DNAs were added. On the other hand, the addition of quadruplex DNAs can increase the fluorescence intensity to high levels, and the increased fluorescence in the presence of intramolecular G-quadruplexes (Hum21 and Oxy28) is higher than that in the presence of intermolecular G-quadruplexes (Hum12 and Oxy12). In addition, when compared with the spectra of mixtures of CV with duplex (or single-stranded) DNAs, there is a distinct band at a wavelength of around 260 nm in the excitation spectra of the mixtures of CV with G-quadruplex DNAs;

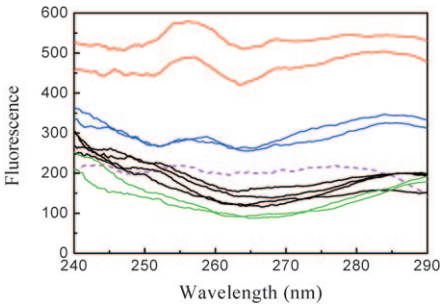

Figure 3. Exitation spectra of free CV and CV+DNA solutions: Free CV (purple, dashed lines), intramolecular G-quadruplexes (red), intermolecular G-quadruplexes (blue), duplex DNAs (black), and single-stranded DNAs (green). The orders (top to bottom) of the DNAs are intramolecular G-quadruplex DNAs: 7.5 μM Hum21 and Oxy28; intermolecular G-quadruplex DNAs: 15 μM Oxy12 and Hum12; duplex DNAs: 360 μM Ct DNA (base concentration), 15 μM AT, LD, and GC; single-stranded DNAs:15 μM ssDNA1 and ssDNA2. [CV] = 15 μM.

this value is the maximum absorbance wavelength of DNA. This result further demonstrates the presence of energy transfer between G-quadruplex DNAs and CV.

Fluorescence titrations and energy-transfer fluorescence titrations by different DNAs: As CV showed a better ability to discriminate G-quadruplexes from duplex and single-stranded DNAs than MG, the fluorescence and energy-transfer fluorescence of CV as a function of DNA concentration was investigated. Figure 4 shows the results of fluorescence and energy-transfer-fluorescence titrations of CV with different DNAs. In the fluorescence results, it can be seen that the fluorescence of CV initially increased greatly with increased concentrations of added G-quadruplexes and then leveled off or slowed down. The binding-stoichiometry ratios were determined from the corresponding fluorescence binding isotherms. When the concentration of CV was fixed at 15 μM, the DNA concentrations (strand concentration) at the intersection points on the titration curves were (8 ± 1) μM for Hum21, (7 ± 1) μM for Oxy28, (15 ± 1) μM for Hum12, and (16 ± 1) μM for Oxy12, respectively. Two strands of Hum12 or Oxy12 can form a quadruplex, so the binding-stoichiometry ratios of CV to the four G-quadruplexes were all 2:1. This 2:1 stoichiometry is consistent with a binding mode based on stacking of the dye onto the two external G-quartets of the quadruplexes.[13] Based on the stoichiometry of 2:1, the fluorescence titration data of the four G-quadruplexes can be well fitted by the corresponding equation (see the Supporting Information), and the association constants (K_a) of the G-quadruplex–CV complexes are (0.036 ± 0.008), (0.019 ± 0.003), (0.0033 ± 0.0008), and (0.0066 ± 0.0003) μM^{-2} for Hum21, Oxy28, Hum12, and Oxy12, respectively. Under the same conditions, addition of duplex and single-stranded DNAs continuously increased the fluorescence of CV and no obvious curve inflexion was observed over the studied concentration range; the association constants between CV and duplex or single-stranded DNAs could not be determined.

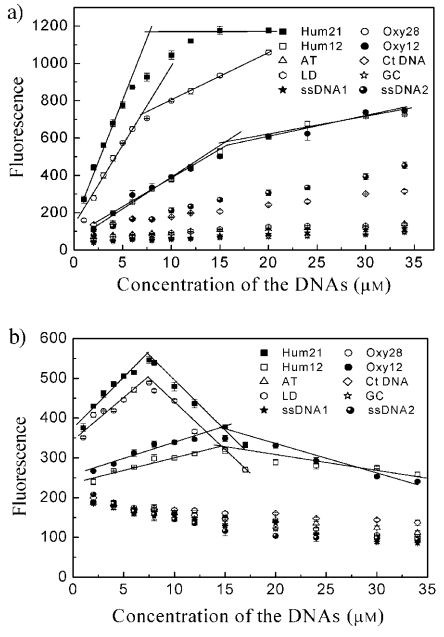

Figure 4. a) Fluorescence and b) energy-transfer fluorescence titrations of CV with different DNAs. [CV] = 15 μM. The DNA concentration is expressed as the strand concentration. The concentration of Ct DNA is converted into the strand concentration of an oligonucleotide with 12 nucleotides. Values are the mean ± standard deviation (SD) from three scans (n = 3).

In the energy-transfer fluorescence results, the fluorescence of CV initially increased with increased concentrations of G-quadruplexes and then reached a maximum at the appropriate G-quadruplex concentration (around 7.5 μM for intramolecular G-quadruplexes and around 15 μM for intermolecular G-quadruplexes). Further increase in the concentration of G-quadruplexes led to an abrupt decrease in the fluorescence of CV. At low DNA concentrations, the CV dye was in excess. The addition of G-quadruplexes was helpful for the formation of G-quadruplex–CV complexes as more and more energy was transferred from DNA to CV, so the fluorescence of CV increased. At higher DNA concentrations, the G-quadruplexes were in excess. Free G-quadruplexes compete with the G-quadruplex–CV complexes to absorb the energy of the excitation light and, therefore, the fluorescence of CV decreased sharply. Under the same conditions, the addition of duplex or single-stranded DNAs led to a steady decrease in the fluorescence of CV. This can be interpreted as the competitive absorbance of excitation energy by the DNAs and CV. In order to demonstrate the competitive absorbance of the excitation energy between excess DNAs and CV (or G-quadruplex–CV complexes), the data in Figure 4b were corrected for the "inner-filter effect" by using a method that has been previously reported (see the Supporting Information).[14] The corrected titration curves display similar change patterns to those in Figure 4a, and an identical binding-stoichiometry ratio of 2:1 between CV and each G-quadruplex can also be observed. These results indicate that energy-transfer fluorescence data are

more suitable for studying the binding-stoichiometry ratios of fluorescent dyes to G-quadruplexes. With the energy-transfer fluorescence results, the inflexions in the titration curves can be identified more clearly than those in the fluorescence titration curves. In addition, G-quadruplex DNAs can be easily distinguished from duplex and single-stranded DNAs according to the trend in the fluorescence change of CV.

Fluorescence titrations of G-quadruplex–CV complexes with C-rich complementary strands: To further demonstrate the ability of CV to distinguish G-quadruplex DNAs from duplex DNAs, the fluorescence of CV was recorded at a fixed Hum21 concentration while the concentration of Hum21c, which is a C-rich strand complementary to Hum21 (Table 1, entry 11), was varied. With an increased Hum21c concentration, both the fluorescence and energy-transfer fluorescence of CV decreased rapidly, which indicates the breakage of the G-quadruplex structure and the formation of the duplex DNA. From the fluorescence titration curve (Figure 5), the percentage of Hum21 that exists in the form

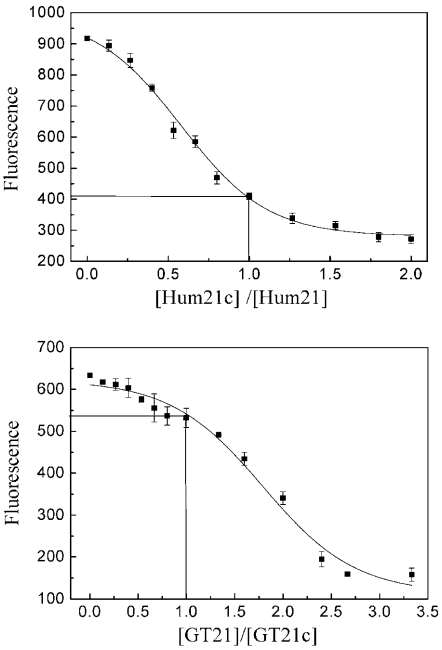

Figure 5. Fluorescence titration of Hum21–CV complex (top) or GT21–CV complex (bottom) with corresponding C-rich complementary strands. [CV] = 15 μM, [Hum21] = [GT21] = 7.5 μM. Values are the mean ± SD (n = 3). The solid lines in the two plots represent nonlinear least-squares fits to the data.

of a G-quadruplex structure can be calculated according to Equation (1), in which F_{max} and F_{min} correspond to the maximum and minimum fluorescence intensities in the curve, respectively, and F is the fluorescence intensity of a selected point in the curve. When the concentration ratio of Hum21 to Hum21c was 1:1, around 20% of Hum21 could fold into the G-quadruplex.

$$\text{G-quadruplex}\ (\%) = \frac{F - F_{min}}{F_{max} - F_{min}} \times 100 \quad (1)$$

Under the same conditions, the fluorescence titration experiment was performed for another G-quadruplex formed by GT21 (Table 1, entry 12). The results show that, when the concentration ratio of GT21 to GT21c (Table 1, entry 13) was 1:1, around 78% of GT21 could fold into the G-quadruplex, which indicates that GT21 is more likely to form a G-quadruplex than Hum21. These findings are consistent with a previous report that the presence of thymine residues in the loops best promotes the quadruplex structure.[15]

The addition of complementary strands can also decrease the energy-transfer fluorescence of CV; this also demonstrates the discrimination abilities of CV toward quadruplex and duplex DNAs. In this paper, energy-transfer fluorescence titration curves were not selected to determine the quadruplex-percentage probabilities of the G-rich oligonucleotides in the presence of complementary strands. As the concentration of complementary strands increased, the energy-transfer fluorescence of CV decreased steadily, and no plateau or minimum fluorescence was observed due to the competitive absorbance of excitation energy by free complementary strands (see the Supporting Information). This phenomenon was more pronounced for GT21 due to the low initial fluorescence intensity in the titration curve. In order to calculate the quadruplex-percentage probabilities, the energy-transfer titration curves would have to be corrected by using the curves of the corresponding single-stranded DNAs.

Fluorescence titration of G-quadruplex molecular beacons by C-rich complementary strands: To demonstrate the accuracy of this method, conformational changes of the two G-rich oligonucleotides as a function of complementary strands were investigated by using the G-quadruplex molecular-beacon method, in which two G-quadruplex molecular beacons (F-Hum21-D and F-GT21-D) were used. A G-quadruplex molecular beacon is a single-stranded oligonucleotide probe with a G-rich sequence.[16] A fluorophore (6-carboxyfluorescein, FAM) is attached to one end of the oligonucleotide and a quencher (4-(4′-dimethylaminophenylazo)benzoic acid, DABCYL) is attached to the other end. When the oligonucleotide is folded into the G-quadruplex structure, the fluorophore is kept in close proximity to the quencher, which causes the fluorescence of the fluorophore to be quenched. In the presence of a C-rich complementary strand, the G-quadruplex molecular beacon will hybridize to the complementary strand and undergo a conformational change that forces the fluorophore and quencher apart and allows fluorescence to occur. By monitoring the fluorescence of the beacon as a function of complementary-strand concentration, a corresponding fluorescence titration curve was obtained (Figure 6). As the concentration of the complementary strand increased, the fluorescence intensity of the beacon increased and reached a plateau, thereby indicat-

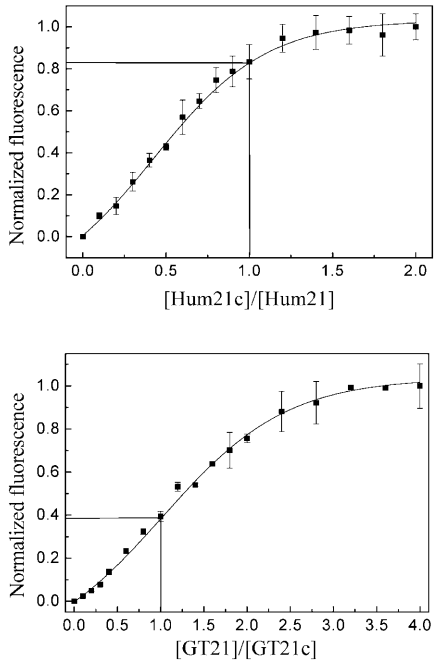

Figure 6. Fluorescence titration of F-Hum21-D (top) or F-GT21-D (bottom) with corresponding C-rich complementary strands. [F-Hum21-D]=[F-GT21-D]=1 μM. Values are the mean±SD (n=3). The solid lines in the two plots represent nonlinear least-squares fits to the data.

ing that the beacon was completely converted from a quadruplex into a duplex structure. From the fluorescence titration curve, the fractions of the beacons engaged in the G-quadruplex structure, as a function of the complementary-strand concentration, were calculated according to Equation (2), in which F_{max} and F_{min} correspond to the maximum and minimum fluorescence intensities in the curve, respectively, and F is the fluorescence intensity of a selected point in the curve.

$$\text{G-quadruplex}\ (\%) = \frac{F_{max}-F}{F_{max}-F_{min}} \times 100 \qquad (2)$$

When the concentration ratio of the beacons to the corresponding complementary strands was 1:1, around 16% of F-Hum21-D and 63% of F-GT21-D were folded into quadruplex structures. This result also demonstrated that GT21 was more likely to form a G-quadruplex than Hum21.

The G-quadruplex percentages calculated by our CV method were higher than those calculated by the G-quadruplex molecular beacon method for both of the G-rich oligonucleotides studied. This may have occurred for two reasons. First, the interaction of CV with G-quadruplexes may increase the stabilities of the quadruplex structures. Second, the attachment of FAM and DABCYL to the oligonucleotides may decrease the stabilities of the G-quadruplex structures. To investigate the influence of CV on the stabilities of the G-quadruplex structures, the melting curves of the two beacons in the absence and presence of CV were determined by recording the fluorescence change of the beacons as a function of temperature. The melting temperatures (T_m) of the G-quadruplex structures were determined from the position of the maximum of the first derivative (see the Supporting Information).[17] The results showed that CV had little influence on the stabilities of the quadruplex structures. That is to say, the influence of CV on quadruplex stabilities may not be the primary factor causing the difference between the two methods.

Competition dialysis assay: Competition dialysis has proved to be a powerful and versatile tool for the study of the binding selectivity of ligands to different DNA structures.[18] In order to compare the binding affinity of CV to G-quadruplex, duplex, and single-stranded DNAs, the DNAs listed as entries 1–10 of Table 1 were dialyzed against the CV dye. The results are shown as a bar graph (Figure 7), in which

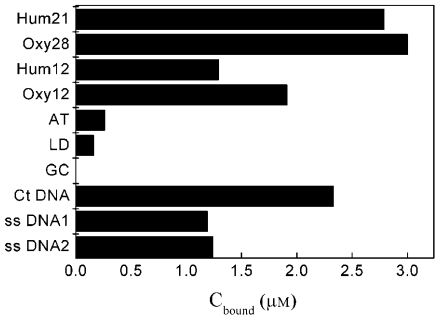

Figure 7. Results obtained by the competition dialysis method for CV. In this experiment, the DNA concentrations added in each dialysis unit were 25 μM Hum21, 25 μM Oxy28, 50 μM Hum12, 50 μM Oxy12, 50 μM AT, 50 μM LD, 50 μM GC, 1200 μM (base concentration) Ct DNA, 50 μM ssDNA1, and 50 μM ssDNA2.

the amount of CV bound to each nucleic acid sample is plotted. The data show that CV binds preferentially to intramolecular G-quadruplexes (Hum21 and Oxy28). It shows a moderate preference for intermolecular G-quadruplexes (Hum12 and Oxy12) and less or no binding to short-stranded duplex DNAs (AT, LD, and GC). These results are quite consistent with those of the fluorescence assays mentioned above. However, CV can also bind to single-stranded DNAs (ssDNA1 and ssDNA2) and long-stranded duplex DNA (Ct DNA) with levels comparable to the binding with intermolecular G-quadruplexes. Similar results were also shown by two positively charged G-quadruplex ligands *meso*-tetrakis(*N*-methyl-4-pyridyl)porphine (H_2TMPyP) and 5,10,15,20-tetrakis[4-(trimethylammonio)phenyl]-21*H*,23*H*-porphine).[18c] Both of these ligands can bind to single-stranded poly(dT). To clarify this issue, more experiments need to be performed. One possible reason may be the electrostatic interaction between negatively charged DNAs and positively charged CV, but this kind of interaction cannot place CV in close contact with the DNA bases and no effective energy transfer occurs.

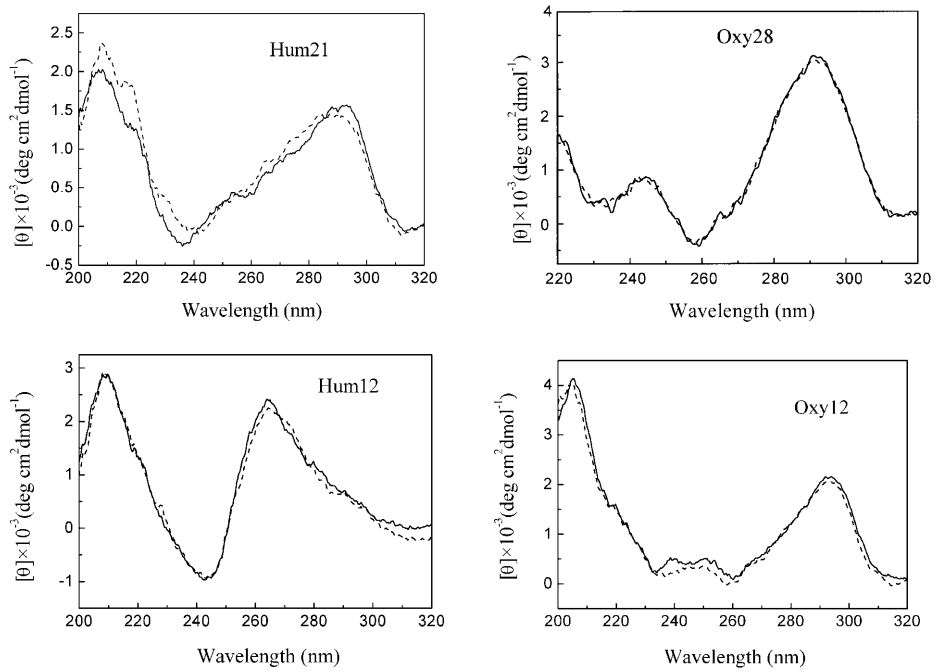

Figure 8. CD spectra of Hum21, Hum12, Oxy28, and Oxy12 in the absence (full line) or presence (dotted line) of CV. [CV] = 15 μM.

Circular dichroism: To demonstrate the formation of the G-quadruplex structures of the G-rich oligonucleotides, circular dichroism (CD) spectra of Hum21, Oxy28, Hum12, and Oxy12 were measured (Figure 8). The formation of quadruplexes was reflected by the appearance of a short-wavelength positive band at around 210 nm.[15] A positive CD peak near 295 nm (for Hum21, Oxy28, and Oxy12) is usually indicative of antiparallel-quadruplex formation, and a positive CD peak near 260 nm (for Hum12) is usually indicative of parallel-quadruplex formation.[19] In addition, no appreciable change in the CD patterns was observed in the presence of CV, which implies that the quadruplex structures were not significantly affected by CV.

Discussion

In this paper, the fluorescence and energy-transfer fluorescence of malachite green (MG) and crystal violet (CV) in the presence of G-quadruplex, duplex, and single-stranded DNAs were compared. For these two dyes, the propeller-like twisting of the phenyl rings with respect to the central triarylmethyl carbon atom increases the vibrational flexibility, and the total polarity of the whole molecule is poor.[20] Thus, the fluorescence yields of these dyes are very low. The stacking of the dyes on the face of the G-quartet increases the rigidity of the dye structures. When the dyes bind to the G-quadruplexes, it is likely that the three phenyl rings of the dyes stack onto the two external G-quartets of the quadruplexes, and the pendent positively charged dialkylamino substituents interact with the negatively charged phosphate backbone and loops. The frame structures of CV and MG are similar; the main difference between them is the number of substituent moieties. The presence of dimethylamino substituents can hinder the intercalative binding of the dyes into stacked base pairs in duplex DNAs and G-quartets in G-quadruplex DNAs, as well as the groove binding of the dyes to duplex DNAs. However, the presence of these substituents can increase the binding stabilities of the dyes with G-quadruplexes if the dyes stack effectively onto the two external G-quartets of the quadruplexes. The absence of the third dimethylamino substituent in MG may allow the dye to partially intercalate between stacked base pairs in duplex DNAs or to bind to the grooves of duplex DNAs through the side of the phenyl ring without dimethylamino substituents, so the fluorescence of MG can increase to a larger extent than that of CV in the presence of duplex DNAs. Therefore, the fluorescence spectrum of MG showed weaker discrimination ability toward quadruplex and duplex DNAs than that of CV.

The findings of this study suggest that CV may be used in the following fields: 1) Discrimination of G-quadruplexes from duplex and single-stranded DNAs; 2) comparison of the G-quadruplex-forming abilities of different G-rich oligonucleotides; 3) measurement of the fraction of G-rich oligonucleotide that engages in G-quadruplex structures in the presence of its complementary sequence; 4) determination of the optimal G-quadruplex-forming conditions for an oligonucleotide in the presence of its complementary sequence; 5) monitoring of the structural changes of quadruplexes that occur due to changing conditions, such as changes in the cation, cation concentration, or ligands, and chemical modification.[19]

Energy-transfer fluorescence spectra have been used to study the binding modes between fluorescent ligands and G-quadruplexes.[10] In this study, energy-transfer fluorescence spectra were used in two new fields. Firstly, energy-transfer fluorescence spectra were used in the discrimination of G-quadruplexes from duplex and single-stranded DNAs and gave better discrimination results than fluorescence spectra. In energy-transfer fluorescence spectra, G-quadruplexes can be discriminated from duplex and single-stranded DNAs not only by fluorescence intensities (Figure 2) but also by trends in fluorescence change as a function of DNA concentration (Figure 4b). Secondly, energy-transfer fluorescence spectra were used in the determination of the binding-stoichiometry

ratios of CV to G-quadruplexes. Energy-transfer fluorescence curves produced more pronounced inflexions than fluorescence titration curves (Figure 4). This method can also be expanded for the study of the interaction between other fluorescent dyes and G-quadruplexes.

Experimental Section

Materials: All nonlabeled oligonucleotides were purchased from Etiwu Biotech. Co. Ltd. (Tianjin, China). The concentrations of these oligonucleotides were represented as single-stranded concentrations unless otherwise noted. Single-stranded concentrations were determined by measuring the absorbance at 260 nm. Molar extinction coefficients were determined by using a nearest neighbor approximation (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer), and the extinction coefficients for the oligonucleotides are listed in Table 1. To ensure the formation of G-quadruplex or duplex structures, the oligonucleotide samples were dissolved in a buffer solution consisting of 25 mM tris(hydroxymethyl)aminomethane–HCl (Tris-HCl; pH 7.0) and 100 mM KCl. The solutions were heated to 90 °C for 5 min, cooled slowly to room temperature, and then incubated at room temperature overnight. The concentration of Ct DNA was represented as the base concentration; the concentration was determined by absorption spectroscopy by using the molar absorption coefficient ($6600\,M^{-1}\,cm^{-1}$) at 260 nm. The solution gave a ratio of UV absorbance at 260 and 280 nm of $\approx$1.83:1, which indicates that the DNA was sufficiently free of protein. G-quadruplex molecular beacons (F-Hum21-D: FAM-$G_3(T_2AG_3)_3$-DABCYL; F-GT21-D: FAM-$G_3(T_3G_3)_3$-DABCYL) were synthesized and purified by Invitrogen Ltd. (Shanghai, China).

Fluorescence spectroscopy and energy-transfer fluorescence spectroscopy: All fluorescence measurements were made by using a Hitachi model RF-4500 spectrofluorimeter. Reaction mixtures with different concentrations of oligonucleotides were mixed with 15 μM CV (or MG) in 25 mM Tris-HCl buffer (pH 7.0) containing 100 mM KCl. To ensure the interaction between G-quadruplexes and the dyes, the mixtures were kept at 30 °C for 1 h before spectrometric detection. In fluorescence spectroscopy determination, the mixtures were excited at 630 nm and monitored from 635 to 800 nm for MG and excited at 580 nm and monitored from 600 to 800 nm for CV. In energy-transfer fluorescence spectroscopy determination, the mixtures were excited at 280 nm and monitored from 635 to 800 nm for MG and from 600 to 800 nm for CV. The fluorescence titration and energy-transfer fluorescence titration measurements were performed by keeping the concentration of CV at 15 μM while varying the DNA concentrations (from 1 to 17 μM for Hum21 and Oxy28, strand concentration; from 24 μM to 4.08 mM for Ct DNA, base concentration; from 2 to 34 μM for other DNAs, strand concentration). The emission at 635 nm was monitored as a function of DNA concentration.

Fluorescence titration of G-quadruplex molecular beacons by complementary strands: The measurements were performed by keeping the concentration of G-quadruplex molecular beacons at 1 μM while varying the concentrations of complementary strands (from 0 to 2 μM for Hum21c and from 0 to 4 μM for GT21c). The fluorescence of FAM was recorded by using a micro fluorescence spectrometer (RX-1000, Yatai, Shangdong, China), which is specifically designed for the fluorescence detection of FAM. All of the solutions were prepared and the measurements were performed in 100 μL PCR tubes (Axygen, USA).

Competition dialysis assay: The buffer (25 mM Tris-HCl (pH 7.0), 100 mM KCl) that was used in the fluorescence assays was also used in the competition dialysis assay. Dialysate solution (200 mL) containing 1 μM CV was placed into a beaker. A volume of 100 μL of each of the DNA samples (Table 1, DNA1–10) was pipetted into a silide-A-lyzer miniature dialysis unit (molecular-weight cutoff of 3500; Thermo Scientific, USA). All of the ten dialysis units were inserted into a float and then placed in the beaker containing the dialysate solution. In order to minimize the effects of evaporation of the dialysate solution, the beaker was covered with preservative film and wrapped in aluminum foil. The dialysate solution was continuously stirred for 24 h at room temperature. DNA samples or dialysate solution (90 μL of each) were then carefully removed to a PCR tube and 10 % (w/v) Triton X-100 (10 μL) was added to dissociate CV from the DNA structures. The total concentration of CV within each dialysis unit (C_t) and the free concentration of CV within the dialysate solution (C_f) were determined spectrophotometrically by using an absorbance wavelength of 595 nm and a constructed standard curve. Appropriate corrections were made for the small dilution resulting from the addition of the Triton X-100 stock solution. The bound-CV concentration (C_b) for each dialysis unit was then determined by difference as $C_b = C_t - C_f$.

Circular dichroism (CD) study: CD spectra were measured on a Jasco J-715 spectropolarimeter at room temperature. Oligonucleotide solutions (7.5 μM for intramolecular quadruplexes and 15 μM for intermolecular quadruplexes) were prepared in 25 mM Tris-HCl buffer (pH 7.0) containing 100 mM KCl. To ensure the formation of quadruplex structures, the solutions were heated to 90 °C for 5 min, cooled slowly to room temperature, and then incubated at room temperature overnight. Spectra were recorded between 200 and 320 nm in 1 mm pathlength cuvettes. Spectra were averaged from 3 scans, which were recorded at 50 nm min^{-1} with a response time of 1 s and a bandwidth of 0.2 nm.

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