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Selective Interactions of Ethidiums with G-Quadruplex DNA Revealed by Surface-Enhanced Raman Scattering

Gilles Breuzard, Jean-Marc Millot,* Jean-François Riou, and Michel Manfait

Unité MéDIAN CNRS UMR 6142, IFR53, UFR de Pharmacie, 51 rue Cognacq-Jay, 51096 Reims Cedex, France

Complexes formed between G-quadruplex (G4)-conformed oligonucleotides and four ethidium derivatives were studied by surface-enhanced Raman spectroscopy (SERS) to detail the topology of complexes that support a G4 stabilization. Ethidium bromide (EB), which presents a weak ability to stabilize oligonucleotides in G4 conformation, displayed no SERS intensity modification when bound to G4, as compared with the free EB. Three ethidium derivatives have been selected due to their higher ability to stabilize G4 than EB. Bound with G4-conformed oligonucleotides, SERS intensity of these three ethidiums decreased by factors of about 6, 3.5, and 15. The high SERS quenching was interpreted as a loss of accessibility of silver colloids for G4-bound ethidiums. This could represent a new selective parameter useful to identify G4-stabilizing molecules. To apraise the role of the oligonucleotide sequence on the interaction mode, complexes were formed with eight G4-conformed oligonucleotides in which the three loops were either 5′-TTA-3′ or 5′-AAA-3′. Spectra of ethidiums were sensitive to both lateral loops, opposite to the 3′ and 5′ G4 ends. The sequence of these loops are believed to be selective in the interaction mode of ethidiums for G4.

Chromosome ends of eukaryotes are stabilized by telomeric sequences that are mainly double-stranded, except for the extreme 3′ part in G-rich single-stranded. These 3′ overhangs are surprisingly long (averaging 130−210 bases) and can adopt in vitro an unusual structure with four strands connected by G-quartets (G4)[1,2] (Figure 1A). Such structures protect against random fusion events and degradation by present nucleases. Today, it is well known that telomeres can control the correct division of sister chromatids during final levels of mitotic and meiotic cycles. The telomeric G-rich single-stranded DNA has been shown to directly inhibit telomerase activity.[3] This enzyme stabilizes 3′ telomeric ends, which include repetitive sequences of G-rich single-stranded DNA that allows cellular proliferation.[4−6]

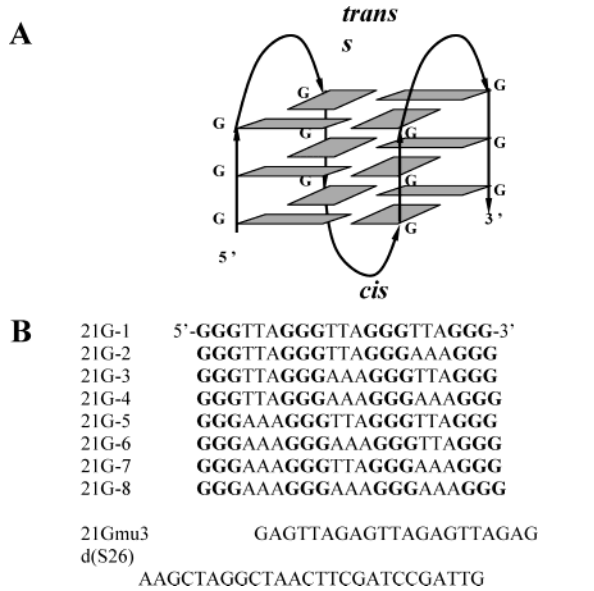

Figure 1. (A) One possible conformation of an intramolecular G-quadruplex formed by the human G-rich telomeric repeat: fold determined from the NMR intramolecular G-quadruplex solution structure.[15] The position of three loops determines the cis side or the trans side. (B) Sequence of different oligonucleotides used in this study: runs of guanine are indicated in boldface type.

Many of the G4 ligands have been shown to exhibit anti-telomerase activity in vitro. These molecules are also able to stabilize G-quadruplexes and to increase the welling temperature of an intramolecular quadruplex structure, as shown by fluorescence and absorbance measurements. Ethidium bromide (EB) has been previously reported to interact with quadruplexes.[7] Recently, ethidium derivatives was shown to provide a higher G4 stabilization than EB and an increased anti-telomerase activity.[8] Although this G4 stabilization might result from the stacking of ethidium either between the planar rings of the G4 structure or with G4 loops, the nature of this interaction is not yet resolved.

* To whom correspondence should be addressed. Tel: +33 3 26 91 35 59. Fax: +33 3 26 91 35 50. E-mail: jm.millot@univ-reims.fr.

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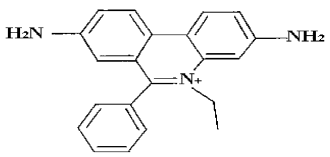
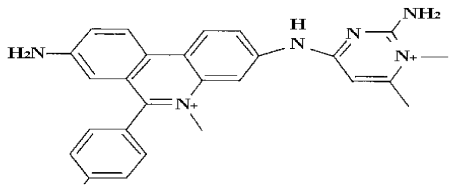
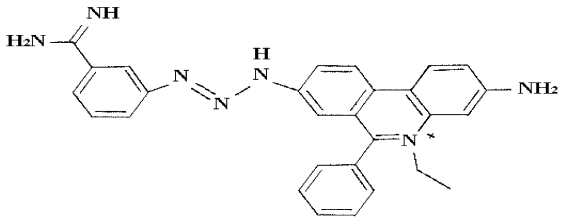


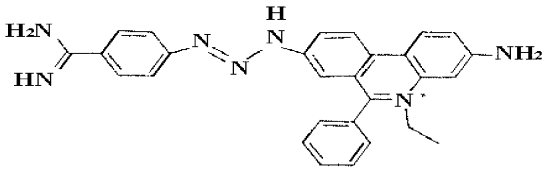
Ethidium Bromide ($\Delta T_{1/2}G4 = 0.6$ °C)



Ethidium 1 ($\Delta T_{1/2}G4 = 9.6$ °C)



Ethidium 2 ($\Delta T_{1/2}G4 = 9.7$ °C)



Ethidium 3 ($\Delta T_{1/2}G4 = 10.7$ °C)

Figure 2. Chemical structures of ethidium bromide and three of its analogues, ethidiums 1−3. The variations in the fusion temperature ($\Delta T_{1/2}$G4) between the free and the ethidium-bound G-quadruplexes are indicated in parentheses, as previously determined by fluorescence energy transfer.[8]

To elucidate the structural basis of intercalation mechanisms, resonance Raman spectroscopy has been widely applied due to its selectivity, which allowed the observation of bands corresponding only to chromophores vibrations. These bands have been described to be sensitive to the structure of drug/DNA complexes.[9] Surface-enhanced Raman scatterring (SERS) is based on the enhancement, by several orders of magnitude, of the Raman intensity of molecules in the vicinity of a roughened metallic surface. This technique has also become attractive for investigating the topology of the drug/DNA complexes due to the short-range feature of the Raman enhancement effect. The colloid was a citrate-reduced silver, which has a negative charge on the surface.[10] Due to the negative charges of the DNA backbone, the consecutive repulsion induces a lack of accessibility of colloid for DNA-complexed molecules and modifies the SERS intensity.

The present report compares the interaction modes of ethidium bromide or three ethidium analogues, with G4 oligonucleotides. SERS spectra of G4-complexed ethidiums are interpreted in connection with the ability of these molecules to stabilize G4.

MATERIALS AND METHODS

Ligand G4. Ethidium derivatives, ethidium bromide (Sigma, St. Louis, MO MW = 394.3) and ethidiums 1−3 (Laboratoire Aventis), are illustrated in Figure 2. Ethidium 2, also known as isomethamidium, was previously prepared and identified as a trypanocidal drug.[8] Ethidium 3 was similarly prepared from 2,7-diamino-10-ethyl-9-phenylphenanthridinium iodide, using 4-benzamidodiazonium salt in place of the 3-benzamidodiazonium salt used to synthesize ethidium 2. Ethidium 1 was identified as the main byproduct in the synthesis of prothidium iodide according to a May and Baker patent (GB 816236). It occurred through substitution of the chlorine atom of 2-amino-4-chloro-6-methylpyrimidine with the 7-amino group of 2,7-diamino-10-ethyl-9-phenylphenanthridinium iodide, instead of substitution with the 2-amino group, as in the case for prothidium iodide synthesis. Solutions of all derivatives were kept at −20 °C in the dark between experiments.

Oligonucleotides. Oligonucleotides were chosen with respect to their sequence and their capacity to conform in G4: 21G-1−8 able to conform in G4 versus 21Gmu3 and d(S26) unable to conform (Figure 1B). All oligonucleotides were obtained from Eurogentec, and salmon testis in sodium salt DNA was from Sigma.

Preparation of Buffers. Tris-HCl and KCl buffers were used in order to conserve conformation and integrity of the different nucleic acids. Buffers Tris-HCl (1 M, pH = 7.5 at 25 °C) was prepared from Tris-base (Eurobio, MW = 121.14, pH = 10.0−11.5 (5% in water)) and Tris-HCl (Mannheim Boerhinger, MW = 157.56) dissolved in distilled water. pH was regulated with a 1 N HCl solution. Moreover, buffers 1 M KCl (Sigma, MW = 74.55) and NaCl (Prolabo, MW = 58.44) were used to conform in G4.

Preparation of G4. The formation of intramolecular G-quadruplex species was carried out as previously described.[8] The 21G-1 and 21G-8 oligonucleotides and their derivatives (21G-2−7) are diluted to 100 μM in 100 mM KCl buffer and 20 mM Tris-HCl, pH = 7.5. This solution was heated to 95 °C during 5 min and cooled to room temperature to favor conformation in G4. To evaluate the role of K^+ or Na^+ ions in the ligand/G4 interaction, oligonucleotides were prepared in a sodium salt using the same protocol.

Preparation of Silver Colloid and Acquisition of SERS Spectra. Ag sols were prepared as described by Lee and Meisel.[9,11] A 90-mg sample of $AgNO_3$ (Alfa, MW = 169.87) was suspended in 500 mL of quartz-distilled water, purged with pure N_2, and heated to 100 °C. A 10-mL sample of a 1% solution of sodium citrate (Sigma, MW = 258.1), purged with N_2, was added dropwise to the boiling solution under vigorous stirring. The solution was kept boiling for 60−90 min. The absorption curve of the brownish suspension showed a maximum at 415 nm; 0.6 M

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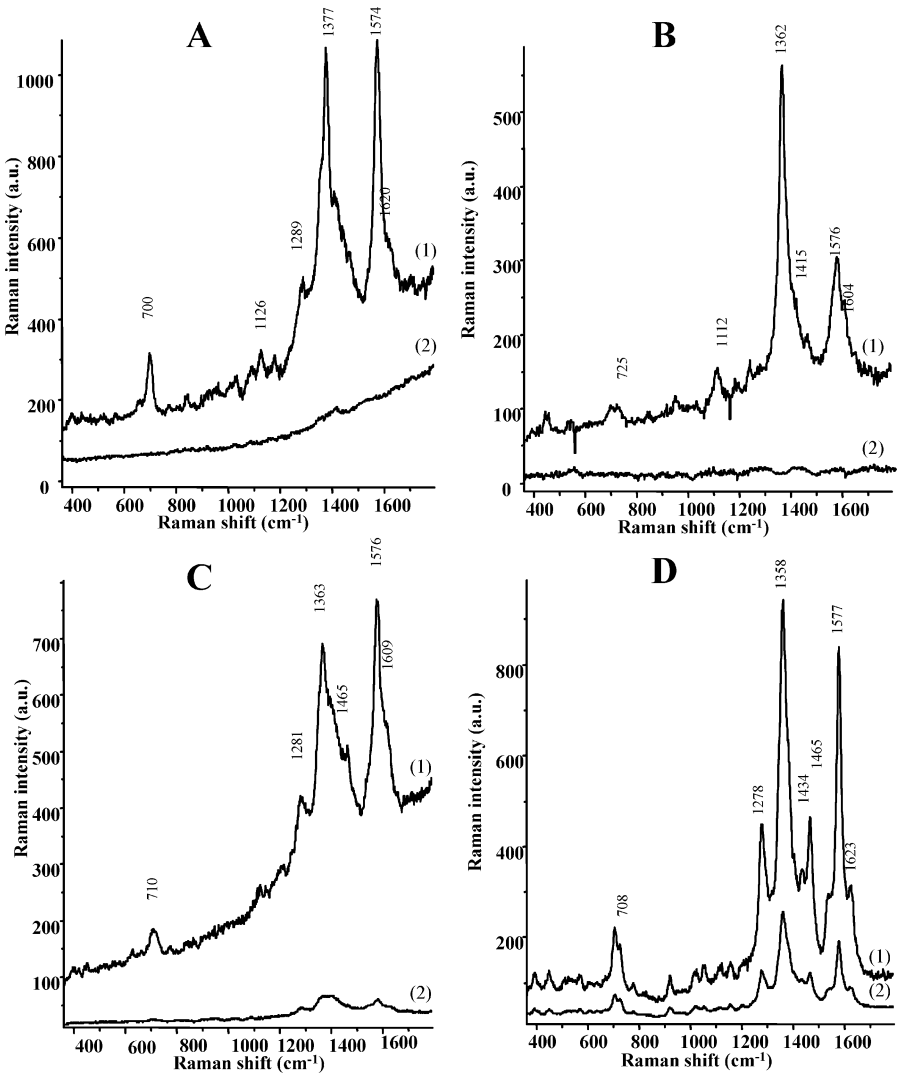

Figure 3. SERS spectra of ethidiums in aqueous solution (1) and their DNA complexes (2). (A) Ethidium bromide, (B) ethidium 1, (C) ethidium 2, and (D) ethidium 3. Final concentrations in the hydrosol were 10^{-7} M for ethidiums and 2×10^{-5} M for double-stranded DNA. Experimental conditions: 40-mW laser power and 20 accumulations of 1 s. each.

solutions of $NaClO_4$ (Sigma, MW = 122.4), $MgSO_4$ (Sigma, MW = 120), or NaCl (Prolabo, MW = 58.44) were used to aggregate Ag sols.

Each presented spectrum is representative of a series of three reproductible spectra. A first series of tests determined aggregation conditions of the colloid. Ethidiums were analyzed to the final 10^{-7} M concentration with Ag sols (diluted at 50% v/v), aggregated by $NaClO_4$, $MgSO_4$, or finally NaCl salts ($[salts]_{final}$ = 0.06 M). $NaClO_4$ salts allowed the highest scattering of Raman diffusion.

Raman Spectrometer. SERS spectra were obtained with a Omars 89 spectrometer (Dilor, France). The diffraction is assured by a 1800 line/mm grating that allowed the analysis of a spectral area of ~400 cm^{-1}. The detection system is constituted with 512 photodiodes coupled with a light amplifier. The excitation used for SERS experiments was the 514.6-nm line of an Ar^+ laser (Spectra Physics 2020-03 model). The theorical spectral resolution was determined at 0.83 cm^{-1}. The spectrometer was linked to a computer that allowed acquisition and mathematical processing of spectra, with homemade software. The power ranged from 40 to 400 mW. In these conditions, no Raman scattering contribution from nucleic acids was observed.

Table 1. Table Summarizing the Main SERS Bands Observed in Ethidium Bromide and Three Analogues (Ethidiums 1−3) and Their Assignments Characterized by Their Vibrational Modes[a]

band position (cm^{-1})				
EB	ethidium 1	ethidium 2	ethidium 3	assignment[b]
700	725	710	708	ring vibration
1126	1112			ν (N═N)
1289		1281	1278	δ (CH_2, CH_3)
1356	1362	1363	1358	ring ≪breathing≫
1377				
1412	1415			
1442			1434	δ (CH_2, CH_3)
1467		1465	1465	δ (CH_2, CH_3)
1574	1576	1576	1577	ν (C═C)
1620	1604	1609	1623	ν (C═C)

[a] Experimental conditions: [ethidium] = 10^{-7} M, laser power 40 mW, and 20 accumulations of 1 s each. [b] ν, stretch; δ, distortion.

RESULTS

The assignments of the main bands of EB and ethidium analogues are presented in Table 1.[13] After comparing molecular

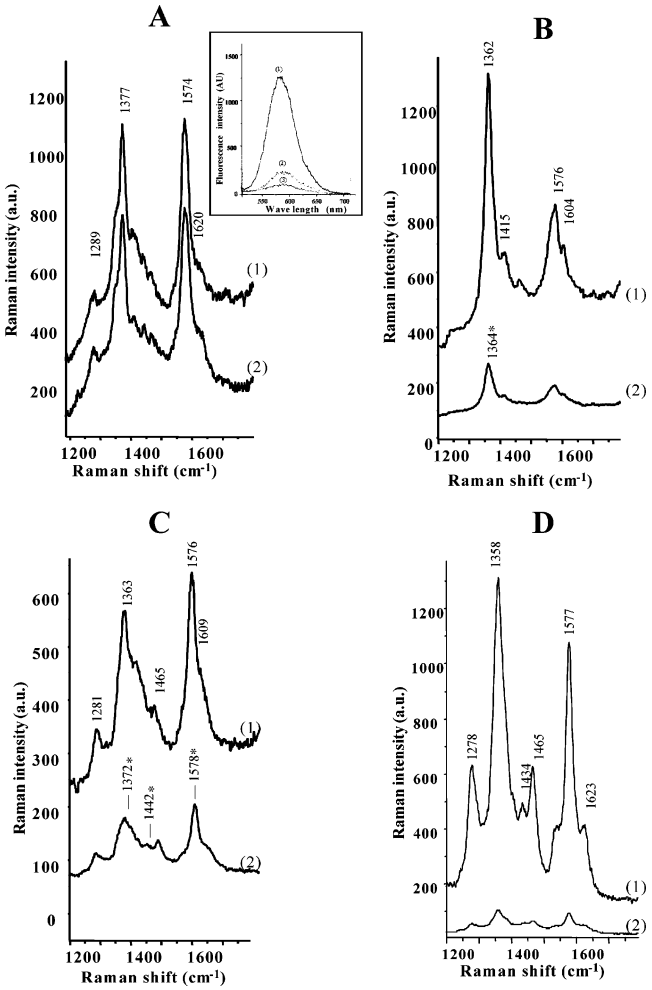

Figure 4. SERS spectra of ethidiums in aqueous solution (1) and their complexes (2) with 21G-1 conformed in G4. (A) Ethidium bromide, (B) ethidium 1, (C) ethidium 2, and (D) ethidium 3. Final concentrations in the hydrosol were 10^{-7} M for ethidiums and 5×10^{-6} M for 21G-1. Bands, noted with asterisk (*) correspond to shifted bands. Experimental conditions were as in Figure 3. Inset: fluorescence emission spectra of EB/DNA complex (spectrum 1), EB/21G-1 complex (spectrum 2), and free EB (spectrum 3). Experimental conditions: 10^{-6} M EB, 5×10^{-6} M DNA, 5×10^{-6} M 21G-1, and $\lambda_{exc} = 500$ nm.

similarities, SERS spectra of different ethidiums (Figure 3) corresponded mainly to the C═C band of the ethidium core (9-phenylphenanthridinium group) structure. Additional bands observed can be used to characterize each ethidium.

When ethidiums (10^{-7} M) were complexed to 2×10^{-5} M double-stranded DNA, a complete (ethidiums 1 and 2 and EB) or a partial (ethidium 3) extinction of Raman scattering was observed (Figure 3). The SERS quenching was interpreted as a loss of accessibility for DNA-complexed ligands to the silver colloids. This was the result of a total or a partial intercalation of ethidiums between successive nucleic bases. Note that, for the DNA-complexed EB, a baseline increase corresponded to a higher fluorescence emission of the complex.

SERS spectra of 10^{-7} M ethidiums bound with 5×10^{-6} M 21G-1 are illustrated in Figure 4. 21G-1-bound ethidiums 1−3 presented a decrease in SERS intensity with factors of about 6, 3.5, and 15, respectively, as compared with the free forms (Table 2). This was interpreted as a decrease of hydrosol accessibility

Table 2. Comparison of SERS Absolute Intensities for Ethidiums Complexed with G4-Conformed Oligonucleotides[a]

relative intensities (%)	G4
ethidium bromide	100 ± 10
ethidium 1	15 ± 5
ethidium 2	30 ± 15
ethidium 3	8 ± 2

[a] Percentage is calculated with respect to the SERS intensity of signal from the free drugs. Each value represents an average of three independent experiments. All spectra were recorded under identical experimental conditions. Ethidium-to-oligonucleotide ratios were 0.02. The band at ~1360 cm^{-1} was used as a reference for intensity measurements.

for ethidium/G4 complexes. Unlike ethidiums 1−3, no significant modification of Raman intensities was observed for EB complexed to 21G-1 (Figure 4A). We applied spectrofluorometry to verify whether EB formed a complex with 21G-1 or double-stranded DNA: the fluorescence emission of EB/21G-1 was 2-fold higher than the free form (inset of Figure 4A). The comparison of SERS spectral profiles of free and 21G-1-bound ethidiums presented the following differences: a shift of the 1362-cm^{-1} band (free) to 1364 cm^{-1} (G4-bound) appeared on the spectrum of ethidium 1 (Figure 4B). Ethidium 1 complexed either with 10^{-5} or 5×10^{-6} M 21G-1 showed similar Raman scattering, indicating a complete bound form. In the 21G-1-bound form, the ethidium 2 spectrum showed an additional band at 1442 cm^{-1} and shifts from 1363- and 1576-cm^{-1} bands to 1372 and 1578 cm^{-1}, respectively (Figure 4C). Between the free ethidium 3 and its complexed form with 21G-1, a very significative decrease of Raman scattering was observed, but no profile modification could be noted (Figure 4D).

The 21Gmu3 and d(S26) oligonucleotides (5×10^{-6} M), unable to conform in G4, were complexed with a 10^{-7} M concentration of the tested ligands (Figure 5). No significative profile or intensity modification was observed between spectra of free (spectrum 1) and complexed (spectrum 2) ethidium 2. Bound with either 21Gmu3 or d(S26), ethidiums 1 and 3 also showed a higher SERS intensity than that from complexes with 21G-1.

To determine whether K^+ or Na^+ ions may influence the topology of ethidiums/G4 complexes, SERS spectra of 10^{-7} M ethidium 1 with 5×10^{-6} M 21G-1 formed in 100 mM KCl (spectrum 1) or NaCl (spectrum 2) were compared (Figure 6). No significative change, concerning intensity or profile, was observed between K^+ or Na^+ conditions, as shown by the difference spectrum (spectrum 3). This suggested that K^+ or Na^+ had no influence on the topology of ethidium 1/G4 complexes.

Loop sequences of G4 were mutated to obtain more insight into the interaction mode of ethidiums with either guanine plates or loops. The 21G-1 and 21G-8 oligonucleotides showed three loops with 5′-TTA-3′ or 5′-AAA-3′ sequences, respectively (Figure 1B). Ethidium 1 (10^{-7}M) was bound to 5×10^{-6} M 21G-1 or 21G-8. Compared with the 1576-cm^{-1} band of ethidium 1/21G-1 (spectrum 1), the SERS spectrum of ethidium 1/21G-8 (spectrum 2) showed a shifted band at 1578 cm^{-1}, which partially hide the 1604-cm^{-1} band (Figure 7). The subtracted spectrum (3) clearly displayed profile differences between both complexes. The spectral resolution was estimated at ~0.83 cm^{-1}. Therefore, a shift

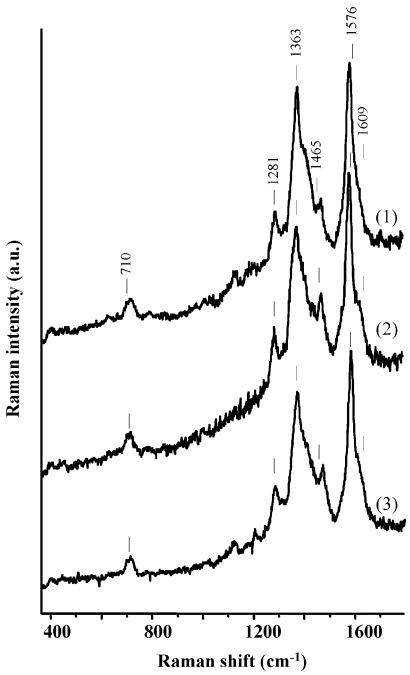

Figure 5. SERS spectra of free ethidium 2 (spectrum 1), ethidium 2/21Gmu3 (spectrum 2), and ethidium 2/d(S26) (spectrum 3) complexes. 21Gmu3 and d(S26) oligonucleotides were unable to conform in G-quartet. Final concentrations in the hydrosol were 10^{-7} M for ethidium 2 and 5×10^{-6} M for 21Gmu3 or d(26). Experimental conditions were as in Figure 3.

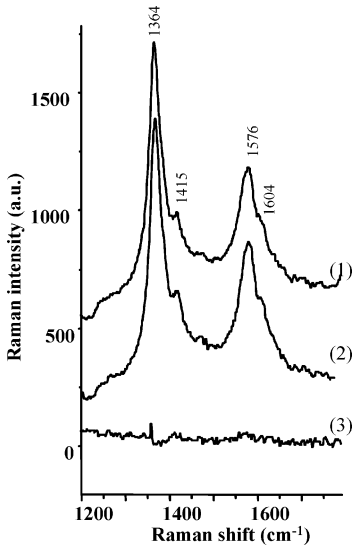

Figure 6. SERS spectra of ethidium 1/21G-1 complex. G4 was formed in K^+ (spectrum 1) or in Na^+ ions (spectrum 2). Spectrum 3 corresponds to the difference spectrum (3 = 2 − 1). Final concentrations in the hydrosol were 10^{-7} M for ethidium 1 and 5×10^{-6} M for G4. Experimental conditions: 400-mW laser power and 20 accumulations of 1 s.

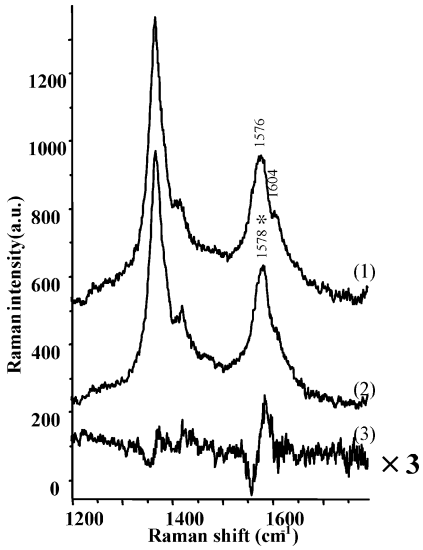

Figure 7. SERS spectra of ethidium 1/21G-1 (spectrum 1) and ethidium 1/21G-8 (spectrum 2) complexes. Spectrum 3 corresponds to the difference spectrum (3 = 2 − 1) with a 3× multiplicative factor. Bands, noted with and asterisk (*) correspond to shifted bands. Experimental conditions were as in Figure 6.

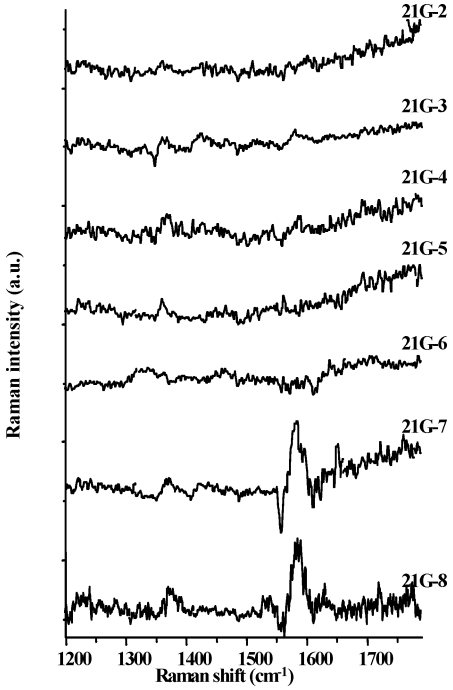

Figure 8. Role of the sequence of G4 loops in the ethidium 1/G4 complex. Subtracted SERS spectra between each ethidium 1/oligonucleotide complex and ethidium 1/21G-1 complex. Experimental conditions were as in Figure 6.

of ~2 cm^{-1} was sufficient to appreciate the difference between 21G-1- or 21G-8-bound ethidium 1 spectra.

To detail the origin of the differences between 21G-1/ethidium and 21G-8/ethidium SERS spectra, eight oligonucleotides including 21G-1 and 21G-8 were selected, in which three loops were either 5′-TTA-3′ or 5′-AAA-3′ (Figure 1B). These sequences showed all combinations of 5′-TTA-3′ or 5′-AAA-3′ for these three loops. For each complex, a difference spectrum was calculated after subtraction from the ethidium 1/21G-1 spectrum (Figure 8). Ethidium 1 bound with 21G-2, 21G-3, 21G-4, 21G-5, or 21G-6 all showed a spectrum similar to that of the 21G-1 complex and then, a difference spectrum near to zero. These six oligonucleotides (21G-1−6) presented at least one 5′-TTA-3′ sequence on their trans loop. The cis loop varied to either 5′-TTA-3′ or 5′-AAA-3′ and did not influence the spectra of the complexes. Besides, spectra of ethidium 1 bound with 21G-7 or 21G-8 were similar and the 1576-cm^{-1} band shifted to 1578 cm^{-1}, as displayed by their difference spectra (Figure 8). Both 21G-7 and 21G-8 presented two 5′-AAA-

3′ sequences on trans loops and differed on their cis loop. It seems that the ethidium 1 spectrum was sensitive to both trans loop sequences, which would imply an interaction of ethidium 1 with these loops.

DISCUSSION

The first high-resolution structures of G-quadruplexes were reported for the sequence $[d(G_4T_4G_4)]_2$ about a decade ago, a telomeric G-rich sequence from *Oxytrichia nova*, by both X-ray crystallography and NMR spectroscopy.[14] Subsequent NMR and crystallography studies have shown that guanine-rich sequences form quadruplexes with a variety of topologies and strand orientations.[15] Telomeric DNA structures, from *O. nova*, have also been probed by UV resonance Raman spectroscopy using 257-nm excitation.[16] The ion-binding sites in quadruplex have been localized experimentally[6] to provide unambiguous information about number and coordination geometry of the monovalent cations that are necessary to form G-quadruplex DNA.[17,18]

At present, a number of G4 ligands have been developed to stabilize the G4 conformation. Sun et al. showed using NMR that anthraquinone analogues, which were originally developed as DNA-interactive agents, were able to bind and to stabilize G4 structures and finally inhibited telomerase.[2,19] Other molecules such as carbocyanine or porphyrin derivatives (e.g., porphyrin PIPER) also showed a single spectroscopic signature when they were bound to guanine-rich sequences.[20−22] Fluorescence resonance energy transfer followed the stabilization of 3′ and 5′ double-labeled-ends G4.[8] For ethidums, it has been described that ethidiums 1−3, but not EB, can stabilize G4 at a higher temperature. Moreover, telomerase response activating protocol tests have shown that ethidiums 1−3 were inhibitors of telomerase, in contrast to EB, which presented a 4−5-fold higher IC_{50} value.[8]

Two sets of SERS spectral parameters enable the analysis of both the topology of high-molecular-weight complexes and this intermolecular interactions. The first feature can be estimated by the SERS absolute intensity. The loss of SERS absolute intensity due to the intercalation of the drug into DNA bases and to the higher distance between the molecule and the hydrosol surface have been well documented in terms of a short-range character of the Raman enhancement.[9,23] The SERS signal of EB almost completely disappeared upon its interaction with DNA. The drug shows a typical deep intercalating mode of binding that has been previously described for aclacinomycin, doxorubicin, intoplicine, *m*- and *o*-AMSA, and many other compounds.[23−25] Since the molecule is deeply intercalated within the DNA double-stranded helix, it becomes undetectable by means of SERS spectroscopy. No SERS signal was observed for ethidiums 1 and 2, when complexed to double-stranded DNA that was compatible with an intercalation model in the double helix. For the DNA-complexed ethidium 3, the residual SERS signal would indicate an uncomplete intercalation between each nucleic base. Previously, EB has been described as unable to stabilize G4 structures.[8] By SERS spectroscopy, no modification of Raman scattering for G4-complexed EB was observed, which correlates with the inability of G4 to be stabilized by EB. For ethidiums 1−3, a significant accessibility decrease of G4-complexes with ethidiums was noted. This decrease of SERS intensity could be associated with a selective G4/ethidium interaction, leading to the G4 stabilization and, finally, the telomerase inhibition. SERS spectra of G4-complexed EB have not reflected any accessibility change. Therefore, the interaction model of EB/G4 complexes was not equivalent to complexes with other ethidiums. The possible binding modes of EB could be an external stacking to G4 structures that was unable to stabilize G4.

The second set of parameters includes SERS frequencies and relative intensities. SERS frequencies can be analyzed in exactly the same way as for normal Raman spectra, to get information on the interaction identity. SERS relative intensities reflect the mode of drug/target interaction; i.e., the bands corresponding to vibrations of functional groups of the drug involved in the interaction with the target are preferably decreased in intensity.[24] For Na^+-containing G-quadruplexes from *O. nova*, NMR analysis have described topologies with a diagonal loop (cis side) and two lateral loops (trans side)[15−17] (Figure 1A). The conformation of quadruplexe DNA has been described to be highly dependent on (i) the type and the concentration of cation (K^+, Na^+), (ii) the rate of cooling to room temperature, and (iii) the concentration of oligonucleotide.[8,25,26] To compare the influence of loop sequence apart from the G-quadruplexe structure, each of eight oligonucleotides (21G-1−8) was prepared, using the following conditions: 100 mM K^+, 100 μM oligonucleotide, 5 min at 95 °C, and 2 h cooling to room temperature. Under these conditions, a mass spectroscopy study displayed the same conformation of G4, whatever the cation used (K^+ or Na^+).[27] In our study, the permutation of the 5′-TTA-3′ with the 5′-AAA-3′ sequence in the diagonal loop (cis side) does not have any effect on the vibration modes of G4-complexed ethidium 1. Nevertheless, the nucleotidic sequence of lateral loops (trans side) modulates vibrational modes of ethidium 1: a significant shift of the 1576-cm^{-1} band is observed when both lateral loops are 5′-AAA-3′ sequences. It was shown that ethidium 1 bound to G4 in equimolar proportion.[8] Consequently, one molecule of ethidium 1 could be in interaction with both two loops on the trans side and not with guanine plates

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(Figure 1), leading to the G4 stabilization. The nature of this interaction between the ligand with G4 was neither an external stacking as for EB nor an intercalating mode as with duplexe DNA.

Recently, a totally different topology of quadruplexes in parallel DNA strands has been reported under crystallizing conditions containing K^+ ions.[18] The three trinucleotide loops linked the top of one strand with the bottom of the other and were extended laterally on the exterior of the quadruplex core. In this model, 21G-1 and 21G-7 differ by the permutation of 5′-TTA-3′ by 5′-AAA-3′ in two opposite loops on both sides of the core. This topology excludes a model of interaction of one ethidium with both opposite loops. Previous studies have described that the antiparallel form was the consequence of Na^+ ion in solution, whereas K^+ ion would induce a transition to a parallel topology in its crystal form. In our case, for G-quadruplex as formed either in K^+ or in Na^+ in solution, SERS spectra of ethidium/21G-1 are totally similar, suggesting identical structures of G-quadruplexes in these different ionic environments.

In conclusion, the decrease in absolute SERS intensity could be associated with a G4 stabilization and could represent a spectroscopic parameter useful to identify new G4-stabilizing molecules. SERS spectroscopy could be useful in providing new information on G4/ligand interaction mode by using low oligonucleotide concentrations.

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