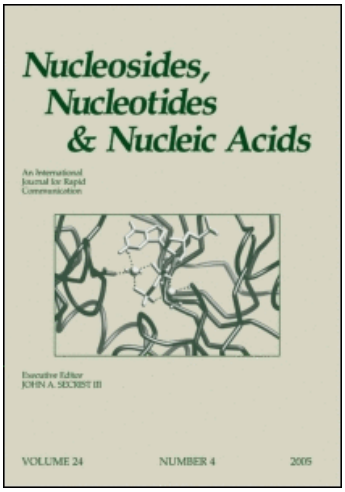

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Noncovalent Interaction of G-Quadruplex DNA with Acridine at Low Concentration Monitored by MALDI-TOF Mass Spectrometry

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To cite this Article Nagesh, Narayana , Krishnaiah, Abburi , Dhople, Vishnu Mukund , Sundaram, Curum S. and Jagannadham, Medicharla V.(2007) 'Noncovalent Interaction of G-Quadruplex DNA with Acridine at Low Concentration Monitored by MALDI-TOF Mass Spectrometry', Nucleosides, Nucleotides and Nucleic Acids, 26: 3, 303 — 315
To link to this Article: DOI: 10.1080/15257770701257590
URL: http://dx.doi.org/10.1080/15257770701257590

Nucleosides, Nucleotides, and Nucleic Acids, 26:303–315, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701257590

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NONCOVALENT INTERACTION OF G-QUADRUPLEX DNA WITH ACRIDINE AT LOW CONCENTRATION MONITORED BY MALDI-TOF MASS SPECTROMETRY

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□ *The telomeric G-rich single-stranded DNA d(T_2G_8) can adopt in vitro G-quadruplex structure, even at low DNA concentration. Studies on stability of telomeric structures, has gained importance recently as the molecules, which can stabilize quadruplex structure, can inhibit cancer progression. In this study, G-quadruplex structure is formed by 1.0 mM NH_4(I) ion. Stability of G-quadruplex complex is studied on interaction with acridine using CD and MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometric experiments were carried out mainly to observe the noncovalent drug-DNA interactions at low concentration. From MALDI-TOF spectrum, it is identified that three ammonium ions are required for the formation of G-quadruplex structure and to provide stability to NH_4(I)-G-quadruplex complex. With MALDI-TOF it is evident that two acridine molecules interact with NH_4(I) G-quadruplex complex. CD studies, shows that stability of NH_4(I) G-quadruplex, decreases and conformation change takes place on interaction with acridine. Interaction with drug reduces mostly due to transformation of G-quadruplex complex to single stranded DNA.*

Keywords G-quadruplex DNA; acridine; noncovalent interactions; MALDI-TOF; CD

INTRODUCTION

G-quadruplex (or) G_4-DNA structures are composed of stacked tetrads, each having planar association of four guanosines arranged in a cyclic manner connected by Hoogsteen hydrogen bonds.[1,2] The cavities of

Received July 21, 2006; accepted December 22, 2006.

We are thankful to Director, CCMB, Hyderabad for allowing us to use the facilities. We are thankful to Dr. K. Thangaraj for the suggestions and discussions during the preparation of this manuscript. We are thankful to Mrs. Mehar Sultana for providing the oligonucleotides.

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these quadruplexes are occupied by either monovalent or divalent cations like Na(I), K(I), NH_4(I), Sr(II) etc., presumably coordinating with eight carbonyl oxygens projected between the strands of tetrads from guanine residues.[3,4] G-quadruplex structures are referred as inter- or intramolecular depending upon the manner in which the guanines are bonded to each other. Formations of inter- and intramolecular structures depend upon the length and concentration of G-quadruplex forming oligonucleotide. It has been reported earlier that at higher DNA concentration, G-rich oligonucleotides prefer to form intermolecular G-quadruplex structure and at lower DNA concentration, they form intramolecular G-quadruplex structure.[5] In addition to these structures, a sequence that contains guanine-rich repeats can form guanine-guanine hairpins, which may in turn dimerize to form a number of G-quadruplex isomers.[6,7] In the present investigation, the oligonucleotide d(T_2G_8), preferably form guanine-guanine hairpin structure.

G-quadruplexes are rich in guanosine content and the over all telomere size in human beings ranges from 15 to 20 kb, at birth to sometimes less than 5 kb in chronic disease states.[8–10] The existence of G-quadruplex structure was found in certain regions of human genome that are functionally important and have potential to form G-quadruplex structures like, telomeric region of DNA,[11,12] the immunoglobulin switch region,[13] *C-myc* promoter,[14] and the triplet repeats region of the fragile X-chromosome.[15] G-quadruplex may indeed have a role in several biological events, particularly when regulatory sequences possess the capacity to form such structures.[16] It was found earlier that some proteins like RAP1 of *Saccharomyces cerevisiae,*[17] b-subunit of telomere-binding proteins (TBP-b) from *oxytricha*,[18] etc., interact with quadruplex structures. Most of these proteins stabilize the G-quadruplex structures by binding to them.

G-quadruplex structures have been shown to directly inhibit telomerase elongation in vitro.[16] Telomerase is expressed in tumor cells, but not in most of the somatic cells. Therefore, a drug that stabilizes quadruplexes could interfere with telomere elongation and can stop replication of cancer cells. Hence, studies on molecules, which interact and show effect on G-quadruplex complex stability, have gained tremendous importance in the recent past. There are reports earlier that small molecules interact specifically with quadruplex structures and stabilize them by stacking externally.[19] We have studied some of the salient features of quadruplex complex stability when it interacts with drugs and ion.[20–22] In the present study, we have utilized MALDI-TOF mass and circular dichroism (CD) spectroscopic techniques to observe the effect of noncovalent interaction between acridine and G-quadruplex DNA on quadruplex stability. MALDI-TOF is used to investigate minimum number of centrally placed cations required for stability of G-quadruplex complex and to explore number of acridine molecules interacting with it, at lower concentration. Due to

experimental limitations, most of the spectroscopic experiments reported previously else where were carried out at higher DNA-drug concentrations. But, studies on drug-DNA noncovalent interactions, especially at lower concentration, may provide much closer and accurate information about in vivo G-quadruplex DNA stability. CD studies were performed to observe the changes in conformation of G-quadruplex DNA after interaction with acridine.

MATERIALS AND METHODS

Synthesis of d(T_2G_8) Oligonucleotide and Formation of G-Quadruplex DNA

Oligonucleotide that has the ability to form G-quadruplex structure, d(T_2G_8) or d(5′ GGGGTTGGGG 3′) was synthesized using ABI 394 DNA synthesizer following phosphoramidite chemistry, on a solid support and deprotected using 25% ammonium hydroxide solution at 55°C for 16 hours. Oligonucleotides were purified using Sephadex G-25 spun column. Purity of d(T_2G_8) was checked by 12% denaturing PAGE after end labeling the oligonucleotide with γ^{32}-P. It was found that oligonucleotide is pure and free from impurities. They were heated to 100°C to denature the DNA strands completely and allowed them to attain 4°C slowly in presence of 1.0 mM NH_4(I) ion, in milli Q water (in the absence of 1.0 mM NH_4(I) ion) as well as with 100 mM K(I). Oligonucleotide incubated in milli Q water is used as a control to study the importance of NH_4(I) ion, as a stabilizing cation. Where as oligonucleotide, d(T_2G_8) incubated at 4°C with 100 mM K(I) ion is used as a control for the formation of G-quadruplex complex as it was reported earlier.[4,5] Alkali metal ions, specially K(I) ion is ideal and efficient in stabilizing the G-quadruplex DNA structures. DNA molecules, which are rich in G residues, move faster than its actual size on nondenaturing PAGE upon formation of folded G-quadruplex structures.[2] From Figure 1 it is evident that oligonucleotide, d(T_2G_8) is moving faster than 10 mer on a 12% nondenaturing PAGE, indicating the formation of compact and folded G-quadruplex structure in presence of 1.0 mM NH_4(I) ion. Incubation at 4°C is crucial for the formation of G-quadruplex structure by d(T_2G_8) oligonucleotide. But, during deprotection step the oligonucleotide, d(T_2G_8), is incubated with NH_4OH at 55°C for 16 hours. As the temperature is not ideal, it is evident that, G-quadruplex structure with NH_4(I) ion does not take place during deprotection.

Sodium hydroxide, 3-hydroxypecolinic acid (3-HPA), ammonium citrate, acridine, and ammonium hydroxide were purchased from Sigma (St. Louis, MO, USA). Oligonucleotide, which is purified by HPLC and having 7.691 KDa molecular mass is purchased from Applied Biosystems (Foster

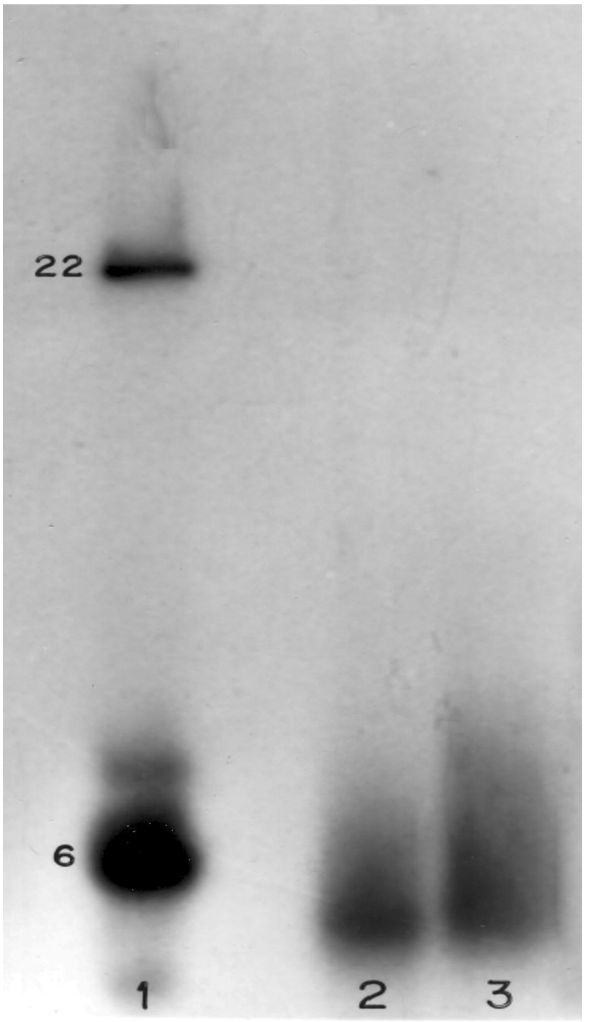


FIGURE 1 Comparison of the electrophoretic mobilities of d (T_2G_8) over 12% nondenaturing acrylamide gel at 4°C. Lane 1, molecular weight marker; lane 2, 100 mM K(I); and lane 3, 1.0 mM NH_4(I).

City, USA) and it is used as a standard for calibrating the MALDI-TOF instrument. For all the experiments, milli Q water was used. CD experiments were carried out using Jasco J 715 spectropolarimeter. Concentration of acridine (λ max 430 nm) was determined by measuring the optical densities at 430 nm by using extinction coefficient 27,000 M^{-1} cm^{-1}.[23] Molecular weight of acridine is considered as 179 Da. For CD work, NH_4(I) G-quadruplex DNA samples were made with sodium phosphate buffer [10 mM phosphate (pH 7) and 0.1 mM EDTA] containing 1.0 mM NH_4(I).

Sample Preparation for MALDI-TOF Studies

3-hydroxy piconlinic acid (10 mg/ml) was prepared in milli Q water. Oligonucleotide, $d(T_2G_8)$ was incubated at 4°C in water as well as in 1 mM $NH_4(I)$ for 16 hours. DNA incubated in water is used to check the purity of the oligonucleotide and also to observe the formation of G-quadruplex structure, in the absence of ammonium ion. About 10 pico moles of G-quadruplex DNA formed by $d(T_2G_8)$ was used in each analysis. 1 μl of the sample was spotted on the MALDI plate and allowed to air dry; 1 μl of the matrix was spotted on the dried sample and allowed to air dry; 3-HPA-matrix solution was always prepared freshly before spotting the samples on the MALDI-TOF plate. To study the interaction between acridine and G-quadruplex DNA, about 2.5 pico moles of acridine drug is added to 10 pico moles of DNA and mixed them thoroughly. Later, 1 μl of the sample was spotted on the MALDI-TOF plate and spectrum was recorded in a similar manner mentioned above.

MALDI Mass Spectrometry

MALDI-TOF experiments are performed using Voyager-DE STR from PerSeptive Biosystems (Framingham, MA, USA). A N_2-laser operating at 337 nm was used for ionizing the samples in the negative ion mode. The accelerating voltage was set at 20 KV and laser source power was adjusted and set to the minimum necessary to generate the signal. About 100 laser shots were given for each spectrum. The mass spectra were acquired in the reflector mode with delayed extraction. The extraction delay time was set at 200 nano seconds. MALDI-TOF experimental conditions were optimized to detect noncovalent interactions. The mass accuracy was in the range of 50 to 500 ppm.

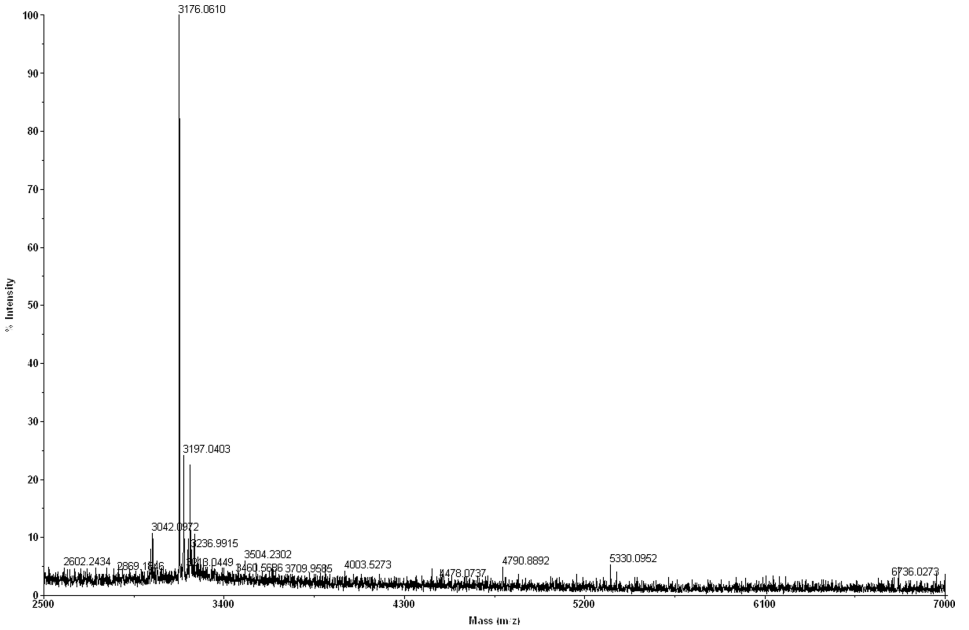
RESULTS AND DISCUSSION

One of the main objectives of this study is to find the efficiency of $NH_4(I)$ ion, a nonmetallic cation with tetrahedral structure, on formation of G-quadruplex structure and to elucidate its stability on interaction with acridine, at low concentration. Noncovalent drug-DNA interaction is studied at lower (at pico mole) concentration. CD experiments were carried out at higher drug-DNA concentration to find changes in G-quadruplex DNA conformation, its stability and to observe noncovalent interactions at higher concentrations.

MALDI-TOF Spectral Studies for Finding the Number of Cations Required for G-Quadruplex Formation and Its Stability

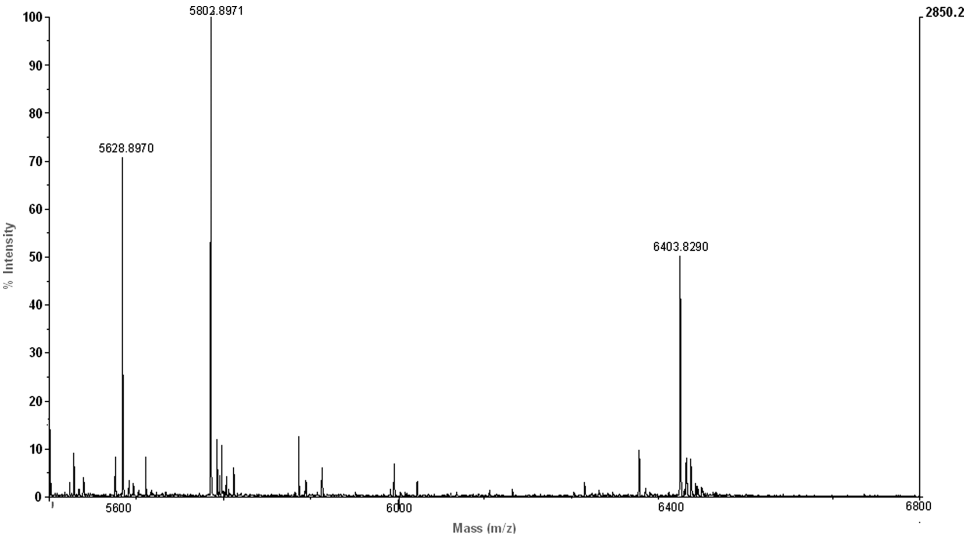
It is evident from Figure 1 that the oligonucleotide d(T_2G_8) has faster mobility on 12% nondenaturing PAGE and d(T_2G_8) oligonucleotide is capable of forming G-quadruplex structure in presence of 1.0 mM NH_4(I) ion. Oligonucleotide d(T_2G_8) is loaded on a 12% nondenaturing gel to check its purity after synthesis. It is clear that the oligonucleotide is pure and showing a single band. It migrates along with a 10 mer on a 12% nondenaturing PAGE, in the absence of stabilizing cation. This observation is not shown in the present investigation as it is made while purifying the d(T_2G_8) oligonucleotide using PAGE. The oligonucleotide d(T_2G_8) is incubated at 4°C in presence and absence of ammonium ions for 16 hours, and after incubation, MALDI-TOF spectrum were recorded. MALDI-TOF spectra show a single peak with m/z corresponding to 3176.0610, indicating the presence of pure and intact d(T_2G_8) oligonucleotide in water (Figure 2a).

Figure 2a and 2b corresponds to MALDI-TOF spectra of oligonucleotide d(T_2G_8) when it is incubated in absence and in presence of 1.0 mM

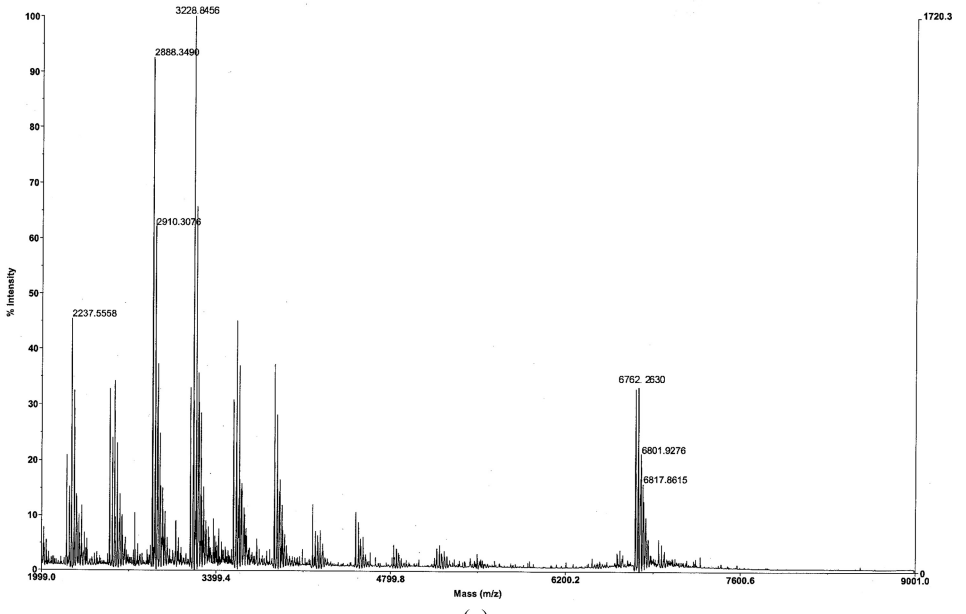


(a)

FIGURE 2 a) MALDI-TOF spectra of 10 pmole of oligonucleotide d (T_2G_8) using 3-HPA matrix after 16 hours of incubating at 4°C in milli Q water.; b) MALDI-TOF spectra of 10 pmole of oligonucleotide d (T_2G_8) using 3-HPA matrix after 16 hours of incubation at 4°C in 1.0 mM NH_4(I) ion; c) MALDI-TOF spectra of NH_4(I)-d (T_2G_8) in 1.0 mM NH_4(I) and after interaction with acridine (1: 0.25 mM). *(Continued)*



(b)



(c)

FIGURE 2 (Continued)

NH_4(I) ion. Figure 2a shows a prominent peak with m/z corresponding to 3176.0610. From the theoretical calculations, the monoisotopic mass of d(T_2G_8) oligonucleotide is 3176. This shows that the oligonucleotide d(T_2G_8) is pure, exists in single stranded form and does not contain any

cation in its cavity. Figure 2b shows a prominent peak with m/z value 6403.8290. The mass difference between 6403.8290 and 6352.1220 (3176 + 3176 = 6352, mass corresponding to two oligonucleotides in the form of hairpin) is 51.707 units, which is equal to three ammonium ions. The results demonstrates that G-quadruplex complex formed by $d(T_2G_8)$, can accommodate three ammonium ions in its cavity. In Figure 2b there are few small peaks besides the 6403.8290 peak, which are either due to the presence of truncated oligonucleotides or due to the formation of sodium adducts. G-rich oligonucleotides can form quadruplex structure preferably with sodium ions than with ammonium ions[27] and the occurrence of sodium adducts are common in many of the MALDI-TOF spectrum.

Studies on G-Quadruplex DNA-Acridine Noncovalent Interaction Using MALDI-TOF Mass Spectrometry

Noncovalent interaction between G-quadruplex DNA and acridine is studied using MALDI-TOF spectroscopy because the aim of the present work is to observe the interaction at low concentration. Though it is suitable to study the drug-DNA interactions using ESI-MS, we have studied the interactions using MALDI-TOF, because it is a novel approach and it is comparatively quick to get the results. There are recent reports where noncovalent peptide-DNA interaction was studied using MALDI-TOF mass spectroscopy.[24] Interaction of acridine with G-quadruplex DNA is considered in this study, because it is already reported that acridine derivatives are good inhibitors of cancer.[25,26]

From the mass data (Figure 2c) it is evident that about two acridine molecules are interacting with each G-quadruplex complex. This is supported by the presence of 6762.2630 peak in Figure 2c. The difference in m/z values (6762.2630–6403.8290 = 358.434), before and after the interaction of G-quadruplex DNA with acridine indicates that the complex after interaction has two acridine molecules (179 + 179 = 358) imbedded in G-quadruplex DNA complex. Details of drug-DNA complex, before and after the interaction are given in the Table 1. It is clear from the mass spectroscopic results that two acridine molecules interact with ammonium G-quadruplex complex, but after interaction, what happens to the stability of G-quadruplex complex is not known. The stability of the complex after interaction with the drug is studied using CD. It is understood that acridine does not interact with DNA in water or in the absence of ammonium ion (data not shown). Hence, it is well demonstrated from Figure 2 c that for the formation of G-quadruplex complex central ion is required and interaction of drug with the complex occurs only after the formation of G-quadruplex complex.

It is clear that m/z values for $d(T_2G_8)$ oligonucleotide remained same (3176) so long as it is in milli Q water or in the absence of 1.0 mM

TABLE 1 Theoretical and experimental m/z values obtained for G-quadruplex DNA before and after interaction with acridine

S. no.	Name of the species	Theoretical Mass (Da) $(M\text{-}H)^-$ (m/z)	Experimental Mass (Da) $(M\text{-}H)^-$ (m/z)	Difference in m/z value	Inference from the results obtained from experiments
1.	G-quadruplex DNA/d(T_2G_8) in water	3177.545	3176.0619	0.4831	Oligonucleotide is pure
2.	G-quadruplex DNA/d(T_2G_8) incubated in 1.0 mM NH_4(I) at 4°C	6403.8462	6403.8290	0.0172	The d(T_2G_8) G-quadruplex complex is formed with two hairpin structures and 3 ammonium ions are incorporated in the cavity
3.	G-quadruplex DNA in 1.0 mM NH_4(I) + acridine		6762.2630 (6762.2630 − 6403.8290 = 358.434)	358.434	G-quadruplex with 2 acridine molecules & 3 ammonium ions in the central cavity.

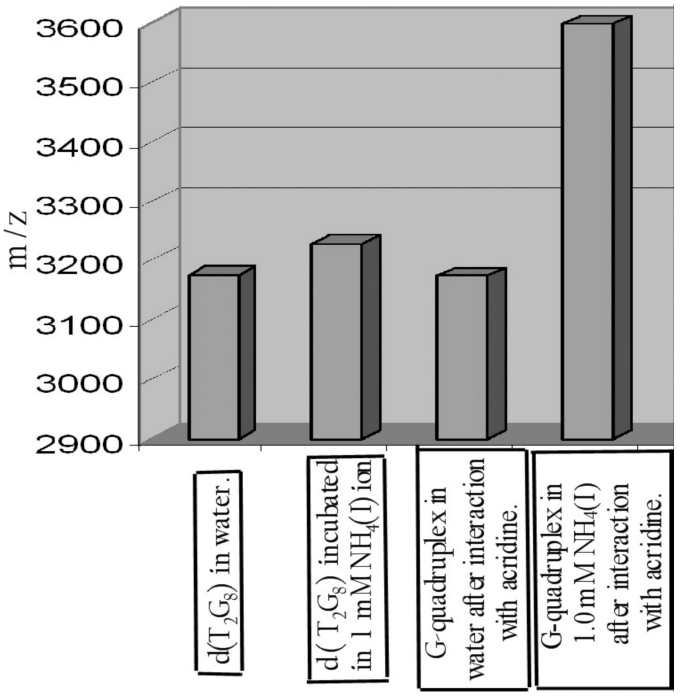


FIGURE 3 Bar diagram showing the variation in m/z values (y axis) for single stranded oligonucleotide, 1.0 mM $NH_4(I)$-G-quadruplex DNA and on its interaction with acridine.

ammonium ion, indicating $d(T_2G_8)$ oligonucleotide is in single stranded form. Where as it showed increase in m/z value indicating the incorporation of ammonium ion in the G-quadruplex DNA cavity formed by two $d(T_2G_8)$ oligonucleotides in the form of hairpin structures, upon incubation at 4°C with 1.0 mM ammonium ion. Noncovalent interaction between G-quadruplex DNA complex with acridine drug has taken place only in presence of 1.0 mM $NH_4(I)$ ion. Results obtained from the MALDI-TOF experiments are graphically represented in the Figure 3.

G-Quadruplex DNA—Drug Interaction Studies Using CD

In order to examine the nature of interaction at higher concentrations and to observe the effect of acridine interaction on G-quadruplex complex stability, experiments were carried out under similar conditions using CD. CD spectra of single stranded $d(T_2G_8)$ oligonucleotide in sodium phosphate buffer is considered as baseline throughout the CD experiments. Acridine does not show interaction with single stranded oligonucleotide. About 1.0 mM $NH_4(I)$ $d(T_2G_8)$ oligonucleotide is used for recording CD spectra (labeled as 1 in Figure 4) and 0.25 mM concentration of drug is allowed to interact with the G-quadruplex complex (labeled as 2 in

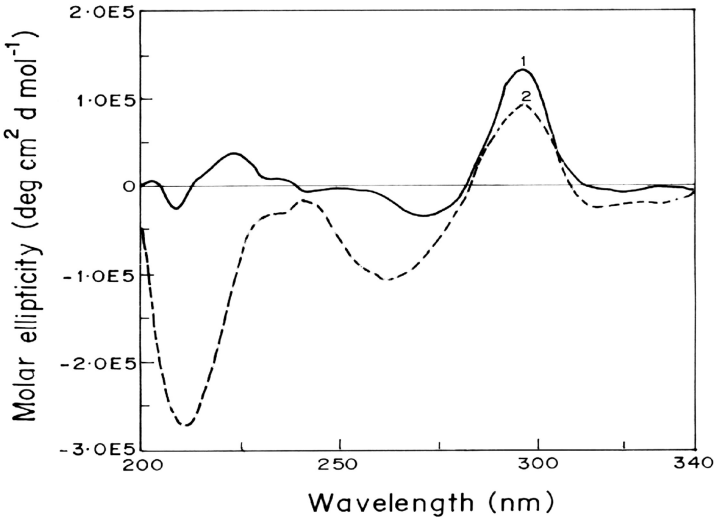


FIGURE 4 CD spectra of NH_4 (I)-d (T_2G_8) in 10 mM sodium phosphate buffer (pH 7.0) 0.1 mM EDTA. Each spectrum corresponds to an average of three scans from which the buffer background was subtracted. Spectra (1) NH_4 (I)-G-quadruplex complex and (2) after the interaction with acridine.

figure 4). It was reported that intramolecular G-quadruplex, G-hairpins, and the G-quadruplexes formed by dimerization of hairpins give a prominent positive CD peak at 293 and a weak negative peak at 265 nm.[28] From Figure 4 it is clear that there is a prominent positive CD peak at 293 nm and negative peak at 265 nm, indicating the existence of G-hairpin structures in the system. To assess the stability of G-quadruplex complex on acridine drug interaction, more emphasis is laid on 293 nm and 265 nm peaks, as they represent the G-quadruplex DNA. As the length of the oligonucleotide molecule is short, there is more probability for the formation of G-hairpin structures. In the CD spectrum (Figure 4), the 293 nm positive peak intensity decreases, when a drug like acridine interacts with NH_4(I)-d(T_2G_8). On addition of acridine drug to G-quadruplex complex, the peak intensity at 293 nm reduces or in other words, upon drug interaction the peak is moving towards baseline, indicating that the complex is slowly started melting and begins to form single stranded structure. Acridine is interacting with G-quadruplex DNA and after the interaction it is undergoing conformational changes, as it is evident from the drug induced CD band at 210 nm. In this process, it looses the central cation and there by the complex stability decreases. There is no much change in the peak intensity at 293 nm on addition of excess of drug to G-quadruplex complex. This shows that the G-quadruplex DNA has no interaction with the acridine drug as G-quadruplex DNA complex is either transformed to single stranded DNA form and further addition of drug does not show any interaction or the binding sites got saturated with the drug. The peak at 210 nm (in the

UV region) in Figure 4 is due to induced CD activity by the bound acridine drug to G-quadruplex complex. A positive peak at 460 nm is observed due to induced CD activity of bound acridine drug to G-quadruplex DNA (data not shown).

CONCLUSIONS

It is evident from experiments that the central ion is very much necessary for the formation of G-quadruplex structure and for the interaction of acridine with G-quadruplex DNA. MALDI-TOF experiments are useful for studying the noncovalent interaction of G-quadruplex DNA with drug and other macromolecules at low concentrations as well as to find the number of interacting molecules and stabilizing cations present in the quadruplex cavity. It can be inferred that $d(T_2G_8)$ G-quadruplex complex can accommodate three ammonium ions in its central cavity and two acridine molecules can intercalate with each $NH_4(I)$-G-quadruplex complex. Acridine does not show any interaction with single stranded $d(T_2G_8)$ oligonucleotide. CD experimental data indicates the presence of G-quadruplex complex formed by two hairpin structures and clarifies that acridine can bring about conformational change in G-quadruplex complex resulting in the conversion of G-quadruplex complex to single stranded form.

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