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Regulation of Telomeric i-motif Stability by 5-methylcytosine and 5-hydroxymethylcytosine Modification

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Abstract

The two important epigenetic markers, 5-methylcytosine (mC) and 5-hydroxymethylcytosine (hmC) in human genome are involved in gene regulation processes. The major epigenetic target, cytosines in a C-rich DNA sequence, was substituted by mC and hmC to investigate the thermal stability and pH sensitivity of the corresponding i-motifs. Circular Dichroism (CD) studies indicate the formation of i-motif at acidic pH (< 6.5) for mC and hmC modified DNA sequences. Thermal denaturation results suggest that DNA i-motifs are stabilized when modified with one or two mCs. However, hypermethylation with mC and single modification with hmC cause destabilization to the structure. Biomimetic crowding agent does not alter the trends of stability effects resulted from mC and hmC modifications, though the corresponding i-motifs show elevated melting temperatures without significant changes in p*K*a values.

Introduction

I-motif is an intercalated tetraplex structure formed by association of hemiprotonated cytosine pairs (C-CH$^+$) in a C-rich DNA oligonucleotide, under acidic to slightly alkaline pH.[1-3] G-quadruplex and i-motifs are widely present in oncogenic regions and human telomeric DNA, thus indicating the possible intramolecular tetraplex structure formtaion during the regulation of oncogene expression at the transcription level.[4-6] Various proteins have been discovered to specifically bind to C-rich telomeric DNA fragments which further suggest the potential biological significance of i-motifs.[7-12] I-motifs are reversibly and rapidly oscillated between a stable intercalated tetraplex and an unfolded structure along with rocking between acidic and basic pH (Figure 1).[13] This renders i-motif a great potential as nanomolecular devices for biosensing and bioimaging.[14-19] Due to the paramount significance of i-motif forming sequences in gene transcription and cell apoptosis, it has become an attractive drug target for chemotherapy and gene transcription modulation.[20, 21] Furthermore, DNA i-motif stabilized by single walled carbon nanotubes (SWCNT) can inhibit telomerase activity both *in vitro* and *in vivo*, and induce telomere uncapping.[22] Considering the pH sensitivity, i-motif structures have been used for reversible dispersion and precipitation of SWCNT, reversible switching of nanocontainer that traps small molecule for release.[23, 24]

Since cytosine is the major epigenetic target in human genome, the ability of cytosine rich DNA sequences to form i-motif and consequent intervention on the down-stream biological processes may be affected by DNA epigenetics. The nucleobases such as 5-methylcytosine (mC) and 5-hydroxymethylcytosine (hmC) have been found in mammalian genome and their biological functions have been of great interest.[25, 26] Substantial studies have shown that mC are involved in genomic imprinting, X-chromosome inactivation, cellular differentiation by inhibiting transcription and gene silencing.[27] Though majority of cytosine methylation occurs in CpG segments, hypermethylation in subtelomere has been observed in cancer cell lines, immortalized by either high activity of telomerase or recombination-based alternative lengthening of telomeres mechanism. It has been hypothesized that mC rich telomere sequences are closely related to the length maintenance of telomere, the transcription of *TERRA* RNA and the abnormal high activity of telomerase in cancer cells.[28, 29] Recent discovery of nucleobase, hmC in human genome, is associated with the cellular development processes and carcinogenesis. It is also evidenced that it may not only behave as an oxidized intermediate to gate an active demethylation process, but also function as a major epigenetic marker.[30-34] Epigenetic modification shows intriguing regulation on the biofunctions of telomeric i-motif, thus making it essential to understand the formation and stability of telomeric i-motif when methylation and/or hydroxymethylation on cytosines occur.

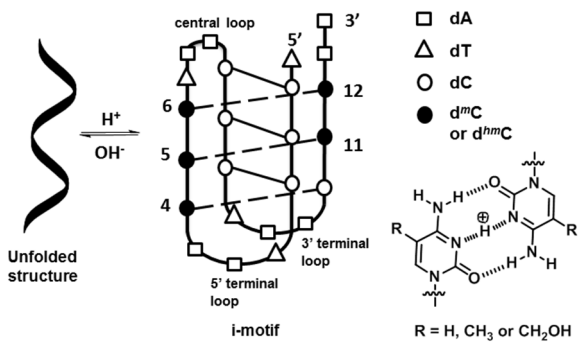

Figure 1. Schematic diagram of C-rich DNA (**cHT)** in i-motif and random coil state. Structure at *bottom right* indicates the hydrogen bonding pattern between a hemiprotonated cytosine base pair. **R** indicates the various substituents at the C5 position of pyrimidine ring.

A recent article on mC and hmC modified DNA i-motif appeared in literature during the preparation of this manuscript, which illustrates the effect on the stability of i-motif structure, when one cytosine is replaced with mC or hmC at a fixed position in i-motif.[35] However, the detailed positional effects of such modifications on the stability of C-rich i-motif have not been studied so far. Hence, in the present study, cytosine bases were substituted with varying number of mC and hmC in different positions of an i-motif forming DNA sequence, **cHT** (Table 1). Circular Dichroism (CD), UV detected thermal denaturation (UV-T_m) experiments were performed at varying pHs, to investigate the folding and stability of i-motif structure. Moreover, folding of mC and hmC modified **cHT** in presence of biomimetic crowding agent were carried out to study the effects of mC and hmC modification on i-motif in cell-like environments.

Results and discussion

A series of DNA sequences *viz.* **Nm** and **Nhm** were synthesized by standard phosphoramidite chemistry, where **N** (= 1, 2 etc.) and **m/hm** indicates the position and the types of modified bases, mC and hmC, respectively[36] (Table 1). In Figure 1, cytosines in **cHT** are annotated as C1 to C12 from 5' to 3' end, respectively. The three loops are named as the 5' terminal loop, central loop and 3' terminal loop from 5' to 3' end sequentially. **cHT** were mono-substituted by 5-methylcytosine at C4, C5 or C6 position to obtain sequences, **4m**, **5m** and **6m**, respectively, as they are on the stretch connecting to two loops (central and 5' terminal loop), which may yield a greater and more evident effect. Modifications on C11 and C12 position at 3'-end of C-stretch attaching to the 3'-terminal loop, yield sequences, **11m** and **12m**, respectively. Bi-modification with mC was done by modifying C5-C11 and C6-C12 base pairs to get **5,11m** and **6,12m**, respectively, which possess the middle and external layer of C-CH$^+$ base pair in i-motif. In order to investigate the effect of hypermethylation, triple modification with mC was done consecutively on C4, C5 and C6 positions of **cHT**, to obtain the sequence, **4,5,6m**. Single modifications with 5-hydroxymethylcytosine at C4, C5, C6, C11 and C12 resulted to **4hm**, **5hm**, **6hm**, **11hm** and **12hm**, respectively. All sequences were synthesized at our laboratory using standard phosphoramidite chemistry and were purified by RP-HPLC and characterized by ESI-MS (Figure S4, S5).

CD Studies

CD spectra for **Nm** and **Nhm** sequences were collected between 230-330 nm at room temperature. At acidic pH (< 6.5), all mC and hmC modified sequences show the characteristic CD signals of an i-motif with a positive peak at ~286 nm and a negative peak at ~255 nm (Figure 2a and Figure S1).[3] The similar CD pattern of modified sequences to that of **cHT** indicates a negligible effect of mC and hmC modification on the global structure of i-motif. At pH 7.3, the characteristic i-motif peak at ~286 nm disappears and a positive peak at ~275 nm and a negative peak at ~249 nm emerge instead, thus indicating formation of a unfolded structure with increasing pH. Further, pH dependent (pH 4.17 to pH 8.10) CD experiments suggest that while moving from acidic to physiological pH, all mC and hmC modified sequences lose their i-motif structure to form a unfolded structure (Figure 2b and Figure S2). Hence, pH sensitivity of i-motif structures was maintained when cytosines were replaced by mC and hmC bases.

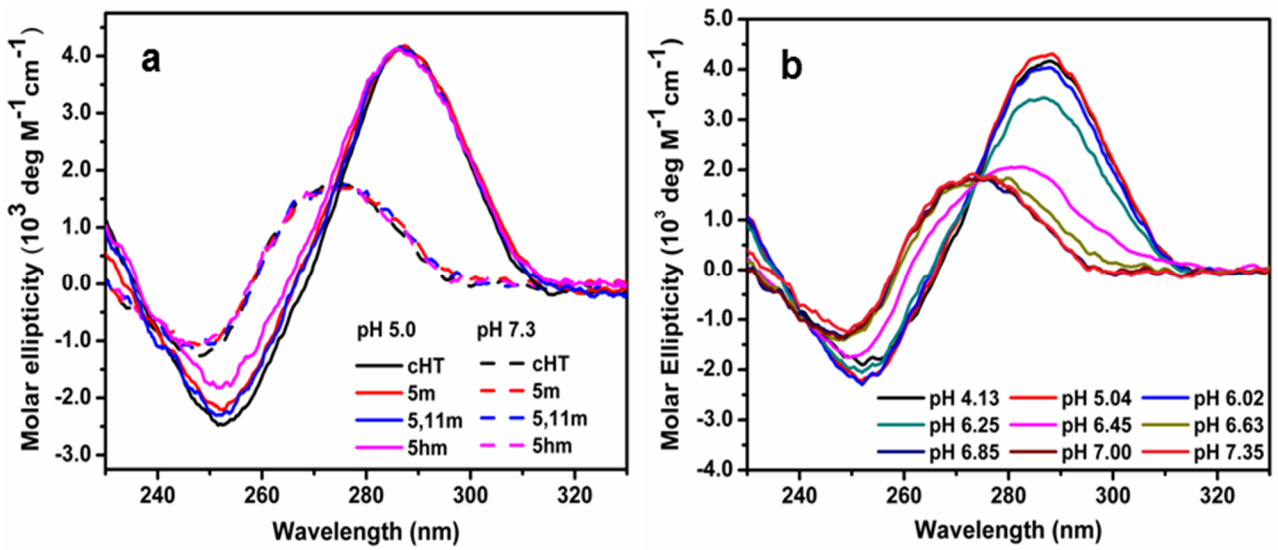

Figure 2. a) CD spectra of **cHT**, **Nm** and **Nhm** series of DNA at pH 5.0 and pH 7.3 and b) pH dependent CD spectra of **5hm**.

pK_a Determination

To investigate the stability of mC and hmC modified i-motifs against pH variation, pK_a values were determined by measuring the change in ellipticity at 286 nm at varying pH. The sigmoidal plot of CD intensity vs pH gives rise to pK_a value. It is found that mC modified i-motifs have similar or higher pK_a than that of unmodified **cHT** (Table 1), whereas, significant decrease in pK_a values were observed for hmC modified i-motifs. Hence, these initial results show stabilizing effects of mC and pronounced destabilization by hmC. In order to validate these findings, UV-detected thermal denaturation experiments were performed with mC and hmC modified DNA sequences to examine the i-motif stability. UV melting temperatures (T_ms), derived from the sigmoidal fitting of UV absorption intensity at 260 nm during temperature elevation, were used to evaluate the thermal stability of i-motifs. The thermal denaturation experiments for all modified and unmodified sequences were conducted at pHs that is more (pH 4) or less (pH 5) acidic than nucleobase p*K*a of cytosine and is closer to p*K*a of natural i-motif (pH 6). Results are summarized in Table 1.

Table 1: The oligonucleotide sequences used in this study

DNA	Sequence[a]	pK_a[b]	UV-T_m[c]		
			pH 4	pH 5	pH 6
cHT	5′-TCCCTAACCCTAACCCTAACCCAA-3′	6.58	56.9	53.7	36.0
4m	5′-TCCCTAA^{m}CCCTAACCCTAACCCAA-3′	6.58	57.1(+0.2)	53.9(+0.2)	36.4(+0.4)
5m	5′-TCCCTAAC^{m}CCTAACCCTAACCCAA-3′	6.66	57.3(+0.4)	54.3(+0.6)	36.9(+0.9)
6m	5′-TCCCTAACC^{m}CTAACCCTAACCCAA-3′	6.63	57.4(+0.5)	54.8(+1.1)	37.8(+1.8)
11m	5′-TCCCTAACCCTAACCCTAAC^{m}CCAA-3′	6.63	57.9(+1.0)	54.5(+0.8)	36.7(+0.7)
12m	5′-TCCCTAACCCTAACCCTAACC^{m}CAA-3′	6.61	58.4(+1.3)	55.2(+1.5)	38.0(+2.0)
5,11m	5′-TCCCTAAC^{m}CCTAACCCTAAC^{m}CCAA-3′	6.68	59.6(+2.7)	55.4(+1.7)	36.2(+0.2)
6,12m	5′-TCCCTAACC^{m}CTAACCCTAACC^{m}CAA-3′	6.69	60.1(+3.2)	56.0(+2.3)	37.9(+1.9)
4,5,6m	5′-TCCCTAA^{m}C^{m}C^{m}CTAACCCTAACCCAA-3′	6.60	55.9(-1.0)	52.7(-1.0)	36.0(0.0)
4hm	5′-TCCCTAA^{hm}CCCTAACCCTAACCCAA-3′	6.38	54.3(-2.6)	51.6(-2.1)	34.5(-1.5)
5hm	5′-TCCCTAAC^{hm}CCTAACCCTAACCCAA-3′	6.36	54.0(-2.9)	51.1(-2.6)	34.5(-1.5)
6hm	5′-TCCCTAACC^{hm}CTAACCCTAACCCAA-3′	6.43	54.8(-2.1)	51.5(-2.2)	35.2(-0.8)
11hm	5′-TCCCTAACCCTAACCCTAAC^{hm}CCAA-3′	6.39	54.2(-2.7)	50.9(-2.8)	34.0(-2.0)
12hm	5′-TCCCTAACCCTAACCCTAACC^{hm}CAA-3′	6.41	54.1(-2.8)	51.2(-2.5)	35.2(-0.8)

[a]^{m}C indiactes 5-methylcytosine and ^{hm}C indicates 5-hydroxymethylcytosine. [b]pK_a is derived from sigmoidal plot of CD intensities at 286 nm at varying pHs. [c] UV-T_ms were obtained by heating the samples from 20 to 90 °C with a ramp rate of 0.5 °C/min at 260 nm. ΔT_m values are indicated in parentheses.

Thermal Denaturation Studies

The thermal denaturation studies were performed by monitoring UV absorbance at 260 nm with continuous elevation of temperature from 20 to 90 °C with a ramp rate of 0.5 °C/min. At pH 5.0, the difference in melting temperature (ΔT_m) for **4m**, **5m**, **6m**, **11m** and **12m** are +0.2, +0.6, +1.1, +0.8 and +1.5 °C, respectively, to that of unmodified sequence, **cHT** (Table 1, Figure 3a). Among the single mC modified sequences, smallest effect on the stability of i-motif is observed when C4 is methylated, which is consistent with previous report.[37] The little influence may be due to C4 situating at the vicinity of dual loop region, which is more rigid and have less tolerance to modification, compared to the region of the single loop side (Figure 1). Modification at C6 and C12 position shows better stabilization to the structure compared to the other single mC modified sequences. At the other two experimental pHs, increase in T_m values were always observed, when different positions of cHT was modified with single mC. For bi-modified sequences, **5,11m** and **6,12m,** higher thermal stability with ΔT_m value of +2.7 and +3.2 °C, respectively, is observed compared to control **cHT**, at pH 5. The stabilization could be attributed to the steric effect and hydrophobicity of the 5-methyl group in mC. The increased local rigidity within the base pairs due to the bulkiness of 5-methyl group and the consequent reduced flexibility of three loops, introduces extra stabilization to methylated i-motif structures.[38-41] The bi-modification was not performed at C4-C10 position, as previous report suggests that the modification near 5' and 3'-terminal loops cast little effect on i-motif stability.[37] It was also mentioned in literature that consecutive mC repeats may appear in living cells.[42] However, triple modification in **4,5,6m** destabilizes the i-motif structure (ΔT_m = -1.0 °C at pH 5), which might be due to the steric hindrance caused by three consecutive bulky methyl groups present on the same C-stretch. Thus, with an exception of triple mC modification, all single and double mC modified i-motifs exhibit higher thermal stability compared to unmodified sequence.

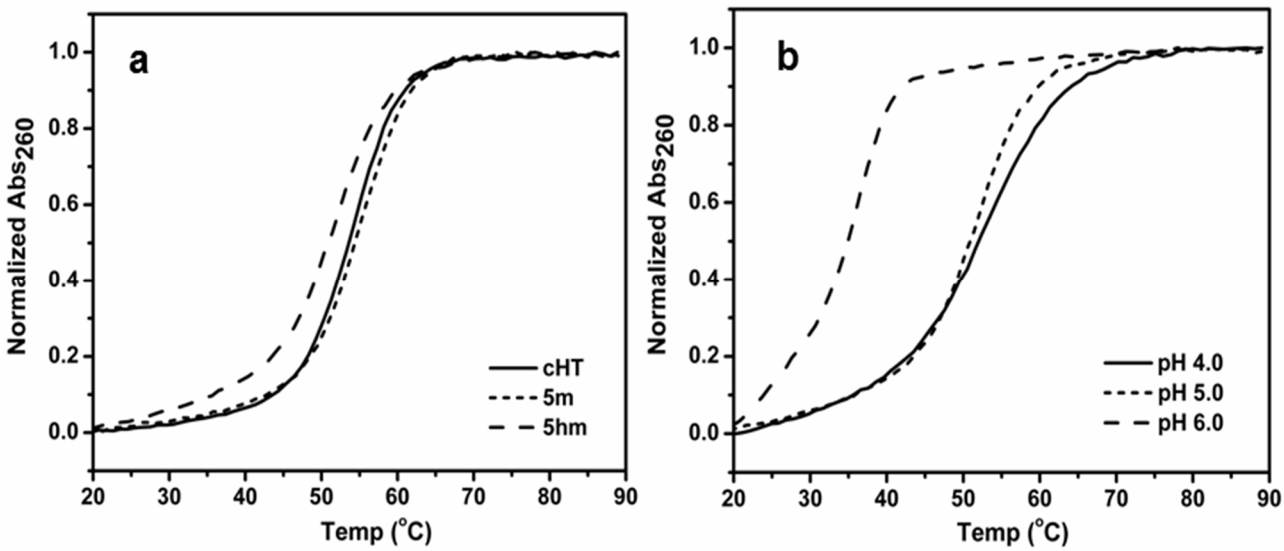

Figure 3. Thermal stabilities of **cHT**, **Nm** and **Nhm** series of DNA. UV-melting curves of a) **cHT**, **5m** and **5hm** at pH 5. b) **5hm** at pH 4.0, pH 5.0 and pH 6.0.

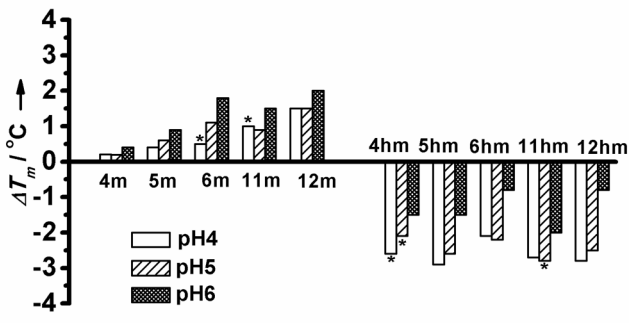

Figure 4. Comparison of difference in melting temperatures (ΔT_ms) for mC and hmC modified sequences at pH 4, pH 5 and pH 6, with standard deviation (SD)≤ 0.4. *SD ≤ 0.8.

Due to the infrequent occurrence of more than one 5-hydroxymethylcytosine in an i-motif forming sequence, only mono-modification with hmC were designed for the present study.[31, 43] However, decrease in thermal stability was apparently observed, when single cytosines were replaced with hmC in **4hm**, **5hm**, **6hm**, **11hm** and **12hm** (Table 1). Under all three pHs, five hmC modified i-motif showed negative ΔT_m. At more acidic pHs (4 and 5), ΔT_m values for **4hm**, **5hm**, **6hm**, **11hm** and **12hm** were larger than -2 °C, while the maximum destabilization were occurred to **5hm** at pH 4 (ΔT_m= -3.1°C) (Figure 4c). Unlike mC modification, **Nhm** i-motifs, though with substitution at different positions, showed similar level of destabilization, except slightly smaller ΔT_m of **6hm**. The destabilization caused by hmC modification might be due to alleviation of local rigidity resulting from the introduction of a polar 5-hydroxymethyl group, which reduces the hydrophobicity and make the solvation of DNA strand easier via forming extra H-bond network with environmental water.[38, 41] Moreover, hmC has a significantly lower nucleobase p*K*a compared to mC and cytosine which indicates that protonation of N3 in hmC is less favourable (Table S1).[43, 44] As a result, hmC modification gives rise to a less stable i-motif structure.

UV-T_m values for cHT, mC and hmC modified i-motifs demonstrate a decrease in melting temperature while going from pH 4 to pH 6, thus indicating the higher thermal stability of i-motifs at more acidic pH, regardless of modification patterns (Table 1, Figure 3b,4). All melting curves exhibit a two-phase transition which suggests that dissociation process is cooperative at all three pHs and no additional intermediates are induced by mC and hmC modifications. The melting curve at pH 6 also exhibits a clear two-phase transition as those at pH 4 and 5, which demonstrates that the unfolding is cooperative even at the pH edge of denaturation. The effect of mC modification on i-motif thermal stability is more pronounced at near-physiological pH. ΔT_m values of all **Nm** i-motifs are much higher

at pH 6 than those at pH 4 and 5. The enhancements were most pronounced in **6m** and **12m**, in which the methylation sites were near central loop and two strand terminals. However, a reverse trend is observed from the destabilization effect of hmC with the increase in pH. ΔT_m values of each **Nhm** reduced significantly from pH 4 to pH 6. All **Nhm** i-motifs show 1~2 °C in $\Delta\Delta T_m = \Delta T_{m,pH6} - \Delta T_{m,pH4}$, except that the phenomena is not well pronounced in **11hm**. Hydroxymethylated i-motifs are least destabilized when pH approaches near physiological conditions. At pH 6, which is more basic than the nucleobase p*K*a, i-motif structure rather tends to become more flexible with less defined secondary structure.[43, 44] Thus, this "loose" structure can more readily accommodate hmC with larger size and high hydrophilicity, which reflects a smaller negative ΔT_m of hmC modified i-motif at pH 6 comparing to those at pH 4 and 5.

Molecular Crowding Effect

It is of great interest to understand how mC and hmC modifications would affect the structural stability of i-motifs under crowding condition. In the interior of cells, DNA i-motifs are surrounded mainly by large biomolecules such as proteins and nucleic acids. In order to mimic the cellular environment *in vitro*, we have used 20% w/w of Ficoll 400 as the crowding agent. Ficoll 400 is a hydrophilic polysaccharide with high molecular weights that are commonly used as a bimolecular mimetic for molecular crowding studies.[45]

Table 2. Acid dissociation constants (pK_a) and UV-melting temperatures (T_ms) at pH 5 under crowding condition.

DNA	pK_a		T_m (°C)	
	-	+[a]	-	+
cHT	6.58	6.57	53.7	55.9
5m	6.66	6.62	54.3	56.3
5hm	6.39	6.43	51.1	53.1

[a] In the presence of 20% (wt%) Ficoll 400.

CD spectra show that i-motifs, **5m** and **5hm** undergo the similar structural transitions in presence of 20% Ficoll 400 solution as that in pure aqueous buffer at pH 5 (Figure S3). Previous literature shows a significant enhancement of ΔT_m in presence of crowding agent.[35, 46] In the presence of Ficoll 400, similar increments in ΔT_m value (Table 2) were observed for both natural and modified i-motifs, though no significant alteration in p*K*a value was observed. The smaller ΔT_m compared to previous report could be due to the fact that the telomeric C-rich sequence used in current study contains CCC repeats, while a repetitive CCT sequence were used in the literature.[46] Two sequences with difference in strand lengths, number of C-repeats and intervening sequences may respond slightly different to the crowding environment. Moreover, the same trend of p*K*a, *i.e.* increased in **5m** and decreased in **5hm** comparing to **cHT,** was observed in the presence of Ficoll 400, which further indicated that pH sensitivity of i-motifs may remain unaltered in cellular environments regardless of mC and hmC modifications. It is interesting to note that the trend on thermal stability of i-motifs, *i.e.* higher T_m by methylation and lower T_m by hydroxymethylation on cytosines, is conserved under crowding conditions. Hence, it could imply that cellular crowdness should have insignificant alteration on mC and hmC regulation of i-motif stability.

Conclusions

In summary, the present study clearly demonstrates that telomeric sequences bearing mC and hmC can form i-motif structure under acidic conditions (pH<6.5). mC substituted DNA i-motifs apart from three consecutive mCs in the same C-stretch, stabilizes i-motif structure. Hypermethylation causes destabilization to the i-motif structure due to the steric hindrance of 5-methyl group present in mC. Single modification with hmC shows significant destabilization to i-motif structure due to its lower p*K*a value, larger molecular size and high hydrophobility compared to mC and cytosine. In presence of biomimetic crowding agent, the similar trend of stabilization and destabilization effects were observed from mC and hmC modifications, respectively. Thus, 5-methylcytosine and 5-hydroxymethylcytosine modifications can modulate the stability of high order telomeric structure, which may open a new insight in epigenetic regulation of carcinoma.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Methods and materials, pH dependent CD spectra of DNA i-motifs, spectra for T_m and p*K*a dertermination for molecular crwoding studies, physical and electronic properties of substituents at C5 position of cytosine, HPLC and ESI-MS of DNAs. See DOI: 10.1039/b000000x/

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