Formation of *i*-motif structure at neutral and slightly alkaline pH†

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Received 21st September 2009, Accepted 4th November 2009
First published as an Advance Article on the web 14th December 2009
DOI: 10.1039/b919600e

It is well known that oligonucleotides containing tracts cytosines can form *i*-motif structures under acidic conditions (pH < 7). However, whether *i*-motif can be formed under normal physiological cellular conditions (pH 7.0–7.5) is yet no conclusive proof. In the present work, using circular dichroism (CD), UV absorption spectroscopies and native polyacrylamide gel electrophoresis (PAGE), we provided the compelling evidence for the formation of *i*-motif structures by four cytosine clusters, $[C_3TA_2]_3C_3$ (HT), $[C_4G]_3C_4TA$ (RET), $C_2T_3C_2T_4C_2T_3C_2$ (CTC) and $GC_2GC_3A_4C_6G$ (Rb), at neutral and slightly alkaline pH at 4 °C. Furthermore, for HT, we also supplied the evidence for the formation of *i*-motif structure by fluorescence resonance energy transfer (FRET) and investigated its folding kinetics. The formation time constants obtained by CD and fluorescence experiments are 214 and 493 s, respectively, indicating that HT can slowly form *i*-motif structure at pH 7.0 and 4 °C. This work implies that *i*-motif structures may possible form *in vivo*.

Introduction

The *i*-motif, a four-stranded structure also called the *i*-tetraplex, is formed by two parallel duplexes intercalating with each other in an antiparallel orientation and each duplex is held together *via* hemiprotonated cytosine$^+$–cytosine base pairs.[1,2] Currently, it is well known that sequences with the potential to form *i*-motifs are frequently found in eukaryotic genomes[3] and some proteins can interact selectively with cytosine-rich sequences,[4–7] therefore, such four-stranded structure could be biologically relevant. For example, previous studies have reported that heterogeneous nuclear ribonucleoprotein K can activate *c-myc* transcription through binding to the C-rich strand in the NHE III_1 (nuclease hypersensitive element) region of the *c-myc* oncogene promoter.[8,9] Moreover, *i*-motif has great intriguing potential application in nanotechnology as nanomolecular devices because its conformational switch could be easily controlled by pH stimulus.[10–12] Recently, based on *i*-motif Krishnan and colleagues constructed a DNA nanomachine for the application of pH sensor inside living cells, which can function between pH 5.5 and 6.8.[13]

The formation of an *i*-motif using different DNA sequences has been studied extensively *in vitro* under acidic conditions,[14–21] and *i*-motif structures of some cytosine-rich sequences have been discussed based on NMR and crystallography results.[1,22–30] Until now, there are very few studies reporting that *i*-motif structure can be formed at neutral pH.[31–34] For instance, Mergny and coworkers have shown that a stretch of cytidines can form *i*-motif at slightly acidic or even neutral pH.[31] A recent work has described that the DNA fragments of fragile X chromosome can fold into *i*-motif structure at neutral pH.[32] It is widely accepted that the structure of *i*-motif will transform into random coil conformation due to the deprotonation of cytosines at higher pH values (pH > 7). However, under normal physiological cellular conditions, an intracellular pH was maintained in the range from 7.0 to 7.5. Unquestionably, it is necessary to investigate whether *i*-motif can be formed at neutral and slightly alkaline pH. Using carboxyl-modified single-walled carbon nanotubes (SWNTs) to reduce the pK_a of cytosine$^+$–cytosine, Qu's group demonstrated that SWNTs can selectively induce human telomeric *i*-motif structure formation at neutral or even slightly alkaline pH.[33,34] Up to now, to our knowledge, there is no report to show that *i*-motif can be formed without the interaction with SWNTs at slightly alkaline pH. We thus decided to explore this challenging problem, particularly since *i*-motif needs to play its leading biological role under slightly alkaline conditions *in vivo*.

In the present work, we used human telomeric sequence (HT) as a model system, and employed spectroscopic techniques (CD, fluorescence and UV absorption spectroscopies) and native PAGE to characterize such cytosine-rich sequence which can form well-defined *i*-motif structure under neutral and slightly alkaline pH at 4 °C. Furthermore, we also demonstrated other cytosine clusters could form *i*-motif structures under physiological pH by CD, UV and native PAGE. Therefore, the compelling evidence presented here suggests that cytosine-rich sequences can fold into *i*-motif structures *in vivo*. Our work may provide a new insight into the formation of *i*-motif structures, which can be formed not only under acidic condition, but also at neutral and slightly alkaline pH.

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† Electronic supplementary information (ESI) available: Supporting figures. See DOI: 10.1039/b919600e

Results

CD spectra suggest *i*-motif formation at neutral and slightly alkaline pH

The changes of CD spectra of HT at several pH values (pH 7.00, 7.05, 7.10, and 7.15) under different temperatures show the similar behavior, therefore, in Fig. 1A only shown the representative results at pH 7.00. The CD spectrum shows a positive band at 286 nm and a negative band centered at 254 nm at 4 °C, indicating the formation of cytosine–cytosine$^+$ base pairs. This is the characteristic spectrum of *i*-motif structures as reported previously.[15,16,20,21] The CD result demonstrates that HT can form *i*-motif structure under neutral conditions at 4 °C like that under acidic conditions (the CD spectra of HT at pH 5.0 were shown in Fig. S1A, ESI†). As the temperature is increased gradually from 4 to 25 °C, the positive band is blue shifted and the magnitude of the ellipticity at 286 nm is decreased. At 25 °C, the CD spectrum shows positive and negative bands at 272 nm and 248 nm, respectively, indicating that the structure of HT is random coil. In Fig. 1A, there are two isoselliptic points at about 244 and 274 nm, which imply that the transition between two discrete conformations (*i*-motif and random coil) is a two state process. This result is in line with the previous report about human *c-myc* promoter sequences.[16] The melting temperature (T_m) at pH 7.0 was obtained by plotting the ellipticity at 286 nm *versus* temperature (Fig. 1A, insert), the T_m was determined to be 13 °C (Table 1), which is in agreement with the result observed by UV melting previously.[31] In addition, the T_m of HT at pH 7.0 almost consists with the results obtained by UV and fluorescence melting (shown below).

Fig. 1B gives the pH dependence of HT structure at 4 °C. As the pH increased gradually from pH 7.00 to 7.15, the positive peak at 286 nm decreases and shifts to about 282 nm, which suggest that *i*-motif structure is gradually transformed into random coil. In fact, we observed the typical CD spectrum of random coil at pH 7.20. The pH dependence of *i*-motif observed here is in accordance with previous widely reported results,[15,16,20,21,35,36] and this property is the foundation of *i*-motif nanomolecular devices.[10–13] The CD spectra shown in Fig. 1A and B demonstrate that, at 4 °C, HT can form *i*-motif structure not only at neutral pH but also at slightly alkaline pH. From these results, the pH 7.15 seems to be the turning point because the HT is the random coil at pH 7.2. At 25 °C, however, the CD spectra at all these pH values show the random coil (Fig. S1B, ESI†).

To further confirm the results that HT can form an *i*-motif structure at slightly alkaline pH, we investigated its conformation in MOPS buffer (3-(*N*-morpholino)propanesulfonic acid). MOPS was chosen because its pK_a is extremely temperature dependent.[37] The buffer was prepared at 25 °C and adjusted its pH value to 7.0. When the temperature decreased to 4 °C, its pH value will increase to about 7.25.[37] As shown in Fig. 1C, the HT is in the conformation of random coil at 25 °C, while it is a *i*-motif structure at 4 °C in MOPS buffer. These results seem to be inconsistent with the results above in cacodylate buffer (its pK_a is almost temperature independent) because we observed that *i*-motif structure can not be formed at 4 °C with pH 7.2. This difference may be result from the different properties between two buffers. Moreover, we performed the similar experiments in ACES (*N*-(2-acetamido)-2-aminoethane sulfonic acid) buffer due to its pK_a is more temperature dependent than that of MOPS.[37] We found the HT at 4 °C does not form *i*-motif structure (the positive peak is at 282 nm and the pH value of ACES at 4 °C is about 7.4, results not shown). Integrating with the CD results of HT in cacodylate, MOPS and ACES buffers, we can affirm that HT can form an *i*-motif structure at slightly alkaline pH at 4 °C. If the pH value higher than a certain degree, HT will not form the *i*-motif structure even at 4 °C. For further proving our conclusion, we used other techniques to validate it at pH 7.0 and 7.1.

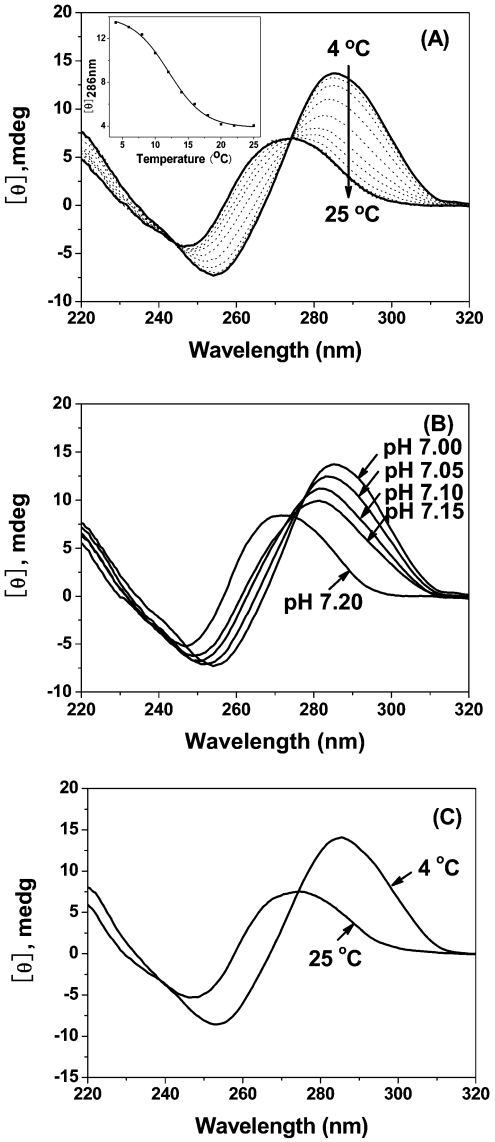

Fig. 1 Circular dichroism spectra of HT recorded at different temperatures at pH 7.0 (A), at 4 °C with different pH (B) in 10 mM cacodylate buffer, and at 4 and 25 °C in 10 mM MOPS (pH was adjusted to 7.0 at 25 °C, and at 4 °C the pH value will change to about 7.25) (C). Inset in Fig. A shows the ellipticity at 286 nm as a function of temperature (pH 7.0).

Table 1 DNA sequences and their molar extinction coefficients used in this article and their corresponding T_ms at pH 7.0 and 7.1 obtained by different methods

Sequence	$\varepsilon_{260}/M^{-1}\ cm^{-1}$	Abbreviation	T_m/°C	
			pH 7	pH 7.1
5′-CCCTAACCCTAACCCTAACCC-3′	171 000	HT	13 (13)[a]	8 (10)
5′-CCCCGCCCCGCCCCGCCCCTA-3′	166 400	RET	16 (15)	13.5 (12)
5′-CCTTTCCTTTTCCTTTCC-3′	138 800	CTC	11 (10)	7 (7.5)
5′-GCCGCCCAAAACCCCCCG-3′	155 800	Rb	8 (7)	—(−)[b]
5′-FAM-CCC(TAACCC)$_3$-TAMRA-3′	238 840	F-HT-T	16.5[c]	12

[a] The values were determined by CD melting method and the values in the bracket were obtained by UV melting at 295 nm. [b] We did not observed the sigmoidal curve of melting. From our experiments, the values should be less than 5 °C. [c] The results obtained by fluorescence melting method.

UV spectra demonstrate *i*-motif formation at neutral and slightly alkaline pH

Absorption spectra of HT at pH 7.0 and 7.1 as a function of temperature show the similar behavior, therefore, in Fig. 2A only the results at pH 7.0 are shown. For example, both absorbances at 265 nm increases and at 295 nm decreases as the temperature are increased from 4 to 25 °C. In addition, both of absorption spectra have isosbestic points at around 275 nm, which indicates the presence of the equilibrium of two different species. By subtracting the low temperature (4 °C) spectrum from the high temperature spectrum (25 °C), we obtained the thermal difference spectrum (Fig. 2B). At pH 7.0 and 7.1, both the thermal difference spectra of HT show major positive peak at 239 nm and negative peak at 294 nm. These thermal difference spectra are the features of *i*-motif structure and consist with the previous results.[38] We also observed that both the positive and negative peaks of HT at pH 7.0 are stronger than those at pH 7.1, which may be result from the structure of HT is destabilized with pH increasing. Furthermore, we measured the unfolding of HT by UV melting at 295 nm as previous reports (Fig. 2B, insert).[31,38,39] The T_ms of HT are 13 and 10 °C at pH 7.0 and 7.1 (Table 1), respectively, and are in line with the results obtained above by CD. The thermal difference spectra here further confirm that *i*-motif structure can form at neutral and slightly alkaline pH.

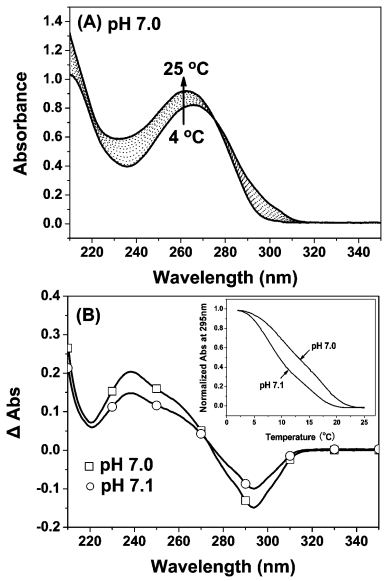

Fig. 2 (A) UV absorption spectrum of HT in 10 mM cacodylate buffer as a function of temperature (pH 7.0); (B) Thermal difference spectra of HT at pH 7.0 (□) and pH 7.1(○) resulting from the subtraction of the 4 °C spectrum from the 25 °C one. The absorption spectra of HT at 7.1 as a function of temperature were shown in Fig. S2.† Inset shows the melting curve of HT at 295 nm at pH 7.0 and 7.1.

HT forms intramolecular *i*-motif structure at neutral and slightly alkaline pH

To further investigate whether the *i*-motif can form at neutral and slightly alkaline pH, we employed the FRET technique owing to it being able to reveal the formation of *i*-motif structure.[40] The HT sequence was covalently attached with a donor (fluorescein) and an acceptor (tetramethylrhodamine) at 5′ and 3′ ends, respectively. As shown in Fig. 3, at 25 °C, both emission spectra of HT at pH 7.0 and 7.1 show the major peak at 515 nm which suggests the random coil conformation. There is a small peak at 580 nm, which may result from the direct excitation at 480 nm of the acceptor dye.[40] Compared to the emission at 25 °C, the emission of donor and acceptor are obviously decreased at 4 °C, but the intensity at 580 nm is stronger than that at 515 nm, which illuminates the result of FRET. The phenomenon of FRET, the emission of donor and acceptor are both obviously decreased, has been also observed by Takenaka and coworkers about the formation of G-quadruplex structure induced by Mg^{2+} and Ca^{2+}.[41] Furthermore, the observed FRET here demonstrate that HT not only form *i*-motif, but also form intramolecular one, which was further supported by native PAGE and concentration melting experiments.

Next, we analyzed the electrophoretic mobility of HT at different pH (pH 5.0, 7.0 and 7.1) at 4 °C on a native polyacrylamide gel. At pH 5.0, 7.0 and 7.1, HT all migrated faster than control oligodeoxythymidilates (T_{21}), indicating HT form compact structures (Fig. 4). Moreover, HT at pH 5.0, 7.0 and 7.1 do not show variations in migration distance, implying that they form the same structures. It is well documented that HT can form intramolecular *i*-motif structure under acidic conditions, such as pH 5.0 used here.[31] Therefore, the structures of HT at pH 7.0 and 7.1 also are the intramolecular *i*-motif structure, which was further confirmed by melting temperature measurement. We detected the melting temperature of HT at pH 7.0 by CD and fluorescence melting experiments and found it does not depend on concentration (Fig. S3, ESI†), suggesting that HT forms a monomer intramolecular structure. This result consists with the conclusion

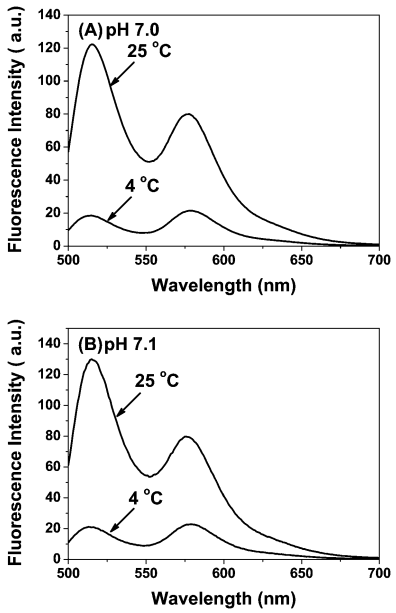

Fig. 3 Fluorescence spectra of F-HT-T at 4 and 25 °C at pH 7.0 (A) and 7.1(B).

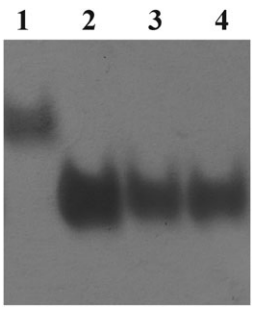

Fig. 4 Native PAGE image of HT at different pH values. Lane 1: T_{21}; lanes (2–4), HT at pH 7.0 (2), 7.1 (3) and 5.0 (4).

drew from FRET above and further proves that HT can form intramolecular *i*-motif structure at neutral and slightly alkaline pH.

Folding kinetics of HT at pH 7.0

The results above clearly show the formation of the *i*-motif structure at neutral and slightly alkaline pH. However, how about the folding kinetics of *i*-motif formation under these conditions? To analyze the kinetics of *i*-motif formation, we chose the CD and fluorescence spectra to study the folding of HT at pH 7.0 (Fig. 5). Concentrated nucleic acids aliquots were directly added into the buffer (kept at 4 °C in advance) and mixed quickly, then equilibrated at 4 °C and collected the spectra of CD or fluorescence at different time intervals. The kinetic data obtained by CD at 286 nm, with a time constant of 214 s, is presented in Fig. 5A and is well fitted by single exponential curve shown in methods section. However, the kinetic data gained from fluorescence at 515 nm also show the first-order reaction and the time constant is 493 s, which is larger than that obtained by CD method (Fig. 5B). This difference may be result from covalent attachment of two dyes

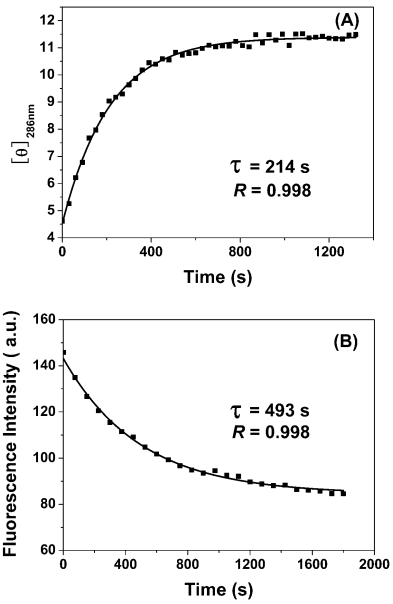

Fig. 5 Time dependence of both CD intensity at 286 nm of HT (A) and fluorescence emission intensity at 515 nm of F-HT-T (B) in 10 mM cacodylate buffer (pH 7.0) at 4 °C. Dots are experimental results and lines are fits with single exponential kinetics equation.

into HT delays the formation of the *i*-motif. From Fig. 5, one may observed that HT can completely form an *i*-motif within 700 s (Fig. 5A), while F-HT-T requires at least 1600 s (Fig. 5B).

Other sequences form *i*-motif structures at neutral and slightly alkaline pH

In view of HT forming an *i*-motif structure at neutral and slightly alkaline pH, it is also of interest to evaluate whether the phenomenon is a prevalent conclusion. To this end, we selected the three well-characterized sequences that have been proved that they can form *i*-motif structures in acidic conditions in the literature.[31,35,36] The sequences are listed in Table 1.

Intriguingly, from the CD and UV thermal difference spectra of RET, CTC and Rb, all exhibit the similar results to HT at neutral and slightly alkaline pH (Fig. 6). The CD spectra of RET, CTC and Rb show positive and negative bands at 286 and 254 nm, respectively, indicating formation of *i*-motif structures under neutral pH at 4 °C (Fig. 6A, C and E). However, when pH increased to pH 7.1, all the spectral features is similarity for three sequences. Their positive peaks, especially for CTC and Rb, are blue shifted and the amplitude are decreased, which imply *i*-motif structures are slightly transform into random coil conformations (they are still *i*-motif structures at pH 7.1, see below). Compared to CTC and Rb, the sequence of RET show little changes as pH increased to 7.1. As shown in Table 1, the stability of RET is stronger than the other two sequences, CTC and Rb. A previous study has demonstrated that the length of cytosine tract had a marked impact on the stability of *i*-motif structures.[31] Consequently, the difference of CD spectra may result from the variation of cytosine contents among these three sequences. The CD results reveal that the three sequences, like HT, can form an *i*-motif structure under normal physiological pH at 4 °C. In agreement with HT

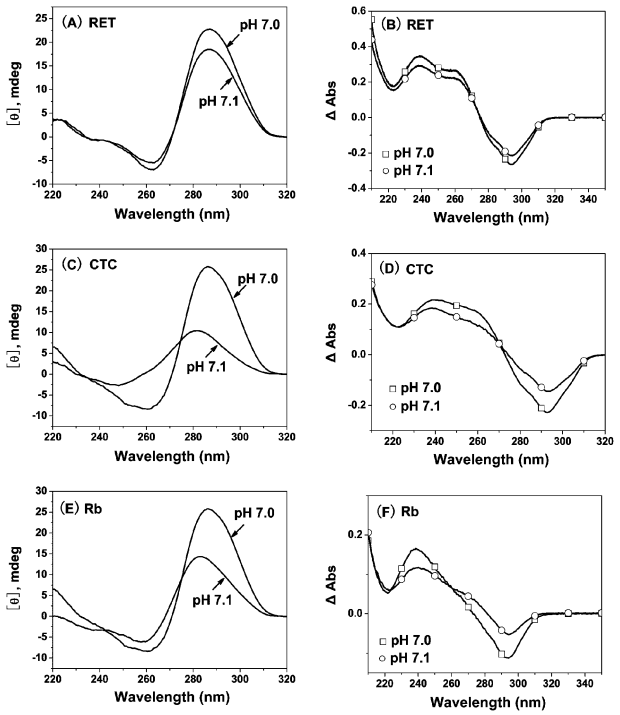

Fig. 6 Circular dichroism spectra and thermal difference spectra of RET (A, B), CTC (C, D) and Rb (E, F) recorded at 4 °C with pH 7.0 (□) and 7.1 (○) in 10 mM cacodylate buffer, respectively.

described above, the CD spectra of three sequences all exhibit positive and negative bands at 272 nm and 248 nm at 25 °C, respectively, suggesting their structures are random coil under neutral and slightly alkaline pH (Fig. S4A, ESI†).

At pH 7.0 and 7.1, similar to HT, all the thermal difference spectra of RET, CTC and Rb show major positive peaks at 239 nm and negative peaks at 294 nm (Fig. 6B, D and F). These thermal difference spectra indicate that such three sequences form *i*-motif structures neutral and slightly alkaline pH, which was further confirmed by native PAGE. RET, CTC and Rb all migrated faster than control T_{21}, and they themselves do not show variations in migration distance at pH 5.0, 7.0 and 7.1 (Fig. 7). These results show that these three sequences form compact structures. Previous reports have well demonstrated that RET, CTC and Rb can form intramolecular *i*-motif structure under acidic conditions.[31,35,36] Therefore, these three sequences also form *i*-motif structures under neutral and slightly alkaline pH at 4 °C. In addition, CTC migrated slower than RET and Rb, which suggest *i*-motif structures of RET and Rb are more compact than that of CTC (Fig. 7). Admittedly, together with CD, thermal difference spectra and native PAGE, the sequences of RET, CTC and Rb can form *i*-motif structures under neutral and slightly alkaline pH at 4 °C.

Discussion

The repetitive cytosine-rich sequences and their complementary, guanine-rich sequences, are widely dispersed in the human genome.[3,42,43] Nowadays, guanine-rich sequences that can form an unusual four stranded structure, the G-quadruplex,

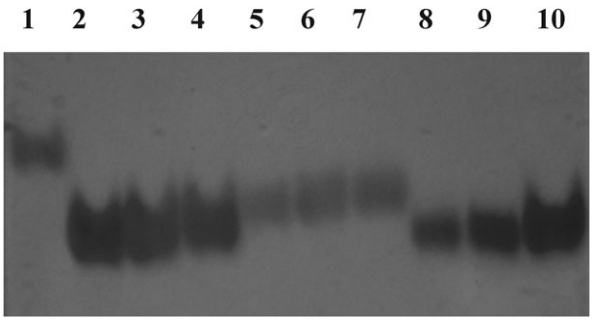

Fig. 7 Native PAGE image of RET, CTC and Rb. Lane 1: T_{21}; lanes (2–4), RET at pH 7.0 (2), 7.1(3) and 5.0 (4); lanes (5–7), CTC at pH 7.0 (5), 7.1(6) and 5.0 (7); lanes (8–10), Rb at pH 7.0 (8), 7.1(8) and 5.0 (10).

are a hot research topic. Compared to the G-quadruplex, the *i*-motif has received less attention. To date, though the *i*-motif may be formed in acidic compartments like endosomes *in vivo*,[13,31] and many proteins, which can interact selectively with cytosine-rich sequences, have been found,[4–9] there is no conclusive proof of *i*-motif existence *in vivo*. This disparity may be owing to fact that formation of the *i*-motif requires acidic to neutral pH, but normal physiological cellular conditions are slightly alkaline.

In the present work, in combination with spectroscopic techniques and native PAGE, we demonstrated that cytosine-rich sequences can form *i*-motif structures at neutral and slightly alkaline pH, which is same as physiological pH. However, we observed the formation of the *i*-motif at low temperature (4 °C used in the work). As shown in the melting study, all the structures are relatively unstable because their T_ms are in the range of about 10 °C. This temperature is much lower than physiological temperature (37 °C). Moreover, most of the studies performed these experiments at room temperature, therefore, they did not observe the phenomena of *i*-motif formation described here. We note that a recent work has shown that the fragile X chromosome (GCC) repeat folds into an *i*-motif at neutral pH, the authors carried out the experiments at 0 °C, which can further support our results.[32]

The marginal stability of *i*-motif at neutral and slightly alkaline pH, which means *i*-motif formation is disfavoured, raised a question: does it occur in biological conditions? Firstly, Qu's group has demonstrated that SWNTs can increase the stability of *i*-motif,[33] and Hurley and coworkers have shown that cationic porphyrins can promote the formation of *i*-motif.[44] Therefore, some ligands may promote the formation and increase the stability of *i*-motif *in vivo*. Secondly, as discussed above, many proteins have been found that can interact selectively with cytosine-rich sequences.[4–9] We deduce that some proteins may increase the stability of *i*-motif at neutral and slightly alkaline pH by interacting with *i*-motif because a number of cellular proteins have been reported that can promote the formation of G-quadruplex structures *in vivo*.[45,46] For example, the yeast meiosis specific protein, Hop1, can specifically recognize G-rich sequences to promote the formation of G-quadruplex structure *in vivo*.[47] In fact, the formation rate of *i*-motif structure is so slow at neutral condition shown by our kinetic data, consequently, some protein may promote, not only increase the stability of

i-motif, the formation of *i*-motif structure as Hop1 for G-quadruplex structure *in vivo*. Thirdly, it is well known that the biochemical milieu includes multiple components *in vivo*. So, except for the influence on the *i*-motif through the direct contact interactions with SWNT, porphyrins and proteins described above, the *i*-motif also can be affected indirectly by other component *in vivo*.[48] Therefore, there is a possibility that the localized changes resulting from biological processes may stabilize *i*-motif. For example, protons can lead to cross talk between *i*-motifs (composed of TC_5) and poly(A)s that share a common solution milieu.[49]

Another important question need to solve is the protonation of cytosine because the parallel duplexes, the component of *i*-motif, are stabilized by hemiprotonated cytosine$^+$–cytosine base pairs. It is known that the pK_a of cytosine at N3 position is less than 5.[31] In theory, cytosine will be deprotonated and results in the instability of *i*-motif, even transition into random coil at higher pH than 7. It is true because there are few reports showing *i*-motif structure can form at neutral pH. However, previous reports have demonstrated that cytosine$^+$–cytosine base pairs can be formed in hairpin and parallel duplex at neutral and slightly alkaline pH.[50–52] Therefore, cytosine$^+$–cytosine base pairs can be formed under normal physiological cellular conditions. Of course, the stability of cytosine$^+$–cytosine base pairs would be weak under such condition. So, we only observed the formation of *i*-motif at low temperature, while at 25 °C, *i*-motif structures are transit into random coil. Accordingly, besides the well known effect of pH, the temperature is also an important factor for the formation of *i*-motif structures.

Conclusions

Previous studies were well demonstrated that cytosine-rich sequences can form *i*-motif structures under acidic conditions. However, in the present work, results of CD, UV and native PAGE supported compelling evidence that all the oligonucleotides used here can form *i*-motif structures at neutral and slightly alkaline pH (pH 7.1 used in this work) at 4 °C. Melting results revealed that these *i*-motif structures are unstable under physiological pH, therefore, their formation may need the assistant of other ingredients *in vivo*. This work may provide a new insight that *i*-motif can be formed not only under acidic condition, but also at physiological pH and imply their possible biological role.

Experimental

Materials

All DNA oligonucleotides were purchased from Sangon (Shanghai, China) and used as received. Table 1 lists all the DNA sequences and their molar extinction coefficient values used in this work. The molar extinction coefficient values were obtained in the web (http://scitools.idtdna.com/analyzer/Applications/oligoanalyzer/).The strand concentrations were determined by measuring the absorbance at 260 nm at a high temperature. The buffer used in the work was 10 mM sodium cacodylate buffer with different pH values unless otherwise stated. All the cuvette-holding chambers of spectroscopic experiments were flushed with a constant stream of dry N_2 gas to avoid water condensation on the cuvette exterior. Water used in all the experiments was distilled and deionized using a Milli-Q A10 water purification system.

CD spectroscopy

CD spectra were recorded on a dualbeam DSM 1000 CD spectrophotometer (Olis, Bogart, GA) equipped with a Peltier temperature control accessory. Each measurement was the average of three repeated scans recorded from 220 to 320 nm with a 10 mm quartz cell. The scanning rate (nm min^{-1}) was automatically selected by the Olis software as a function of the signal intensity to optimize data collection. A background CD spectrum of corresponding buffer solution was subtracted from the average scan for each sample. T_m of *i*-motif was obtained by the molar ellipticity at 286 nm *versus* temperature profiles. After each temperature changes, samples were allowed to stand for 10 min for the next measurements. The folding kinetics of *i*-motif was determined by the intensity of CD spectra at 286 nm as time course. The data obtained were fitted by Origin 7.0 according to single exponential kinetics equation as follows:

$$\theta_{286\ \mathrm{nm}} = Ae^{-t/\tau} + C \qquad (1)$$

where τ is the time constant of the decay, A is amplitude, t is the time after initiating measurements, and C is the CD intensity at $t = \infty$.

Fluorescence spectroscopy

Fluorescence spectra were taken on a spectrofluorometer model FLS 920 (Edinburgh Analytical Instruments, UK) equipped with a Peltier temperature control accessory. Spectra were collected from 500 to 700 nm while exciting at 480 nm, with both excitation and emission slits being 1 nm. In these measurements, the path lengths of the quartz cell used were 0.2 cm in the excitation direction and 1 cm in the emission direction. The contribution from direct excitation of TAMRA was neglected. All emission spectra were corrected with background fluorescence and instrument response. The fluorescence kinetic of *i*-motif folding was monitored by the decrease in the fluorescence intensity at 515 nm as a function of time. The experimental curves were fit by means of eqn (1).

UV absorbance spectroscopy

UV experiments were carried out on a Shimadzu 2450 spectrophotometer (Shimadzu, Japan) equipped with a Peltier temperature control accessory. All UV/Vis spectra were measured in a sealed quartz cell with a path length of 1.0 cm. Absorption spectra were recorded in the 220–350 nm range with a data interval of 0.1 nm. Melting curves of samples were measured at 295 nm from 2 °C to 25 °C with the temperature gradient of 0.2 °C min^{-1}.

Native polyacrylamide gel electrophoresis

Native gel electrophoresis was carried out on acryamide gel (20%) and run at 4 °C at 50 V (about 5 V cm^{-1}) in 1×TBE buffer (pH 7.0) buffer containing 20 mM KCl. The samples

were treated at 4 °C overnight before loading. After electrophoresis, the gel was stained with stains-all (Sigma) and visualized under white light and photographed by personal digital camera.

Acknowledgements

This work was supported by The National Natural Science Foundation of China (grant numbers 20673110, 20621063).

Notes and references

1 K. Gehring, J. L. Leroy and M. Gueron, *Nature*, 1993, **363**, 561–565.
2 M. Gueron and J. L. Leroy, *Curr. Opin. Struct. Biol.*, 2000, **10**, 326–331.
3 P. Catasti, X. Chen, S. V. Mariappan, E. M. Bradbury and G. Gupta, *Genetica*, 1999, **106**, 15–36.
4 E. Marsich, A. Piccini, L. E. Xodo and G. Manzini, *Nucleic Acids Res.*, 1996, **24**, 4029–4033.
5 E. Marsich, L. E. Xodo and G. Manzini, *Eur. J. Biochem.*, 1998, **258**, 93–99.
6 L. Lacroix, H. Lienard, E. Labourier, M. D. Mergny, J. Lacoste, H. Leffers, J. Tazi, C. Helene and J. L. Mergny, *Nucleic Acids Res.*, 2000, **28**, 1564–1575.
7 J. F. Cornuel, A. Moraillon and M. Guéron, *Biochimie*, 2002, **84**, 279–289.
8 M. Takimoto, T. Tomonaga, M. Matunis, M. Avigan, H. Krutzsch, G. Dreyfuss and D. Levens, *J. Biol. Chem.*, 1993, **268**, 18249–18258.
9 E. F. Michelotti, G. A. Michelotti, A. I. Aronsohn and D. Levens, *Mol. Cell Biol.*, 1996, **16**, 2350–2360.
10 D. Liu and S. Balasubramanian, *Angew. Chem., Int. Ed.*, 2003, **42**, 5734–5736.
11 T. Liedl and F. Simmel, *Nano Lett.*, 2005, **5**, 1894–1898.
12 P. Alberti, A. Bourdoncle, B. Saccà, L. Lacroix and J. L. Mergny, *Org. Biomol. Chem.*, 2006, **4**, 3383–3391.
13 S. Modi, M. G. Swetha, D. Goswami, G. D. Gupta, S. Mayor and Y. Krishnan, *Nat. Nanotechnol.*, 2009, **4**, 325–330.
14 J. L. Leroy, M. Guéron, J. L. Mergny and C. Hélène, *Nucleic Acids Res.*, 1994, **22**, 1600–1606.
15 G. Manzini, N. Yathindra and L. F. Xodo, *Nucleic Acids Res.*, 1994, **22**, 4634–4640.
16 T. Simonsson, M. Pribylova and M. Vorlickova, *Biochem. Biophys. Res. Commun.*, 2000, **278**, 158–166.
17 S. Nonin-Lecomte and J. L. Leroy, *J. Mol. Biol.*, 2001, **309**, 491–506.
18 N. Khan, A. Avino, R. Tauler, C. González, R. Eritja and R. Gargallo, *Biochimie*, 2007, **89**, 1562–1572.
19 J. L. Mergny and L. Lacroix, *Nucleic Acids Res.*, 1998, **26**, 4797–4803.
20 M. Kaushik, N. Suehl and L. A. Marky, *Biophys. Chem.*, 2007, **126**, 154–164.
21 V. Mathur, A. Verma, S. Maiti and S. Chowdhury, *Biochem. Biophys. Res. Commun.*, 2004, **320**, 1220–1227.
22 S. Ahmed, A. Kintanar and E. Henderson, *Nat. Struct. Biol.*, 1994, **1**, 83–88.
23 C. Kang, I. Berger, C. Lockshin, R. Ratliff, R. Moyzis and A. Rich, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 3874–3878.
24 S. Nonin and J. L. Leroy, *J. Mol. Biol.*, 1996, **261**, 399–414.
25 X. Han, J. L. Leroy and M. Guéron, *J. Mol. Biol.*, 1998, **278**, 949–965.
26 S. Nonin-Lecomte and J. L. Leroy, *J. Mol. Biol.*, 2001, **309**, 491–506.
27 K. Kanaori, N. Hibayama, K. Gohda, K. Tajima and K. Makino, *Nucleic Acids Res.*, 2001, **29**, 831–840.
28 M. Canalia and J. L. Leroy, *Nucleic Acids Res.*, 2005, **33**, 5471–5481.
29 N. Esmaili and J. L. Leroy, *Nucleic Acids Res.*, 2005, **33**, 213–224.
30 J. Dai, A. Ambrus, L. H. Hurley and D. Yang, *J. Am. Chem. Soc.*, 2009, **131**, 6102–6104.
31 J. L. Mergny, L. Lacroix, X. Han, J. L. Leroy and C. Hélène, *J. Am. Chem. Soc.*, 1995, **117**, 8887–8898.
32 P. Fojtík and M. Vorlícková, *Nucleic Acids Res.*, 2001, **29**, 4684–4690.
33 X. Li, Y. Peng, J. Ren and X. Qu, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 19658–19663.
34 C. Zhao, J. Ren and X. Qu, *Chem.–Eur. J.*, 2008, **14**, 5435–5439.
35 Y. Xu and H. Sugiyama, *Nucleic Acids Res.*, 2006, **34**, 949–954.
36 K. Guo, A. Pourpak, K. Beetz-Rogers, V. Gokhale, D. Sun and L. H. Hurley, *J. Am. Chem. Soc.*, 2007, **129**, 10220–10228.
37 H. Fukada and K. Takahashi, *Proteins: Struct., Funct., Genet.*, 1998, **33**, 159–166.
38 J. L. Mergny, J. Li, L. Lacroix, S. Amrane and J. B. Chaires, *Nucleic Acids Res.*, 2005, **33**, e138.
39 J. L. Mergny and L. Lacroix, *Nucleic Acids Res.*, 1998, **26**, 4797–4803.
40 J. L. Mergny, *Biochemistry*, 1999, **38**, 1573–1581.
41 H. Ueyama, M. Takagi and S. Takenaka, *J. Am. Chem. Soc.*, 2002, **124**, 14286–14287.
42 J. L. Huppert and S. Balasubramanian, *Nucleic Acids Res.*, 2005, **33**, 2908–2916.
43 A. K. Todd, M. Johnston and S. Neidle, *Nucleic Acids Res.*, 2005, **33**, 2901–2907.
44 O. Y. Fedoroff, A. Rangan, V. V. Chemeris and L. H. Hurley, *Biochemistry*, 2000, **39**, 15083–15090.
45 T. M. Bryan and M. B. Jarstfer, *Methods*, 2007, **43**, 332–339.
46 M. Fry, *Front. Biosci.*, 2007, **12**, 4336–4351.
47 K. Muniyappa, S. Anuradha and B. Byers, *Mol. Cell. Biol.*, 2000, **20**, 1361–1369.
48 J. VÖlker and K. J. Breslauer, *Annu. Rev. Biophys. Biomol. Struct.*, 2005, **34**, 21–42.
49 J. VÖlker, H. H. Klump and K. J. Breslauer, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 7694–7699.
50 A. Yu, M. D. Barron, R. M. Romero, M. Christy, B. Gold, J. Dai, D. M. Gray, I. S. Haworth and M. Mitas, *Biochemistry*, 1997, **36**, 3687–3699.
51 M. Vorlíčková, I. Kejnovská, M. Tumová and J. Kypr, *Eur. Biophys. J.*, 2001, **30**, 179–185.
52 I. Kejnovská, M. Tumová and M. Vorlíčková, *Biochim. Biophys. Acta.*, 2001, **1527**, 73–80.

