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Influence of 5-bromodeoxycytosine substitution on triplex DNA stability and conformation

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Abstract

Three triple-helical hairpin DNAs with substitution of 5-bromocytosine for cytosine in different strands have been investigated by molecular mechanics and Raman spectroscopy. The stability of the three substituted triplexes were compared with the corresponding unsubstituted triplex DNA by the molecular mechanics method. Base stacking interactions and strand–strand interactions of each triplex were analyzed in detail. Sugar conformations in these triplexes have been determined by both vibrational spectroscopy and molecular dynamics simulation. The hairpin triplexes with substitution occurring in strand I or both in strands I and III have the main sugar conformation of C3′-endo, while the triplex with substitution occurring in strand III is the combination of C3′-endo and C2′-endo sugar conformation. Theoretical results are basically in agreement with experiments.

Keywords: Stability; Conformation; Hairpin triplex; 5-Bromodeoxycytosine

1. Introduction

There is increasing evidence that DNA triple helices may be biologically important, especially with the identification of several triplex-prone sequences in a variety of eukaryotic gene sequences [1–5]. Therefore it is of importance to increase the stability of triplex DNA in the neutral condition. There are many factors that could influence the stability of the DNA triple helix formation with regard to sequence effects, base composition, pH, cations, etc. [6,7]. Base modifications have also been introduced into triple-helix forming oligonucleotides to further extend the range of recognition sequences. Cytosine methylation plays a critical role in modulation of cellular signal transduction and regulation. Methylation of cytosine at the fifth position clearly increases the stability of duplex and triplex DNA [8–13]. Substitution of 5-bromouracil for thymine

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also extends triple helix stability toward higher pH values, and combination of both substitutions, Br^5U and M^5C, strongly increased the cleavage efficiency of oligonucleotides tethered to Fe-EDTA at higher pH values [9].

It is reported that the thermal stability of double-stranded polynucleotides are enhanced when halogenated pyrimidines are substituted for the naturally occurring bases due to a stacking pattern which permits intimate contact between the halogen atom and the adjacent base [14–16]. It has also been demonstrated that for duplex DNA, association of 5Br-nucleosides occurs to a greater extent than that of an unmodified component [17]. However, there are not much studies on substitution of 5-bromocytosine for cytosine in three-stranded DNAs.

In this paper we have synthesized three kinds of triplex DNAs with cytosines replaced by 5-bromocytosines in different strands. The stability of the three substituted triplexes and the corresponding unsubstituted triplex were systematically investigated by a molecular mechanics method. The conformations of the three bromocytosine triplexes were studied by both FT-Raman spectroscopy and molecular dynamics simulation. Oligonucleotide specificity could provide a method for artificial repression of gene expression and viral diseases, thus the results could be helpful in understanding the factors influencing triple helix formation in vivo.

2. Methods

2.1. Theoretical methods

2.1.1. General

The initial structures of three triple-helical DNAs were built by using parameters from the Arnott fiber diffraction model [18]. This is based on the winding of the Hoogsteen-binding pyrimidine strand into the major groove of an A-type DNA duplex in an orientation parallel to that of the purine strand. All calculations were performed on Silicon Graphic R3000 Indigo. A CFF91 forcefield was used for molecular minimization, dynamics and analysis [19–21]. Visualization and molecular modeling were performed using the InsightII interface.

Hydrogen atoms on the C5 position of cytosine were replaced by bromine, the charge of bromine atoms were assigned in a CFF91 forcefield. The N3 atom of each cytosine was hemi-protonated and the hydrogen atom has a distance of 1.04 Å to the N3 atom. Each triplex has 28 nucleotides and each residue has a negative charge of -1 in the CFF91 forcefield. Na^+ counterions were coordinated to each phosphate group in the three strands of the triplex to maintain the electroneutrality of the entire system. At the initial point, positive counterions of Na^+ were placed around DNA at bifurcating positions of the O–P–O angle at a distance of 3.5 Å from both phosphate and oxygen atoms [22–25].

2.1.2. Minimization

Atomic coordination energy minimization and molecular dynamics simulation were performed using Discover (version 2.9.5) software package (Biosym Technologies). First, the entire triplex was minimized by the steepest descendent (100 steps) and conjugate gradient (200 steps), respectively, with fixing of the end residues of all strands. Then, the restraints were removed and the triplex was further minimized to 1000 steps by conjugate gradient. After that, Na^+ counterions were added and the entire system was soaked with a 10-Å layer of water [26]. In order to remove artifacts introduced by the addition of water molecules, the system was energy minimized with the positions of all heavy atoms fixed using the method of steepest descents for 500 steps followed by 500 steps of conjugate gradients minimization. Next, all constraints were removed and the triplex/water/counterions coordinates were minimized by steepest 300 steps and conjugate 500 steps, the rms derivative of each system was less than 0.5 kcal/mol-Å at the end of minimization.

2.1.3. MD simulations

The triplex/water/counterions system from the minimization results were calculated by MD simulation at a constant temperature and pressure (300 K and 1 bar). A constant dielectric of

1.0 was used [27]. During the simulation, no constraints were applied to the entire system so that all water molecules were free to move from their initial positions. The trajectories were calculated using a group-based non-bond cutoff. We carried on without constraints for 10 ps to equilibrate each system, and this was followed by 30-ps runs under the same conditions for data collection and analysis [22–25,28–37]. The initial velocities were assigned to the atoms with a random-number generator using a Maxwell–Boltzmann distribution for the target temperature. The Verlet algorithm was used to integrate the equations of motion, with an integration time step of 1 fs (0.001 ps). Snapshots were collected every 0.2 ps for 30 ps.

2.2. Experimental methods

Four oligonucleotides: $5' - d(T^{Br}C)_3 - d(T)_4 - d(CT)_3$-3′, $5' - d(TC)_3 - d(T)_4 - d(^{Br}CT)_3$-3′, 5′-$d(T^{Br}C)_3 - d(T)_4 - d(^{Br}CT)_3$-3′ and $-d(AG)_3$-3′ were synthesized with a DNA synthesizer (Applied Biosym Model 391). The oligomers were purified after being deblocked and cleaved from the solid support. The three triplexes were prepared by mixing equi-mole $-d(AG)_3$-3′ and the former three oligomers, respectively in 200 mM NaCl and 10 mM of sodium phosphate buffer (pH = 4.5). The solution was kept for 15 min at 80°C and then slowly cooled to room temperature and stored for approximately 10 h at 4°C. FT-Raman spectra were collected with a Bruker RFS1000 FT-RAMAN spectrometer equipped with a diodo-pumped Nd/YAG laser operating at 1064 nm. The laser power at the sample was 200 mW, and 2000 scans were accumulated for all spectra at a resolution of 4 cm^{-1}. The samples were dissolved in sodium phosphate buffer containing 200 mM of NaCl at pH = 4.5. The concentration was approximately 10 mM.

3. Results and discussion

Sequences of three triplex DNAs with substitution of 5-bromocytosine for cytosine in different strands are shown in Fig. 1. In the third, i.e. Hoogsteen-binding strand, the cytosines or 5-

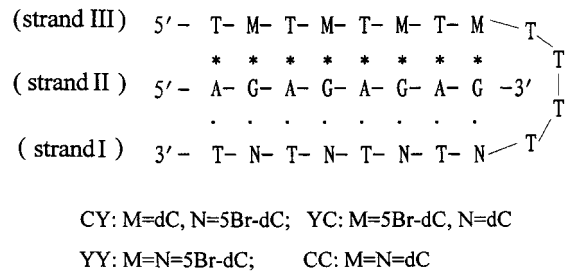

Fig. 1. Sequences of the four triplex DNAs (·, Watson–Crick H-bond; ∗, Hoogsteen H-bond).

bromocytosine are all hemi-protonated. The $d(TC)_4 - d(T)_4 - d(CT)_4 + d(AG)_4$ triplex with 5-bromocytosine instead of cytosine in strand I is simply defined as CY, in strand II as YC and both in strand I and III as YY. The corresponding triplex without substitution on cytosines is called CC.

In this section, the results are presented in two parts. First we studied the stability of the four triplexes CC, CY, YC and YY by molecular mechanics calculation. The stability of the three substituted triplexes were compared with triplex CC by analyzing strand energy, base stacking interaction and strand–strand interaction. The interaction between counterions Na^+ and triplex DNA is also discussed. In the second part, the conformations of three substituted triplexes are determined from the results of FT-Raman spectroscopy and molecular dynamics simulation. As examples, the final computation DNA conformation of CC and YY including solvent and ions are presented in Fig. 2.

3.1. Stability comparison of the four triplex DNAs

The results of the molecular mechanics calculation revealed that conformational energies of the four triplexes CC, CY, YC, YY are −4319.2, −6953.0, −4246.0 and −3922.4 kcal/mol, respectively. It is obvious that the stability order is CY > CC > YC > YY, which means that substitution of 5-bromocytosine for cytosine in different strands makes triplex DNA a different stability. Triplex CY, in which cytosines are substituted by 5-bromocytosines in strand I, is much more stable than the unsubstituted triplex CC. While triplex

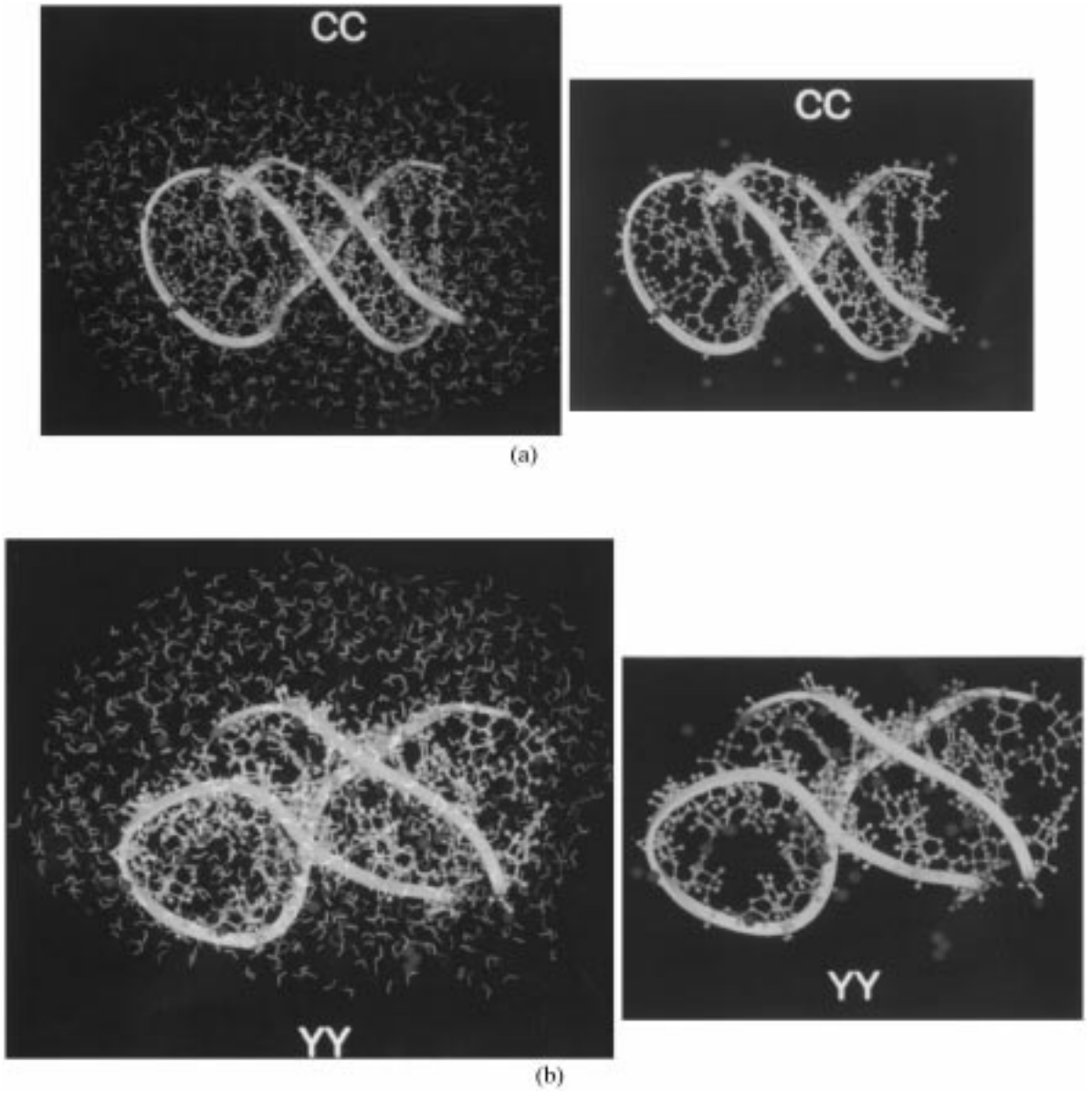

Fig. 2. Computational conformations of triplex DNA CC and YY with- and without water solvent (the bigger spheres represent Na^+). (a) CC conformation (left, with water; right, without water). (b) YY conformation (left, with water; right, without water).

YC and YY, in which substitution occurs in strand III or both in strands I and III, respectively, are a little less stable than triplex CC. The results of triplex YC are similar to the former conclusion that substitution of either bromine [38] or 1-propyne [39] at the C5 position of cytosine in third-strand pyrimidine reduces the apparent stability of the corresponding DNA triplexes. In the following paragraphs, the stability of four triplexes are analyzed in detail.

The changes of three strand energies without the -TTTT- loop for every triplex are all shown in Fig. 3. In triplex CY, the three strand energies are all much lower than the corresponding ones

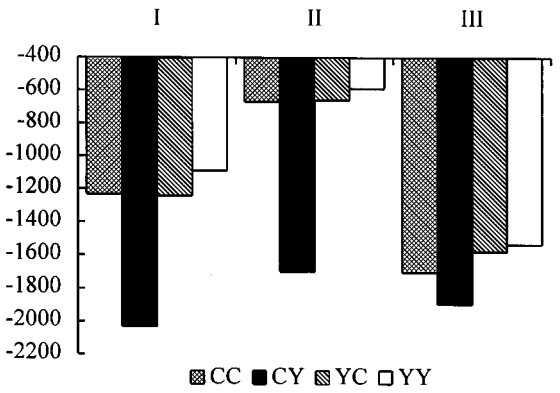

Fig. 3. Strand energies of the four triplex DNAs (Unit: kcal/mol).

in triplex CC. It is interesting to observe that the energy of strand II decreases most instead of the substituted strand I. Strands I and II reduce 780.9 and 1031.1 kcal/mol, respectively. However, the energy of strand III only reduces 181.3 kcal/mol. These results indicate that Watson–Crick region is more stable than the Hoogsteen region in triplex CY. It has been reported that by using 5Br substitution of the deoxycytosine nucleotide in the *Not*I octanucleotide sequence, a considerable improvement in duplex stability is achieved [40]. The stable Watson–Crick region in triplex CY with 5Br-cytosine substitution in strand I is similar to the duplex DNA with 5Br-cytosine substitution in the pyrimidine strand. For triplex YC, in which cytosines are substituted by 5-bromocytosine in strand III, strand energies of I and II change little and that of strand III increase by 134.2 kcal/mol compared to those of triplex CC. Therefore, in triplex YC, the Hoogsteen region become less stable than the Watson–Crick region. In triplex YY, all of the three strand energies increase comparing to those of triplex CC. Strand energies of I, II and III increase 145.4, 78.0 and 177.4 kcal/mol, respectively, which means the entire triplex YY becomes less stable than triplex CC. Variation of strand energy is mainly from electrostatic energy, and van der Waals energy changes little (data not shown). For example, the strand III energy of triplex YY increased from -1710.7 to -1533.2 kcal/mol compared to that in triplex CC, the van der Waals energy changed by -5 kcal/mol, while the electrostatic energy changed 196.3 kcal/mol. It may be reasonable to predict that substitution of 5-bromocytosine for cytosine changes the charge distribution of the nucleotides, which make the interatomic electrostatic interaction variable.

Stacking interactions between successive base-pairs provide a crucial link between sequence, structure, and hence the relevant properties. The stacking energies of strands I–III in triplex CC are 214.2, 213.4 and 245.5 kcal/mol, respectively (Table 1). When cytosines are replaced by 5-bromocytosines in strand I of triplex CY, the stacking energies of all three strands decrease significantly, especially strand III. The results show that 5Br cytosine in strand I makes the stacking interaction stronger, therefore all of the three strands become more stable. In triplex CY, electrostatic energies from the three strand base stacking interaction decrease significantly, which are the main contributors to improve the strand stability. When cytosines are replaced by 5-bromocytosine in strand III, the stacking energies of three strands in triplex YC all become a little higher than those in triplex CC. In YC, the stacking energies of strand I and II increase approximately 10 kcal/mol, respectively, and that of strand III increase 34.4 kcal/mol in comparison with those in triplex CC. The variation in YC is much less than that in CY compared to the triplex CC. In YC, the van der Waals energy of strand I increases by 6.2 kcal/mol and the electrostatic energy increases by 3.3 kcal/mol, showing that the increment of stacking energy is mainly from van der Waals energy. When the substitution occurs in both strands I and III of triplex YY, the stacking energies of strands I and II decrease 16.8 and 69.1 kcal/mol, respectively, and that of strand III changes little. In the three stacking energies, van der Waals interaction in YY become a little stronger than that in CC, especially for strand III. According to the analysis, it was found that the van der Waals stacking interaction of the substituted strands all become stronger than the corresponding unsubstituted ones. In summary, the base stacking interaction in triplex CY with substitution in strand I increases greatly, while it decreases a little with

Table 1
Strand stacking energy of the four triplex DNAs (Unit: kcal/mol)

Triplex	Strand	E_{total}	E_{vdw}	E_{elec}
CC	I	214.2	−46.0	260.2
	II	213.4	−50.0	263.4
	III	245.5	−46.8	292.3
CY	I	−44.4	−50.0	5.6
	II	−61.9	−42.4	−19.5
	III	−228.8	−42.6	−186.2
YC	I	223.7	−39.7	263.4
	II	223.3	−51.1	274.4
	III	279.8	−55.7	335.5
YY	I	197.4	−49.5	246.9
	II	144.4	−51.6	196.0
	III	248.2	−55.7	303.9

substitution in strand III of triplex YC. When strands I and III are both simultaneously substituted by 5-bromocytosine in triplex YY, the stacking interaction becomes a little stronger.

Strand–strand interactions are closely related to the formation of triplex DNA. The stronger the strand–strand interaction, the more stable the triplex DNA. Strand–strand interactions of the four triplexes are all shown in Table 2. In triplex CC, the attractive interaction between strands I and II is the strongest and the repulsive interaction between strands II and III is the strongest. The most flexible DNA strand is obviously the Hoogsteen-binding pyrimidine strand, namely strand III. When cytosines are substituted by 5-bromocytosines in strand I of triplex CY, the interactive energy between strands II and III decreases by approximately 259.8 kcal/mol, which indicates that the attraction of strands II–III is appreciably strengthened and the Hoogsteen region becomes more stable. The repulsive interaction between strands I and III decreases, while that between strands I and II increases a little. The variation shows attraction between strands II and III greatly enhances the stability of the entire triplex CY. In triplex YC, the strand–strand interactions have no noticeable changes compared to those of triplex CC. The biggest difference of strand–strand interactions between triplex YC and CC is the energy of strands II–III, which decreases by approximately 18.4 kcal/mol in triplex YC as compared with that of triplex CC. The variation in triplex YY is somewhat similar to that in CY. The interactive energy of strands II–III decreases 24 kcal/mol, while that between strands I and II increase 21.8 kcal/mol. From the analysis of strand–strand interactions of the four triplex DNAs, substitution in strand I greatly increases the stability of triplex DNA and substitution in strand III or in both strands I and III make less changes on triplex stability. This conclusion is in agreement with the results from analysis of strand stacking interaction.

The coordinations of counterions along the DNA backbone are also investigated. As counterions are all without any bond restrictions, it might be expected that large position fluctuations are found for counterions (shown in Fig. 2). In triplex CY, the initial position of Na^+ has the same distances of 3.5 Å with two phosphorus oxygens. After molecular dynamics simulation, counterion positions changed. Approximately seven Na^+ out of 28 are observed to have moved more than 4 Å away from the initial positions. The distances between Na^+ and the related two oxygens are no longer the same. The average

Table 2
Strand–strand interactive energy of the four triple DNAs (Unit: kcal/mol)

Triplex DNA	Strand–strand	E_{total}	E_{vdw}	E_{elec}
CC	I–II	−15.2	−33.0	17.8
	II–III	704.7	−62.2	766.9
	I–III	77.3	−5.1	82.4
CY	I–II	60.7	−30.1	90.8
	II–III	444.9	−75.3	520.2
	I–III	33.7	−7.6	41.3
YC	I–II	−16.9	−24.9	8.0
	II–III	686.3	−74.2	760.5
	I–III	84.4	−6.2	90.6
YY	I–II	6.5	−33.2	39.7
	II–III	680.7	−73.4	754.1
	I–III	74.0	−7.8	81.8

distance between Na^+ and O1p is 4.22 Å, the other distance is 3.67 Å. It is known that the stability of the triplex is greatly increased by the presence of polyvalent cations [41] and polycationic species [2,42]. In our study, the interactive energies of Na^+ and the four triplex, CC, CY, YC and YY are −7420.3, −7272.8, −7501.9 and −7366.2 kcal/mol, respectively, showing that the addition of Na^+ greatly enhances the stability of the four triplex . Counterions could stabilize the triplex structure by reducing the inter-strand phosphate–phosphate repulsion. The interactive energies between DNA and Na^+ are completely free from electrostatic attraction. However, counterions could not change the relative stability of the four triplexes.

It has been reported that substitution at position 5 of pyrimidines could alter the hydrophobic driving force, base stacking and the electronic complementary of the Hoogsteen pyrimidine–purine base pairing for triple strand formation. Dervan et al. [4] suggested that the electro-withdrawing bromo substitution for uracil increases the acidity at N3H (better hydrogen donor) and decreases the electron-donating properties of the carbonyl lone pair (poor hydrogen acceptor). However, it seems this idea does not fit to 5-bromocytosine, because we obtain two completely contrary results when 5-bromocytosine substitution occurred in strands I or III of the triplex CC. Triplex CY is much more stable than CC, while YC is less stable than CC. In the double-stranded polynucleotide with halogenated pyrimidine, the stability is enhanced due to the stacking pattern which permits intimate contact between the halogen atom and the adjacent base. Base stacking increases the stability of triplex CY, but contributes little to triplex YC and YY. From the above analysis, it seems that electrostatic interaction is generally the most important factor in triplex stability.

3.2. Conformations of the three substituted triplex DNAs

In this part, the sugar conformations of DNA triplexes effected by substitution of 5-bromocytosines for cytosines in different strands were studied by both experiment and theory.

Raman spectra of the three 5-bromocytosine substituted triplexes YC, CY and YY in the 1800–600 cm^{-1} region were all shown in Fig. 4, respectively. For triple-helical DNA, the band at 805–816 cm^{-1} is indicative of C3′-endo sugar conformation while at 835 cm^{-1} it is indicative of C2′-endo sugar conformation [43,44]. The vibration in 812, 816.5 and 815.7 cm^{-1} are assigned to C3′-endo sugar conformation while the vibration in 827.9, 833.5 and 840.2 cm^{-1} are assigned to C2′-endo sugar conformation of the three triplex YC, CY and YY, respectively. Comparing the relative intensity of the two vibrations in each spectrum, it is obvious that C3′-endo is the main sugar conformation in triplex YY or CY; while C2′-endo and C3′-endo sugar conformations are equivalent in YC.

According to the references, a Raman peak occurs at 777 cm^{-1} in the spectrum of the C3′-endo sugar conformation of the triplex only [45,46]. In three triplexes, YC, CY and YY, the band at 778.4, 778.0 or 778.4 cm^{-1} appear, respectively. Therefore, all of the triplex YC, CY and YY have some characteristics of C3′-endo sugar conformation. The band at 639 cm^{-1} is generally assigned to C3′-endo/anti dA residues. From Fig. 4, the spectra of triplex YY or CY has a clear peak at 639.7 cm^{-1}, while it is not clearly observed in triplex YC. The results show that triplex CY and YY have more A-type conformation than triplex YC.

In general, the C2′-endo sugar pucker of thymine occurs at 1210 cm^{-1} and C3′-endo occurs at 1230 cm^{-1} [47]. In triplex YY, the band at 1223.1 cm^{-1} can be assigned to C3′-endo sugar pucker of thymine and the band at 1210 cm^{-1} do not appear. Both bands at 1207 cm^{-1} and 1228 cm^{-1} appear in triplex CY, corresponding to the C2′-endo and C3′-endo of thymine, respectively. In triplex YC, 1210.5 cm^{-1} is assigned to C2′-endo and 1230 cm^{-1} to C3′-endo. However, the bands at 1207 cm^{-1} and 1230 cm^{-1} are both weak shoulder peaks.

We have further analyzed the conformational sugar subpopulations as a function of substitution

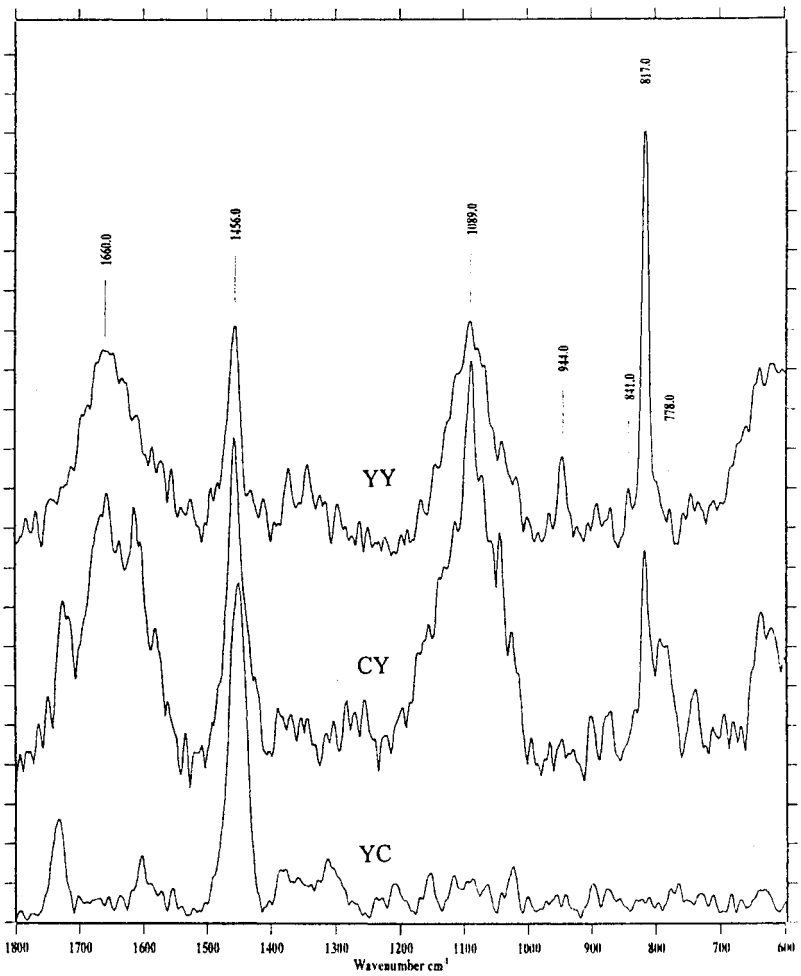

Fig. 4. FT-Raman spectra between 1800 and 600 cm^{-1} of the three triplexes YY, CY and YC at 5 mM concentration in phosphate buffer containing 200 mM NaCl pH = 4.5 at room temperature.

in different strands in the DNA triple helix by molecular dynamics simulation. The nucleotide numbers with different sugar conformations of the three triplexes are shown in Table 3. In the Arnott triplex structure, X-ray diffraction data has shown there is only one kind of sugar pucker, C3′-endo. However, 5-bromocytosine substituted triplexes exhibit polymorphic conformational behavior due to the presence of multiple sugar puckers. The predominant sugar population in triplex CY and YY are C3′-endo, consistent with an A-DNA conformation. In triplex YC, the subpopulation of sugar conformation C3′-endo and C2′-endo are similar to each other, which indicates that YC triplex is not the complete C3′-endo or C2′-endo sugar conformation but the combination of two ones. The results are in agreement with Raman spectra of the three triplexes. For pyrimidine strands I and III, all of the three triplexes have more C3′-endo sugar conformation than C2′-endo; while for purine strand II, there are more C3′-endo than C2′-endo in triplex YY and CY, however, the case is just the opposite in triplex YC. In Raman spectra of triplex YC, several characteristic bands of guanosine sugar conformation at 1312.6, 1328, 1338, 1345, 1355 and 1376 cm^{-1} merge into one strong broad line, which means there are many sugar pucker of dG

[48]. From theoretical analysis, the relatively strong band at 1312 cm^{-1} may be assigned to a diagnostic band of C2′-endo conformation of dG. There are many experimental and theoretical studies on triplex DNAs. One of the main questions is the sugar conformation. Arnott presented A-DNA conformation of triplex from the fiber diffraction results, namely the sugar pucker is C3′-endo. A study of the $d(TC)_5d(GA)_5d(CT)_5$ triplex found evidence that the sugar conformations of pyrimidine strands are C3′-endo and those of purine strand are generally C2′-endo [49]. However, in triplex $d(T)_6d(A)_6d(T)_6$, all three strands had the C3′-endo conformation [50]. Here in the study on hairpin triplex $d(TC)_4d(T)_4d(CT)_4 \bullet d(AG)_4$ with substitution of 5Br-dC for dC in different strands, the result about sugar conformations is not in agreement with both of the above. In triplex YY and CY, most of the sugar conformations are found as C3′-endo; however, in triplex YC, the number of nucleotides with C2′-endo sugar conformations is similar to that with C3′-endo. Therefore, it can be concluded that sugar conformations vary with different triplex systems.

4. Conclusions

The stability of three triple-helical DNAs with substitution of 5-bromocytosine for cytosine occurring in different strands are compared with that of unsubstituted DNA triplex by molecular minimization. The results show that triplex CY, in which the cytosines are replaced by 5-bromocytosine in strand I, is much more stable than the unsubstituted triplex CC. Triplex YC and YY, in which substitution occur in strand III or both strands I and III, respectively, are a little less stable than triplex CC. In triplex CY, base stacking interactions are greatly stronger than those of other three triplexes. Strand–strand interaction is also an important contributor to improve the stability of triplex CY. 5-Bromocytosine substitution in strand III deceases the base stacking interaction and has not substantially changed strand–strand interactions compared with those of triplex CC. In triplex YY substitution, occurring in both strands I and III, base stacking interaction of strands I and II increases a little and the strand–strand interactions have not much variation. In summary, substitution of 5Br-dC for dC in strand I dramatically increases the triplex stability. However, substitution occurring in strand III or both strands I and III decrease the triplex stability a little. The counterions of Na^+ greatly enhance the stability of four triplexes.

Table 3
The numbers of different sugar conformations in the three triplex DNAs

Triplex	Strand	C3′-endo	C2′-endo	O4′-endo	Others
CY	I	3	1	2	2
	II	5	1	1	1
	III	5	1		2
	Loop	1		3	
YC	I	4	3		1
	II		4		4
	III	4		3	1
	Loop	2	1	1	
YY	I	5	1		2
	II	4	1	1	2
	III	4		2	2
	Loop	2			2

The results of both Raman spectroscopy and molecular dynamics simulation show that the three triplexes all exhibit polymorphic conformation because of the presence of multiple sugar puckers. Triplex CY and YY have the C3′-endo sugar pucker predominantly, while triplex YC has the equivalent sugar conformations C3′-endo and C2′-endo. The theoretical results are in agreement with the Raman spectroscopy results.

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