Bioorganic & Medicinal Chemistry Letters 27 (2017) 5424–5428

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Hybrid DNA i-motif: Aminoethylprolyl-PNA (pC_5) enhance the stability of DNA (dC_5) i-motif structure

Chandrasekhar Reddy Gade, Nagendra K. Sharma *

School of Chemical Sciences, National Institute of Science Education and Research (NISER), HBNI-Mumbai, Bhubaneswar, Jatni 752050, Odisha, India

ARTICLE INFO

Article history:
Received 9 August 2017
Revised 1 November 2017
Accepted 2 November 2017
Available online 3 November 2017

Keywords:
Hybrid i-motif
Aep-PNA
DNA
Ion-ion interaction
Enhanced stability

ABSTRACT

This report describes the synthesis of C-rich sequence, cytosine pentamer, of *aep*-PNA and its biophysical studies for the formation of hybrid DNA:*aep*-PNAi-motif structure with DNA cytosine pentamer (dC_5) under acidic pH conditions. Herein, the CD/UV/NMR/ESI-Mass studies strongly support the formation of stable hybrid DNA i-motif structure with *aep*-PNA even near acidic conditions. Hence *aep*-PNA C-rich sequence cytosine could be considered as potential DNA i-motif stabilizing agents *in vivo* conditions.

Since after discovery of DNA duplex structure, DNA are also known to form *triplex*, *tetraplex*, and other many more DNA polymorphs.[1] Importantly, DNA forms two type of *tetraplex* structures from the repeated G-/C-rich sequences: (i) the G-quadruplex (G_4), which encompasses of stacked guanine tetrads stabilized by monovalent cations (Na^+/K^+); and (ii) the i-motif, a cytosine-rich structure comprising of two hemi-protonated parallel duplexes intercalated in an antiparallel orientation (Fig. 1).[2,3] The role of those DNA tetraplexes structures are reportedly considered in the maintenance of telomere and gene regulation. Recently, the occurrence of i-motif are noticed in regulatory regions of the human genome such as centromeres, telomeres, and oncogene promoter regions.[4] These structures are also considered as potential materials for nanotechnology research.[5,6]

Structurally, the DNA i-motif is reportedly a compact structure with short base-pairing distances (~3.1 Å), helical twist (~12–16°) between adjacent C:C$^+$H base pairs and close sugar-sugar contacts, C—H—O interactions (Fig. 1).[3] However, the repulsive interactions also exist between the charged *C-imino* protons and phosphates groups of DNA strands, which intend the destabilization of that i-motif structure. *In vitro*, the formation of DNA i-motif structures are occurred only under acidic conditions, near acidic pH range (3.5–5.5) and the maximum stability are noticed at pH equal to the pKa of the cytosine N-3. Though DNA i-motifs are stable at neutral pH conditions *in vivo* presumably due to other cellular factors such as negative super helicity, cellular proteins and molecular crowding conditions.[7] Recently, the formation of DNA i-motif are noticed *in vitro*, at neutral pH conditions, in presence of metal ions (Cu^{2+}/Ag^+).[8] Furthermore, the stable i-motif structures have been achieved by sugar/phosphate backbone modified DNA analogues which are following-Thio-phosphoramidates,[9] RNA,[10] Locked nucleic acid (LNA),[11] and 2′-fluoro DNA analogue.[12]

Nevertheless the stabilization of DNA i-motifs are also explored by hybridization with similar C-rich sequence of DNA analogues, and with the sequence specific DNA binding small synthetic molecules.[13] In repertoire of structurally backbone modified DNA analogues, Peptide nucleic acid (PNA), consisting aminoethylglycinate (*aeg*) backbone, has emerged as a potential DNA analogue because of its promisable binding affinities with DNA duplex/triplex structures.[14] The C-rich sequence (TC_5/TC_8) of *aeg*-PNAs are also explored in the formation of stable i-motif.[15,16] Moreover modified PNA as Alanyl-PNA, alanine backbone instead of *aeg*, are reportedly known the formation of the stable i-motif from its TC_8 sequence.[17] Further, *aeg*-PNA are successfully employed for the formation of hybrid DNA (or RNA) i-motif structure which are more stable than respective DNA (or RNA) i-motif.[18,19] The stabilization of PNA:DNA (or PNA:RNA) hybrid i-motif are presumably considered because the hybridized *aeg*-PNA strand diminish the ion-ion repulsive interaction between negatively charged DNA's phosphate backbone in that hybrid i-motif.

These results inspired us to design the hybrid DNA i-motif from the positively charged backbone containing DNA or PNA analogue which could enhance the stability of DNA i-motif structure near

* Corresponding author.
E-mail address: nagendra@niser.ac.in (N.K. Sharma).

https://doi.org/10.1016/j.bmcl.2017.11.004
0960-894X/

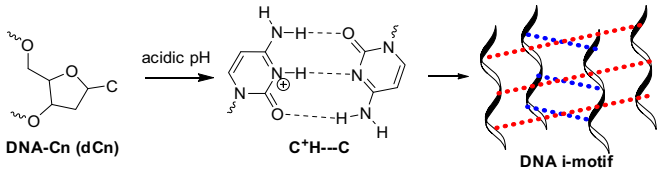


Fig. 1. DNA cytosine (dC), hydrogen bonding in C+H–C, and i-motif structure.

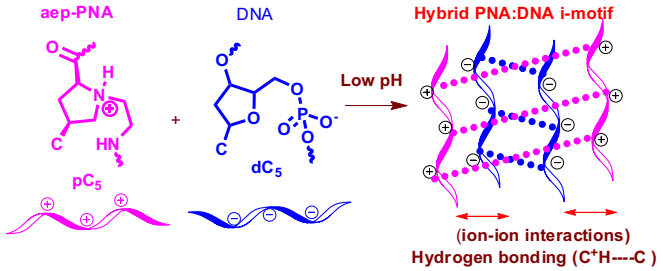


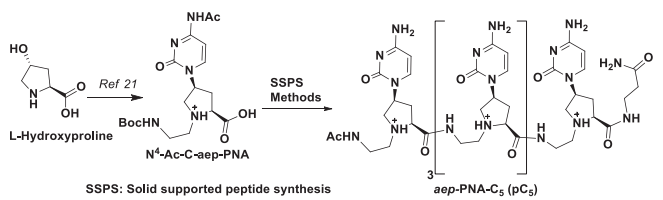
Fig. 2. Proposed hybrid i-motif formation.

acidic conditions, possibly due to ion-ion attractive interaction. Since aminoethylprolyl (*aep*) modified PNA analogues, conformationally constrained chiral PNA, is reported and found that *aep*-PNA is strongly stabilized hybrid DNA duplexes/triplexes structure with complementary DNA.[20–23] The G-rich sequence of *aep*-PNA are also explored in the formation of stable G-quadruplex.[24] Thus we planned to employ *aep*-PNA, cytosine rich sequence, to explore the formation of stable hybrid DNA i-motif structure by introducing ion-ion attractive interactions between amino backbone of *aep*-PNA and phosphate backbone of DNA (Fig. 2). Herein, we describe the synthesis and biophysical studies of *aep*-PNA cytosine pentamer (pC_5) for hybrid DNA i-motif structure with DNA dC_5 by CD/UV/NMR/ESI-MS techniques.

Results and discussion

We began with the synthesis of N^4-acetyl *aep*-PNA cytosine (*aep*-PNA-C) monomer from L-4-Hydroxyproline by following the reported procedure (see in Suppl. Materials).[21] This monomer was employed for the synthesis of *aep*-PNA C_5 (pC_5), designed i-motif forming sequence, by solid support peptide synthesis methods using MBHA resin (Scheme 1). After cleavage from the resin, oligopeptide pC_5 was isolated by gel filtration (Sephadex G-15), and then purified by HPLC. The purified pC_5 was characterized by ESI-Mass studies (see in Supplementary Materials). This PNA pC_5 was used to prepare the hybrid DNA:PNAi-motif structure with DNA-C_5 (dC_5) and performed a comparative biophysical studies such as CD/UV/NMR/ESI-Mass studies of DNA:PNA hybrid i-motif structure. The DNA (dC_5) was purchased and directly used to perform biophysical experiments.

CD studies. We recorded the CD spectra of the annealed PNA pC_5, DNA dC_5, and hybrid pC_5:dC_5 (1:1) samples in sodium acetate buffer



Scheme 1. Synthesis of *aep*-C-monomer and *aep*-PNA-C_5.

(100 mM, pH4.5) at 10 °C (Fig. 3). The CD spectra of DNA i-motif structure gives maxima (~296 nm) and minima (~261 nm) respectively. We observed similar CD spectrum with control DNA dC_5. We were unable to observe the characteristic CD signal of i-motif with alone *aep*-PNA (pC_5) even at low pH (4.5). The CD spectrum of *aep*-PNA pC_5 did not match with the CD of control DNA (dC_5). The CD spectra of pC_5, however, give only minima at ~263 nm under that pH (4.5) condition. This CD signature resembled to the self-duplex type of secondary structure. This self-duplex possibly forms from the PNA pC_5 and cytosine protonated (N^3-atom of cytosine) PNA pC_5 $(pC^+\text{-}H)_5$ by hydrogen bonding. The repulsive interactions between positively charged prolyl amine of pC_5 PNA backbone might prevent the formation of stable tetraplex structure. Interestingly, the CD spectrum of hybrid dC_5:pC_5 (1:1) showed characteristic CD signature with the consisting maxima (~300 nm) and minima (~261 nm) like control dC_5.Importantly, the marginal red shift (~4.0 nm) was observed in the CD maxima of hybrid sample. The comparative CD spectral analyses strongly support the hybridization of pC_5 with dC_5 which lead to the formation of hybrid i-motif pC_5:dC_5 structure.

To find the appropriate pH conditions for the formation of hybrid i-motif, we recorded the pH dependent CD spectra of dC_5/hybrid dC_5:pC_5 (1:1) (Fig. 3). The characteristic CD signal of control i-motif forming DNA dC_5 are diminished with increasing pH values (Fig. 4A). The similar pH dependent CD signals depletion are observed with hybrid (pC_5:dC_5) (Fig. 4B). These results indicate that the hybrid pC_5:dC_5 (1:1) also forms DNA i-motif type of structure, as like control, only at low pH range (4.0–6.0).

Further the stability of hybrid i-motif structure was examined by the temperature dependent CD-spectra analyses. We recorded the temperature dependent CD spectra of annealed hybrid pC_5:dC_5 (1:1) and control dC_5 at acidic pH 4.5. The characteristic i-motif CD signals (maxima & minima) of hybrid structure were significantly depleted in cooperative manner with increasing the temperature as like control dC_5 (Supplementary Materials). The stability of DNA structures (duplex, triplex and *tetraplex*) are measured in term of their T_m (Thermal melting) values, which are extracted from respective sigmoidal UV/CD-melting profiles.[25] The CD thermal melting profiles of hybrid pC_5:dC_5, at wavelength 299 nm, is appeared as negative sigmoidal as like control dC_5. Such melting profiles are characteristic for DNA i-motif structures. Thus, hybrid pC_5:dC_5 also forms DNA i-motif type of structure and their profiles indicate that hybrid i-motif structure is more stable than lone DNA.

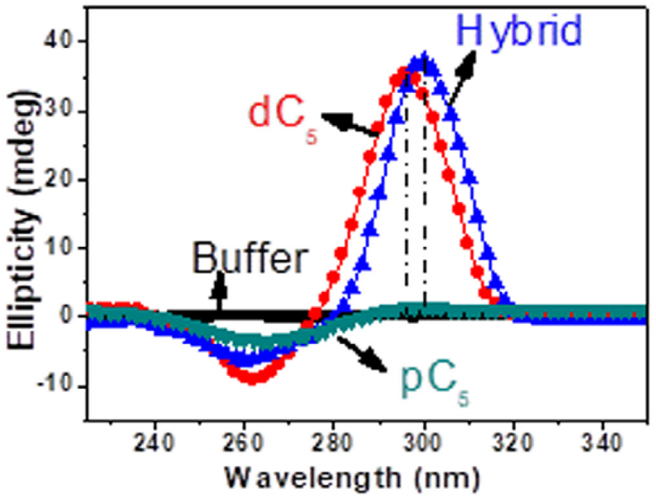


Fig. 3. CD-Spectra of pC_5 (45.0 μM)/dC_5 (45.0 μM)/hybrid dC_5:pC_5 (1:1), 22.5 μM each.at pH 4.5 (10 °C).

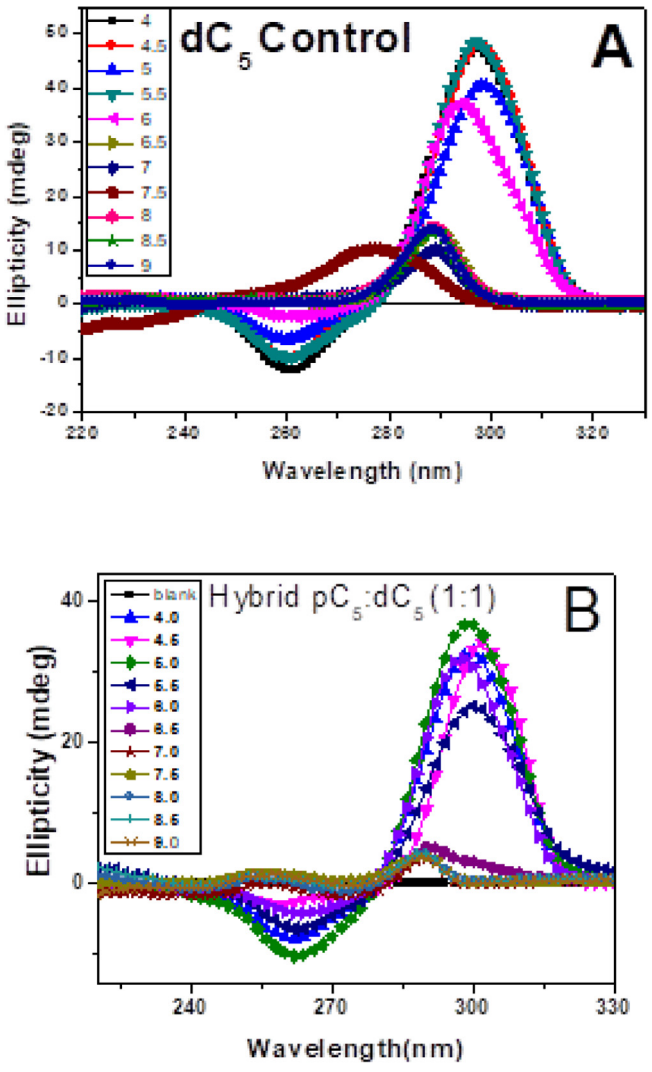


Fig. 4. pH dependent CD spectra of dC_5(45.0 μM) (A); and hybrid pC_5:dC_5, 22.5 μM each (B).

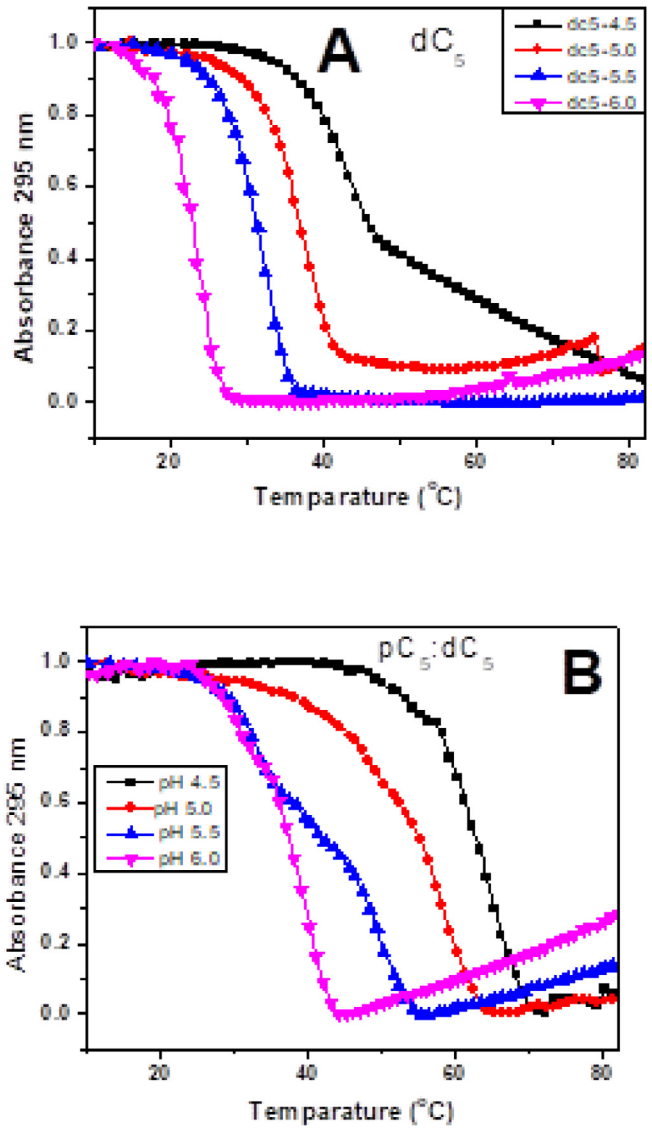


Fig. 5. Thermal UV-melting profiles at different pH at λ_{295nm}: (A) DNA dC_5; (45.0 μM) (B) DNA dC_5: PNA pC5 hybrid (1:1), 22.5 μM each.

Thermal UV-studies

The temperature dependent UV studies are one of the inexpensive and accurate methods to determine T_m of DNA structures. DNA duplexes/tetraplex structures have characteristic sigmoidal melting curves at different wavelengths. The positive sigmoidal melting profiles at 260 nm are known for duplexes/triplexes/tetraplexes while the negative sigmoidal melting profile at wavelength 300 nm are characteristic for DNA tetraplexes (G-quadruplex and i-motif).[25] The first derivative curve of those sigmoidal curves give accurate Tm of specific DNA structures. Pleasantly, we performed the UV melting experiments for annealed pC_5/dC_5/hybrid pC_5:dC_5 (1:1) sample at λ_{295nm} under acidic conditions (pH 4.0–6.0), and then plotted their respective thermal melting profiles (Fig. 5 & SI). At pH 4.5, the melting profiles of dC_5 is appeared as negative sigmoidal curves (at λ_{295nm}) which are characteristic for the denaturation of i-motif structure (Fig. 5A). The similar melting profiles, negative sigmoidal curves, are also observed with dC_5 under other pH conditions: pH 5.0, 5.5, and 6.0. The melting profiles of hybrid dC_5:pC_5 at different pH conditions (pH 4.5–6.0) are depicted in Fig. 5B which also exhibit i-motif's characteristic negative sigmoidal curve (at λ_{295nm}) under acidic conditions. In contrast, the melting profiles of pC_5, at pH range 4.5–6.5, exhibit positive sigmoidal types curve (at λ_{295nm}) (Supplementary Materials) which is non-characteristic for i-motif structure. The positive sigmoidal curves for lone PNA pC_5 are appeared under acidic pH conditions possibly because of the self-duplex formation from protonated and non-protonated cytosine of pC_5 C^+H:C) via hydrogen bonding. However, we could not observe the sigmoidal curve (at λ_{295nm}) with dC_5/pC_5/hybrid dC_5:pC_5 at neutral pH 7. Importantly, the UV-melting profiles of hybrid dC_5:pC_5 and control dC_5 are characteristically similar to that of DNA i-motif structure.

Thus *aep*-PNA (pC_5) is hybridized with DNA (dC_5) and formed hybrid i-motif structure as like control DNA (dC_5) under acidic conditions. The stability of those hybrid i-motifs was compared with control DNA (dC_5) i-motif by DNA denaturation temperature (T_m) value. We, then, extracted the T_m values of hybrid (dC_5:pC_5) and dC_5 under different pH conditions from their respecting UV-melting profiles (see Table 1). At pH 4.5, the Tm value of hybrid (dC_5:pC_5) i-motif is remarkably higher than control dC_5 with temperature difference (ΔT_m) ~15.0 °C (Table 1, Entry 1). This difference is almost 2.5 folds higher than that of reported T_m (~6–7.0 °C) of the unmodified DNA:PNA hybrid i-motif structure.[19]

At other different pH conditions, the ΔT_ms of hybrid dC_5:pC_5 and control dC_5 are ~18.0 °C, ~9.0 °C, and ~13.0 °C at respective pHs 5.0, 5.5 and 6.0 (Table 1, Entry 2–4). Importantly, more than

Table 1
pH dependent UV-T_m/$\Delta T_m$of dC_5/Hybrid dC_5:pC_5.

Entry	pH	Tm (°C) of dC_5	Tm (°C) of dC_5:pC_5(1:1)	ΔT (°C)*
1	4.5	46.73	62.00	15.27
2	5.0	36.13	54.06	17.93
3	5.5	31.26	40.11	8.85
4	6.0	22.66	35.82	13.16

* ΔT_m is difference in T_m of dC_5 and dC_5:pC_5 (1:1).

one transitions in UV-melting profile of hybrid at pH 5.5 are observed. It may appeared due to other complexes such as hybrid triplex and duplex structure. These UV-melting studies strongly support the formation of stable hybrid DNA:*aep*-PNAi-motif structure under acidic conditions at least by 9.0 °C. In contrast, the melting profiles of annealed-pC_5 at different acidic pH range (4.5–6.0) did not match with characteristic i-motif structure.

NMR studies

We further examined i-motif formation by hybrid pC_5:dC_5 i-motif structure under acidic conditions by ^{1}H NMR studies and compared with the control dC_5i-motif structure. Since *imino* proton of protonated cytosine (N^3-$\underline{H}^{\pm}$) is involved in hydrogen bonding with N^3 of non-protonated cytosine in DNA i-motif structure, and

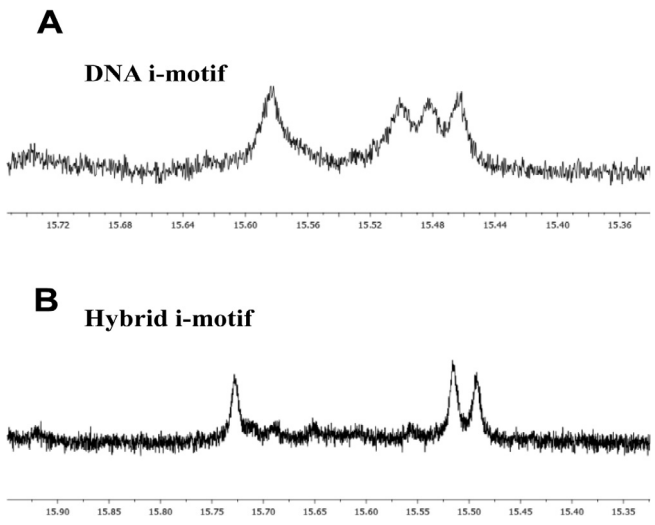


Fig. 6. ^{1}H NMR of hydrogen bonded *imino* N-H of protonated cytosine at pH 4.5 at 25 °C (700 MHz):(A) DNA dC_5(300 μM); (B) hybrid dC_5:pC_5 (1:1), each 200 μM.

^{1}H NMR signal of that proton is characteristically appeared at δ 15.0–16.00 (ppm) in DNA i-motif structure.[26] Herein, we recorded the ^{1}H NMR spectrum of annealed hybrid pC_5:dC_5 (1:1) and control annealed-dC_5 under acidic pH (4.5) conditions at temperature 25 °C. The ^{1}H NMR spectrum of *imino*-regions of hybrid dC_5:pC_5 and control dC_5 are depicted in Fig. 6A & B respectively. The ^{1}H NMR spectrum of both control dC_5 and hybrid dC_5:pC_5 exhibit prominent signals at δ 15.0–16.00 ppm which are characteristic peaks for DNA i-motif structure. However no characteristic ^{1}H NMR signal (δ 15.0–16.00) are noticed in the NMR spectra of annealed-pC_5 (data not shown). These NMR results strongly support the hybrid DNA:*aep*-PNAi-motif formation as like control DNA (dC_5) i-motif structure.

ESI-MS studies

Further, to confirm the above hybrid i-motif structure, we recorded the mass spectra of control dC_5 and hybrid pC_5:dC_5 (1:1) sample before/after annealing under acidic (pH 4.5) conditions. The mass spectra are provided in Supplementary Materials, while the selective mass spectra of annealed- hybrid (pC_5:dC_5) are depicted in Fig. 7. Further, the prominent mass peaks of dC_5/pC_5/hybrid (dC_5:pC_5), are extracted from their respective mass spectrum and summarized in Table 2. The mass spectra of un-annealed dC_5 or pC_5 showed mass peaks of respective monomeric unit as M_p (molecular mass of pC_5) and M_d (molecular mass of dC_5) (Table 2, Entry 1 & 2). The selected region of mass spectra of annealed dC_5/pC_5 are provided in Supplementary Materials. Fig. 7, the mass spectra of annealed hybrid dC_5:pC_5 (1:1) exhibit prominent mass peaks at 1104.98 (*m*/*z*) which matches to mass of hemi-protonated hybrid tetraplex dC_5:pC_5 (1:1) (Table 2, Entry 3). We also observed quintuple charged sodium adducts of hybrid tetraplexes (1104.98 + ~4.4) with mass difference of ~4.4 units (Supplementary Materials). These mass data confirm the formation of hybrid

Table 2
ESI-Mass analysis of pC_5/dC_5/hybrid dC_5:pC_5.

Entry	pC_5/hybrid pC_5:dC_5 (calculated Mass)	Mass (Observed)*
1	dC_5 (M_d) 1383.2760	1382.05 $(M_d-H)^-$*
2	pC_5(M_p) 1375.6900	1376.71 $(Mp+H)^+$
3	Annealed-pC_5:dC_5 (1:1); (pH 4.5)	1104.98 $(2M_p+2M_d+10H)^{5+}$; 1036.80 $(2M_d+M_p+5H)^{4+}$; 1382.39,$(2M_d+M_p+5H)^{3+}$; 1384.75 $(2M_d+5H)^{2+}$

* M_d: Molecular mass of DNA dC_5; Mp: Molecular mass of pC_5.

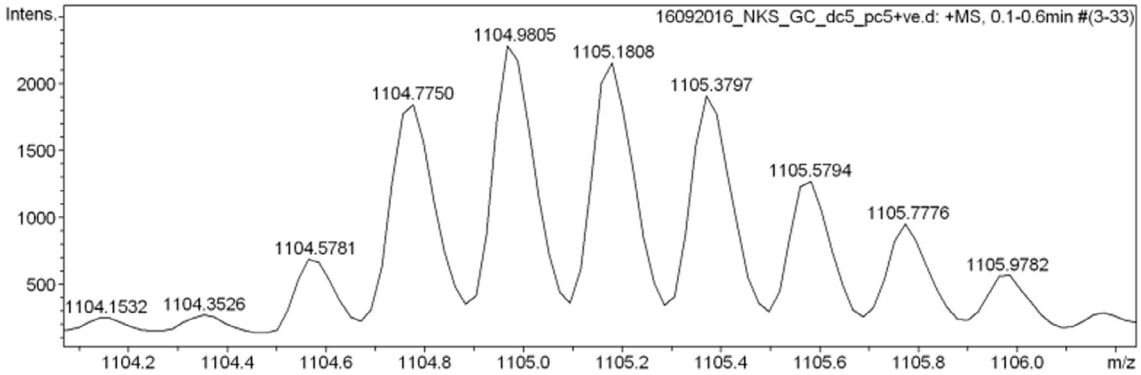


Fig. 7. ESI-MS of hybrid i-motif dC_5:pC_5 (1:1).

pC_5:pC_5i-motif structure. The mass peak of annealed hybrid dC_5:pC_5 also exhibit at *m*/*z* 1036.80 and 1382.39 which match to the mass of protonated hybrid triplex as $2dC_5$:pC_5 (Table 2, Entry 3). The appearance of mass peak of hybrid trimer is probably occurred due to the separation of one pC_5 strand from hybrid tetraplex. In literature, formation of triplex from DNA i-motif structure are also noticed during nano-ESI mass analysis.[16] Another mass peak is also prominent at 1384.75 which match to mass of protonated self-duplex dC_5-dimer (Table 2, Entry 3). These mass analyses confirmed the formation of hybrid pC_5:dC_5 (1:1) i-motif structure under acidic conditions along with few other species (hybrid triplex & DNA self-duplex). We recorded the mass spectrum of annealed-dC_5 with negative mode ESI method but observed only mass peak of monomeric dC_5 and its ions along with sodium salts (Supplementary Materials). Presumably the i-motif structure of lone dC_5 are not stable under that conditions.

In summary, we have successfully completed the synthesis of *aep*-PNA-C_5 (pC_5) and accomplished the comparative biophysical studies by CD/UV/NMR/ESI-Mass. The repeated C-rich sequence of modified PNA, *aep*-PNA, forms hybrid DNA:*aep*-PNA (1:1) i-motif and enhanced the stability of that i-motif structure. The remarkable stability of that hybrid i-motif is most probably due to additional strong ion-ion attractive interactions between *tertiary amine* backbone of *aep*-PNA and *phosphate* backbone of DNA under near acidic condition. Importantly, the formation of i-motif by only *aep*-PNA (pC_5) is not observed under acidic conditions. Hence this type of hybrid i-motif analogues could be potential candidate for the regulation of specific gene expressions, and other applications related to i-motif.

Acknowledgment

This paper is dedicated to Prof. K. N. Ganesh for his 64th birthday.

A. Supplementary data

Synthetic procedures for the *aep*-C monomer, the NMR and HRMS of all the compounds also provided. Synthetic procedures for *aep*-C_5 pentamer, HPLC chromatogram and mass spectrum are provided. Temperature dependent CD spectra of DNA and hybrid i-motif, UV-Vis, spectral data for pC_5, are provided.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.11.004.

References

1. Choi J, Majima T. *Chem Soc Rev*. 2011;40:5893–5909.
2. Gehring K, Leroy JL, Gueron M. *Nature*. 1993;363:565.
3. Kang CH, Berger I, Lockshin C, Ratliff R, Moyzis R, Rich A. *Proc Nat Acta Sci*. 1994;91:11636–11640.
4. Kendrick S, Kang H-J, Alam MP, et al. *J Am Chem Soc*. 2014;136:4161–4171.
5. Dong Y, Yang Z, Liu D. *Acc Chem Res*. 2014;47:1853–1860.
6. Wilner OI, Willner I. *Chem Rev*. 2012;112:2528–2556.
7. Brooks TA, Hurley LH. *Nat Rev Cancer*. 2009;9:849–861.
8. Day HA, Huguin C, Waller ZAE. *Chem Commun*. 2013;49:7696–7698.
9. Brazier JA, Fisher J, Cosstick R. *Angew Chem Int Ed*. 2006;45:114–117.
10. Phan AT, Guéron M, Leroy J-L. *J Mol Biol*. 2000;299:123–144.
11. Kumar N, Nielsen JT, Maiti S, Petersen M. *Angew Chem Int Ed*. 2007;46:9220–9222.
12. Assi HA, Harkness VRW, Martin-Pintado N, et al. *Nucleic Acids Res*. 2016;44:4998–5009.
13. Day HA, Pavlou P, Waller ZAE. *Bioorg Med Chem*. 2014;22:4407–4418.
14. Nielsen PE, Egholm M, Berg RH, Buchardt O. *Science*. 1991;254:1497–1500.
15. Sharma NK, Ganesh KN. *Chem Commun*. 2005;4330–4332.
16. Krishnan-Ghosh Y, Stephens E, Balasubramanian S. *Chem Commun*. 2005;5278–5280.
17. Diederichsen U. *Angew Chem Int Ed*. 1998;37:2273–2276.
18. Chakraborty S, Modi S, Krishnan Y. *Chem Commun*. 2008;70–72.
19. Modi S, Wani AH, Krishnan Y. *Nucleic Acid Res*. 2006;34:4354–4363.
20. Kumar VA, Ganesh KN. *Acc Chem Res*. 2005;38:404–412.
21. D'Costa M, Kumar VA, Ganesh KN. *Org Lett*. 2001;3:1281–1284.
22. D'Costa M, Kumar VA, Ganesh KN. *Org Lett*. 1999;1:1513–1516.
23. Sharma NK, Ganesh KN. *Tetrahedron*. 2010;66:9165–9170.
24. Sharma NK, Ganesh KN. *Org Biomol Chem*. 2011;9:725–729.
25. Mergny J-L, Lacroix L. *Nucleic Acid Res*. 1998;26:4797–4803.
26. Phan AT, Mergny JL. *Nucleic Acid Res*. 2002;30:4618–4625.