Research Article

# Effect of Divalent ions on the structure and stability of c-tetraplex

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**Abstract**

Most of the important genomic regions, especially the G,C rich gene promoters consist of sequences, potentially able to form G,C-tetraplexes on both the DNA strands. In current studies we used three C-rich oligonucleotides (11Py, 21Py and HTPy), of which the 11Py and 21Py are located at various transcriptional regulatory elements of human genome while the HTPy sequence is a C-rich strand of human telomere sequence. These C-rich oligonucleotides formed i-motif structures, verified by Circular Dichroism (CD), UV absorption melting experiments, and native gel electrophoresis. The CD spectra revealed that 11Py and 21Py form i-motif structures at acidic pH values 4.5 and 5.7, in the presence of 100 mM NaCl and remain unstructured at pH 7.0. However, 21Py can form stable i-motif structure even at neutral pH when 1 mM $MgCl_2$ was added to the solution. UV-thermal melting studies suggested the stabilization of i-motif structure of 21Py at pH 5.7 in the presence of $Na^+$ or $K^+$ with increasing the concentration of $MgCl_2$ or $CaCl_2$ from 1 mM to 10 mM. Significant shift in the CD peak of HTPy sequence is observed as the positive peak from 286 nm shifted to 276 nm while the negative peak from 265 nm to 254 nm. There is inevitable necessity of 1 mM $Mg^{2+}$ to form i-motif structure at neutral pH. Under similar ionic conditions and neutral pH, all the three sequences can form stable i-motif structures (11Py, 21Py) or altered i-motif /homoduplex structures (HTPy) in the presence of $MgCl_2$ and cell mimicking molecular crowding conditions of 40 wt% PEG 200. It is suggested that presence of $Mg^{2+}$ ions and molecular crowding agents induce and stabilize i-motif structures at physiological solution environment.

**Keywords:** c-tetraplex, i-motif, thermal melting, CD (Circular Dichroism)

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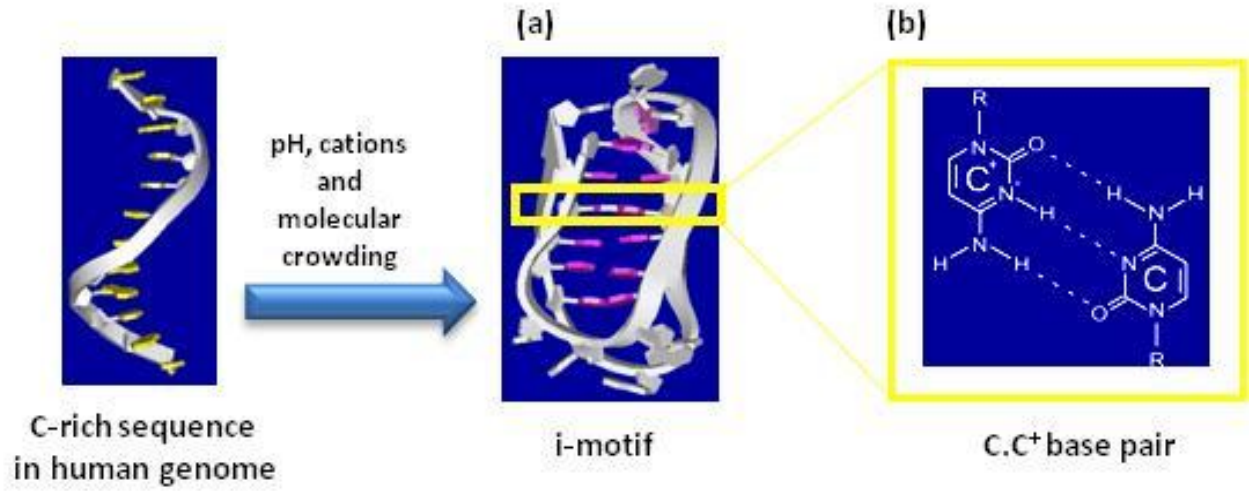
## Introduction

DNA sequences containing consecutive runs of guanines (G) and cytosines (C) are prevalent in the human genome and normally found at the regulatory regions of various genes in humans including telomere of eukaryotic chromosomes. It is interesting that these repetitive G-rich and C-rich sequences can combine to form DNA double helix by Watson-Crick base-pairing while each single strand can dissociate to form self-associated tetraplex structures [1, 2]. Structural studies using NMR, X-ray Crystallography and Circular Dichroism (CD) well documented that repetitive G-rich and C-rich DNA sequences can fold to form intermolecular or intramolecular tetraplex DNA structures interconnected with loop regions of varying lengths [3-6]. G-rich DNA sequences can form G-quadruplex structure consisting of stacked G-tetrads stabilized by Hoogsteen bonding in the presence of monovalent cations either $K^+$ or $Na^+$ [3, 4]. C-rich DNA sequences can form second type of quadruplex structure called i-motif either at slightly acidic or neutral pH [5]. This so called i-motif is a tetramer of equivalent strands in which C. $C^+$ base pair of two parallel duplexes face each other and stabilized by three hydrogen bonds [6] (shown in **scheme 1**). The structure and stability of these four stranded structures strongly depend on the sequence and solution conditions, such as temperature, pH, and metals ions especially $K^+$ and $Na^+$ [6-8]. Recent reports suggest that G-qaudruplex forming motifs in oncogenes can form G-qaudruplex structures in the presence of physiological monovalent ions and $Mg^{2+}$ conditions which can enhance the thermal stability of these structures. These results highlight the fact that those G-rich sequences in oncogenes favor the formation of G-quadruplex by Hoogsteen bonding rather than canonical Watson–Crick base-pairing to form duplex [9]. However, in *Escherichia coli* the intracellular concentration of the biomolecules is in the range of 300-400 g/L [10] which forms molecularly crowding environment. This is distinct from typical biomolecular concentrations of less than 1 g/L which forms dilute conditions. Therefore, to carry out the cellular functions most of the biochemical reactions in a living cell progress under the crowded environment of various biomolecules which occupies 20-40% of the total volume [11-14]. Most of the studies on i-motif structure and stability have been done in dilute solutions at acidic pH containing monovalent cations with little or no $Mg^{2+}$ and co-solute or molecular crowding agent present at neutral pH. As both G-rich and C-rich strands face same microenvironment of molecular crowding containing different cations, therefore, in the present work we investigated the potential of some C-rich oligonucleotides to form stable i-motif structures at physiological pH, ionic conditions

and cell-mimicking molecular crowding environments. We used human telomeric sequence (HTPy) as a model system and other two C-rich oligonucleotides of varied lengths namely 11Py - 5′-CCTCCCCCTCC-3′ and 21Py- 5′-TTTCCCC**CCTCCCCCTCC**CCC-3′, located in the transcriptional regulatory region of most of the genes. We employed spectroscopic techniques (CD, UV-thermal denaturation) and native PAGE to characterize the formation of i-motif structure. Our data suggests that these C-rich sequences form i-motif structure at acidic pH, however $Mg^{2+}$ can promote the i-motif formation even at neutral pH, depending on the length of the sequence. Cell mimicking molecular crowding conditions of 40 wt% PEG 200 were found to be most suitable for i-motif formation for all the three oligonucleotides. This finding suggests that intracellular conditions where hydration becomes less favorable may facilitate the folding of C-rich sequences into i-motif structure at physiological pH and cation concentration.




**Scheme 1** Schematic representation of c-rich single strand into i-motif structure (a) and $C.C^+$ base pairing (b)

## Materials and Methods

*DNA Sequences.* PAGE purified DNA sequences were purchased from Life Technologies (Delhi, India). The single-strand concentrations of the DNA oligonucleotides were determined by measuring the absorbance at 260 nm at high temperature using a spectrophotometer (1800; Shimadzu, Tokyo, Japan) connected to a peltier thermo-programmer. The single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data using the nearest neighbor approximation [15-16].

### *Circular Dichroism (CD) Measurements*

CD spectrophotometer (JASCO J-815) was used to record the CD spectra of the samples using a 0.1 cm path length quartz cell at room temperature. The cell holder was thermostated by a JASCO PTC-348 temperature controller and to avoid water condensation on the cuvette exterior, the cuvette-holding chamber was flushed with a constant stream of dry N2 gas. The CD spectrum was averaged of at least three scans from 200 nm to 350 nm. Samples were prepared in 30 mM sodium cacodylate buffer (pH 5.7 or pH 7.0) containing 0.5 mM EDTA, 100 mM NaCl or 100 mM KCl with or without 1 mM $MgCl_2$ or 10 mM $MgCl_2$, or 1 mM $CaCl_2$ at 0 wt% PEG 200 or 40 wt% PEG 200. Before measurement, the samples were heated to 95$^o$C, gently allowed to cool down up to room temperature followed by incubation at 4$^o$C overnight to ensure the target C-rich sequences fold to form i-motif structures or other stable higher order structures.

### *UV Melting Analysis*

To investigate the thermal stabilities of the non-protonated and protonated structures of 21Py, 11Py and HTPy sequences, UV absorbance melting profiles were obtained in 30 mM sodium cacodylate buffer (pH 5.7 or pH 7.0) containing 0.5 mM EDTA, 100 mM NaCl or 100 mM KCl with or without 1 mM $MgCl_2$ or 1 mM $CaCl_2$ at 0 wt% PEG 200 or 40 wt% PEG 200 and melting profiles were recorded at 265 nm and 295 nm. UV absorbance was measured with a Shimadzu 1800 spectrophotometer equipped with the temperature controller. The melting temperature (*T*m) values for DNA structures were obtained from the UV melting curves as described previously [16]. The heating rate was 0.5 $^o$C min$^{-1}$.

***Nondenaturing Gel Electrophoresis***

Electrophoresis was carried out on 15% polyacrylamide gels (19:1 acrylamide/bisacrylamide) under non denaturating conditions. DNA samples of 15 $\mu$M were mixed with ice-cold loading buffer. Samples were prepared in 30 mM sodium cacodylate buffer (pH 5.7) containing 0.5 mM EDTA, 100 mM $Na^+$ or 100 mM $Na^+$ + 1 mM $Mg^{2+}$. Finally, 8 $\mu$L aliquot of the mixed solution was loaded. The electrophoresis was run at 5 V $cm^{-1}$ at 25$^o$C in 1x TBE buffer. The gel was stained using Stains-All (Sigma-Aldrich) and imaged using Gel-Doc (Biorad).

***Sequence Design***

We carried out the sequence analysis of 11Py and 21Py in human genome data base and identified the location of these sequences in human genome using BLAST. Bioinformatics suggest that these target sequences are frequently present in the regulatory regions of various genes such as 3′-UTR of c-jun proto-oncogene, neuroplastin isoform b precursor, CD276 antigen isoform a precursor, melanoma associated antigen G1, tight junction protein ZO-1 isoform b, protein CASC5 isoform 2, protein GRINL1A isoform 2, retinal dehydrogenase 2 isoform 2, transducin-like enhancer protein 3 isoform g, arpin isoform 2, transient receptor potential cation channel subfamily, coronin-2B isoform 1 and 2 etc. Therefore, it is sufficiently important to examine the functional significance of these repetitive elements by studying their structural behavior. With the aim of elucidating the structural polymorphism of DNA oligonucleotides composed of C-rich sequences. We have investigated the following three C-rich oligonucleotides (a) 11Py (5′-CCTCCCCCTCC-3′) (b) 21Py (5′- TTTCCCC**CCTCCCCCTCC**CCC) and (c) HTPy (5′-CCCTAACCC TAACCCTAACCCT-3′). The HTPy represents the human telomeric repeat. As these sequences are important biological targets, we studied their solution structure in cell-mimicking physiological conditions to understand their biological implications.

## Results and Discussion

***Effect of monovalent (NaCl) and divalent cations ($MgCl_2$) on i-motif structure***

CD experiments were used to establish the presence of i-motif DNA structure in solution. It is well known that CD spectrum for a typical i-motif DNA structure exhibits a positive peak at (or near) 286 nm followed by a negative peak at 265 nm [17] while the B-DNA like structure exhibits a positive peak at (or near) 275 nm and negative peak at 254 nm [11, 18]. **Figure 1a** shows the CD spectra of 4 $\mu$M 11Py in sodium cacodylate buffer at varied pH (4.5, 5.7 and 7.0), containing 100 mM NaCl. At acidic pH, the CD spectrum of 11Py showed signature peak of i-motif formation [19, 20]. Nevertheless, the positive peak at 286 nm sharply decreased depicting a loss of secondary structure at pH 7.0. This could be due to the deprotonation of cytosines reflecting in a decrease of the $C.C^+$ base pairing. Similarly, the CD spectra of 21Py under similar conditions suggested the formation of i-motif structure at acidic pH, which consequently got unstructured at pH 7.0 (Figure 1b). Overall, these results indicate that both 11Py and 21Py sequences have a strong preference for the formation of i-motif structure at acidic pH in the presence of 100 mM NaCl, consistent with previous structural studies of i-motif [5-7]. The CD spectra of HTPy in the presence of 100 mM NaCl showed the maximum ellipticity at 275 nm and the minimum at 254 nm, suggesting the formation of a structure other than i-motif form. We interpret this to the formation of some parallel stranded homoduplex comprising of $C{\cdot}C^+$ base pairing, but lacking the intercalated nature of i-motif structure. This result is in agreement with the previous report that the insertion of G or T within C rich sequence may shift the equilibrium from native i-motif structure to parallel DNA structure with $C{\cdot}C^+$ pairing [21]. However, the positive peak at 275 nm sharply decreased with the increase in pH of the solution, possibly due to the deprotonation of C at pH 7.0 (Figure 1c).

Further, to see the effect of $Mg^{2+}$ on i-motif structure CD spectra of C-rich sequences were recorded at different concentrations of $Mg^{2+}$. CD spectra of 11Py displayed characteristic CD signature of i-motif only at acidic pH, in the presence of 100 mM NaCl and 1 mM $MgCl_2$ (Figure 1d) / 10 mM $MgCl_2$ at pH 7.0 (**Figure 2a**). Increase in pH did not stabilize i-motif form of 11Py even in the presence of 10 mM concentration of divalent cation ($Mg^{2+}$) (Figure 2a). Similar studies were carried out for the information on the structural status of 21Py, which is just an extended version of 11Py and contains 11Py as its integral part. Interestingly, CD results of 21Py displayed prominent CD signatures characteristic of i-motif and the molar ellipticity did not change due to the changes in pH. Physiological cationic concentrations of NaCl and $MgCl_2$ (Figures 1e)/10 mM $MgCl_2$ at pH 7.0 stabilized the i-motif form (Figures 2b). Interestingly, under similar solution conditions CD spectra of HTPy did not display signatures of i-motif form but exhibited homoduplex formation possibly mediated by the $C{\cdot}C^+$ base pairing at acidic and even at neutral pH (Figures 1f) /10 mM $MgCl_2$ at pH 7.0 (Figures 2c). Therefore, based on CD findings, we propose that $Mg^{2+}$ may promote the formation of i-motif or other self associated structures. Also, there lies a possibility of end to end stacking interactions of terminal C's at 3′ terminus of 21Py sequence. However, it may depend on the length of the C-rich sequences [22].

As it is well known that i-motif structure is a tetramer of equivalent strands, which represents the novel feature of intercalated C.C$^+$ base pairs of two parallel-stranded duplexes, therefore, more the length, more will be the repulsion due to the negative charges of phosphates. As the ionic strength of $Mg^{2+}$ ion is more in comparison to monovalent cation ($Na^+$ or $K^+$), therefore, $Mg^{2+}$ is likely to contribute to the partial neutralization of negative charges of the molecule, which in turn promotes the dimerization. As the i-motif structure is a dimer of two parallel-stranded duplexes, therefore, $Mg^{2+}$ is a suitable cation for promoting the formation of i-motif structure. Therefore longer the sequence more will be the stabilization due to enhanced cation binding.

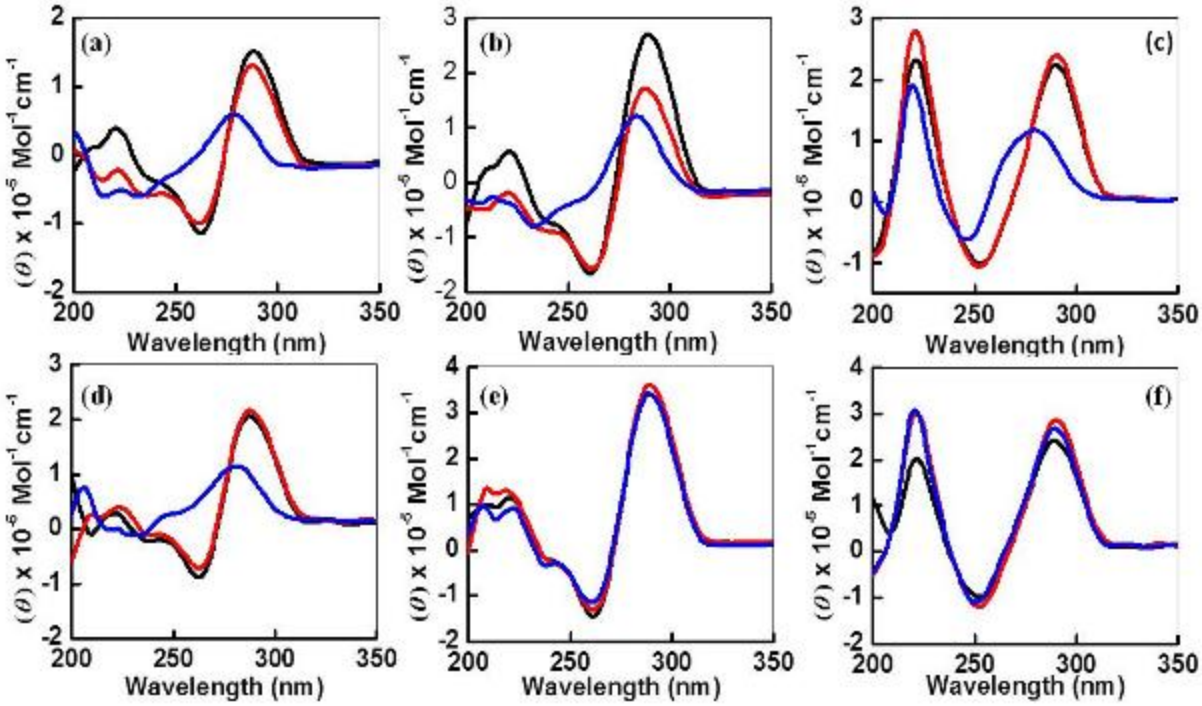



**Figure 1** CD spectra of 4$\mu$M 11Py (a,d), 21Py (b,e), HTPy (c,f) in 30 mM sodium cacodylate buffer (pH 4.5) (black), (pH 5.7) (red), (pH 7.0) (blue), 0.5 mM EDTA, 100 mM NaCl (a, b and c) and 100 mM NaCl + 1 mM $MgCl_2$ (d, e and f) at 25$^o$C respectively

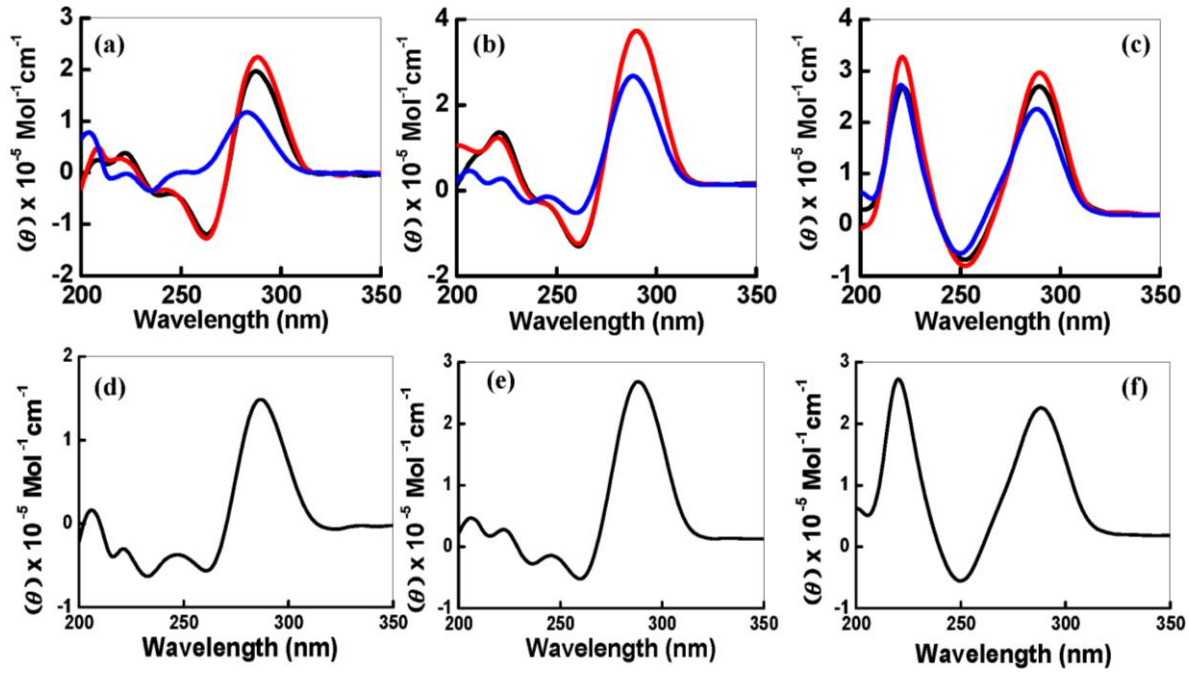



**Figure 2** CD spectra of 4$\mu$M 11Py (a), 21Py (b) and HTPy (c) in 30 mM sodium cacodylate buffer (pH 4.5) (black), (pH 5.7) (red), (pH 7.0) (blue), 100 mM NaCl + 10 mM $MgCl_2$ and 0.5 mM EDTA at 25$^o$C. 11Py (d), 21Py (e), and HTPy (f) in 30 mM sodium cacodylate buffer (pH 7.0), 100 mM NaCl + 1 mM $MgCl_2$, 0.5 mM EDTA and 40 wt% PEG 200 at 25$^o$C respectively

To study the effect of divalent cation on i-motif structure in detail, we also recorded the CD spectra of 21Py at acidic pH 5.7 in the presence of increasing concentration of $MgCl_2$ (1 mM, 3 mM, 5 mM, 7 mM, 10 mM) (Figure S1a) or $CaCl_2$ (1 mM, 3 mM, 5 mM, 7 mM, 10 mM) (Figure S1b). CD results of 21Py displayed prominent CD signatures characteristic of i-motif structure without any significant changes in their molar ellipticity in both the ionic conditions which clearly indicates that both the divalent ions ($Mg^{2+}$ or $Ca^{2+}$) have similar effect on i-motif structure of 21Py.

***Effect of Molecular Crowding on i-motif structures***

To determine the effect of molecular crowding, the CD spectra of all three sequences were recorded in buffer containing physiological ionic concentrations (100 mM NaCl and 1 mM $MgCl_2$) and 40 wt% PEG 200 at pH 7.0. CD spectra of 11Py (Figure 2d) and 21Py (Figure 2e) showed prominent i-motif signatures as 286 nm positive and at 265 nm negative peaks at pH 7.0. Under similar solution condition the sequence HTPy showed signatures of $C{\cdot}C^+$ homoduplex structure (Figure 2f). In all the cases, CD signatures were more prominent in the presence of 40 wt% PEG 200. These results support our speculation that the presence of divalent cation $Mg^{++}$ and PEG 200 (40 wt %) can induce and stabilize the i-motif structure in C-rich sequences at physiological pH, which were not formed otherwise.

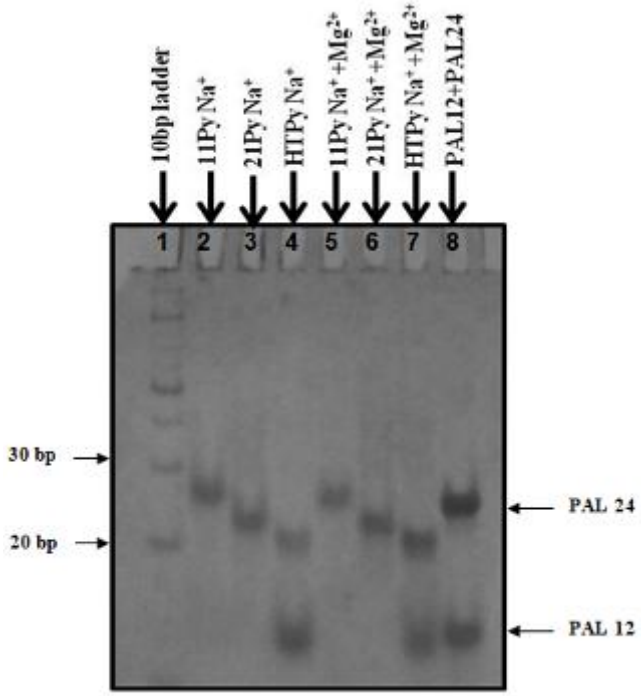



**Figure 3** Native gel electrophoresis of 11Py, 21Py and HTPy sequences in 30 mM sodium cacodylate buffer (pH 5.7), 100 mM $Na^+$, 11Py (Lane 2), 21Py (Lane 3), HTPy (Lane 4) and 100 mM $Na^+$ + 1 mM $Mg^{++}$, 11Py (Lane 5), 21Py (Lane 6), HTPy (Lane 7) respectively. 10 bp ladder in (Lane 1) and PAL 12 and PAL 24 (M) in (Lane 8) were used as size markers

The exact structural status of 11Py, 21Py and HTPy in terms of molecularity (size) on the basis of its mobility on native PAGE was achieved by comparing with the gel mobility of control oligomer band of 10bp ladder (Lane 1) and palindromic sequences (PAL 12 and PAL 24) (Lane 8) (**Figure 3**). Samples were prepared in sodium cacodylate buffer pH 5.7 containing 100 mM $Na^+$ or 100 mM $Na^+$ and 1 mM $Mg^{2+}$ and loaded as 11Py (Lane 2 and Lane 5), 21Py (Lane 3 and Lane 6) and HTPy (Lane 4 and Lane 7). The samples were incubated overnight at 4$^o$C. The molecularity of 11Py was compared with 10 bp ladder and palindromic sequences (PAL 12), 11Py migrated equivalent to tetramolecular (tetramer) i-motif geometries in both ionic conditions. 21Py migrated as dimeric i-motif in similar solution conditions. The same band of the proposed dimeric i-motif was found to be migrating little faster in the presence of 100 mM $Na^+$ and 1 mM $Mg^{2+}$ than the control sequence PAL-24 in Lane 8. These differences in the mobility can be attributed to the compact i-motif structure whose mobility is usually higher than the control PAL-24 which contains 12 (G′s). Thus, the gel mobility of structural species of 21Py suggests that there exist only a bimolecular i-motif topology for 21Py. Next, we checked the gel mobility of HTPy under similar solution conditions which migrated as two bands. The mobility of the upper band was close to PAL 24bp and 20bp in 10bp ladder while the lower band was close to PAL 12 and 10bp in 10bp ladder. This indicates that HTPy migrated as dimeric i-motif

while the lower band corresponds to single strand species. There was no observable difference in the mobility of the bands in all three sequences in the presence and absence of $Mg^{2+}$ which indicates that there is no difference in the structural states of these sequences. These results are consistent with the previously published report which has demonstrated the effect of different cations on oligomer structures at one pH in native PAGE [23].

***Stability of i-motif structure***

It is well known that the thermal denaturation of i-motif shows to hyperchromism at 265 nm and to hypochromism at 295 nm [11]. Therefore, the melting profiles of each of the C-rich oligonucleotides were recorded under various experimental conditions with or without co-solutes. *T*m values were calculated from the first derivative of the absorbance versus temperature profiles and are tabulated in **Table 1** and **Table 2**. To check the effect of monovalent cations, samples were prepared in sodium cacodylate buffer of pH 5.7 or pH7.0 containing either and 100 mM NaCl or 100 mM KCl (**Figure 4**). None of the investigated sequence exhibited a melting profile at pH 7.0 (data not shown). This could be due to the inability to form a stable structure under these conditions consistent with our CD results. We evaluated the $T_m$ values by curve fitting, and the $T_m$ values in the melting curves were (24.5$^o$C), (44.5$^o$C), and (37.5) in the presence of both monovalent cations ($Na^+$ or $K^+$) in case of 11Py (Figure 4a), 21Py (Figure 4b) and HTPy (Figure 4c) respectively at 265 nm and at 295 nm. It is clearly suggested that low pH condition favored the C-protonation and hence the formation of i-motif structures. However, we observed the significant changes in $T_m$ values of (~10$^o$C to 11$^o$C) in the presence of 100 mM NaCl or 100 mM KCl with and without 1 mM $MgCl_2$ or 1 mM $CaCl_2$ respectively (**Figure 5a** and 5b). The denaturation and renaturation data of 11Py was recorded and given in (Figure S2). These results clearly indicate the significant effect on the stabilization of i-motif structure of 11Py by the divalent cations. It is noteworthy that the effect of divalent cations ($Mg^{2+}$ or $Ca^{2+}$) was found to be almost comparable on the stability of i-motif structure of 11Py. Interestingly, 11Py exhibited a melting profile at pH 7.0 in the presence of 100 mM NaCl + 1 mM $MgCl_2$ and 40 wt% PEG 200 with $T_m$ value 30.0$^o$C (Figure 6a and 6b). This result clearly suggests that the molecular crowding induce the i-motif structure at physiological cationic conditions and physiological pH. The effect of divalent ions is well documented on G-quadruplex. Miyoshi et. al., 2003 reported the structural transition in antiparallel G-quadruplex to parallel G-quadruplex and hence into parallel stranded G-wire structure in the presence of $Ca^{2+}$ [24]. Mita et. al., 2006 reported that the $Mg^{2+}$ ions neutralize the negative charges of the molecule, which in turn promotes the dimerization of the G-quadruplex [25]. As the topology of i-motif is concerned, it is a structure which consists of two parallel-stranded $C.C^+$ base-paired helices zipped together in an antiparallel orientation. Therefore, the stabilization of i-motif structure in the presence of divalent ions is consistent with the previously published reports on the effect of divalent ions on G-quadruplex.

**Table 1** Thermodynamic Parameters for the Structural Formations of i-motif structure at 265 nm[a]

| Sequence & conditions | $\Delta H^o$ (kcal mol$^{-1}$) | $T\Delta S^o$ (kcal mol$^{-1}$) | $\Delta G^o_{25}$ (kcal mol$^{-1}$) | $T_m$ ($^o$C) |
|---|---|---|---|---|
| 11Py (pH 5.7, 100mM $Na^+$) | -78 | -75.7 | -2.3 | 24.5 |
| 11Py (pH 5.7, 100mM $K^+$) | -72 | -69.9 | -2.1 | 23.9 |
| 11Py (pH 5.7, 100mM $Na^+$+ 1mM $Mg^{++}$) | -75.7 | -73.4 | -2.3 | 34 |
| 11Py (pH 5.7, 100mM $K^+$+ 1mM $Mg^{++}$) | -72.4 | -70.3 | -2.1 | 35.1 |
| 11Py (pH 5.7, 100mM $Na^+$+ 1mM $Ca^{++}$) | -72.5 | -70.3 | -2.2 | 34.6 |
| 11Py (pH 5.7, 100mM $K^+$+ 1mM $Ca^{++}$) | -72.6 | -70.4 | -2.2 | 34.3 |
| 21Py (pH 5.7, 100mM $Na^+$) | -40.5 | -36.7 | -3.8 | 44.5 |
| 21Py (pH 5.7, 100mM $K^+$) | -36.9 | -33.4 | -3.5 | 44 |
| 21Py (pH 5.7, 100mM $Na^+$+ 1mM $Mg^{++}$) | -38 | -34.3 | -3.7 | 57 |
| 21Py (pH 5.7, 100mM $K^+$+ 1mM $Mg^{++}$) | -29.3 | -26.5 | -2.7 | 56.6 |
| 21Py (pH 5.7, 100mM $Na^+$+ 1mM $Ca^{++}$) | -32.8 | -29.7 | -3.1 | 55.5 |
| 21Py (pH 5.7, 100mM $K^+$+ 1mM $Ca^{++}$) | -33.9 | -30.6 | -3.2 | 56.6 |
| HTPy (pH 5.7, 100mM $Na^+$) | -61.4 | -58.1 | -3.2 | 37.5 |
| HTPy (pH 5.7, 100mM $K^+$) | -59.4 | -56.3 | -3.1 | 37.5 |
| HTPy (pH 5.7, 100mM $Na^+$+ 1mM $Mg^{++}$) | -58.2 | -55.1 | -3.1 | 41.6 |
| HTPy (pH 5.7, 100mM $K^+$+ 1mM $Mg^{++}$) | -56 | -52.7 | -3.3 | 43.7 |
| HTPy (pH 5.7, 100mM $Na^+$+ 1mM $Ca^{++}$) | -58 | -54.8 | -3.2 | 42.5 |
| HTPy (pH 5.7, 100mM $K^+$+ 1mM $Ca^{++}$) | -57.7 | -54.2 | -3.5 | 43.9 |

[a]All experiments were carried out in a buffer containing 30 mM sodium cacodylate (pH 5.7), 1m M NaCl, 1mM KCl, 1m M NaCl + 1mM $MgCl_2$, 1mM KCl+1mM $MgCl_2$, 1m M NaCl + 1mM $CaCl_2$, 1mM KCl+1mM $CaCl_2$ and 0.5 mM EDTA. Values are obtained from the curve fitting. Melting temperatures were measured at a total strand concentration of 4$\mu$M. The standard deviation in $T_m$ is in range of ± 1$^o$C.

**Table 2** Thermodynamic Parameters for the Structural Formations of i-motif structure at 295 nm[a]

| Sequence & conditions | $\Delta H^o$ (kcal mol$^{-1}$) | $T\Delta S^o$ (kcal mol$^{-1}$) | $\Delta G^o 25$ (kcal mol$^{-1}$) | $T_m$ ($^o$C) |
|---|---|---|---|---|
| 11Py (pH 5.7, 100mM $Na^+$) | -54.8 | -53.4 | -1.4 | 24.5 |
| 11Py (pH 5.7, 100mM $K^+$) | -58.8 | -57.3 | -1.5 | 23.9 |
| 11Py (pH 5.7, 100mM $Na^+$ + 1mM $Mg^{++}$) | -48.2 | -47.2 | -1.1 | 34 |
| 11Py (pH 5.7, 100mM $K^+$ + 1mM $Mg^{++}$) | -55.2 | -53.7 | -1.5 | 35.1 |
| 11Py (pH 5.7, 100mM $Na^+$ + 1mM $Ca^{++}$) | -55.5 | -54 | -1.6 | 34.6 |
| 11Py (pH 5.7, 100mM $K^+$ + 1mM $Mg^{++}$) | -55.3 | -53.8 | -1.5 | 34.3 |
| 21Py (pH 5.7, 100mM $Na^+$) | -40.5 | -36.7 | -3.8 | 44.5 |
| 21Py (pH 5.7, 100mM $K^+$) | -58.8 | -57.3 | -3.5 | 44 |
| 21Py (pH 5.7, 100mM $Na^+$ + 1mM $Mg^{++}$) | -34.8 | -31.3 | -3.5 | 57 |
| 21Py (pH 5.7, 100mM $K^+$ + 1mM $Mg^{++}$) | -27.2 | -24.8 | -2.4 | 56.6 |
| 21Py (pH 5.7, 100mM $Na^+$ + 1mM $Ca^{++}$) | -33.4 | -30.4 | -3 | 56.3 |
| 21Py (pH 5.7, 100mM $K^+$ + 1mM $Ca^{++}$) | -31.3 | -28.3 | -3 | 56.4 |
| HTPy (pH 5.7, 100mM $Na^+$) | -68.1 | -64.3 | -3.7 | 37.5 |
| HTPy (pH 5.7, 100mM $K^+$) | -64.6 | -61.1 | -3.6 | 37.5 |
| HTPy (pH 5.7, 100mM $Na^+$ + 1mM $Mg^{++}$) | -61.8 | -58.5 | -3.3 | 41.6 |
| HTPy (pH 5.7, 100mM $K^+$ + 1mM $Mg^{++}$) | -57.4 | -54 | -3.5 | 44 |
| HTPy (pH 5.7, 100mM $Na^+$ + 1mM $Ca^{++}$) | -63.3 | -59.7 | -3.6 | 42.6 |
| HTPy (pH 5.7, 100mM $K^+$ + 1mM $Ca^{++}$) | -59.5 | -55.9 | -3.6 | 43.9 |

[a]All experiments were carried out in a buffer containing 30 mM sodium cacodylate (pH 5.7), 1m M NaCl, 1mM KCl, 1m M NaCl + 1mM $MgCl_2$, 1mM KCl+1mM $MgCl_2$, 1m M NaCl + 1mM $CaCl_2$, 1mM KCl+1mM $CaCl_2$ and 0.5 mM EDTA. Values are obtained from the curve fitting. Melting temperatures were measured at a total strand concentration of 4$\mu$M. The standard deviation in $T_m$ is in range of ± 1$^o$C.

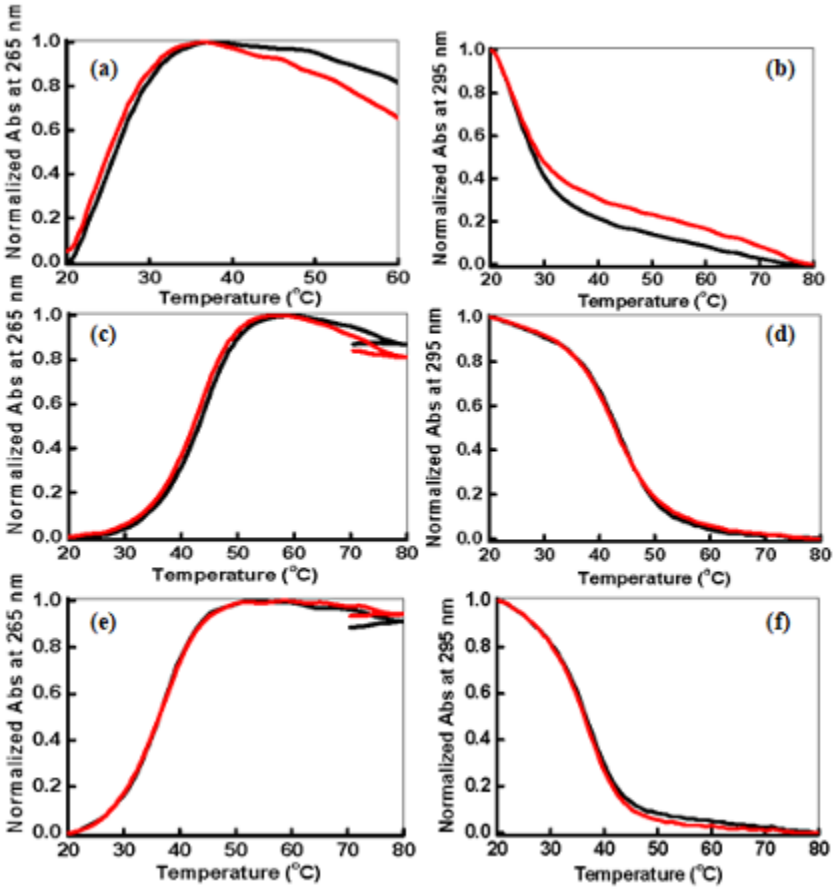



**Figure 4** Normalized UV melting curves of 4$\mu M$ 11Py (a and b), 21Py (c and d) and HTPy (e and f) in 30 mM sodium cacodylate buffer (pH 5.7), 0.5 mM EDTA, 100 mM NaCl (black) and 100 mM KCl (red) at 265 nm (a, c and e) and at 295 nm (b, d and f) respectively.

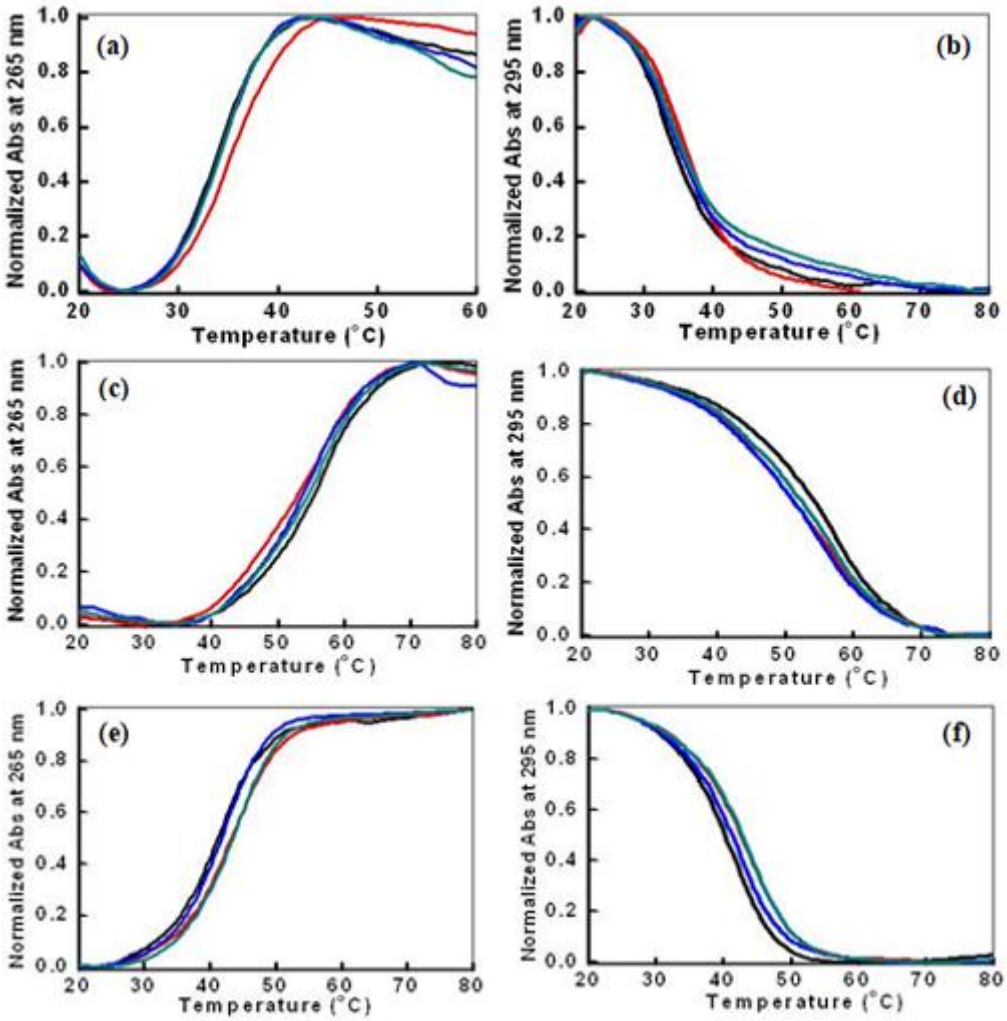



**Figure 5** Normalized UV melting curves of 4$\mu M$ 11Py (a and b), 21Py (c and d) and HTPy (e and f) in 30 mM sodium cacodylate buffer (pH 5.7), 0.5mM EDTA, 100 mM NaCl + 1 mM $MgCl_2$ (black), 100 mM KCl + 1 mM $MgCl_2$ (red), 100 mM NaCl + 1 mM $CaCl_2$ (blue) and 100 mM KCl + 1 mM $CaCl_2$ (green) at 265 nm and at 295 nm respectively

UV melting curves of 4$\mu$M 21Py were also acquired at 265 nm and 295 nm under similar solution conditions. Figure 4c and 4d show the melting profiles of 21Py in the presence of 100 mM NaCl or 100 mM KCl at pH 5.7 respectively. The $T_m$ value of the 21Py was 44.5$^o$C and 44.0 $^o$C in the presence of 100 mM NaCl or 100 mM KCl. However, there was significant increase in $T_m$ values by 11 $^o$C to 12$^o$C in the presence of 100 mM NaCl or 100 mM KCl with and without 1 mM $MgCl_2$ or 1 mM $CaCl_2$ (Figure 5c and 5d). The denaturation and renaturation data of 21Py was recorded and given in Figure S3. To confirm the effect of divalent ions on i-motif structure and stability of 21Py, we also recorded the melting data with an increasing concentration of $Mg^{2+}$ or $Ca^{2+}$ (1 mM, 3 mM, 5 mM, 7 mM, 10 mM) (Figure S4). The $T_m$ values were calculated as 44.5$^o$C, 44.5$^o$C, 54.1$^o$C, 56.0$^o$C, 56.5$^o$C with an increasing concentration of $Mg^{2+}$ and 46.1$^o$C, 47.6$^o$C, 55.5 $^o$C, 56.0 $^o$C, 56.6 $^o$C with an increasing concentration of $Ca^{2+}$ respectively.

Thus, based on $T_m$ values obtained in the presence of $Mg^{2+}$ or $Ca^{2+}$, it is reasonable to conclude that $Mg^{2+}$ or $Ca^{2+}$ significantly stabilized the i-motif structure of 21Py. Interestingly, 21Py has $T_m$ value of 47.5$^o$C in the presence of 100 mM NaCl and 40 wt% PEG 200 at physiological pH 7.0 (**Figure 6a** and 6b). This further suggests that the cell mimicking molecular crowding conditions are favorable for the structuration of 21Py into i-motif structure. These results strongly suggest that the length of the C-rich sequence and the arrangement of C′s in C-rich sequence motif plays an important role in the formation of i-motif structure in physiological ionic conditions and cell-mimicking molecular crowding conditions. We studied the effect of both monovalent and divalent cations in one system to understand the role of cations on the structural polymorphism of C-rich oligonucleotides inside the cell. As $Na^+$ or $K^+$ are transported inside the cell in an antiport manner so at a time either $Na^+$ or $K^+$ is available, but divalent cations are always available at a free intracellular concentration of 1 mM. Therefore, we have recorded the CD and thermal melting data in the presence of monovalent and divalent ions.

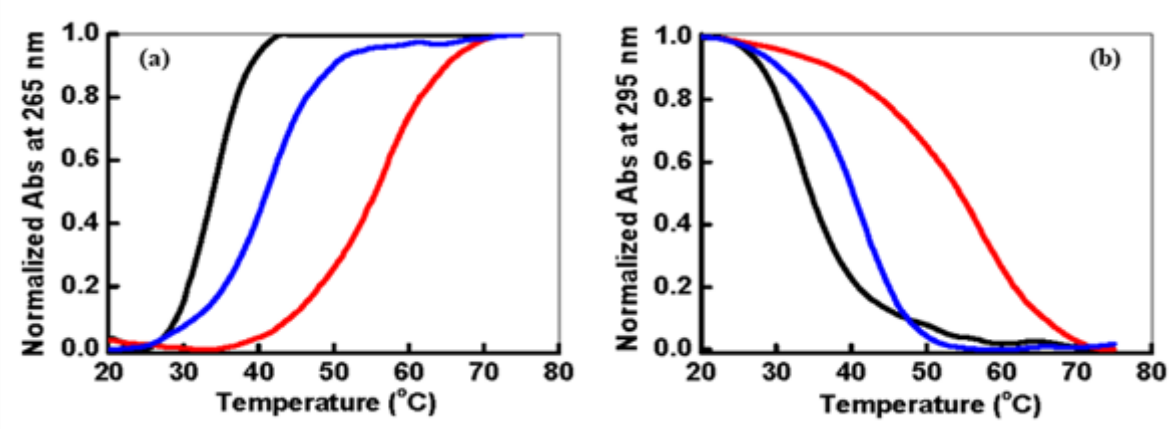



**Figure 6** Normalized UV melting curves of 4$\mu M$ 11Py, 21Py, and HTPy in 30 mM sodium cacodylate buffer (pH 7.0), 0.5mM EDTA, 100 mM NaCl + 1 mM $MgCl_2$ with 40 wt% PEG 200, 11Py (black), 21Py (red) and HTPy (blue) at 265 nm and at 295 nm respectively

Further we studied the effect of cations and molecular crowding on HTPy. Figure 4e and 4f show the UV melting profiles of HTPy in the presence of 100 mM NaCl or 100 mM KCl at pH 5.7. The $T_m$ value of HTPy was 37.5 $^oC$ in the presence of NaCl or KCl respectively. However, there was significant increase in $T_m$ values by 5 $^oC$ to 6$^oC$ in the presence of 100 mM NaCl or 100 mM KCl with and without 1 mM $MgCl_2$ or 1 mM $CaCl_2$ (Figure 5e and Figure 5f). The denaturation and renaturation data of HTPy was recorded and given in Figure S5. Moreover, the $T_m$ value was 36.6$^o$C under physiological ionic conditions and molecular crowding conditions of 40 wt% PEG 200. It is well known that many C-rich sequence motifs could adopt a folded conformation at low pH, as protonation of the cytosine at N3 position of cytosine is a prerequisite step to form $C.C^+$ base pair [26]. Later, several proteins have been identified that could shift the pKa of the formation of i-motif to a more basic value and hence can stabilize the i-motif structure at either near neutral or neutral pH [27]. Recent reports suggest that molecular crowding environment raise the p*K*a value of cytosine at N3 position which can fold the $(CCT)_8$ triple repeat DNAs or C-rich sequences located in promoter region of *c-myc* at neutral pH into i-motif structure [11, 18]. Reports also suggest the formation of stable i-motif structure using single-walled carbon nanotubes as a mimicking agent [14]. To the best of our knowledge this is the first report where using CD and UV-thermal melting studies, it is shown that divalent ions ($Mg^{2+}$ or $Ca^{2+}$) may act as a regulatory element for i-motif formation and can subsequently affect the expression of genes with G,C rich regions.

To assess the origin of the observed stabilities of i-motif structure of 11Py, 21Py and HTPy, the thermodynamic parameters of their formations, such as the enthalpy change ($\Delta H^o$), entropy change ($\Delta S^o$), and free energy change at 25$^o$C ($\Delta G^o$25), were estimated in the presence of various cations (Table 1 and Table 2). The free energy change ($\Delta G^o$25) at 25$^o$C of i- motif structure of 11Py was calculated as -2.3 kcal/mol or -2.2 kcal/mol in the presence of 100 mM NaCl with and without 1 mM $MgCl_2$ or 1 mM $CaCl_2$ while -2.1 kcal/mol or -2.2 kcal/mol the presence of 100 mM KCl with and without 1 mM $MgCl_2$ or 1 mM $CaCl_2$ respectively. The free energy change ($\Delta G^o$25) at 25$^o$C of i-motif structure of 21Py was varied within a range of -3.1 kcal/mol or -3.8 kcal/mol in the presence of different cations. The free energy change ($\Delta G^o$25) at 25$^o$C of i- motif structure of HTPy was varied within a range of -3.1 kcal/mol or -3.5 kcal/mol in the presence of different cations.

## Biological Significance and Conclusions

The most common form of DNA at neutral pH and physiological ionic concentrations is right-handed double helical structure is B-form. Many physical factors affect the B-form DNA duplex structure like water, metal ions, pH, temperature etc. Metal ions can bind tightly to DNA to neutralize the negative charge on phosphate groups of DNA as partially dehydrated or fully hydrated and this binding can be direct or indirect with DNA. Magnesium is the second most important cation in cell together with sodium and potassium and the fourth most abundant positively charged ion in the human body. The $Mg^{2+}$ ions play important role as cofactor in enzymatic activity in most of the biochemical reactions and in various neurological functions. Most recent studies indicate that $Mg^{2+}$ ion are required in the stabilization of not only the DNA duplex structure but also can influence the G-quadruplex structure, which is thought to play an important role in chromatin organization in cell nucleus. Magnesium not only stabilizes DNA structures, it also functions as a cofactor in the repair of DNA damage by environmental mutagens. Combined with ATP, magnesium also assists in the healthy production of RNA, responsible for reading DNA and manufacturing the

proteins used in our body. A significant proportion of cellular calcium resides in the nucleus in free ionic form as well as in bound form. The estimated average physiological free (ionic) calcium concentrations range from nanomolar to micromolar. Calcium is known to affect protein–DNA interactions by regulating secondary modifications such as phosphorylation of various transcription factors with consequences for gene transcription or DNA replication. There are several reports which suggests that both divalent cations ($Mg^{2+}$ and $Ca^{2+}$) promotes the formation of parallel G-quadruplex and assembly of parallel G-quadruplex into higher order G-wire structure. However, both of these structures have also been observed in promoter sites of various genes, satellite DNAs and telomere. Structurally, these polymorphic structures seem to play important biological roles in the regulation of transcription and translation. Therefore, manipulation of these processes is of crucial concern for pharmaceutical industries to develop novel therapeutics as well as for a range of innovative nanotechnological applications. There are several studies reported the effect of cation and molecular crowding environment on G-quadruplex structure but there are fewer studies on the structural polyporphism of i-motif structure. Recent reports suggest that G-quadruplex forming motifs located in promoter region of oncogenes can form thermally stable structure in the presence of monovalent ions and $Mg^{2+}$ ion. From such studies, it was observed that at physiological conditions of pH, salt concentration and temperature, C-rich and G-rich strands can form a normal WC duplex, whereas pH between 4.5 and 5.0 the i-motif and G-quadruplex become the most stable and abundant forms of quadruplex structures. As both G and C-rich sequences experience the same microenvironment of cations and molecular crowding, present study on C-rich sequences, where we discussed about the effect of cations at physiological ionic conditions (100 mM $Na^{+}$ and 1 mM $Mg^{2+}$) and molecular crowding conditions ( 40 wt% PEG 200) is relevant towards understanding the structural polymorphism of DNA in biological systems. To the best of our knowledge this is the first report where using CD and UV-thermal melting studies, it is shown that $Mg^{2+}$ can stabilize the i-motif structure under cell mimicking molecular crowding conditions at neutral pH. Results suggest that divalent cations ($Mg^{2+}$ or $Ca^{2+}$) calcium within its physiological intranuclear concentration specifically regulates the i-motif structure and can subsequently affect the expression of genes with G,C rich regions.

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**Supporting Materials**

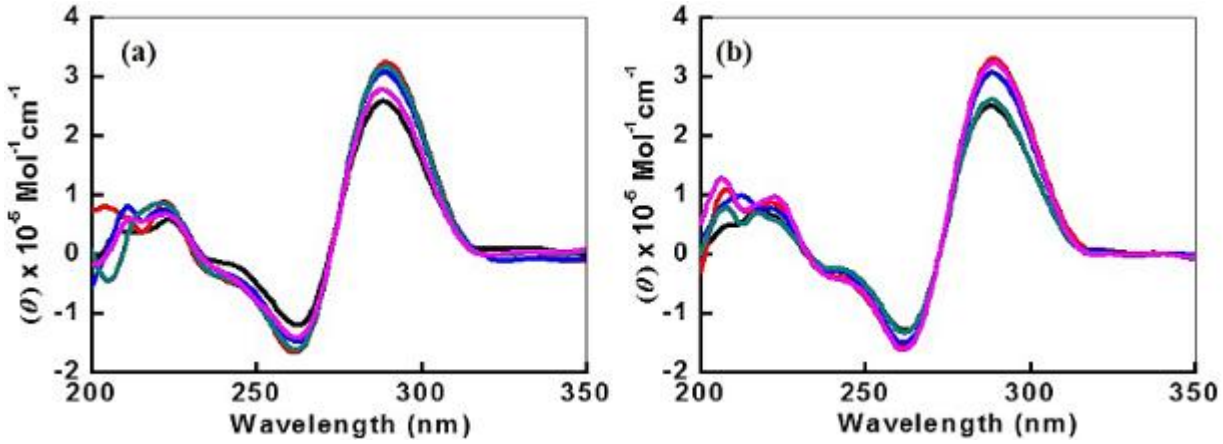


**Figure S1** CD spectra of 4$\mu M$ 21Py in 30 mM sodium cacodylate buffer (pH 5.7), 0.5 mM EDTA, 1 mM (black), or 3 mM (red), or 5 mM (blue), 7 mM (green), 10 mM (pink) $MgCl_2$ (a) and 1 mM (black), or 3 mM (red), or 5 mM (blue), 7 mM (green), 10 mM (pink) $CaCl_2$. (b)

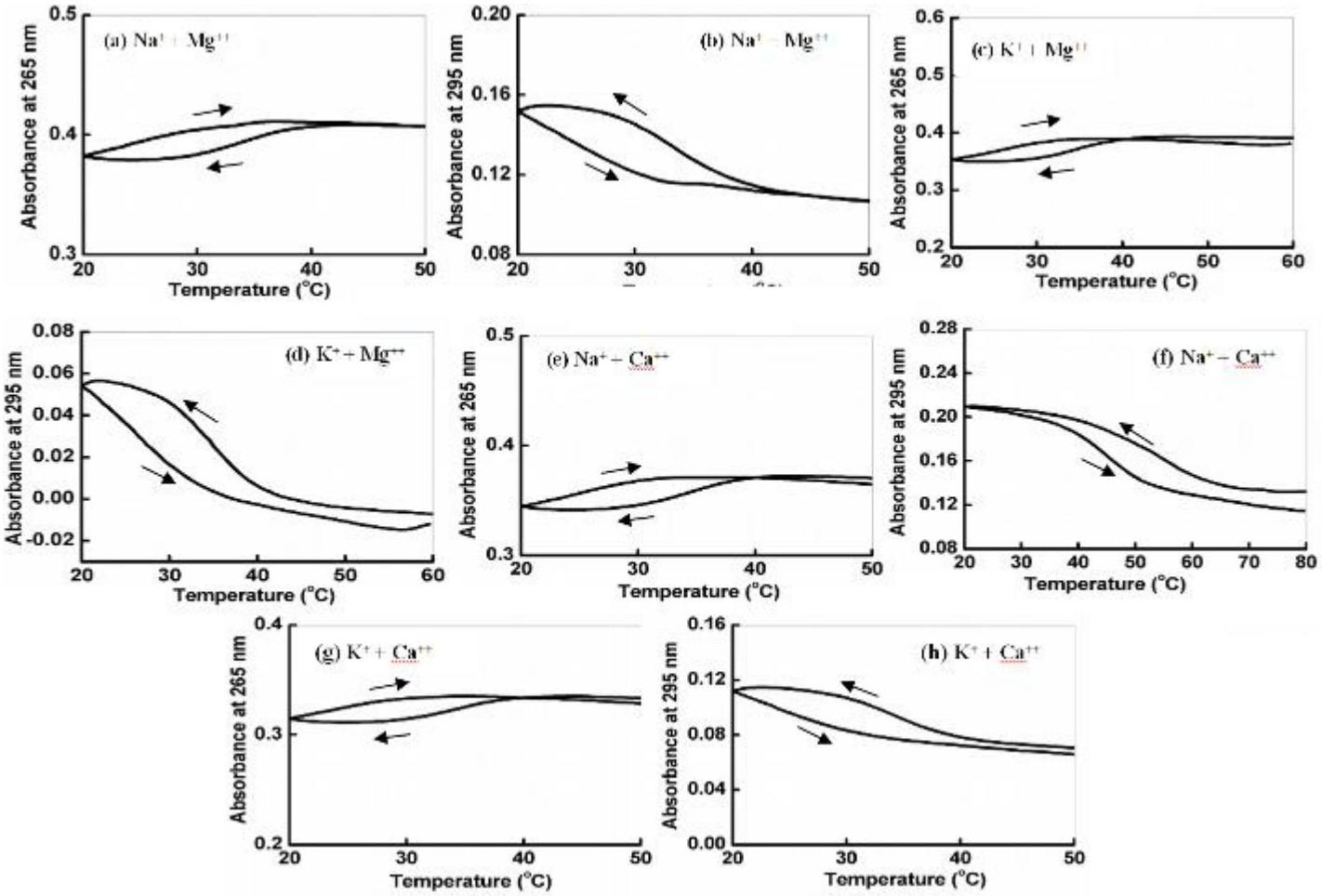


**Figure S2** Thermal denaturation and renaturation data of 4$\mu$M 11Py sequence at 265 nm (a, c, e, and g) and at 295 nm (b, d, f and h) respectively. Samples were prepared in 30 mM sodium cacodylate buffer of pH 5.7 containing 100 mM NaCl + 1 mM $MgCl_2$ (a and b), 100 mM KCl + 1 mM $MgCl_2$ (c and d), 100 mM NaCl + 1 mM $CaCl_2$ (e and f) and 100 mM KCl + 1 mM $CaCl_2$ (g and h) respectively

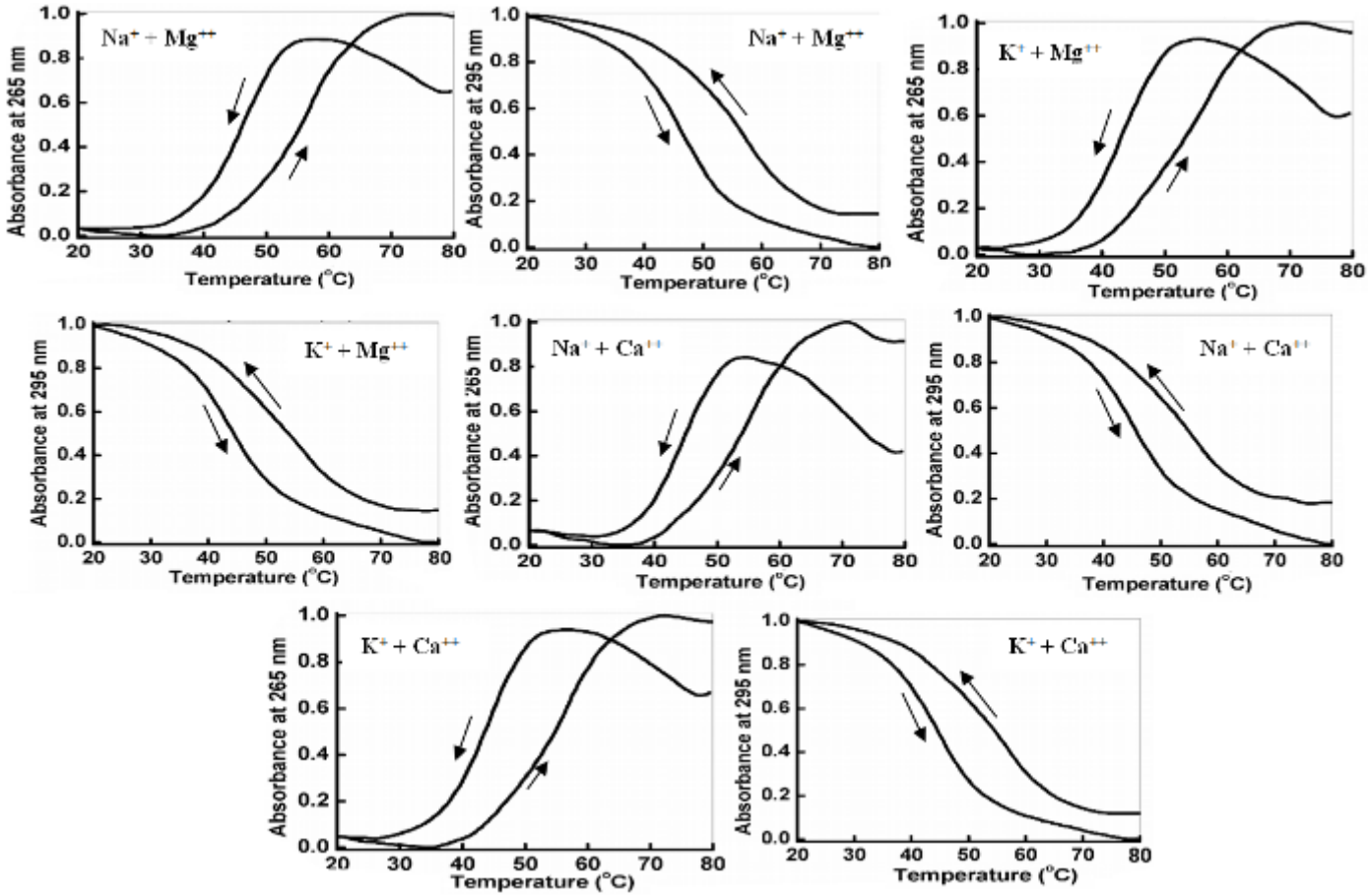


**Figure S3** Thermal denaturation and renaturation data of 4$\mu$M 21Py sequence at 265 nm (a, c, e, and g) and at 295 nm (b, d, f and h) respectively. Samples were prepared in 30 mM sodium cacodylate buffer of pH 5.7 containing 100 mM NaCl + 1 mM $MgCl_2$ (a and b), 100 mM KCl + 1 mM $MgCl_2$ (c and d), 100 mM NaCl + 1 mM $CaCl_2$ (e and f) and 100 mM KCl + 1 mM $CaCl_2$ (g and h) respectively

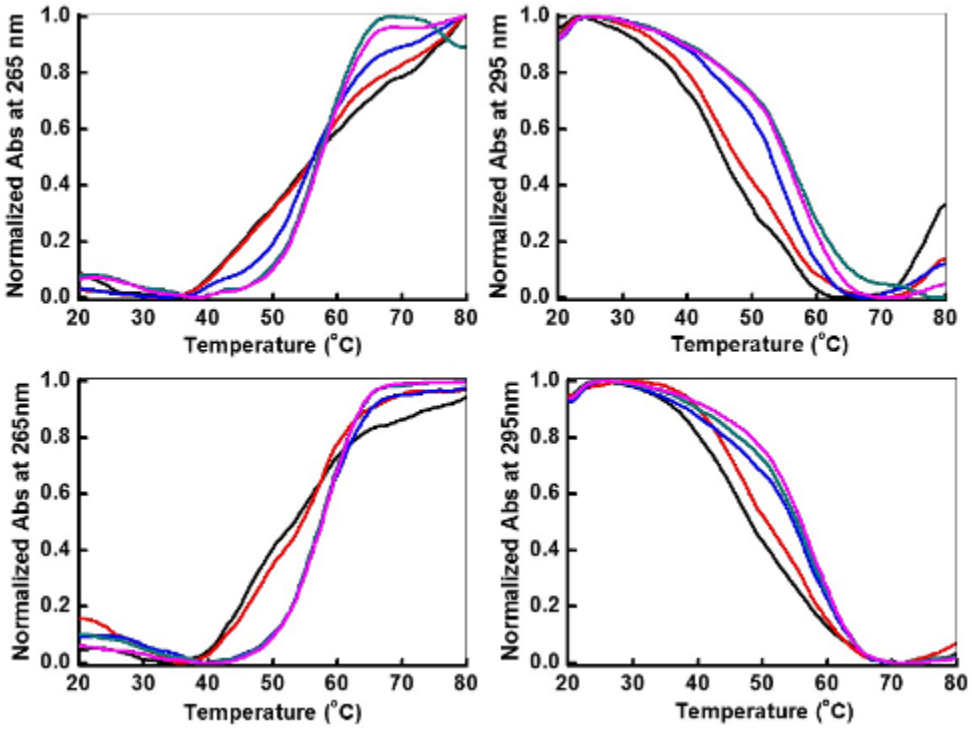


**Figure S4** Normalized UV melting curves of 4$\mu M$ 21Py in 30 mM sodium cacodylate buffer (pH 5.7), 0.5 mM EDTA, 1 mM (black), or 3 mM (red), or 5 mM (blue), 7 mM (green), 10 (pink) $MgCl_2$ or 1 mM (black), or 3 mM (red), or 5 mM (blue), 7 mM (green), 10 (pink) $CaCl_2$ respectively

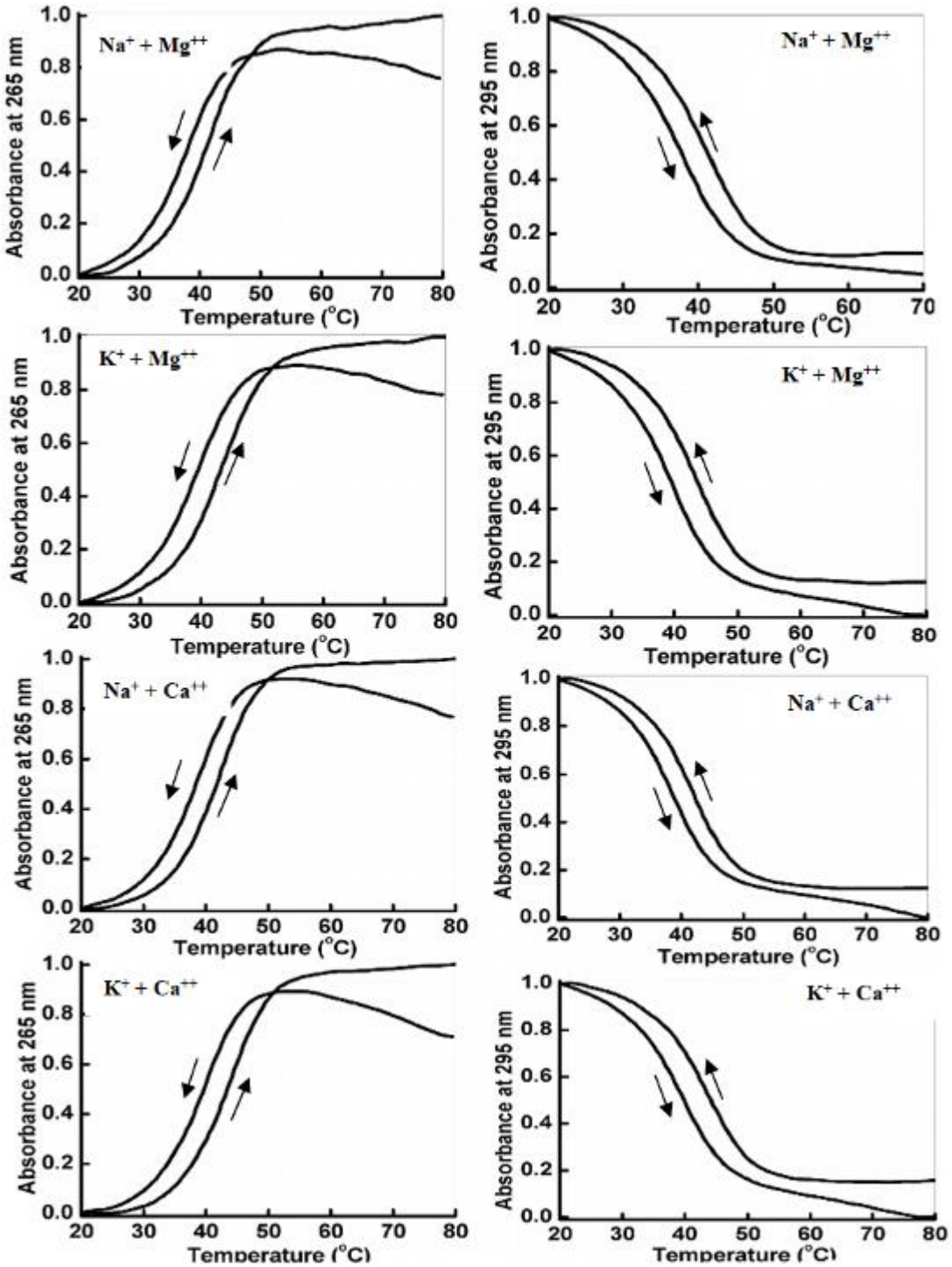


**Figure S5** Thermal denaturation and renaturation data of 4$\mu$M HTPy sequence at 265 nm (a, c, e, and g) and at 295 nm (b, d, f and h) respectively. Samples were prepared in 30 mM sodium cacodylate buffer of pH 5.7 containing 100 mM NaCl + 1 mM $MgCl_2$ (a and b), 100 mM KCl + 1 mM $MgCl_2$ (c and d), 100 mM NaCl + 1 mM $CaCl_2$ (e and f) and 100 mM KCl + 1 mM $CaCl_2$ (g and h) respectively.