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Studies on the thymine- mercury-thymine base pairing in parallel and anti-parallel DNA duplexes

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The stable T-Hg-T base pair in nucleic acids has been widely applied for Hg^{2+} biosensors. Based on the N-Hg binding mechanism of mercury ion to nucleobases, the N-Hg-N bond was studied in other base pairs (T-T, T-G, G-G, C^+- C^+) with two imino groups within the base pairing face, in the context of parallel DNA duplexes. In the six C^+-C^+ pairs - defined parallel DNA duplexes at pH 5.0, the parallel T-Hg-T was suggested to be a very weak base pair, it was not stable enough to support a long T-Hg-T stem, unlike the anti-parallel T-Hg-T base pair. No interaction between Hg^{2+} and the parallel T-G, G-G, and C^+-C^+ pairs was observed. In anti-parallel dsDNA, the stabilizing effect of T-Hg-T was significant, but with position and sequence dependence, and it was mostly less stable than natural A-T base pair. An anti-parallel T-Hg-T stem is preferred over a parallel stem in the functional nucleic acids in the design of Hg^{2+} biosensors. It seems that N-Hg-N bond is only accommodated in the T-T pair within DNA duplex, different from the free bonding mode between individual nucleobases, nucleosides, or nucleotides. These results are helpful for the design of the T-Hg-T stem in the Hg^{2+} biosensors based on functional nucleic acids.

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Introduction

In the research on the metal ion-nucleic acid interactions, metal ion-mediated artificial base pairs have been explored, pyrimidine bases have been found to be capable of specifically binding Hg^{2+},[1] Ag^{+},[2] or Cu^{2+} [3], at the level of nucleobases, nucleosides, nucleotides or nucleic acids. Biosensors based on these artificial base pairs are being developed for various analytes including these metal ions, with the combination of many signaling methods.[4-6] Some metal-base interactions were supposed to be one of the driving forces in the functions of nucleic acids.[7] Especially, the mercury-nucleobase interaction has been extensively studied for understanding the toxicity of mercury contamination on human health.[8]

As mercury contamination is closely related to genetic toxicity, mecurated nucleobases and base pairs have been studied for the binding mechanism. It is suggested that the Hg^{2+} binds to nitrogen atom, amino and imino groups around nucleobases, and the relative affinities were found to be in the order of T > G >> A, C.[9] Especially, the preference of mercury ion for the imino group of thymine residue leads to the formation of a very stable T-Hg-T base pair in a DNA duplex context, compared to the mismatched T-T. This T-Hg-T base pair has been confirmed by various methods and its bond properties studied by ^{1}H,[1, 10] ^{15}N,[10a, 11] ^{199}Hg NMR,[8] MALDI-TOF,[1] CD,[10b, 12] FRET,[13] Raman spectroscopy,[14] and very recent X-ray analysis.[15] The calculated thermodynamic parameters for the formation of the T-Hg-T base pair were in accord with the measured values by ITC (isothermal titration calorimetry), [16, 17] and the reaction pathway to the formation of T-Hg-T was ever proposed.[16] In a T-Hg-T base pair, the N-Hg-N bond and base stacking, and even Hg-Hg stacking were suggested to contribute to the thermal stability.[15]

Based on the stable T-Hg-T base pair, water soluble Hg^{2+} biosensors were thus developed for environmental pollution assays. In a functional nucleic acid (aptamers, DNAzymes, and molecular beacons),[4,5] one or two stretches of consecutive T residues were incorporated, the presence of Hg^{2+} can produce a T-Hg-T base pair stem and induce tertiary structural transition, and the functions of the nucleic acids were turned-on or turned-off , by which the quantitative evaluation of Hg^{2+} was obtained. In order to obtain an expected and unique conformational change in a functional nucleic acid structure by the binding of Hg^{2+}, the affinity of various metal ions to mismatched T-T in the context of anti-parallel duplex DNA and the thermal stability of T-Hg-T relative to other base pairs have been studied. Different evaluation results were reported about the thermal stability of the T-Hg-T base pair relative to the A-T base pair. In a duplex with a long stem of dT, the T-Hg-T was much more stable than the A-T pair,[4] and in the other duplex DNA with random sequences, the T-Hg-T is comparable to A-T in thermal stability.[17] In addition, the ionic property of the N-Hg-N bond and no hydrogen bonding in the T-Hg-T base pair might confer a parallel T-Hg-T base pair. Ono et al reported this base pair in a specific parallel DNA duplex with covalent linkages at the two 5'-ends, a parallel T-Hg-T was suggested, with a T_m increase of 6 °C.[18]

With above information about the T-Hg-T base pair, and its importance for the design of nucleic acid-based Hg^{2+} biosensors, we designed two kinds of anti-parallel DNA duplexes with different numbers and positions of the T-Hg-T base pair, for a full description of its thermal stability. Especially, based on the ionic property of N-Hg bond and imino proton exchange for its formation, N-Hg-N bond was studied in the parallel DNA duplexes formed by two separate sequences, to study whether Hg^{2+} could form stable parallel base pairs with C^{+}-C^{+}, T-T, T-G or G-G, in which there are two imino groups in the base pairing faces for proton exchange with Hg^{2+}. The research about the thermal stability and directionality of the T-Hg-T base pair will be helpful for the design of the sequences of nucleic acid-based Hg^{2+} biosensors.

Results and Discussion

Two kinds of DNA duplexes were used for the evaluation of the formation of the parallel and anti-parallel T-Hg-T base pairs, in which they were introduced in different positions and numbers (random or consecutive). Anti-parallel T-Hg-T base pair and its long stem have been often applied in Hg^{2+}-biosensors, here two anti-parallel DNA duplexes 12-bp **AP01** and 16-bp **AP12**, were used for a full description of their thermal contribution and conformational effect on nucleic acid structures. The possibility of other mercurated base pairs was also examined in this context. Parallel DNA duplexes **P01+P02** were constructed with parallel C^{+}-H-C and parallel T-A base pairs, and confirmed by its characteristic CD and T_m behaviours. Parallel T-Hg-T was evaluated in this context. In addition, parallel T-G and G-G with two imino groups in the base pairing face were also evaluated. The effect of parallel and anti-parallel T-Hg-T base pairs were studied with T_m measurement and CD spectra, with different concentration ratio of Hg^{2+} to mismatched T-T or other base pairs.

The T-Hg-T base pair in anti-parallel DNA duplexes

In the anti-parallel duplex **AP01** (Table 1),[19] when an A-T pair was replaced by a mismatched T-T in **AP02** and **AP03**, a significant decrease of T_m was observed. When the T-T pair

Table 1 Thermal stability of mismatched base pairs in anti-parallel DNA duplex **AP01** in the presence of mercury ion of different concentrations [a]

duplex	Hg^{2+} / T-Hg-T			
	0	0.5	1	2
5'-d(TAG GTC AAT ACT)-3' **AP01** 3'-d(ATC CAG TTA TGA)-5'	47.3	47.2	47.0	45.8
5'-d(TAG G**T**C AAT ACT)-3' **AP02** 3'-d(ATC C**T**G TTA TGA)-5	33.2	37.8	43.7	42.7
5'-d(TAG GTC **T**AT ACT)-3' **AP03** 3'-d(ATC CAG **T**TA TGA)-5'	33.2	35.2	39.8	40.6
5'-d(TAG GTC **G**AT ACT)-3' **AP04** 3'-d(ATC CAG **T**TA TGA)-5'	39.8	39.6	39.0	38.0
5'-d(TAG GTC **G**AT ACT)-3' **AP05** 3'-d(ATC CAG **G**TA TGA)-5'	34.7	34.5	34.2	33.8
5'-d(TAG GTC **TT**T ACT)-3' **AP06** 3'-d(ATC CAG **TT**A TGA)-5'	23.5	29.2	35.9	36.0
5'-d(TAG GTC **GG**T ACT)-3' **AP07** 3'-d(ATC CAG **TT**A TGA)-5'	30.7	31.4	29.6	26.7
5'-d(TAG GTC **C**AT ACT)-3' **AP08** 3'-d(ATC CAG **T**TA TGA)-5'	30.0	30.1	30.1	30.0
5'-d(TAG GTC **C**AT ACT)-3' **AP09** 3'-d(ATC CAG **C**TA TGA)-5'	28.6	28.3	27.2	26.5
5'-d(TAG GTC **G**AT ACT)-3' **AP10** 3'-d(ATC CAG **A**TA TGA)-5'	36.1	36.4	36.1	35.3
5'-d(TAG GTC **A**AT ACT)-3' **AP11** 3'-d(ATC CAG **A**TA TGA)-5'	31.1	31.1	29.7	28.6

[a] Conditions for T_m measurement: equal molar sequences (4 μM) were combined in the buffer consisting of 100 mM $NaNO_3$, 10 mM $Mg(NO_3)_2$, and 10 mM sodium cacodylate (pH 7.5), with different concentrations of Hg^{2+}. UV absorbance of the complexes was recorded at 260 nm when cooling at 1ºC/min in a 10-mm cuvette. Three measurements were conducted for each T_m, with standard error of 0.2 ºC.

was saturated with Hg^{2+}, a significant but different increase of T_m was obtained from the T-Hg-T formation, 10.5ºC for **AP02** and 6.6ºC for **AP03**. But the T-Hg-T base pair could not drive the thermal stability of **AP02** and **AP03** to the level of canonical **AP01**. It seems that the T-Hg-T pair was not as stable as the A-T pair, and its thermal contribution (T_m increase) was position-dependent. Therefore, another random DNA duplex **AP12** was designed to confirm the position-dependence of thermal contribution of the T-Hg-T base pair.

As presented in Table 2, one mismatched T-T in **AP12** led to a significant decrease of T_m (**AP13** and **AP14**), their thermal stability increase by a complete T-Hg-T formation was also distinct, 4.7 ºC and 4.6 ºC for one T-Hg-T base pair in **AP13** and **AP14**, respectively. In the case of duplexes with two incorporations of the T-Hg-T (**AP15**, **AP16**, **AP17**, and **AP18**), the stabilizing effect of each T-Hg-T were different from each other, the T_m increases ranged from 1.0 to 6.15 ºC.

It is worthwhile to be noted that the anti-parallel

Table 2 Thermal stability evaluation of mismatched base pairs based on anti-parallel DNA duplex **AP12** in the presence of mercury ion of different concentrations [a]

duplex	Hg^{2+} / T-Hg-T			
	0	0.5	1	2
5'-d(TGA GTC CAT AGG TAC C)-3' **AP12** 3'-d(ACT CAG GTA TCC ATG G)-5'	58.0		58.0	58.7
5'-d(TGA GTC CA**T** AGG TAC C)-3' **AP13** 3'-d(ACT CAG GT**T** TCC ATG G)-5'	51.3	52.5	56.0	57.0
5'-d(TGA GTC C**TT** AGG TAC C)-3' **AP14** 3'-d(ACT CAG G**T**A TCC ATG G)-5'	53.2	55.5	57.8	58.5
5'-d(TGA GTC C**TT** AGG TAC C)-3' **AP15** 3'-d(ACT CAG G**TT** TCC ATG G)-5'	49.1	51.7	54.2	55.4
5'-d(TGA GTC CAT AGG **TT**C C)-3' **AP16** 3'-d(ACT CAG GTA TCC **TT**G G)-5'	52.4	53.4	54.4	54.7
5'-d(TGA GTC CA**T** AGG T**T**C C)-3' **AP17** 3'-d(ACT CAG GT**T** TCC A**T**G G)-5'	44.5	52.6	56.8	58.6
5'-d(TGA GTC C**TT** AGG **T**AC C)-3' **AP18** 3'-d(ACT CAG G**T**A TCC **TT**G G)-5'	46.3	48.9	55.1	56.6
AP19 5'-d(TGA GTC C**TT TTT T**AG GTA CC)-3' 3'-d(ACT CAG G**TT TTT T**TC CAT GG)-5'	40.0	43.9	50.5	53.7
AP20 5'-d(TGA GTC **TT**C **TT**A G**TT** GTA CC)-3' 3'-d(ACT CAG **TT**G **TT**T C**TT** CAT GG)-5'	28.2	37.0	47.4	51.3

[a] Conditions for T_m measurement: equal molar sequences (3 μM) were combined in the buffer consisting of 100 mM NaNO3, 10 mM Mg(NO3)2, and 10 mM sodium cacodylate (pH 7.5), with different concentrations of Hg^{2+}. UV absorbance of the complexes was recorded at 260 nm when heating at 0.5ºC/min for AP12-AP18 in a 10-mm cuvette. For AP19 and AP20, the cooling and heating rate of 0.1 ºC/min was used. Three measurements were conducted for each Tm, with standard error of 0.2 ºC.

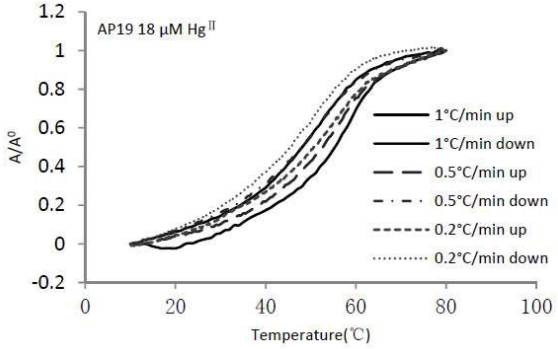


Fig.1 Melting curves of anti-parallel duplex AP19 with different rates of temperature changes.

T-Hg-T base pair was not more stable than the A-T base pair. In the present anti-parallel DNA duplexes, the less stable T-Hg-T was more frequently observed, except in the duplexes **AP13**, **AP14** and **AP17**, in which T-Hg-T stability was close to that of the A-T base pair in the presence of one to two fold of Hg^{2+}.

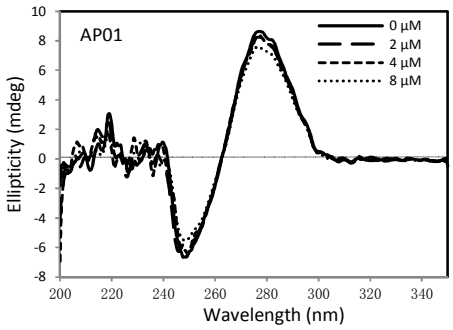
In many reported Hg^{2+} biosensors based on nucleic acids, a stem of at least six consecutive T-Hg-T pairs was often designed in DNAzymes, aptamers, or molecular beacons. Here, the effect of such a stem of consecutive six T-Hg-T pairs in a duplex DNA (**AP19**) was investigated. As shown in Table 2, an Hg^{2+} concentration-dependent T_m change was observed, until all the T-T pairs were saturated with Hg^{2+} to form a stable T-Hg-T stem, by which the concentration of Hg^{2+} was corresponded to the strength of the signal of the biosensors. The different T_m values between **AP19** and **AP20** also demonstrated that the thermal contribution of the T-Hg-T base pair was position-dependent. In addition, as shown in Fig. 1, their association curve and dissociation curve could not overlap until at the rate of 0.1º C/min for the cooling and heating processes, indicating the slow formation process of the long T-Hg-T stem.

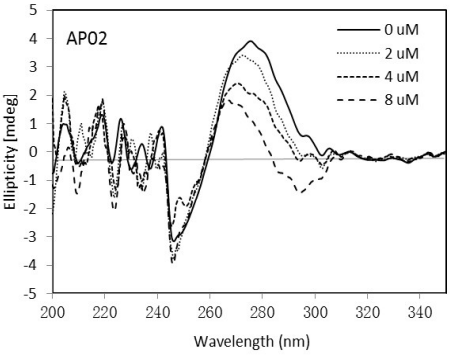
The CD spectrum also showed the significant effect of Hg^{2+} on duplex conformation. As shown in Fig. 2. The B-DNA conformation of duplexes **AP01** and **AP12** was not affected by Hg^{2+}, indicating that Hg^{2+} could not interact with complementary base pairs or nucleobases under present conditions. While, the effect of Hg^{2+} on the CD spectra of **AP02**, **AP03**, **AP06**, and **AP15** with one or two T-Hg-T base pairs was observed, with a significant decrease and red-shift of the positive lobe around 275 nm. In the presence of up to equal molar ratio of [Hg^{2+}] to [T-T], these duplexes adopted the B-DNA form, indicating the well-compatibility of the T-Hg-T in the context of B-DNA duplexes.[4, 14b] In the case of **AP19** and **AP20** with consecutive or random six T-Hg-T pairs (Fig. 3), the amplitude of the positive lobe at around 275 nm also decreased with red shifts, and the crossover changed accordingly. It was obvious that this CD change was Hg^{2+} concentration dependent, without significant distortion of the B-DNA, a B-like DNA duplex was kept.

With extra Hg^{2+}, the positive band around 275 nm further decreased, and even inverted to be negative with **AP19** and **AP20**, as reported earlier with other DNA duplexes,[10b, 10d] a Z-like duplex DNA was observed, or a B-form geometry was still the conformation in spite of CD indicating a left-handed helix. Furthermore, the decreased T_m values with two-fold Hg^{2+} were often observed in some cases. These facts reflected the disruption on the regular duplex structures by Hg^{2+}, through their interactions.

From these evaluation results, we learnt that the thermal contribution of the T-Hg-T pair is both position and sequence dependent. Therefore, the intrinsic properties of the T-Hg-T base pair were studied and a comparison was made with canonical base pairing. On the one hand, the T-Hg-T base pair is different from that of canonical Watson-Crick base pairs in terms of size of the base stacking plane and the bonds in the base pair. The smaller pyrimidine-pyrimidine base stacking plane and one N-Hg-N bond permit more rotation of the T-Hg-T pair. On the other hand, the calculated and measured inter-atomic distance between Hg atom and N3 of thymine was about 2.1 Å, and about 4.2 Å for the N-Hg-N bond in a T-Hg-T base pair of an anti-parallel duplex.[14, 15] The N-Hg-N bond is a little shorter than a hydrogen bond in natural base pairs, which is suggested to be the structural reason for the distortion of duplex conformation by T-Hg-T insertion. As confirmed by the crystal structure of a DNA duplex containing two consecutive T-Hg-T base pairs,[15] the propeller twist angles of the T-Hg-T base pairs (-22 º and -20 º) were remarkably larger than that of canonical Watson-Crick base pairs (-1 º), and the C1'-C1' distances of the T-Hg-T base pairs (9.5-9.6 Å) are 1 Å shorter than those in the canonical Watson-Crick base pairs (ca. 10.7 Å). These different structural parameters of the T-Hg-T base pair in the duplex context was suggested to result in the distortion of the canonical B-duplex consisting of Watson-Crick base pairs.

As is known, in the DNA or RNA duplexes, the sequence composition is critical for the duplex stability and conformations, three basic types have been reported (B, A, and Z) for anti-parallel duplexes, because the two canonical base pairs A-T and G-C are different from each other in hydrogen bonding and base stacking behaviours. Similarly, the local stems in a duplex are also different from each other. When the third base pair T-Hg-T with significantly different structural parameters was introduced between various base pairs or stems within duplex **AP01** or **AP12**, different local changes could be produced, and obvious T_m and CD changes are often observed. Therefore, the effect of the T-Hg-T base pair is not only position-dependent in a duplex, but also duplex-dependent, as will be seen with parallel duplexes later. In general, both the intrinsic stability of the base pair and its compatibility with neighbouring base pairs or local environment and the whole conformation are the main determinants for its effect on a specific duplex.





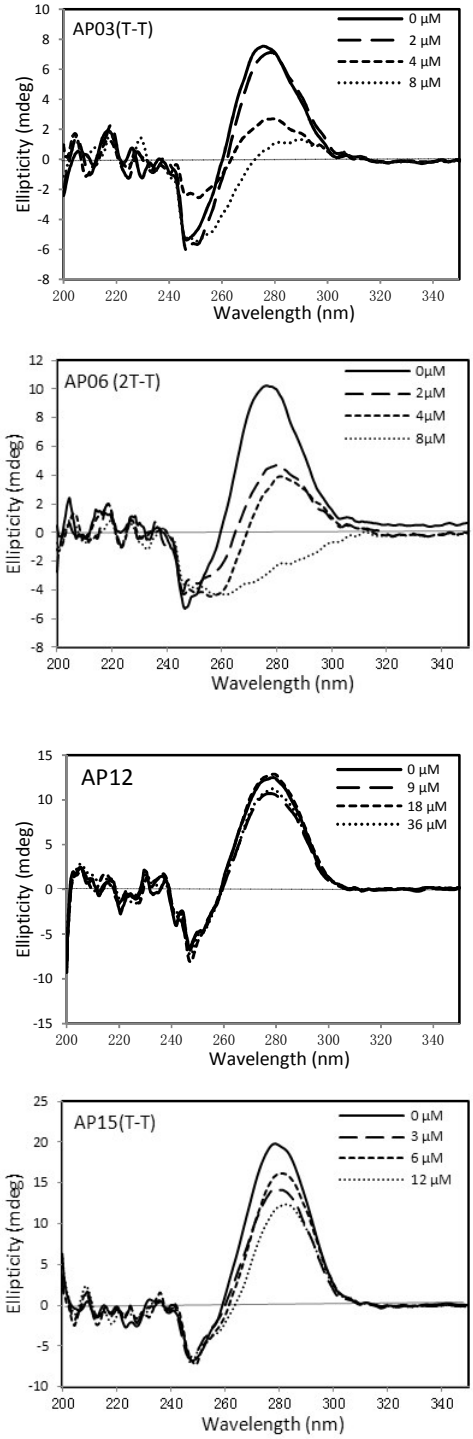
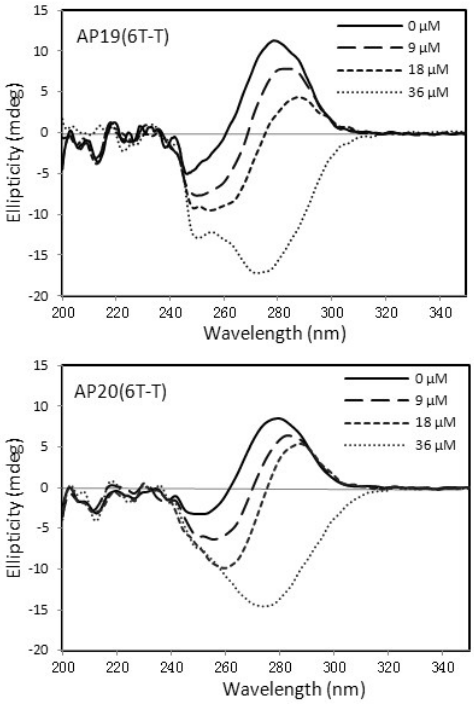


Fig. 2 CD spectra of anti-parallel duplexes containing T-T and T-Hg-T base pairs with the effect of Hg^{2+}. The concentrations of all the samples were the same as those for T_m measurement.

Both the T_m and CD spectra demonstrated that the T-Hg-T formation is suitable for the B-DNA duplex context, but its effect is dependent on the position and sequence as well as the number of T-T mismatches, besides environmental factors (pH, ionic strength, et al). Therefore, in the designing of a Hg^{2+} biosensor with T-T-rich nucleic acids, the position of the T-Hg-T stem in a functional nucleic acid have to be considered carefully, because of its slow formation process and the conformational change, which are related to the response time and the linear relationship between Hg^{2+} concentration and the signalling intensity of the Hg^{2+} biosensors.

As shown in Scheme 1, there exists two imino groups in the base pairing face of the mismatched G-T and G-G pairs, and the G-Hg-T and G-Hg-G base pairs have been observed at the level of monomers, with the preference of T-Hg-T > G-Hg-G > T-Hg-G.[20] Here, these base pairs were examined in the context of DNA duplex (Table 1). Firstly, the duplexes with one or two mismatched T-G (**AP04** and **AP07**) were more stable than the corresponding duplexes with one or two mismatched T-T pairs in **AP03** and **AP06**, respectively, reflecting the contribution of the wobble pairing and the base-stacking of the T-G pair.[21] But, no effect of Hg^{2+} on T-G pair was observed in the present context. Especially, the T-G pair is almost as stable as a T-Hg-T pair, in the case of one incorporation. Although it could not exclude the formation of T-Hg-G, while in the case of two incorporations, the stability of T-G in **AP07** was not driven to the level of T-Hg-T in **AP06**, it seems that T-G was not bridged by Hg^{2+}. Secondly, the G-G pair in **AP05** was a little more stable than the T-T pair in **AP03**, the larger base stacking of G-G than that of T-T might be one of the factors responsible for the T_m difference. Similarly, no effect of Hg^{2+} was observed



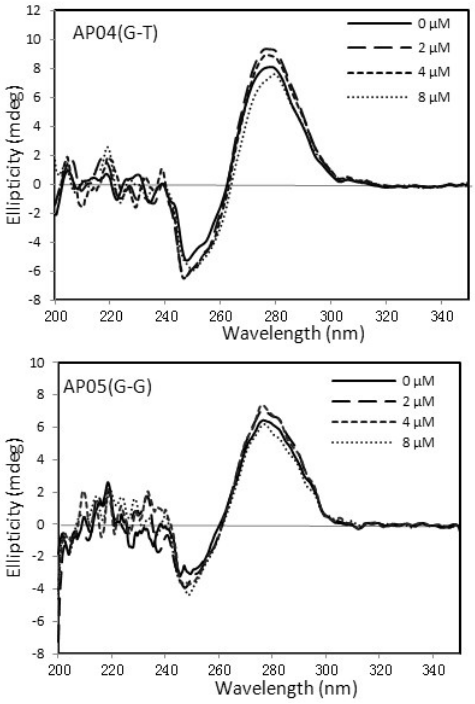
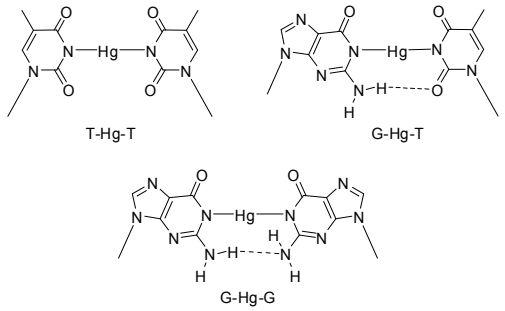


Fig. 3 The effect of Hg^{2+} on the CD of anti-parallel duplexes containing T-T stem and other mismatched base pairs. The concentrations of all the samples were the same as those for T_m measurement.

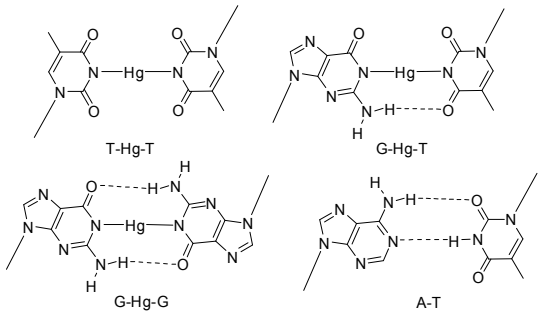


Scheme 1 The possible B-Hg-B base pairs in anti-parallel dsDNA.

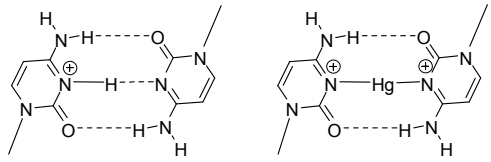
on the G-G pair. The CD spectra (Fig. 3) also showed that Hg^{2+} had no effect on these duplexes, as observed for the parent duplex **AP01**. It seems that Hg^{2+} ion could not bridge the two central imino groups in T-G and G-G to form a new N-Hg-N bond (Scheme 1) in the duplex context. It was probably that this N-Hg-N bond and the inner hydrogen bond could not be compatible with each other for a stable base pair, or probably the new G-Hg-T and G-Hg-G pairs were too large to be well accommodated in the duplex structure. With regard to other mismatched base pairs C-T, C-C, G-A, and A-A (Table 1), no positive effect of Hg^{2+} was observed by T_m measurement (Table 1), as early reported.[17] These facts confirmed that Hg^{2+} is unique for mismatched T-T pair in the context of an antiparallel DNA duplex. Its N-Hg-N bonding, the base pair stacking and Hg atom stacking are the factors for its contribution to the stability of DNA duplexes, and the N-Hg-N bond and the size of the base pair corresponds well to normal purine-pyrimidine base pair.

The possibility of B-Hg-B base pairs in parallel DNA duplexes

Considering the ionic property of the N-Hg-N bond,[14] and the absence of hydrogen-bonding in the T-Hg-T base pair, it seems that there is no directionality problem related to the T-Hg-T base pairing (Scheme 2). Similarly, parallel G-Hg-T and G-Hg-G base pairs were also suggested. Parallel T-Hg-T was reported to form in a parallel dsDNA (psDNA) constructed by covalent linkage.[18] Therefore, the existence of these parallel B-Hg-B base pairs was investigated in parallel dsDNA formed between two separate sequences, 5'-d(CCC ATA ATA ATT TAT CCC)-3' (**P01**) and 5'-CCC TAT TAT TAA ATA CCC-3' (**P02**), in which six C^+-H-C clamps on either end and the reverse Watson-Crick A-T base pairs allow the parallel-stranded duplex formation.[22, 23] In addition, cytosine could be protonated in acidic pH and a C^+-H-C base pair forms in a parallel dsDNA. Therefore, it is interesting to study whether another base pairing C-Hg-C (Scheme 3) could possibly form, through the proton replacement between the two protonated cytosine bases by one Hg^{2+} ion.



Scheme 2 B-Hg-B formation in parallel dsDNA.

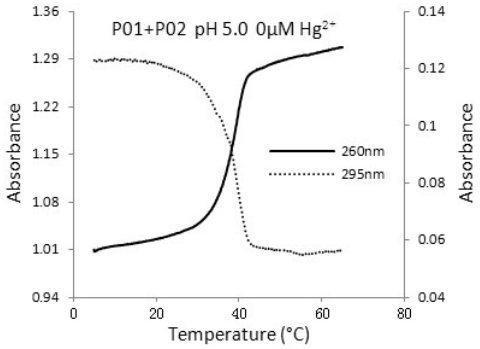


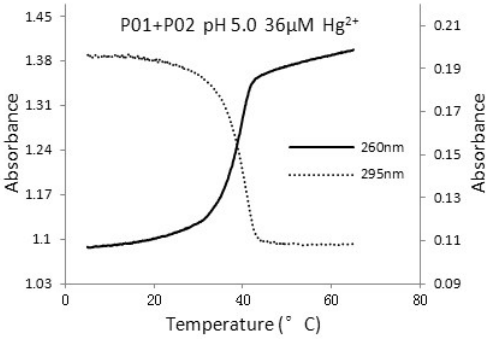
P01 5'-d (CCC ATA ATA ATT TAT CCC)-3'
P02 5'-d (CCC TAT TAT TAA ATA CCC)-3'

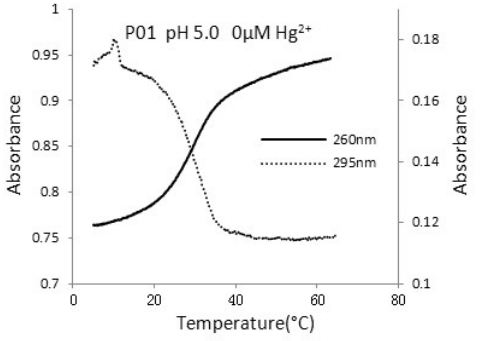
Scheme 3 C^+-H-C and C-Hg-C base pairs.

In the complex **P01+P02**, the two stems of three consecutive cytidine residues at both ends meet the least requirement for the formation of an i-motif consisting of two parallel stranded and C-C^+ base paired duplexes held together in an antiparallel orientation. The denaturation curves of the complex **P01+P02** exhibited a hyperchromism at 295 nm and an inverted transition at 260 nm during the folding process (Fig. 4), and its T_m increased with pH changing from 6.0 to 4.0 (Table 3), which are in full agreement with the existence of the C^+-H-C base

pairs and the i-motif as previously reported.[24, 25] It was thus suggested that the complex **P01+P02** could form the hemi-protonated cytosine–cytosine pairs in the complex and further i-motif structure under the present conditions. Single sequence **P01** behaved similarly, as testified by the same UV absorbance changes at 295 and 260 nm (Fig. 4). But the big difference in T_m between the complex **P01+P02** and the sequence **P01** indicated that they existed in different states at pH 5.0, although they have the same ends for hemiprotonated cytosine–cytosine pairs and i-motif structure. It is important to be noted that the mutated complexes **P01+P03**, **P02+P04**, **P03+P04**, **P01+P08**, **P01+P09**, **P09+P10** (Table 3 and Table 4) had lower T_m than that of the complementary complex **P01+P02**. These results constituted the strong evidence that **P01+P02** formed a parallel duplex, and further i-motif structure at pH 5.0.







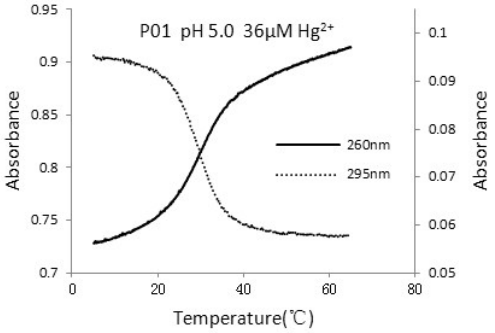


Fig. 4 Melting curves of parallel duplex **P01**+**P02** and single sequences, UV recording at 295 (right y axis) and 260 nm (left y axis). The concentrations of all the samples were the same as those for T_m measurement.

Table 3 Thermal stability of parallel DNA duplex **P01+P02** and single **P01** at pH 4.0 - 6.0 in the presence of mercury ion of different concentrations [a]

sequences	Hg^{2+} (μM)	pH 4.0	pH 5.0	pH 6.0
5'-d(CCC ATA ATA ATT TAT CCC)-3' (**P01**) 5'-d(CCC TAT TAT TAA ATA CCC)-3' (**P02**)	0	44.8	38.2 (38.3)	24.1
	18	44.9	38.4	23.2
	36	45.2	38.0 (38.2)	22.8
	48	44.5	37.7	23.0
5'-d(CCC ATA ATA A**T**T TAT CCC)-3' (**P01**) 5'-d(CCC TAT TAT T**T**A ATA CCC)-3' (**P03**)	0		33.6	
	18		33.9	
	36		34.0	
5'-d(CCC TA**T** TAT TAA ATA CCC)-3' (**P02**) 5'-d(CCC AT**T** ATA ATT TAT CCC)-3' (**P04**)	0		34.3	
	18		34.6	
	36		34.9	
5'-d(CCC TA**T** TAT T**T**A ATA CCC)-3' (**P03**) 5'-d(CCC AT**T** ATA A**T**T TAT CCC)-3' (**P04**)	0		26.7	
	18		27.0	
	36		27.3	
5'-d(CCC TAA TTT TTT ATA CCC)-3' (**P05**) 5'-d(CCC ATT AAA AAA TAT CCC)-3' **P06**)	0		38.6	
	36		38.4	
5'-d(CCC TAA **TTT TTT** ATA CCC)-3' (**P05**) 5'-d(CCC ATT **TTT TTT** TAT CCC)-3' (**P07**)	0		27.6	
	36		28.0	
5'-d(CCC ATA ATA ATT TAT CCC)-3' (**P01**)	0		28.1	
	36		28.3	
5'-d(CCC ATT **TTT TTT** TAT CCC)-3' (**P07**)	0		26.3	
	36		26.0	

[a] Conditions for T_m measurement: equal molar sequences (3 μM) were combined in the buffer consisting of 1.2 M NaCl and 20 mM sodium cacodylate with different pH and different concentrations of Hg^{2+}. UV absorbance of the complexes was recorded at 260 nm or 295 nm when heating at 0.1ºC/min in a 10 mm cuvette. The data in parenthesis were the T_m measured at 295 nm. Three measurements were conducted for each T_m, with standard error of 0.2 ºC.

Table 4 Thermal stability of parallel DNA duplexes and single sequences at pH 5.0 in the presence of mercury ion of different concentrations [a]

Sequences	Hg^{2+} (μM)			
	0	6	12	48

P01+P08				
5'-d(CCC ATA ATA A**TT** TAT CCC)-3'	30.6	30.3	31.4	31.9
5'-d(CCC TAT TAT T**TT** ATA CCC)-3'	(29.7)	(29.6)	(30.1)	(30.7)
P08	28.9	28.5	28.1	28.5
5'-d(CCC TAT TAT T**TT** ATA CCC)-3'	(29.1)	(28.0)	(28.0)	(28.6)
P01+P09				
5'-d(CCC ATA ATA A**TT** TAT CCC)-3'	34.2	34.2	33.7	34.4
5'-d(CCC TAT TAT T**GG** ATA CCC)-3'				
P09+P10				
5'-d(CCC TAT TAT T**GG** ATA CCC)-3'	36.4	36.4	36.2	36.3
5'-d(CCC ATA ATA A**GG** TAT CCC)-3'				

[a] see footnote under Table 3. Three measurements were conducted for each T_m, with standard error of 0.2 ºC.

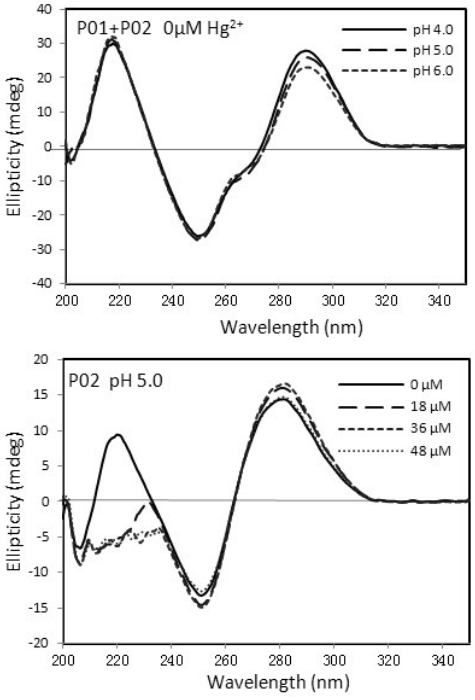
The other evidence was their CD spectrum. As shown in Fig. 5, for the complex **P01+P02** at pH 4.0, 5.0, and 6.0, the strong positive band at 287 nm and a crossover at 274 nm demonstrated the existence of the i-motif structure (Fig. 5).[26] Upon heating, a sequential conformational change was observed, especially for the positive lobe shifting from 287 nm to 280 nm, which is characteristic of an i-motif dissociation with blue shift and sharp magnitude decrease of the positive band.[27] It was thus suggested that the positive lobe shift corresponded to the two steps of conformational change of the complex **P01+P02**, in which the i-motif was firstly decomposed and then the parallel duplex melted to single sequences. In the case of single **P02** at pH 5.0, its positive lobe located at 278 nm, when heated to 40 ºC and 60 ºC, only one blue shift and amplitude decrease occurred for the positive lobe, indicating that an i-motif was dissociated to unstructured single sequence. Together with the melting curves at 295 nm (Fig. 4) of the single sequences, it was suggested that **P02** could form an i-motif structure, and the T_m reflected its thermal stability under present conditions. Single sequences **P01** and **P08** had very close thermal stability (Table 3, Table 4, and Supporting information).

With these two kinds of i-motifs formed by single sequence or complementary complexes, the influence of Hg^{2+} on i-motif and parallel base pairs was investigated. In the case of i-motif structure formed by single sequence **P01**, **P02**, or **P08**, no significant change on their T_m and CD was observed upon increasing Hg^{2+} concentration, the same T_m at 295 and 260 nm and CD spectra (the positive band at 287 nm and the negative band at 250 nm) were always observed (Table 3, Table 4, Fig. 5). Even the conformational change upon heating was the same between the absence and the presence of Hg^{2+}. It seems that Hg^{2+} could not interrupt the C^+-H-C base pair or induce any significant change on the i-motif structure.

In the case of parallel dsDNA **P01+P02**, Hg^{2+} could not interact with A-T or C^+-H-C, at pH from 4.0 to 6.0. Even at pH 4.0, most cytosine residues were protonated, no interaction between Hg^{2+} and protonated cytosine were observed in the present context, based on the almost the same T_m and CD spectra. In addition, the heating-induced conformational change of **P01+P02** in the presence of Hg^{2+} was the same as in the absence of Hg^{2+} (Fig. 5), conformational transition occurred from i-motif to parallel duplex and finally to single sequences during the heating process, as indicated by the positive band shift from 286 nm to 276 nm and to 270 nm, respectively.

In the complex **P01+P02**, when one or two mismatched T-T pair was incorporated, the lower T_m was always obtained (Table 3) (**P01+P03**, **P02+P04**, **P03+P04**, and **P01+P08**). The addition of Hg^{2+} to the complexes **P01+P03**, **P02+P04** and **P03+P04** led to a slight increase of T_m (0.1 - 0.3 ºC). In the case of the duplex **P01+P08** with two mismatched T-T (Table 4), its T_m decreased accordingly. The addition of Hg^{2+} led to a T_m increase of 0.8 ºC and 1.3 ºC measured at 260 nm, 0.4 ºC and 0.65 ºC were supposed for each T-Hg-T when the Hg^{2+} reached to 12 μM and 48 μM (Table 4), respectively. Their CD spectra indicated that there was no significant influence on the complex structures (Fig. 6 and Supporting information).

In order to further evaluate the effect of Hg^{2+} on the T-T pair in parallel duplex context, a stem of consecutive six T residues was designed in **P05**, its complex with complementary **P06** is as stable as the duplex **P01+P02** (Table 4), and single **P07** behaved like **P02** (Supporting information). When a consecutive six T-T pairs was supposed in complex **P05+P07**, the destabilizing effect of six T-T mismatched pairs was significant, resulting in a T_m decrease of -11 °C. However, no increasing effect of Hg^{2+} was observed for this complex, and no significant change for the CD spectra (Fig. 6). It was thought that no parallel T-Hg-T stem formed in this parallel duplex DNA



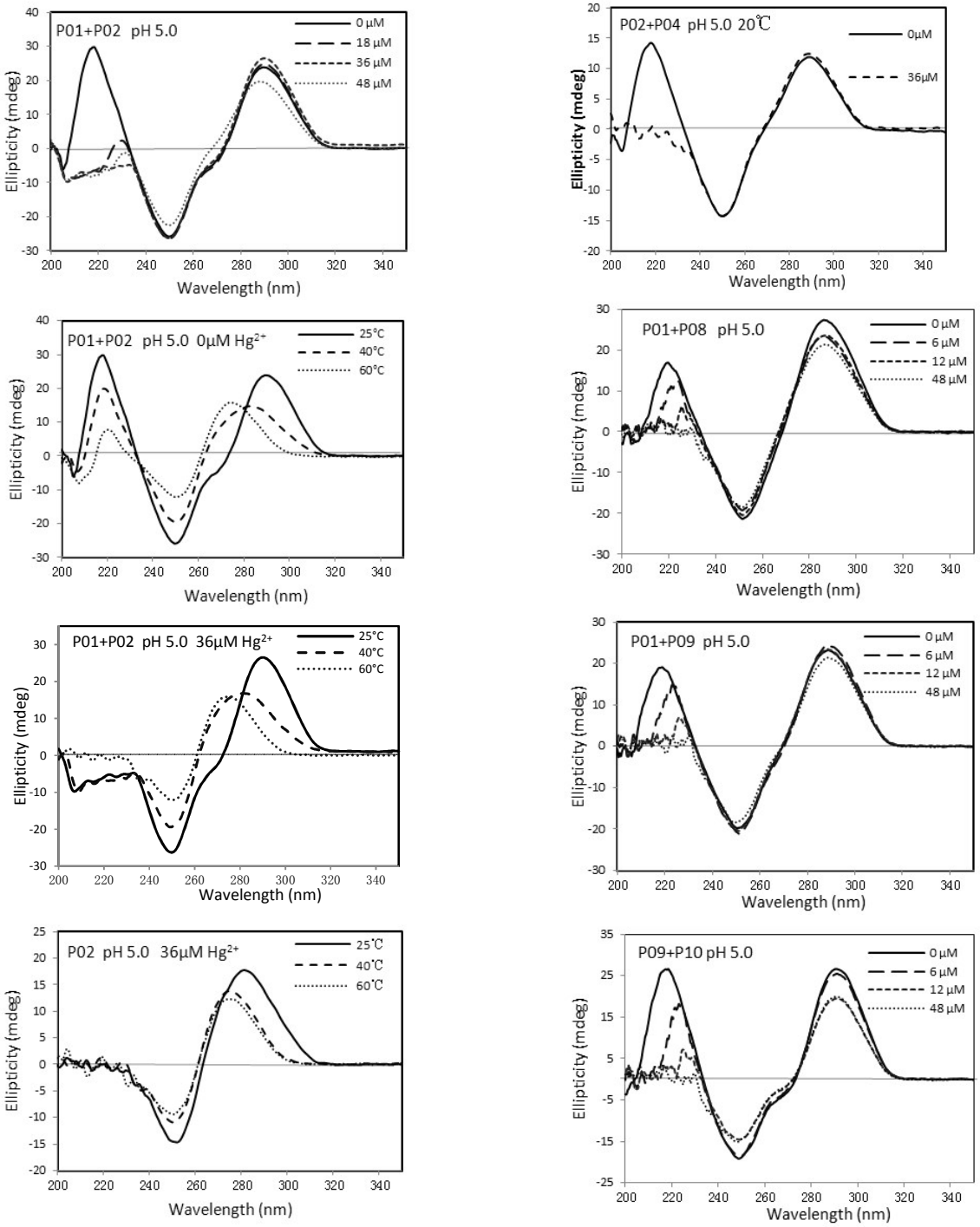


Fig. 5 CD spectra of the parallel duplex P01+P02 and the single sequences P01 and P02 in the absence or presence of Hg^{2+}. The concentrations of all the samples were the same as those for Tm measurement.

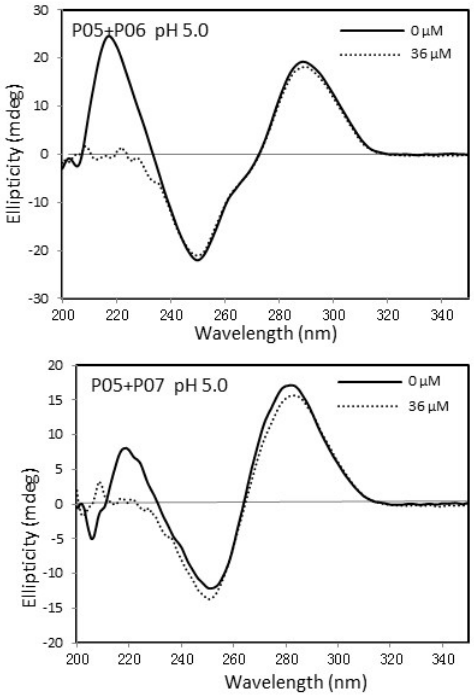


Fig. 6 CD spectra of parallel duplexes in the absence or presence of Hg^{2+}. The concentrations of all the samples were the same as those for T_m measurement.

under present conditions. This observation confirmed that parallel T-T was at least not a stable base pair. It is not stable enough to form a long parallel T-Hg-T stem. We thought that the tertiary environment in the parallel duplex DNA might not meet the spacial requirement for the N-Hg-N bond formation.

It is worthwhile to be noted that Hg^{2+} did not cause significant change on the positive band around 287 nm and the negative band at 246 nm of all the parallel duplexes, but an amplitude decrease on the other positive band at 215 nm with a red-shift was observed to be Hg^{2+} concentration dependent, no matter whether the duplex contained mismatched T-T or not. Furthermore, this change (amplitude decrease and red-shift) were different from that with heating-induced change (Fig 5 and Fig. 6). It was probably that the interaction between Hg^{2+} and nucleic acids led to such changes.

In C^+-H-C base pair, there are three hydrogen bonds that hold it together, its N3-N3 hydrogen bonds in the center of the cytosine pairs have a bond length of 2.76± 0.08 Å, and the hydrogen bond lengths involving the exocyclic amino group N4 and oxygen O2 have similar lengths of 2.75 ± 0.1 Å, this stereo compatibility leads to a stable C^+-H-C base pair.[29] The longer N-Hg-N could be unfavourable for the other two hydrogen bonding in C^+-H-C base pair. Although it has been observed that Hg^{2+} could bind with all the nucleobases at the level of individual residues, both the base pair properties and tertiary structural requirement might determine the strong preference of Hg^{2+} for mismatched T-T in an anti-parallel duplex DNA. DNA chain slippage mechanism including base tilting and contraction of DNA strands was proposed for the compatability of T-Hg-T into the context of anti-parallel dsDNA.

In the context of **P01+P09** and **P09+P10** with mismatched T-G and G-G pairs, respectively, no effect on thermal stability (Table 4) and CD spectra (Fig. 6) was observed when Hg^{2+} was applied, indicating that no Hg^{2+}-related base pair formed. The fact that Hg^{2+} can interact with imino groups of guanine and protonated cytosine at the level of nucloebase, nucleoside, and nucleotide, demonstrated that these two nucleobases could perform imino proton exchange with Hg^{2+}. In the context of both anti-parallel and parallel duplexes, T-G and G-G are more stable than T-T, maybe they are stable enough and could not be interrupted by Hg^{2+}, acting as a natural base pair. In addition, there exist several hydrogen bonds in these base pairs, these hydrogen bonds and the N-Hg-N bond might not be cooperative to allow new bond formation in such a tertiary structure. The mercurated base pair formation and stability are dependent on its bonding compatability within the base pair and stereo compatabiltiy in the tertiary structures.

Conclusions

With thermal stability evaluation and CD spectra, it was demonstrated that T-Hg-T is a unique base pair in DNA duplexes, no interaction was observed between Hg^{2+} and other mismatched base pairs T-G, G-G, and C^+-C^+ with two imino groups at the base pairing face in parallel and anti-parallel duplex context. Due to the thymine-mercury-thymine base stacking and one N-Hg-N bonding, T-Hg-T base pair has no directionality problem, but with significantly different thermal stability when located in the anti-parallel or parallel DNA duplexes. The compatibility between the N-Hg-N bond and hydrogen bonds within the base pair, and the compatibility between the B-Hg-B base pair and the neighbouring base pairs in a local stem or the whole helical conformation are critical for the effect of the new base pair, like the performance of the T-Hg-T base pair in different DNA structures.

The anti-parallel T-Hg-T base pair was much more stable than its parallel counterpart. Especially, the thermal stability of the T-Hg-T base pair was position-dependent, either in anti-parallel or parallel DNA duplexes. Therefore, in the design of Hg^{2+}-biosensers with functional nucleic acids, an anti-parallel T-Hg-T stem is preferred, and the position and number of the T-Hg-T base pair need to be examined for an expected and sharp conforamtional change of functional nucleic acids, and rapid and accurate quantification of mercury ion could be realized.

Experimental Section

Materials

Sodium nitrate and sodium magnesium, and sodium cacodylate were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (Beijing, China) and standard mercury in 2-5% nitric acid from AccuStandard. All the buffers of different pH were prepared in stock solutions, with or without Hg^{2+}.

Oligodeoxynucleotides

All oligodeoxynucleotides (ODNs) were synthesized on a DNA 392 synthesizer on 1 μM scale. The CPG beads with DMTr-off sequences were sealed in conc. aq. ammonia. After heating at 55 ºC for 18 h, the solution was filtered and the filtrate was

concentrated for denaturing polyacrylamide gel electrophoresis (20%) (8 M urea). The product was extracted from gel with TBE buffer. SEP-PARK column was used to extract ODNs and desalting. After washing the column with bidistilled water, the ODNs were eluted with methanol/water (70/30, v/v) and lyophilized, stored at -30 ºC. All the DNA sequences were dissolved in deionized water and quantified by UV-absorption measurement at 260 nm at 60 ºC, with the extinction coefficients of A (15 400), G (11 500), C (7 500), and T (8 800).

T_m measurement

With a Cary Bio100 UV-Vis spectrophotometer equipped with a temperature controller, the UV absorbance change of DNA complexes at 260 nm and 295 nm was recorded versus heating and/or cooling processes between 65 ºC and 5 ºC. The concentration of the samples was indicated under the tables. For the heating process, the sample was pre-cooled from 90 ºC to 4 ºC naturally and stored at 4 ºC overnight. Different temperature ramp range of 0.1-1.0 ºC min-1 was used, depending on the kinds of DNA complexes. Firstly, different cooling rates (1, 0.5, 0.2. 0.1 ºC/min) were used, until the cooling curves of UV absorbance vs, temperature could overlap and no difference of Tm values were observed, and then, the same heating rate was conducted to confirm the real reversibility of the duplex formation. Therefore, different rates were used for different duplexes. The first derivative analysis of the melting curves was conducted for the melting temperature of each DNA complex.

Circular Dichroism (CD)

The samples after T_m evaluation were used for CD measurement. It was conducted on a MOS-450 spectropolarimeter (Biologic, France), using a quartz cell of 10 mm path length. The samples were pre-cooled from 90 ºC to 4 ºC naturally and stored at 4 ºC overnight. Scanning was performed over a wavelength range of 200-350 nm, with a speed of 100 nm/min. The spectra were the averaged results of three accumulations and smoothed using a 25-point adaptive smoothing algorithm.

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Studies on the thymine- mercury-thymine base pairing in parallel and anti-parallel DNA duplexes

Gaofeng Liu, Zhiwen Li, Junfei Zhu, Yang Liu, Ying Zhou and Junlin He

Parallel and anti-parallel T-Hg-T base pairs have different thermal stability and conformational influence on DNA duplex structures.